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Hedgehog-EGFR cooperation response genes determine the oncogenic phenotype of basal cell carcinoma and tumor-initiating pancreatic cancer cells

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Transaction Report:

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

1st Editorial Decision 01 July 2011

Thank you for the submission of your manuscript "Hedgehog-EGFR cooperation response genes determine the oncogenic phenotype of basal cell carcinoma and pancreatic cancer cells" to EMBO Molecular Medicine. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees find the study potentially interesting, but they also raise a number of serious concerns that should be satisfactorily addressed in a major revision. The referees request a number of additional controls and details (including statistics) and also some further experiments:
- addressing the role of FGF in activating oncogenic RAS,
- solving the 3D versus 2D culture issue raised by all three reviewers,
- adding *in vivo* data to show, like in *in vitro*, that the CRGs are indeed dual targets of HH-GLI and EGFR.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can address the issues that have been raised within the space and time constraints outlined below. Please note that it is EMBO Molecular Medicine policy to allow a single major round of revision only and that it is therefore important to address the raised concerns at this stage.

We generally allow three months as standard revision time. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
EMBO Molecular Medicine

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

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

Coordination between Hh and other signals in tumorigenesis have previously been suggested, but this work examines genetically and pharmacologically the interaction with EGF signaling. The initial work is done in a mouse model and is convincing. The subsequent xenograft and cell culture work is more speculative.

Referee #1 (Other Remarks):

The manuscript by Eberl et al., describes the synergistic activation by Hh/EGFR signaling of what they call core response genes (CRGs) in BCC and pancreatic tumors *in vitro* and in skin or xenografts. The data, especially the data indicating the genetic requirement for EGFR in skin SmoM2-induced tumorigenesis are interesting, but somewhat arbitrary criteria were used to select the CRGs, the experiments are poorly described in the main body of the text, the experimental approaches often appear inconsistent (see below), and the figures are not well labeled.

Major points:

- As presented, the hypothesis that there is synergy between Hh and EGFR signaling is tenuous in pancreatic tumor cells, and more data are needed to support this connection. In particular, is EGF signaling simply required for cell survival in the skin? Does drug/RNAi inhibition of Hh or EGFR signaling affect CRG gene expression? In addition, does activation of these pathways (similar to the experiments in 3A) also lead to synergy of CRG expression in pancreatic tumor cells?

- Figure 2C needs significantly more than 3 mice per genotype for the Kaplan Meier graph.

- Was afatinib tested in the SmoM2 mice to inhibit EGFR? It's not clear why the authors decided to switch model systems and use the CLEG mice for these studies, as the CLEG model is less relevant to human BCC.

- Figure 5C comparing gene expression of cells in 3D vs. 2D culture is not valid, and the results could be due to artifacts of cell culture conditions. Instead, the authors should compare 3D cultures that have few macrospheres vs. 3D cultures that are enriched for macrospheres (as in Fig. S3b) to deduce whether there is a concomitant increase in CRG expression. Another possibility is to compare early 3D cultures that have not yet selected for macrosphere growth, versus late 3D cultures where the bulk of the cells is contained in macrospheres.

Minor points:

- Page 6. The description of SmoM2 should reference the study by Mao and McMahon, Cancer Research 2006.
- Page 6. Notation "EGFR-delta-ep" should be fully defined in the text.
- Page 7. The CLEG2 mice should be better described.
- Page 7. The authors need to clearly define what they mean by oncogenic "cooperation" and "synergism" in the main text.
- Page 7: "We have shown that EGFR signaling converges at the promoters of selected direct Gli target genes" - Unclear. Converges on what? How exactly is EGFR inducing Gli1 target genes? Please clarify.

- Page 7. Please describe in the main text how EGFR signaling was induced for the experiments.
- Page 8 - It's unclear what was meant by "time-resolved genome-wide expression profiling." Also, the filter parameters were not clearly defined, e.g. what did "early activation by Hh/Gli-EGFR" mean? Did the genes need to be activated by either? Both? What cell lines were used? Were these 7 targets the only ones that met the criteria?
- Page 8. It's unclear why the reference to Regl et al., was included here.
- Page 9. The pancreatic macrosphere assay selecting for cancer stem cells needs a citation in the main text if it has been previously described.
- Page 9. The names of the pancreatic cancer cell lines should be mentioned in the main text.
- Page 9. Please clarify why IL1R2 is an indicator of Hh-EGFR synergistic signaling in the main text.
- Page 10. The text describing the inhibition studies in Fig. 6B is confusingly written.

- Fig 1. The schematic for treatment in D/E should be moved to the top of the figure for clarity.
- Fig 1. Genotype nomenclature differs in Fig 1A vs. other panels in the figure.
- Fig 2A. Y-axis is labeled, "relative tumor growth" - relative to what?
- Fig 2B. Y-axes in 2A/2B should be consistent.
- Fig 2B. What was the knock-down efficiency of the shEGFR?
- Fig 3. What cells were used? Why did 3A use Gli1 expression to induce Hh signaling, but 3C use Gli2 expression? Please add relevant error bars to Fig 3 especially to the controls of Fig 3C.
- Fig 3C. Please specify how the experiments were controlled. Did the authors use untransfected cells? Empty vector?
- Fig. 4A. Why was expression for CXCR4 not determined?
- Fig 4B. This figure compares the expression of tumor cells grown *in vitro* vs. *in vivo*, and the findings add little to rest of the study and could possibly be omitted.
- Fig 4C. Why were these 3 genes chosen over the other CRGs?
- Fig 4C. What's the control?
- Fig 6A. The reference to this figure does not appear in the main text.
- Fig 6A. What's the Gli1 % knock-down by RNAi?
- Fig 6A/B. The axes should be consistent.
- Fig. 6B. Why is erlotinib used in these experiments while afatinib is used previously as the EGFR inhibitor?
- Fig 7. What is the control in these experiments?
- Fig 7F. What was the RNAi knock-down efficiency for Smo?
- Fig 8. As depicted, this model is complicated and adds little information. Also, it does not account for the cell-autonomous role of Smo/EGFR signaling described in the earlier BCC portion of this study.
- Numerous misspellings throughout the text.

- Fig. S2. What cells were used here? How was EGFR signaling induced? How long were the cells treated before testing? Given that the data in S2-C were negative, does this figure really need to be included in this study?

Referee #2 (Comments on Novelty/Model System):

As detailed in my report the data are interesting but more statistical analysis is required, and in those case where this analysis is presented more details need to be provided. The finding that HH/GLI1 acts synergistically with EGFR in oncogenesis is not novel (and in fact has been published by this group before) but the *in vivo* studies and the identification of CPG's is novel. A number of models systems are used and are appropriate.

Referee #2 (Other Remarks):

This manuscript investigates the co-operation between the HH/GLI and EGFR signalling pathways in oncogenesis, and identifies a set of HH-EGFR co-operation response genes (CRGs) synergistically regulated by HH and EGFR. The fact that these pathways co-operate in oncogenesis has been shown previously, but the novel aspect of this manuscript lies in the *in vivo* studies and the demonstration that the CRGs play a major role in this effect.

The manuscript contains some interesting data, however in many instances there is insufficient information supplied on some of the experimental approaches and analyses to accurately assess the validity of the findings. In particular, as detailed below, the authors need to provide more evidence of statistical significance in a number of their experiments. If the authors can adequately address these issues the manuscript would be more suitable for publication.

SPECIFIC COMMENTS:
Page 5. The authors state that they have demonstrated an "essential *in vivo* requirement for EGFR in Hh/Gli-driven BCC....". While EGFR synergises with HH/GLI is it essential for tumorigenesis or does it enhance it? In the experiments described in figure 1 tumours still form in the Hh/Gli model in the absence of EGFR, but are smaller and fewer in number. Or are these small lesions not true BCCs? The authors should address this.

Figure 2: There are no details relating to the level of knock-down achieved in the EGFR shRNA experiments. It is stated in the materials and methods that functionality of all shRNAs was validated by qPCR and Western blotting. However, the authors should state what overall level of knock-down was achieved for each shRNA employed (this applies to other experiments using shRNA), and consider adding these data to the supplementary information. They should also provide details of the lentiviral vector used.

Figure 2: What do the error bars represent in A and B?

Page 8: State which cells were used in the expression profiling experiments even though it can be found in the materials and methods.
Use full titles the first time the CRGs are mentioned.

Figure 3A: How many technical and biological replicates were performed for each gene analysed by qRT-PCR? The authors should include error bars (and state what they represent). Presuming there are sufficient biological replicates, statistical analysis should be performed and presented (with details of the test used). Is the fold-change relative to untreated cells?

Figure 3C: Why were the GLI binding sites in Sox9 and 2 not subject to *in vitro* mutagenesis like the other molecules analysed?

Figure 4: What do the error bars represent? How many technical and biological replicates were performed in the qPCR experiments? Why Lrg5 and nanog? Why were different sets of genes analysed in A and B?
In the ASZ001 model Gli1 is said to not be up regulated in the tumour relative to the control 2D cells (which presumably already show enhanced Hh signaling due to loss of PTCH1). Based on this the authors propose that the tumour up-regulation of CRGs drives BCC development. I am not convinced that the 2D cells are the right baseline control to use here since you are essentially comparing isolated cells with a complex *in vivo* tumour tissue. The authors should explain in more detail why they are making this comparison and why it is valid.

Page 9: The statement that "inhibition of all three CRG in ASZ001 cells significantly reduce tumour uptake in mice" could be interpreted as knockdown of all three at once, whereas figure 4 shows effects due to individual knockdown of the CRG's. The authors should re-word this.

The effect of individual knockdown of the CRGs tested on *in vivo* tumour growth is quite dramatic. Did the authors test other CRGs and see no effect or were these the only ones tested?

Figure 5: Again, state the number of replicates analysed and present error bars where appropriate.
The level of GLI1 activation is relatively low in Panc-1 and L3.6pl cells. The other universal marker of hedgehog signalling levels is Ptch1 and the authors should show levels of this marker as well.
The use of the term "tumour bulk" to refer to the cells grown in 2D cultures is confusing.
Label panels A and B with the particular cell line used.

Figure 6:
Why are the units used in A and B different (rel cfu vs rel no. of macrospheres)?

Please provide numbers of replicates and include statistics to indicate which changes are statistically significant (panels A and B). State what the error bars represent (A, B and C).

Three different pancreatic cell lines have been used for the studies in fig 5 and the two aspects shown in fig 6. If data were comparable in all cell lines why not show the data from one line only? At the very least the authors should label the figures to make it clear which cell line has been used for each experiment (even though the info can be found in the figure legends).

Figure 7: Give details of the numbers of mice analysed for each molecule, and what the errors bars represent. What cell line was used in this study? How many cells were injected?

Discussion, Page 11: The authors talk about the benefits of targeting SMO and EGFR for BCCs. Given that they show that the HH/GLI1 effect in pancreatic tumours is independent of SMO, they should mention alternatives for targeting these tumour types such as through inhibition of GLI and EGFR.

GENERAL COMMENTS:
Throughout the manuscript there are different combinations of CRGs analysed and cell lines used for different experiments. It is sometimes difficult to know if other molecules were tested and showed no effect, or if only some molecules were tested in different experiments. Same for cell lines, as mentioned above. The authors should address this to make it easier for the reader to follow.

The affect of knockdown of individual CRGs on oncogenesis in a number of approaches presented is dramatic. The authors should discuss what this means in terms of the potential synergistic effects of these genes versus their individual tumour promoting capabilities independent of HH/GLI and EGFR regulation.

Referee #3:

This is an interesting and potentially important paper on target genes regulated by HH-Gli and EGFR signaling.

Introduction

Description of GLi function and their action in HH signaling is mouse centric. A more balanced discussion of results in different species is required, highlighting possible differences in the use of the three different GLI proteins.

Refs to HH and EGF signaling in cancer should also include those from the Toftgard lab and Clement et al 2007, Palma et al etc. Kasper et al is inappropriate for metastasis.

Yauch et al suggested paracrine signaling, but did not prove it. This whole discussion on paracrine signaling is strange since if any the authors here demonstrate cell-autonomous requirement of GLI and targets!

Ref to the integration of oncogenic RAS-MEK, PTEN/AKT is Stecca et al., 2007. This is missing.

As oncogenic RAS/MEK/AKT enhance GLI1bs-luciferase reporter (Stecca et al., 2007) Jun would seem not to be required. How do the authors reconcile these findings?

There seems to be a rather jumbled presentation of ideas and references. Reading the introduction it is not clear where the authors want to take the reader. Wherever this is, the refs need to be clarified and not be selective.
Again, Clement et al. showed synergism between temozolomide and cyclopamine, and this is, again, ignored.

Results

Why is the model of BCC in mice restricted to the ears? If the results deleting EGFR also seen in the back?

Is the reduction in the size of the tumors (Fig. 1) related to a possible reduction in the size of spheres as described below?

Why do the Gleg2 mice die after complete inhibition of EGFR (Fig. 2C)?

ASZ001 is a strange cell line. It adds little.

Use of Gli2deltaN is inappropriate. This is an artificial form of Gli2 that does not allow one to distinguish Gli function, and it is used since the full-length GLI2 does not work.

The first paragraph of the second part of the Results should be deleted as it belongs to discussion.

The section on targets is very nice. However, the CRG nomenclature is confusing and should be deleted, keeping to the names of the genes used for each assay.

The histograms in Fig 3 need statistics and error bars. How many different cells were used for this? Is the same result obtained with BCC and pancreatic cells?

When dealing with the regulation of targets, the use of Gli2deltaN is inappropriate. Full-length forms should be used. Results with the former form cannot be taken to mean Gli2 action.

Is SOX9 a stemness gene? No refs are given here or elsewhere in many instances.

Is expression of FGF19, TGFA, SOX2, SOX9 and CXCR4, etc in the same cells that express GLI2 or GLI1 in BCCs *in vivo* in mice and humans? This is an essential issue to resolve at a single-cell level.

Since GLI1 is said to be unchanged *in vitro* and *in vivo*, can the authors show the data and can they test if activity is changed other than in expression levels?

What are the controls for *in vivo* blockade of JUN, SOX2 and CXCR4? These seem to be missing.

What is the level of kd in tumors *in vivo*? This is essential as the varying results could explain the different tumor inhibition results (Fig. 4C).

What are the refs for the 'emerging role of HH-GLI in cancer stem cells'?

What are the refs for the 'stemness genes Oct4, NANOG, CD133' in human tumors?

What are the controls for the increased expression of HH-GLI and HH-GLI/EFGR targets in spheres? This needs a lot more work and the jump to pancreatic cancer seems unwarranted. Why pancreatic cancer and BCC? What do they have in common? What is being tested here?

It is said that blockade of HH or EGFR signaling compromises colony formation. Does it also affect size? Is this effect restricted to *in vitro* conditions? Since sphere growth usually requires FGF and EGF, is this result not obvious?

Fig. 6B needs to be expanded to include levels of knock-down by the shRNAs. The interpretation depends on this.

It is surprising that each of the identified genes is required for sphere formation. What are the controls? How many shRNAs were used per target?

Why do the authors mention macrospheres? Are microspheres affected? Do macropsheres pass for multiple passes?

The 2D-3D culture seems cumbersome Why not use standard spheres as with other cancers?

Are the pancreatic cancer cells used for xenografts human or mice? It is not clear in the text.

What is the level of kd of SMOH in the xenografts that are insensitive? This is essential to know in order to interpret the result.

Does co-inhibition of one or more of the target genes result in a greater inhibition? This is an important issue to resolve.

Discussion

The authors need to show that the target genes regulated by HH-GLI and EGFR are indeed dual targets *in vivo* in mouse BCCs and in human samples, as well as in spheres. Otherwise, the results could be unrelated to the direct integration of signaling and due to *in vitro* artifacts. This is essential before possible acceptance.

Does inhibition of any of these target genes inhibit the expression of the others *in vivo*? This could explain the surprising effect that blockade of any of them inhibits tumor growth. Controls are missing.

The effects of FGF would seem to relate to the action of FGF in regulating GLI2 in xenopus (Brewster et al., 2000). Does blocking of FGF signaling yield the same result as blocking EGF? This can easily be done using inhibitors to FGFR.

It would be good if the authors were to focus on the molecular effects observed and less on therapy as this is not a therapy paper. The opening paragraph of the discussion is not appropriate for the results in the paper.

The second paragraph of the discussion is incomplete. EGFR signaling includes the action of RAS-MEK/AKT. This was first shown by Stecca et al, 2007. The concept of combinatorial therapy is already explored in other papers not referenced, e.g. in Stecca et al and Clement et al. It is the novelty of the target genes - and not ideas about combinatorial pathway integration - is what makes this paper interesting. Another example: top of page 12 when discussing oncogenic RAS there is no reference and this was shown by Stecca et al. Thus, this reviewer cannot escape the feeling that there is a strategic deletion of previous work.

Overall, the data is interesting and extend previous work by the Aberger and other labs. There is the exciting finding that specific targets may mediate the effects of HH and EGFR. However, it is not clear if these are specific to any input that activates oncogenic RAS, such as FGF. It is also not clear if integration occurs *in vivo* and in the different cells or cancer types used. There is a disturbing lack of key references and an over interpretation of novelty and translation to a clinical setting. In addition, key controls appear to be missing.

1st Revision - Authors' Response 05 November 2011

We very much appreciate the in-depth reviews and constructive comments provided by all referees. In consideration of the issues raised by the reviewers we re-submit a substantially revised version of our manuscript. Before providing a point-by-point response we briefly summarize the major and most important points of the revision made to strengthen the quality of the work, also considering the editor's statements (see below).

1) *addressing the role of FGF in activating oncogenic RAS*
   We added additional data showing that the expression of cooperation response genes (CRG) requires the specific combination of HH/GLI and EGFR activation but cannot be elicited by e.g. combined FGF/GLI or RAS/GLI signaling.

Unlike EGFR/GLI signaling, activation of other receptor tyrosine kinases pathways including FGF, HGF and VEGF do not induce synergistic activation of HH-EGFR cooperation response genes (see new Figure 4B). Similarly, combined activation of GLI and oncogenic RAS was not sufficient to induce cooperation response gene expression, though it significantly enhanced EGF-independent canonical GLI target expression (i.e. PTCH and HHIP)(new Figure 4B), in line with previous reports (Stecca et al., 2007, Riobo et al., 2006).
Referring to the regulation of GLI expression by FGF signaling (Brewster et al., 2000), we show that inhibition of FGF19, which signals via FGFR4, does not affect GLI2 or GLI1 levels (suppl. information Fig. S9A). Consistent with the requirement of FGF19 for TIC growth *in vitro* and tumor growth *in vivo*, treatment of pancreatic cancer cells with a potent FGFR inhibitor reduced the growth of tumor-initiating macrospheres in 3D cultures (suppl. information, Fig. S9B)

2) *adding in vivo data to show, like in in vitro, that the CRGs are indeed dual targets of HH-GLI and EGFR*
We now show that SmoM2 expression in transgenic mice (tamoxifen treated K5creER;SmoM2;EGFR+/+) induces HH-EGFR CRG expression. By contrast, genetic deletion of EGFR in tam-treated K5creER;SmoM2;EGFRfl/fl mice prevents CRG activation, demonstrating an *in vivo* requirement of EGFR signaling for the regulation of HH/GLI targets (Figure 5D). We also show that high-level CRG expression in allograft tumors of PTCH-/- BCC cells correlates with activation of EGFR/Mek/Erk/Jun signaling (Fig. 5C and suppl. Fig. S6). Furthermore, we added data showing that HH/GLI-EGFR cooperation regulates CRG expression also in pancreatic cancer cells (Figure 6E). Importantly, we added data showing CRG expression in HH-driven mouse and human BCC (suppl. information, Figure S7).

3) *solving the 3D versus 2D culture issue raised by all three reviewers*
We apologize for the insufficient and perhaps confusing description of both the rationale for the use of a modified 3D tumor sphere (macrosphere) assay to characterize tumor-initiating pancreatic cancer cells and the methodology applied. Given the space restrictions for the manuscript length, we now describe in detail in the suppl. information the isolation and characterization of tumor-initiating cells (TICs). We further would like to emphasize that we consistently use the term tumor-initiating cells because we provide solid evidence that macrospheres of human pancreatic cancer cells are highly enriched for TICs as determined by limiting dilution xenograft experiments, which is considered the gold standard for the characterization of TICs (Note that as few as 100 cells from isolated single macrospheres are sufficient for engraftment and tumor growth in all experiments (4/4), Fig. 6B). As we do not address their *in vivo* self-renewal and differentiation capacity (this is not the focus of this study), we are reluctant to call these cells cancer stem cells, although TICs from macrospheres show expression of stem cell genes and characteristics of self-renewal *in vitro* (Fig. 6C and suppl. information, Fig. S8A-C). For these reasons, and in consideration of our vitro and *in vivo* analyses of HH-EGFR CRG function in TICs, we believe that it is correct to claim that HH/GLI-EGFR regulated CRG determine the malignant phenotype of tumor-initiating pancreatic cancer cells.
Please find below a short excerpt of the rationale for using this approach:
"Most of the studies of pancreatic TICs used distinct surface marker combinations or enzyme-activities to enrich for TICs by cell sorting. The obvious heterogeneity of pancreatic TIC populations and their inconsistent marker profile poses significant problems and limitations for their isolation and analysis….
The lack of a universal pancreatic TIC marker and the obvious discrepancy of the TIC identity in pancreatic cancer cells prompted us to use in this study a TIC enrichment technique that is based on the growth characteristics of TICs rather than surface marker expression or enzyme activity, i.e. high clonogenic growth and (tumor) sphere formation under non-adherent conditions (Simeone, 2008)….."

4) *we provide an explanation for the observation that individual inhibition of CRG function (by RNAi) each had a striking effect on the in vivo tumor growth of pancreatic cancer cells (e.g. shJUN, shSOX9, shCXCR4, shFGF19, Fig. 8)*
Although it has been shown that cooperation response gene sets (e.g. those regulated by cooperation of p53 loss and oncogenic RAS, McMurray et al., 2008, Nature) can be highly

enriched for determinants of the malignant phenotype, it is unclear whether regulatory interactions among CRGs may contribute to or account for this observation. By knocking down individual HH-EGFR CRG (FGF19, CXCR4, SOX9 and JUN) in pancreatic cancer cells and analyzing the effect on the expression of unperturbed CRG, we provide evidence for the existence of a small regulatory network of cooperation response genes downstream of HH/GLI-EGFR signaling (new Fig. 9A, B). For instance, inhibition of FGF19 resulted in a marked decrease of CXCR4 and vice versa, suggesting mutual positive regulation of these CRG. Also, SOX9 depletion resulted in a significant decrease in JUN expression. Such regulatory mechanisms can explain the dramatic phenotype of individual gene inhibitions on tumor growth.

5) We provide detailed information on controls and statistical data

6) We provide data on knockdown efficiencies of all shRNA constructs used (suppl. information, Fig. S10)

7) As a consequence to the additional experiments and more detailed description of the experiments, the introduction and discussion had to be made more concise in order to comply with the space restrictions of EMBO Mol Med.

Point-by-point response:

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

Coordination between Hh and other signals in tumorigenesis have previously been suggested, but this work examines genetically and pharmacologically the interaction with EGF signaling. The initial work is done in a mouse model and is convincing. The subsequent xenograft and cell culture work is more speculative.

Referee #1 (Other Remarks):

The manuscript by Eberl et al., describes the synergistic activation by Hh/EGFR signaling of what they call core response genes (CRGs) in BCC and pancreatic tumors in vitro and in skin or xenografts. The data, especially the data indicating the genetic requirement for EGFR in skin SmoM2-induced tumorigenesis are interesting, but somewhat arbitrary criteria were used to select the CRGs, the experiments are poorly described in the main body of the text, the experimental approaches often appear inconsistent (see below), and the figures are not well labeled.

Authors' response:

We have carefully revised the description of the experiments and provide a much more detailed results section and figure legends. Also, additional information has been added to the suppl. information section to comply with the space restrictions for publication in EMBO Mol Med.

Major points:

- As presented, the hypothesis that there is synergy between Hh and EGFR signaling is tenuous in pancreatic tumor cells, and more data are needed to support this connection.

Authors´ response: We added data showing that like in keratinocytes combined activation of GLI1 and EGFR signaling induces CRG expression in pancreatic cancer cells (Figure 6E). In addition, we show that like in human keratinocytes, EGF treatment of pancreatic cancer cells induces EGFR/MEK/ERK/JUN activation characteristic of cooperative EGFR signaling (for comparison see Figure 4A).

In particular, is EGF signaling simply required for cell survival in the skin?

Authors´ response: We addressed this issue by analyzing epidermal cells from tamoxifen-treated K5creER;SmoM2;EGFR+/+ and K5creER;SmoM2;EGFRfl/fl mice for apoptosis. As shown in suppl. Figure S4 deletion of EGFR does not increase apoptosis.

Does drug/RNAi inhibition of Hh or EGFR signaling affect CRG gene expression?

Authors´ response: To address this issue, we measured CRG expression in EGFR knockdown allografts but had to realize that at the time of tumor harvesting (i.e. 4 weeks post inoculation in nude mice) the knockdown of EGFR was no longer detectable (data available upon request). Consequently, we could not see a consistent reduction of CRG expression in shEGFR tumor samples harvested 4 weeks post inoculation. Note that at the beginning of the allograft experiment shEGFR cells show a clear reduction in tumor growth *in vivo*, but once engrafted appear to grow at a rate that is nearly comparable to control knockdown cells (see Figure 2B). We think that this is either due to silencing of the shRNA expression construct or by overgrowth of cells without sufficient EGFR knockdown (we injected the bulk of shRNA transduced BCC cells, not clonal isolates). Knockdown of EGFR protein expression before grafting is shown in Figure 2C.

To demonstrate *in vivo* regulation of CRG by dual HH/GLI and EGFR signaling in cancer, we took another approach. We analyzed expression of HH-EGFR CRG in epidermal cells of tamoxifen-injected K5creER;SmoM2;EGFR+/+ and K5creER;SmoM2;EGFRfl/fl mice and found that deletion of EGFR prevents induction of CRG (Figure 5D, also see above, summary of revised manuscript). We conclude that HH-EGFR cooperation response genes are controlled by both HH/GLI and EGFR during *in vivo* BCC development.

In addition, does activation of these pathways (similar to the experiments in 3A) also lead to synergy of CRG expression in pancreatic tumor cells?

Authors´ response: Yes, we added experiments showing activation of CRG expression in pancreatic cancer cells in response to combined GLI1/EGFR activation (see Figure 6E)

- Figure 2C needs significantly more than 3 mice per genotype for the Kaplan Meier graph.

Authors´ response: We agree with this statement. Unfortunately, we have not been able to breed enough additional K5cre;Cleg2 mice during the 3 month revision period. We therefore decided to omit these data and hold the view that this does not weaken our conclusions about the requirement of EGFR signaling for HH/GLI-driven BCC development *in vivo*. Also in light of the concerns raised by reviewer #3 and #1 arguing that GLI2deltaN is an artificial construct and Cleg2 mice are less relevant to BCC development, we felt that removing the treatment data is acceptable.

- Was afatinib tested in the SmoM2 mice to inhibit EGFR? It's not clear why the authors decided to switch model systems and use the CLEG mice for these studies, as the CLEG model is less relevant to human BCC.

Authors´ response: We would also have preferred the afatinib treatment of SmoM2 mice. However, tumor growth in SmoM2 mice is very slow compared to Cleg2 mice (about 15 weeks in SmoM2 versus 3-4 weeks) and mice do not tolerate p.o. afatinib treatment for much longer than two weeks. It would therefore be difficult to find significant reduction in tumor growth between afatinib and control treated K5creER;SmoM2 mice. Also, K5creER;SmoM2 mice do not succumb to tumor development, making Kaplan-Meier analysis inappropriate.

- Figure 5C comparing gene expression of cells in 3D vs. 2D culture is not valid, and the results could be due to artifacts of cell culture conditions. Instead, the authors should compare 3D cultures that have few macrospheres vs. 3D cultures that are enriched for macrospheres (as in Fig. S3b) to deduce whether there is a concomitant increase in CRG expression. Another possibility is to compare early 3D cultures that have not yet selected for macrosphere growth, versus late 3D cultures where the bulk of the cells is contained in macrospheres.

Authors´ response: we apologize again for the poor and confusing description of the 3D cultures

used to enrich for and isolate tumor-initiating cells. As stated above and in more detail in the manuscript (see Fig. 6A, B and suppl. information including Fig. S8A, B, C), we used the 3D culture system to isolate from 3D cultures single macrospheres highly enriched for tumor-initiating cells. We measured CRG expression in these isolated macrospheres enriched for TICs and compared their expression level to 2D cultured cells (not enriched for TICs) using 2D cultured cells as sort of baseline reference.

All we conclude from the expression analysis is that pancreatic TICs express HH-EGFR cooperation response genes and that their levels correlate with the tumor-initiating capacity of cells (i.e. higher in tumor-initiating macrospheres compared to non-enriched 2D cultured cells). Only by inhibiting CRG function can we show that HH-EGFR CRGs also play an etiologic role in the clonogenic growth of tumor-initiating (macrosphere) cells and in tumor initiation and *in vivo* growth of pancreatic cancer cells.

Minor points:

- Page 6. The description of SmoM2 should reference the study by Mao and McMahon, Cancer Research 2006.

Authors´ response: The reference has been added to the manuscript (now page 15 in the revised version)

- Page 6. Notation "EGFR-delta-ep" should be fully defined in the text.

Authors´ response: this has been removed, for the sake of consistency, the designation of transgenic mice now reads K5creER;SmoM2;EGFR+/+ and K5creER;SmoM2;EGFRfl/fl throughout the manuscript.

- Page 7. The CLEG2 mice should be better described.

Authors´ response: A detailed description of K5cre;Cleg2 mice has been added to the suppl. information due to space restrictions in the main manuscript.

- Page 7. The authors need to clearly define what they mean by oncogenic "cooperation" and "synergism" in the main text.

Authors´ response: We added a brief definition at the beginning of the introduction: …involves cooperation (i.e. synergistic interaction)

- Page 7: "We have shown that EGFR signaling converges at the promoters of selected direct Gli target genes" - Unclear. Converges on what? How exactly is EGFR inducing Gli1 target genes? Please clarify.

Authors´ response: In Kasper et al., 2006 and Schnidar et al., 2009 we could demonstrate that cooperation response genes are directly regulated by GLI i.e. GLI is binding to the promoter of CRGs. Simultaneous EGFR signaling synergistically induces promoter activation, at least in part by recruiting JUN/AP1 to GLI target promoters (see Schnidar et al). Therefore EGFR and HH signaling converge at the promoters of selected direct GLI target genes. This does not apply to EGF-independent, canonical GLI targets such as PTCH, HHIP or BCL2.

- Page 7. Please describe in the main text how EGFR signaling was induced for the experiments.

Authors´ response: page 7 top includes the requested information, …. that GLI1 and EGFR stimulation (by EGF treatment (10ng/mL))

- Page 8 - It's unclear what was meant by "time-resolved genome-wide expression profiling." Also, the filter parameters were not clearly defined, e.g. what did "early activation by Hh/Gli-EGFR" mean? Did the genes need to be activated by either? Both? What cell lines were used? Were these 7 targets the only ones that met the criteria?

Authors´ response: "time-resolved genome-wide expression profiling." Means that several time points were analyzed (time course). We have removed the term and added more clearly that cells were stimulated for the time indicated (4.5, 9 or 18h); we also say that early activation means

activated within 18h of treatment; As stated in the text, we used human HaCaT keratinocytes for the expression analysis of cooperation response genes. Cooperation response genes display synergistic activation by both signals (GLI1 and EGFR), meaning that the expression level of a given CRG in the GLI1/EGFR double stimulated samples is higher than the sum of the expression level in the single treatment samples (i.e. in GLI1 only and EGF only cells). Also see materials and methods, definition of synergy scores. The seven genes described deemed the most promising and interesting genes related to cancer, but there have been several more hits from the genome wide screen.

- Page 8. It's unclear why the reference to Regl et al., was included here.

Authors´ response: Thank you, has been removed

- Page 9. The pancreatic macrosphere assay selecting for cancer stem cells needs a citation in the main text if it has been previously described.

Authors´ response: This modified version of a tumor sphere assay has not been published previously. Pancreatic sphere cultures have been described and the reference is given in the suppl. information.

- Page 9. The names of the pancreatic cancer cell lines should be mentioned in the main text.

Authors´ response: We have added the cell line names were appropriate.

- Page 9. Please clarify why IL1R2 is an indicator of Hh-EGFR synergistic signaling in the main text.

Authors´ response: we have modified this, now reads as follows: the known HH-EGFR target gene Il1r2 (Kasper et al, 2006b),

- Page 10. The text describing the inhibition studies in Fig. 6B is confusingly written.

Authors´ response: Like for many other parts of the revised manuscript we have tried to make it more concise and clearer.

- Fig 1. The schematic for treatment in D/E should be moved to the top of the figure for clarity.

Authors´ response: done

- Fig 1. Genotype nomenclature differs in Fig 1A vs. other panels in the figure.

Authors´ response: nomenclature now is consistent throughout the manuscript

- Fig 2A. Y-axis is labeled, "relative tumor growth" - relative to what?

- Fig 2B. Y-axes in 2A/2B should be consistent

Authors´ response: before the start of treatment mice were randomized in two groups and their average tumor volume set to 1. Relative tumor growth represents the change of tumor volume compared relative to the mean tumor volume of the two groups (treatment and controls) at the beginning of treatment. We decided to display the data in this way as we find that it is easier to see the different growth rates and kinetics between the two cohorts.

- Fig 2B. What was the knockdown efficiency of the shEGFR?

Authors´ response: now shown in Figure 2C

- Fig 3. What cells were used? Why did 3A use Gli1 expression to induce Hh signaling, but 3C use Gli2 expression? Please add relevant error bars to Fig 3 especially to the controls of Fig 3C.

Authors´ response: HaCaT cells in 3A, 293 cells for luciferase assays; Figure 3C is now shown with GLI1, GLI2deltaN is shown in suppl. information, Figure S5

- Fig 3C. Please specify how the experiments were controlled. Did the authors use untransfected cells? Empty vector?

Authors´ response: expt controlled by empty vector and normalized lacZ co-transfection (see materials and methods and references therein)

- Fig. 4A. Why was expression for CXCR4 not determined?

Authors´ response: is included in the revised version, note that due to additional data, Fig 4 now is Fig.5 in the revised manuscript. The CRG sets measured in K5cre;Cleg2 and BCC allografts now match.

- Fig 4B. This figure compares the expression of tumor cells grown in vitro vs. in vivo, and the findings add little to rest of the study and could possibly be omitted.

Authors´ response: we think that the correlation of *in vivo* growth and high-level CRG is interesting, in particular as we now show that EGFR much more active in the allograft compared to the 2D cultured cells (see new Fig. 5C and suppl. information, Fig. S6).

- Fig 4C. Why were these 3 genes chosen over the other CRGs?

Authors´ response: Our aim was to inhibit all CRGs but we had problems finding functional shRNAs against the other CRGs. This worked better for the human constructs.

- Fig 4C. What's the control?

Authors´ response: ASZ001 cells transduced with lentiviral non-target control shRNA. Details about controls are given in the text/figure legend.

- Fig 6A. The reference to this figure does not appear in the main text.

Authors´ response: 6A is 7A in the revised version and has been added to the main text.

- Fig 6A. What's the Gli1 % knockdown by RNAi?

Authors´ response: >80%, see suppl. information, Fig. S10;

- Fig 6A/B. The axes should be consistent.

Authors´ response: has been corrected

- Fig. 6B. Why is erlotinib used in these experiments while afatinib is used previously as the EGFR inhibitor?

Authors´ response: Afatinib is very efficient for *in vivo* studies in mice as it blocks EGFR/ErbB family members irreversibly. Like afatinib, erlotinib works well *in vivo* with higher selectivity for EGFR/HER1. We basically get the same results with erlotinib, afatinib and also gefitinib.

- Fig 7. What is the control in these experiments?

Authors´ response: now Figure 8: controls were done with tumor cells transduced lentivirally with non-target control shRNA, this is now described in the text/figure legend.

- Fig 7F. What was the RNAi knockdown efficiency for Smo?

Authors´ response: comparable to GLI1, >80%, see suppl. information, Fig. S10

- Fig 8. As depicted, this model is complicated and adds little information. Also, it does not account for the cell-autonomous role of Smo/EGFR signaling described in the earlier BCC portion of this study.

Authors´ response: we agree and have drawn a very simplified illustration also including novel data on regulatory interactions between selected CRG

- Numerous misspellings throughout the text.

Authors´ response: we carefully checked and hope that there are no misspellings anymore

- Fig. S2. What cells were used here? How was EGFR signaling induced? How long were the cells

treated before testing? Given that the data in S2-C were negative, does this figure really need to be included in this study?

Authors´ response: this is now Fig. S4, ChIP was done in HaCaT cells, reporter assays in HEK 293 cells. Treatment time was 18h (this information has been added to the figure legend). Based on published data (Palma et al., 2004, 2005) the most obvious mode of HH-EGFR cooperation is synergistic activation of cell cycle targets. We therefore think that it is interesting to show also negative data, suggesting that HH-EGFR cooperation does not simply activate cell proliferation to promote transformation and tumorigenesis.

Referee #2 (Comments on Novelty/Model System):

As detailed in my report the data are interesting but more statistical analysis is required, and in those case where this analysis is presented more details need to be provided. The finding that HH/GLI1 acts synergistically with EGFR in oncogenesis is not novel (and in fact has been published by this group before) but the *in vivo* studies and the identification of CPG's is novel. A number of models systems are used and are appropriate.

Referee #2 (Other Remarks):

This manuscript investigates the co-operation between the HH/GLI and EGFR signaling pathways in oncogenesis, and identifies a set of HH-EGFR co-operation response genes (CRGs) synergistically regulated by HH and EGFR. The fact that these pathways co-operate in oncogenesis has been shown previously, but the novel aspect of this manuscript lies in the in vivo studies and the demonstration that the CRGs play a major role in this effect.

The manuscript contains some interesting data, however in many instances there is insufficient information supplied on some of the experimental approaches and analyses to accurately assess the validity of the findings. In particular, as detailed below, the authors need to provide more evidence of statistical significance in a number of their experiments. If the authors can adequately address these issues the manuscript would be more suitable for publication.

Authors´ response: We have added statistical data where appropriate and added additional data to calculate statistical significance of the findings. Figures have been adjusted accordingly and the statistical significance described in the respective figure legends.

SPECIFIC COMMENTS:
Page 5. The authors state that they have demonstrated an "essential in vivo requirement for EGFR in Hh/Gli-driven BCC....". While EGFR synergizes with HH/GLI is it essential for tumorigenesis or does it enhance it? In the experiments described in figure 1 tumors still form in the Hh/Gli model in the absence of EGFR, but are smaller and fewer in number. Or are these small lesions not true BCCs? The authors should address this.

Authors´ response: The residual tumors on the ears still express BCC markers such as K17, Sox9 and Gli1 (see new sup. information, Fig S3). So at least at the molecular level these small lesions resemble BCC-like tumors. This is somewhat different when looking at dorsal skin rather than ears. As published by the Dlugosz lab, SmoM2 expression induces basaloid hamartoma-like lesions and basaloid hyperproliferations. Deletion of EGFR efficiently prevents the formation of these tumors in dorsal skin (this is novel data shown in Fig. S2, suppl. information)

Figure 2: There are no details relating to the level of knockdown achieved in the EGFR shRNA experiments. It is stated in the materials and methods that functionality of all shRNAs was validated by qPCR and Western blotting. However, the authors should state what overall level of knockdown was achieved for each shRNA employed (this applies to other experiments using shRNA), and consider adding these data to the supplementary information. They should also provide details of the lentiviral vector used.

Authors´ response: knockdown of EGFR is now shown in Figure 2C. Knockdown efficiency of the other shRNA constructs is shown in suppl. information Figure S10. Details of the lentiviral vectors used are provided in the materials and methods section (e.g. shRNA clone numbers, purchased from SIGMA mission shRNA clone library, reference for human shCXCR4 is also provided)

Figure 2: What do the error bars represent in A and B?

Authors´ response: Standard error of the mean (SEM), this is now indicated for all figures in the respective figure legend.

Page 8: State which cells were used in the expression profiling experiments even though it can be found in the materials and methods.
Use full titles the first time the CRGs are mentioned

Authors´ response: information on the cell type for expression profiling (HaCaT keratinocytes) has been added to the main text describing the screening for downstream mediators of HH-EGFR cooperation. CRG has been spelled out when mentioned first time (page 6)

Figure 3A: How many technical and biological replicates were performed for each gene analyzed by qRT-PCR? The authors should include error bars (and state what they represent). Presuming there are sufficient biological replicates, statistical analysis should be performed and presented (with details of the test used). Is the fold-change relative to untreated cells?

Authors´ response: 3 biological replicates with two technical replicates per biological experiment. We added this information to the respective figure legend. in Error bars (SEM) have been added. Fold change is relative to untreated control cells, we added this information to the figure legend.

Figure 3C: Why were the GLI binding sites in Sox9 and 2 not subject to in vitro mutagenesis like the other molecules analyzed?

Authors´ response: we have added data for the mutated Sox9 promoter. Unfortunately, we had technical problems to finish the mutagenesis of Sox2 during the three-month revision period. Direct regulation of Sox2 by GLI1/2 is in agreement with a previous report (Takanaga et al., 2009), which we mention, in the main text on page 7.

Figure 4: What do the error bars represent? How many technical and biological replicates were performed in the qPCR experiments? Why Lrg5 and nanog? Why were different sets of genes analyzed in A and B?

Authors´ response: Figure 4 now is Figure 5. Error bars represent SEM (now added to the figure legend) please note the log10 scale, which is why error bars appear to be very small. We have removed Lgr5 and Nanog from Fig 5A, as this is indeed confusing and does not add relevant information. We have added additional CRG measurements to make the gene sets comparable between A und B. In A and B, we have measured 5 tumors each in two technical replicates. For comparison, we determined gene expression in 4 independent normal skin samples (A) and 2 independent ASZ001 2D cell culture samples, each measured in two technical replicates. As for basically all other data, we have calculated statistical significance and indicate the p-values in the graphs and figure legends.

In the ASZ001 model Gli1 is said to not be up regulated in the tumor relative to the control 2D cells (which presumably already show enhanced Hh signaling due to loss of PTCH1). Based on this the authors propose that the tumour up-regulation of CRGs drives BCC development. I am not convinced that the 2D cells are the right baseline control to use here since you are essentially

comparing isolated cells with a complex in vivo tumour tissue. The authors should explain in more detail why they are making this comparison and why it is valid.

Authors´ response: We fully agree that simply by comparing CRG levels in ASZ001 allograft tumors with those in ASZ001 BCC cells cultured *in vitro* does not allow the conclusion that up-regulation of CRG expression in the allograft drives tumor growth. What we describe is the correlation of elevated CRG expression and *in vivo* tumor growth. In the first version we took this observation as evidence for EGFR activation during *in vivo* tumor growth of ASZ001 cells, as CRG activation requires both GLI activity and EGFR signaling. As GLI1 expression is comparable between the 2D culture and the allograft samples, we suggested that the increase in CRG expression is due to the second signal (EGFR) activated during tumor growth. This is an important prerequisite as we propose in this study that the combined activation of HH/GLI and EGFR drives CRG expression, which in turn promotes tumor growth. To make this more convincing we added additional data showing selective activation of EGFR and Mek/Erk/Jun in the allograft tumors, while 2D cultured cells with lower CRG levels only show a very moderate activation of the EGFR/Mek/Erk/Jun signaling cascade (Figure 5C and suppl. information Fig. S6). From this results together with data on the regulation of CRGs we draw the following conclusion: "These data suggest activation of EGFR signaling during *in vivo* tumor growth of ASZ001 BCC cells (page9)".

Only by also inhibiting HH-EGFR function (i.e. individual knockdowns of Jun, Sox2 and Cxcr4, Figure 5E) do we provide evidence for a role of CRG up-regulation in driving BCC growth.

Page 9: The statement that "inhibition of all three CRG in ASZ001 cells significantly reduce tumour uptake in mice" could be interpreted as knockdown of all three at once, whereas figure 4 shows effects due to individual knockdown of the CRG's. The authors should re-word this.

Authors´ response: has been changed to: Figure 5E shows that individual inhibition of Jun, Sox2 or Cxcr4 in BCC cells significantly reduced tumor take of nude mice, suggesting that Jun, Sox2 and Cxcr4 constitute critical downstream mediators of oncogenic HH-EGFR signaling important for *in vivo* tumor growth of BCC cells

The effect of individual knockdown of the CRGs tested on in vivo tumour growth is quite dramatic. Did the authors test other CRGs and see no effect or were these the only ones tested?

Authors´ response: Currently, the CRGs described in this study are the only ones tested so far. Only knockdown of the non-CRG SMO did not show a reduction in tumor growth of pancreatic cancer cells. Although it has been described by McMurray et al., (2008, Nature (see reference in the manuscript) that oncogene cooperation response genes are highly enriched for determinants of the malignant phenotype, we were also surprised by the dramatic inhibitory effect of individual knockdowns. In response to a question of reviewer #3 we tested the possibility of regulatory interaction between selected HH-EGFR CRG. We found that inhibition of CXCR4 reduces FGF19 levels and vice versa. Also, Sox9 inhibition resulted in reduced JUN levels. The data are shown in Figure 9. We propose that mutual, positive regulatory interactions e.g. between CXCR4 and FGF19 may account for the striking phenotype observed in either knockdown experiment.

Figure 5: Again, state the number of replicates analyzed and present error bars where appropriate. The level of GLI1 activation is relatively low in Panc-1 and L3.6pl cells. The other universal marker of hedgehog signaling levels is Ptch1 and the authors should show levels of this marker as well. The use of the term "tumour bulk" to refer to the cells grown in 2D cultures is confusing. Label panels A and B with the particular cell line used.

Authors´ response: Figure 5 now is Figure 6. We added error bars (SEM), replicate numbers (to figure legend) and labeled panel A and B as requested. We replaced the term tumor bulk with 2D tumor cell bulk. We analyzed Ptch mRNA expression but did not find up-regulation. Rather unexpectedly, individually isolated tumor-initiating macrospheres of all three pancreatic cancer lines showed reduced Ptch expression (data available upon request). Whether this is a consequence of repression of canonical, autocrine HH/PTCH/SMO signaling in pancreatic cancer cells by Ras-

Dirk1b signaling (Lauth et al., 2010, Nature structural & molecular biology) is unclear and its interpretation would require a series of experiments that we think is beyond the scope of this work.

Despite the lack of PTCH up-regulation in tumor-initiating spheres, we provide evidence for GLI1 activity as knockdown of GLI1 has a dramatic impact on both *in vitro* sphere growth (Figure 7A) and *in vivo* tumor growth (Fig.8A)

Figure 6:
Why are the units used in A and B different (rel cfu vs rel no. of macrospheres)?

Authors´ response: This has been adjusted to rel. no. of macrospheres in both graphs (now in Figure 7)

Please provide numbers of replicates and include statistics to indicate which changes are statistically significant (panels A and B). State what the error bars represent (A, B and C).

Authors´ response: statistical significance and the information on replicates and error bars have been added to the figure and the figure legend.

Three different pancreatic cell lines have been used for the studies in fig5 and the two aspects shown in fig6. If data were comparable in all cell lines why not show the data from one line only? At the very least the authors should label the figures to make it clear which cell line has been used for each experiment (even though the info can be found in the figure legends).

Authors´ response: cell line information has been added to Figures 6 and 7 (formerly 5 and 6)

Figure 7: Give details of the numbers of mice analyzed for each molecule, and what the errors bars represent. What cell line was used in this study? How many cells were injected?

Authors´ response: (now Figure 8) Information on the cell line (L3.6sl), cell number (10^6) and number of mice (n=8) has been added to the figure legend, the same applies to statistical significance.

Discussion, Page 11: The authors talk about the benefits of targeting SMO and EGFR for BCCs. Given that they show that the HH/GLI1 effect in pancreatic tumors is independent of SMO, they should mention alternatives for targeting these tumour types such as through inhibition of GLI and EGFR.

Authors´ response: We briefly discuss this option as sort of outlook for future studies at the end of the discussion.

GENERAL COMMENTS:
Throughout the manuscript there are different combinations of CRGs analyzed and cell lines used for different experiments. It is sometimes difficult to know if other molecules were tested and showed no effect, or if only some molecules were tested in different experiments. Same for cell lines, as mentioned above. The authors should address this to make it easier for the reader to follow.

Authors´ response: We apologize that this has not been made clear in the first version of our manuscript. We think that - in response to the valuable reviewers´ comments (also see changes in response to reviewers #1 and #3) - we now have improved most issues raised.

The affect of knockdown of individual CRGs on oncogenesis in a number of approaches presented is dramatic. The authors should discuss what this means in terms of the potential synergistic effects of these genes versus their individual tumour promoting capabilities independent of HH/GLI and EGFR regulation.

Authors´ response: By adding data shown in Figure 9 of the revised manuscript, we provide an explanation of the dramatic inhibitory effects of individual knockdowns and discuss the findings in the results and discussion section; this has also been integrated in the model of HH-EGFR signaling in cancer (Figure 9B). To comply with the space restriction of EMBO Mol Med it is very difficult to provide a more extensive discussion of the data.

Referee #3 (Other Remarks):

This is an interesting and potentially important paper on target genes regulated by HH-Gli and EGFR signaling.

Introduction

Description of GLi function and their action in HH signaling is mouse centric. A more balanced discussion of results in different species is required, highlighting possible differences in the use of the three different GLI proteins.

Authors´ response: In response to the comments of referee #3 (see below) we have modified and partially re-written the introduction. We are confident that this revised version presents our ideas and the references more clearly. As we added a substantial amount of new data and (as requested) more detailed description of our results (also see response to the other referees), the introduction to HH signaling and GLI function needs to be very short and concise. It mainly relates to mouse and human GLI regulation (which are the only species used in our study), space restrictions hardly allow a more elaborate introduction.

Refs to HH and EGF signaling in cancer should also include those from the Toftgard lab and Clement et al 2007, Palma et al etc.... Kasper et al is inappropriate for metastasis.
Yauch et al suggested paracrine signaling, but did not prove it. This whole discussion on paracrine signaling is strange since if any the authors here demonstrate cell-autonomous requirement of GLI and targets! Ref to the integration of oncogenic RAS-MEK, PTEN/AKT is Stecca et al., 2007. This is missing.

Authors´ response: We have carefully revised the references, all relevant and seminal papers relating to the topic should now be included. However, it is impossible to cite all the work on HH/GLI regulation and signal modification as space is restricted by EMM. We agree that the discussion of paracrine vs autocrine HH signaling is not relevant to our study so we decided to remove the part on autocrine/paracrine signaling in the introduction, also to comply with the space restrictions by EMM.

As oncogneic RAS/MEK/AKT enhance GLI1bs-luciferase reporter (Stecca et al., 2007) Jun would seem not to be required. How do the authors reconcile these findings?

Authors´ response: We present additional data in this revised version (new additional Figure 4) that address this important question.

We added additional data showing that the expression of cooperation response genes (CRG) requires the specific combination of HH/GLI and EGFR activation but cannot be elicited by e.g. combined FGF/GLI or RAS/GLI signaling.

Unlike EGFR/GLI signaling, activation of other receptor tyrosine kinases pathways including FGF, HGF and VEGF do not induce synergistic activation of HH-EGFR cooperation response genes (see new Figure 4B). Similarly, combined activation of GLI and oncogenic RAS was not sufficient to induce cooperation response gene expression, though it significantly enhanced EGF-independent canonical GLI target expression (i.e. PTCH and HHIP)(new Figure 4B), in line with previous reports (Stecca et al., 2007, Riobo et al., 2006).

There seems to be a rather jumbled presentation of ideas and references. Reading the introduction it is not clear where the authors want to take the reader. Wherever this is, the refs need to be clarified and not be selective. Again, Clement et al. showed synergism between temozolomide and cyclopamine, and this is, again, ignored.

Authors´ response: as stated above, we have carefully revised the references. Clement et al is cited multiple times.
Results

Why is the model of BCC in mice restricted to the ears? If the results deleting EGFR also seen in the back?

Authors´ response: it is not restricted to ears but the BCC-like phenotype is most pronounced on the ears. This is an unexplained effect that may relate to the *in vivo* distribution and/or efficacy of tamoxifen. We added data (suppl. information Fig. S2) showing that SmoM2 induces basaloid hyperplasia and basaloid hamartoma-like lesions in dorsal skin. Like in ears, genetic deletion of EGFR dramatically reduces the development of these lesions.

Is the reduction in the size of the tumors (Fig. 1) related to a possible reduction in the size of spheres as described below?

Authors´ response: This is difficult to answer as in Fig 1 we study BCC while the tumor-initiating sphere assays relate to tumor-initiating pancreatic cancer cells. Whether reduced BCC development is due to a requirement of EGFR and CRG in stem cells representing the cellular origin of BCC (eg Lgr5+ cells) is unclear though we have unpublished data that support this idea.

On the molecular level, we provide several lines of evidence suggesting that these phenotypes are indeed related as they are caused by the same molecular players i.e. HH-EGFR cooperation response genes.

Why do the Gleg2 mice die after complete inhibition of EGFR (Fig. 2C)?

Authors´ response: Although pharmacological inhibition of EGFR by tyrosine kinase inhibitors such as afatinib works well *in vivo*, complete irreversible inhibition is unlikely as with most drugs. Also, it is well possible that cells are able to overcome the inhibition after some time of treatment via activation of bypass pathways, although we do not have evidence for this. However, as referee #1 requested significantly more mice for the Kaplan-Meier analysis and the time for revision did not allow us to breed enough K5cre;Cleg2 mice, we suggest to remove this figure.

This is the response to the issue raised by referee #1: (*Figure 2C needs significantly more than 3 mice per genotype for the Kaplan Meier graph)*

Authors´ response: We agree with this statement. Unfortunately, we have not been able to breed enough additional K5cre;Cleg2 mice during the 3 month revision period. We therefore decided to omit these data and hold the view that this does not weaken our conclusions about the requirement of EGFR signaling for HH/GLI-driven BCC development *in vivo*. Also in light of the concerns raised by reviewer #3 and #1 arguing that K5cre;GLI2deltaN is an artificial construct and Cleg2 mice are less relevant to BCC development, we felt that removing the treatment data is acceptable.

ASZ001 is a strange cell line. It adds little.

Authors´ response: One of the major limitations in the field of BCC research is the lack of human

BCC lines that reliable represent BCC with the characteristic genetic alterations such as loss of Ptch. Also, to the authors´ knowledge, growing primary BCC from human patient material has not been shown reliably and reproducibly. Thus, Ptch-deficient cells isolated from mouse BCC are not ideal but at least genetically resemble the majority of human BCC and they can be used to grow BCC-cell derived tumors *in vivo*. On the molecular level, ASZ001 cells show ligand dependent activation of HH signaling (Gli1 mRNA and protein expression) and sensitivity of Smo inhibitors (data available upon request). In summary, ASZ001 cells provide essentially the only option to study the requirement of the gene in question for *in vivo* tumor growth of established BCC cells.

Use of Gli2deltaN is inappropriate. This is an artificial form of Gli2 that does not allow one to distinguish Gli function, and it is used since the full-length GLI2 does not work.

Authors´ response: We added data showing activation of CRG promoters by GLI1 (Fig. 3C). The GLI2deltaN data were moved to the suppl. information, Figure S5.

The first paragraph of the second part of the Results should be deleted as it belongs to discussion.

Authors´ response: this has been modified accordingly.

The section on targets is very nice. However, the CRG nomenclature is confusing and should be deleted, keeping to the names of the genes used for each assay.

Authors´ response: we apologize for the confusing nomenclature. We now mention the gene names rather than just saying HH-EGFR CRG.

The histograms in Fig3 need statistics and error bars. How many different cells were used for this? Is the same result obtained with BCC and pancreatic cells?

Authors´ response: error bars and data of statistical significance have been added to figure 3. Yes, activation of cooperation response genes is also true for pancreatic cancer cells. In Figure 6E we show activation of CRG expression by combined GLI1 and EGFR stimulation. Furthermore, EGF treatment of pancreatic cancer cells elicits the same signaling cascade as in human keratinocytes (i.e. EGFR/RAS/MEK/ERK/JUN activation, compare Figure 4A and Figure 6D).

When dealing with the regulation of targets, the use of Gli2deltaN is inappropriate. Full-length forms should be used. Results with the former form cannot be taken to mean Gli2 action.

Authors´ response: We added data showing activation of CRG promoters by GLI1 (Fig. 3C). The GLI2deltaN data were moved to the suppl. information, Figure S5.

Is SOX9 a stemness gene? No refs are given here or elsewhere in many instances.
Authors´ response: Sox9 is expressed in hair follicle stem cells; deletion of Sox9 severely affects skin stem cell fate. The reference is given on page 13, Vidal et al., 2005.

Is expression of FGF19, TGFA, SOX2, SOX9 and CXCR4, etc. in the same cells that express GLI2 or GLI1 in BCCs in vivo in mice and humans? This is an essential issue to resolve at a single-cell level.

Authors´ response: To address this important point we performed a series of immunohistochemical and qPCR analyses of mouse and human BCC and primarily focused on the latter to support the relevance of our findings to human disease. We show high level, BCC-specific JUN and SOX9 protein expression and significantly elevated mRNA levels of SOX2, FGF19, CXCR4, SPP1, GLI1 and PTCH in human BCC samples (suppl. Figure S7 A´, B´, D). In addition, we show Jun, Sox9 and

Cxcr4 protein (Figure S7, A-C) and Gli1 mRNA expression (suppl. Figure S3C) in SmoM2-induced mouse BCC-like lesions. Note that there is no close homologue of FGF19 in mice. The limited availability of specific antibodies and the limited time for this revision did not allow us to analyze expression of all CRG in BCC at the single cell level.

In summary and in agreement with the other data, mouse and human BCC express HH-EGFR cooperation response genes.

Since GLI1 is said to be unchanged in vitro and in vivo, can the authors show the data and can they test if activity is changed other than in expression levels?

Authors´ response: in Figure 5B, we show equal Gli1 mRNA expression in 2D ASZ and ASZ allograft tumors. We added data showing comparable Gli1 protein expression in ASZ BCC cells cultured in 2D or ASZ allograft tumors (Figure 5C). Testing Gli1 activity in allograft tumors is difficult. However, we added novel data to Figure 5B showing that like Gli1 the mRNA levels of the EGF-independent Gli target Bcl2 are also unchanged between *in vitro* cultured ASZ cells and ASZ allograft tumors, pointing to comparable Gli activator activity in cultured cells and allografts.

Please note that the elevated level of HH-EGFR CRG correlates with the activation of EGFR/Mek/Erk/Jun cascade in the ASZ allograft tumors (Figure 3C and suppl. Figure S6).

What are the controls for in vivo blockade of JUN, SOX2 and CXCR4? These seem to be missing.

Authors´ response: We apologize for not clearly describing the RNAi controls. In all RNAi inhibition experiments, we used control cells transduced with non-target control shRNA constructs. This information has been added to the respective figure legends.

What is the level of KD in tumors in vivo? This is essential as the varying results could explain the different tumor inhibition results (Fig. 4C).

Authors´ response: the knockdown efficiencies of all shRNA constructs are now shown in suppl Figure S10. To control the specific and efficient RNAi inhibition of each target, we re-validated the knockdown of each shRNA construct before grafting the tumor cells to nude mice. However, at the time when we harvested the tumors from control and specific knockdowns (i.e. when CRG knockdown cells have also formed tumors and before mice had to be sacrificed), the knockdown of the targets was no longer detectable. We think that this is either due to silencing of the shRNA expression construct or by overgrowth of cells without sufficient CRG knockdown (we injected the bulk of shRNA transduced cancer cells, not clonal isolates). Unfortunately, this limitation does not allow us to answer this question.

What are the refs for the 'emerging role of HH-GLI in cancer stem cells'?
What are the refs for the 'stemness genes Oct4, NANOG, CD133' in human tumors?

Authors´ response: As stated above, we have carefully revised the references to make sure the most relevant literature is cited while at the same time keeping to the space restrictions. As for the role of HH/GLI in tumor-initiating cancer stem cells we cite on page 3 and page 10: (Clement et al, 2007; Dierks et al, 2008; Mueller et al, 2009; Peacock et al, 2007; Varnat et al, 2009; Varnat et al, 2010; Zhao et al, 2009).

What are the controls for the increased expression of HH-GLI and HH-GLI/EFGR targets in spheres?

Authors´ response: we compare the expression of HH/GLI-EGFR target genes in isolated spheres highly enriched for tumor-initiating pancreatic cancer cells to control cells cultured under non-sphere i.e. adherent conditions. Control cells are not enriched for tumor initiating cell as determined by limiting dilution xenograft assays shown in Figure 6B.

This needs a lot more work and the jump to pancreatic cancer seems unwarranted. Why pancreatic cancer and BCC? What do they have in common? What is being tested here?

Authors´ response: As written in the summary of the major points of our revision (see above), we apologize for the insufficient and perhaps confusing description of both the rationale for the use of a modified 3D tumor sphere (macrosphere) assay to characterize tumor-initiating pancreatic cancer cells and the methodology applied. Given the space restrictions for the manuscript length, we now describe in detail in the suppl. information the isolation and characterization of tumor-initiating cells (TICs). We further would like to emphasize that we consistently use the term tumor-initiating cells because we provide solid evidence that macrospheres of human pancreatic cancer cells are highly enriched for TICs as determined by limiting dilution xenograft experiments, which is considered the gold standard for the characterization of TICs (Note that as few as 100 cells from isolated single macrospheres are sufficient for engraftment and tumor growth in all experiments (4/4), Fig. 6B). As we do not address their *in vivo* self-renewal and differentiation capacity (this is not the focus of this study), we are reluctant to call these cells cancer stem cells, although TICs from macrospheres show expression of stem cell genes and characteristics of self-renewal *in vitro* (Fig. 6C and suppl. information, Fig. S8A-C). For these reasons, and in consideration of our vitro and *in vivo* analyses of HH-EGFR CRG function in TICs, we believe that it is correct to claim that HH/GLI-EGFR regulated CRG determine the malignant phenotype of tumor-initiating pancreatic cancer cells.

Please find below a short excerpt of the rationale for using this approach:

"Most of the studies of pancreatic TICs used distinct surface marker combinations or enzyme-activities to enrich for TICs by cell sorting. The obvious heterogeneity of pancreatic TIC populations and their inconsistent marker profile poses significant problems and limitations for their isolation and analysis….

The lack of a universal pancreatic TIC marker and the obvious discrepancy of the TIC identity in pancreatic cancer cells prompted us to use in this study a TIC enrichment technique that is based on the growth characteristics of TICs rather than surface marker expression or enzyme activity, i.e. high clonogenic growth and (tumor) sphere formation under non-adherent conditions (Simeone, 2008)..."

As for the analysis of HH-EGFR cooperation see page 10:

Given the substantial overlap of HH/GLI and EGFR signaling in many human cancers we set out to address whether HH-EGFR cooperation and the respective cooperation response gene signature are also deployed in cancers other than BCC. Here, we studied the role of HH-EGFR cooperation in pancreatic adenocarcinoma cells, which represent one of the most aggressive and still incurable malignancies with a documented role of HH/GLI and EGFR signaling (Li et al, 2007; Nolan-Stevaux et al, 2009; Pasca di Magliano et al, 2006; Yauch et al, 2008).

It is said that blockade of HH or EGFR signaling compromises colony formation. Does it also affect size? Is this effect restricted to in vitro conditions? Since sphere growth usually requires FGF and EGF, is this result not obvious?

Authors´ response: The important finding is that the formation of tumor-initiating cells is basically complete inhibited by combined targeting of EGFR (or JUN) and GLI. Single inhibition of either GLI1 or EGFR only is much less efficient (see Figure 7B). To our mind, this result is not obvious but supports our conclusions about the promoting effect of HH/GLI-EGFR signaling on tumor-initiating pancreatic cancer cells. As we have concentrated in this study on the functional analysis of mediators of HH-EGFR cooperation (i.e. cooperation response genes), we have not analyzed the effect of combined inhibition of HH-EGFR signaling *in vivo*. However, based on the very good correlation of tumor-initiating sphere growth *in vitro* and tumor growth *in vivo* in our study, we propose that this effect is not restricted to *in vitro* conditions.

Fig. 6B needs to be expanded to include levels of knock-down by the shRNAs. The interpretation depends on this.
Authors´ response: levels of knockdowns are shown in suppl. Figure S10.

It is surprising that each of the identified genes is required for sphere formation. What are the controls? How many shRNAs were used per target?

Authors´ response: yes indeed, we were also surprised by this dramatic effect of each cooperation response gene. To find a possible explanation for this, we added experiments suggesting positive regulatory interactions between selected cooperation response gene such as CXCR4 and FGF19 (see Figure 9A and model in 9B). In all RNAi experiments, control cells were transduced with non-target control shRNA (from SIGMA mission shRNA clone set). This is now specified in the main text/figure legends. shRNAs used for specific knockdowns and control knockdown are listed in the materials and methods section. We either used commercially validated shRNAs or self-validated shRNAs selected from commercial shRNA pools of 5 constructs (Mission shRNA clone set). shCXCR4 has been published by the Weinberg lab, the respective reference is given in the materials and methods section.

We have used the most efficient shRNA construct for the experiments.

Why do the authors mention macrospheres? Are microspheres affected? Do macropsheres pass for multiple passes?

Authors´ response: This is explained in the more detailed in the suppl. information about the modified 3D sphere assay. As 3D cultures consist of very small and rare large spheres we termed the latter macrospheres, also to refer to their high clonogenic capacity. The effects described are mainly affecting macrospheres. Yes, macrospheres can be passages multiple times. For more details see suppl. information text and Figure S8.

The 2D-3D culture seems cumbersome Why not use standard spheres as with other cancers?

Authors´ response: the modified sphere assay were cells are embedded in matrix is very stable, reproducible, allows easy isolation of single spheres, long-term observation of spheres as they do not move like in floating cultures, easy quantification by automated sphere counting, and most importantly, the assay very efficiently enriches for tumor-initiating cells (note that in our expts as few as 100 cells were sufficient for engraftment in 4/4 cases, Figure 6B).

Are the pancreatic cancer cells used for xenografts human or mice? It is not clear in the text.

Authors´ response: they are human; we have tried to make this clearer in the revised version.

What is the level of KD of SMOH in the xenografts that are insensitive? This is essential to know in order to interpret the result.

Authors´ response: >80%, similar to the efficiency of GLI1 knockdown; see suppl. Figure S10.

Does co-inhibition of one or more of the target genes result in a greater inhibition? This is an important issue to resolve.

Authors´ response: *in vitro*, we show that co-inhibition of JUN (by shRNA) and GLI by GANT61 treatment very efficiently blocks tumor-initiating spheres, much more than single inhibition. It will be important to address this question. A major challenge is that given the fact that we identified 5 CRGs involved in tumor growth downstream of HH/GLI-EGFR signaling, the number of combinations with EGFR/GLI inhibition is huge and requires a systematic analysis that we plan to do in future experiments.

Discussion

The authors need to show that the targets genes regulated by HH-GLI and EGFR are indeed dual targets in vivo in mouse BCCs and in human samples, as well as in spheres. Otherwise, the results

could be unrelated to the direct integration of signaling and due to in vitro artifacts. This is essential before possible acceptance.

Authors´ response: To demonstrate *in vivo* regulation of CRG by dual HH/GLI and EGFR signaling we analyzed expression of HH-EGFR CRG in epidermal cells of tamoxifen-injected K5creER;SmoM2;EGFR+/+ and K5creER;SmoM2;EGFRfl/fl mice and found that deletion of EGFR prevents induction of CRG (Figure 5D, also see above, summary of revised manuscript). We conclude that HH-EGFR cooperation response genes are controlled by both HH/GLI and EGFR during *in vivo* BCC development. We also added data showing CRG expression in HH-driven mouse and human BCC (suppl. information, Figure S7).

We present novel data showing that like in keratinocytes combined activation of GLI1 and EGFR signaling induces CRG expression in human pancreatic cancer cells used in the sphere assays (Figure 6E). In addition, we show that like in human keratinocytes, EGF treatment of pancreatic cancer cells induces EGFR/MEK/ERK/JUN activation characteristic of cooperative EGFR signaling (for comparison see Figure 4A).

Does inhibition of any of these target genes inhibit the expression of the others in vivo? This could explain the surprising effect that blockade of any of them inhibits tumor growth. Controls are missing.

Authors´ response: We are grateful for this suggestion as the additional experiments performed in response to this question added - as we think - very interesting insight into the existence of regulatory networks of CRGs downstream of HH-EGFR signal integration. We found that inhibition of CXCR4 reduces FGF19 levels and vice versa. Also, Sox9 inhibition resulted in reduced JUN levels. The data are shown in Figure 9A, and integrated in the model of HH-EGFR and CRG function in 9B. We propose that mutual, positive regulatory interactions e.g. between CXCR4 and FGF19 may account for the striking phenotype observed in either knockdown experiment.

The effects of FGF would seem to relate to the action of FGF in regulating GLI2 in xenopus (Brewster et al., 2000). Does blocking of FGF signaling yield the same result as blocking EGF? This can easily be done using inhibitors to FGFR.

Authors´ response: Referring to the regulation of GLI expression by FGF signaling (Brewster et al., 2000), we show that inhibition of FGF19, which signals via FGFR4, does not affect GLI2 or GLI1 levels (suppl. information Fig. S9A). Consistent with the requirement of FGF19 for tumor sphere growth *in vitro* and tumor growth *in vivo*, treatment of pancreatic cancer cells with a potent FGFR inhibitor reduced the growth of tumor-initiating macrospheres in 3D cultures (suppl. information, Fig. S9B)

We added additional data showing that the expression of cooperation response genes (CRG) requires the specific combination of HH/GLI and EGFR activation but cannot be elicited by e.g. combined FGF/GLI or RAS/GLI signaling.

Unlike EGFR/GLI signaling, activation of other receptor tyrosine kinases pathways including FGF, HGF and VEGF do not induce synergistic activation of HH-EGFR cooperation response genes (see new Figure 4B). Similarly, combined activation of GLI and oncogenic RAS was not sufficient to induce cooperation response gene expression, though it significantly enhanced EGF-independent canonical GLI target expression (i.e. PTCH and HHIP)(new Figure 4B), in line with previous reports (Stecca et al., 2007, Riobo et al., 2006).

It would be good if the authors were to focus on the molecular effects observed and less on therapy as this is not a therapy paper. The opening paragraph of the discussion is not appropriate for the results in the paper.

Authors´ response: We have modified both the introduction and discussion and shifted the focus, although the results of the study related to the topic of drug targets and novel treatments. We tried to find a better balance between this and the molecular effects described.

The second paragraph of the discussion is incomplete. EGFR signaling includes the action of RAS-MEK/AKT. This was first shown by Stecca et al, 2007. The concept of combinatorial therapy is already explored in other papers not referenced, e.g. in Stecca et al and Clement et al. It is the novelty of the target genes - and not ideas about combinatorial pathway integration - is what makes this paper interesting. Another example: top of page 12 when discussing oncogenic RAS there is no reference and this was shown by Stecca et al. Thus, this reviewer cannot escape the feeling that there is a strategic deletion of previous work.

Authors´ response: as stated above, references were carefully revised and should now be cited at the correct sites. However, we have to reject that we strategically deleted references of previous work related to the topic. We fully appreciate the seminal work mentioned by referee #3. In fact, Stecca et al., 2007, Clement et al., Palma et al., 2004, 2005 have been referenced already in the first version of the manuscript, but not repeatedly in the text, which we do now.

Overall, the data is interesting and extend previous work by the Aberger and other labs. There is the exciting finding that specific targets may mediate the effects of HH and EGFR. However, it is not clear if these are specific to any input that activates oncogenic RAS, such as FGF. It is also not clear if integration occurs in vivo and in the different cells or cancer types used. There is a disturbing lack of key references and an over interpretation of novelty and translation to a clinical setting. In addition, key controls appear to be missing.

Authors´ response: We added additional data showing that the expression of cooperation response genes (CRG) requires the specific combination of HH/GLI and EGFR activation but cannot be elicited by e.g. combined FGF/GLI or RAS/GLI signaling.

Unlike EGFR/GLI signaling, activation of other receptor tyrosine kinases pathways including FGF, HGF and VEGF do not induce synergistic activation of HH-EGFR cooperation response genes (see new Figure 4B). Similarly, combined activation of GLI and oncogenic RAS was not sufficient to induce cooperation response gene expression, though it significantly enhanced EGF-independent canonical GLI target expression (i.e. PTCH and HHIP)(new Figure 4B), in line with previous reports (Stecca et al., 2007, Riobo et al., 2006).

For regulation of CRG expression by dual HH/GLI-EGFR signaling *in vivo* see response above and data in Figure 5D.