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LncRNA-PAGBC acts as a microRNA sponge and promotes gallbladder tumorigenesis

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

1st Editorial Decision 28 February 2017

Thank you for the transfer of your research manuscript to EMBO Reports. I now went through the referee reports from The EMBO Journal.

All referees acknowledge the potential interest of the findings. Nevertheless, all three referees have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn. As the reports are below, I will not detail them here.

However, we think that it will be essential to allow publication in EMBO Reports that the major points of referee #3 (e.g. the demonstration that natural targets of miR-133b and miR-511 are globally deregulated by lncRNA-PAGBC, and that miR-511-5p is indeed expressed in gallbladder cancer cells at meaningful levels) are addressed with further data. Also the four major points by referee #1 are important, in particular the points regarding the specificity of the microarray analysis (point 1) and the regulation of linc01133 (point 3).

Given the constructive referee comments, we would like to invite you to revise your manuscript

with the understanding that all referee concerns must be addressed in a complete point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

The manuscript by Liu and colleagues entitled "lncRNA-PAGBC promotes tumorigenesis by binding to miR-133b and miR-511 in gallbladder cancer cells" is timely, original and provides interesting insights into the role of a lncRNA in GBC. However, multiple issues need to be experimentally addressed prior to publication.

Major issues:

1) Microarray analysis – Specificity: It seems impossible that more than 16000 genes are significantly deregulated and relevant between the tumor and normal samples analyzed. Since not enough details are provided on the selection of "differentially expressed" genes, this needs to be specified and the analysis should be repeated with more stringent criteria. Most of the hits - also the ones chosen to be verified by qPCR - seem to be moderately differentially expressed around twofold. Within the 9153 lncRNAs differentially expressed, how did linc01133 rank?

2) Prior studies on linc01133: The authors claim that linc01133 has never been studied before (p. 5) - however, there are four studies in PubMed on this lncRNA which should be added to the discussion. Also, the authors should compare their proposed mechanisms to the two other mechanisms published (SRSF6, EZH2) and experimentally test whether these may also explain the detected phenotypes.

3) Regulation of linc01133: The median expression of linc01133 seems to be similar in normal and tumor tissue (figure 1G). Hence, linc01133 seems to be upregulated only in a subgroup of patients. This needs to be analyzed and defined better. Did the authors perform also pairwise analysis and compared tumors with and without upregulation? Also, the figure 1G should be moved after 1B.

4) RNA Affinity Purification: For the RAP experiment, one or two additional RNA controls should be added. Has Ago2 been found in the RAP?

Minor issues:

5) Multivariate regression analysis: It is not explicitly stated which parameters were included in the multivariate regression analysis. Since linc01133 was associated with stage (p. 5), was it an independent prognostic marker even if corrected for stage, so within tumors of the same stage?

6) Migration assays: The migration assays were executed in the absence of mitomycin C although the genes tested had detectable effects on proliferation. One of these experiments should be repeated to ensure that.

7) MicroRNA target sites – Specificity: The authors have identified 1000 potential microRNA binding sites on a 1000 nt transcript. This argues strongly in favor of a lack of specificity. How did the authors select the six microRNAs then?

8) MicroRNA targets – Specificity: The authors have predicted again hundreds of potential targets for the two selected miRNAs. How did the authors select the two targets then?

9) shRNAs: The use of only two shRNAs is critical given the prevalence of off-target effects. Why was the shRNA 1 not included in any additional experiments?

10) Quantification & statistics: Quantifications and statistical analysis of the Western blot replicates

as well as the mouse luciferase (figure 3C) should be provided.

11) Overexpression & ceRNA: It should be discussed that the overexpression is 10000fold while the regulation in tumor is about twofold - and even 10000fold overexpression of the lincRNA only downregulates the microRNAs by twofold. While most of the experiments are well-controlled and the overall mechanism is plausible, it should be more critically discussed that these findings as well as others leave some open questions about the simple ceRNA competition hypothesis.

Referee #2:

The paper by Wu et al describes a lncRNA which correlates with poor prognosis of GBC. It stimulates growth and metastasis by sequestering two microRNAs and by that activate AKT/mTOR pathway. Interaction with PABPC1 is required for its stability.

Generally, the authors provide compelling evidence and very well controlled experiments to substantiate the interpretation. However, before acceptance I would recommend the authors to edit the manuscript by professionals to take out the many typo's and to improve much the English language and grammar.

Further, in Fig.1 D and F: I miss a control lane with siRNA to show that the signals are specific to PAGBC, as they assume.

In Fig. 2A I was expecting to see also a rescue experiment with an shRNA-resistant lncRNA. In Fig 5C and D, I miss control lanes of mutant lncRNA+microRNA for each condition.

Figs 6A, D, G: It is difficult to spot the relevant differences for each panel. I suggest to highlight the relevant blots - but also to take care to have the full ones as PDFs in supplementary. Also quantification of multiple repeats in panels B and E is required.

Fig.7 which describes the interaction of lnc-PAGBC with PABPC1 is somewhat out of the focus of the story, and the results are not very convincing. Panel A, judging from the picture clearly the pointed bands appear also in the control lane, and other bands appear in the control but less in the sense. Therefore, this result should either be improved or taken out. Panel C shows over exposed bands. The authors need to show a less exposed blots and also quantify several experiments to reach the conclusion that there is indeed no difference. Panel F should include a rescue experiment with siRNA-resistant PABPC1 to be convincing that it is not a result of off target effects.

Referee #3:

In this manuscript, Wu et al. identify a long non-coding RNA (lncRNA), termed lncRNA-PAGBC, which is upregulated in human gallbladder cancer patients compared to the normal organ. Over-expression of lncRNA-PAGBC in the NOZ human gallbladder cancer cell line enhanced tumor growth in immunodeficient mice. Conversely, silencing lncRNA-PAGBC by shRNA reduced tumor growth and experimental liver metastasis. By using bioinformatics predictions and cell assays, the authors show that lncRNA-PAGBC binds two miRNAs, miR-133b and miR-511. In particular, they observed that lncRNA-PAGBC reduces the activity of miR-133b and miR-511 through a competing endogenous RNA (ceRNA)-like effect. In the absence of lncRNA-PAGBC, heightened miR-133b and miR-511 levels suppress the AKT/mTOR pathway by targeting SOX4 and PIK3R3, respectively. This ceRNA cross talk between miR-133b/miR-511 and lncRNA-PAGBC may regulate cancer cell proliferation and invasion.

Main criticisms:

1. The authors propose that lncRNA-PAGBC may limit miR-133b and miR-511 activity by competing with endogenous target mRNAs. However, at least according to recent studies, lncRNA target sites are much less abundant than other miRNA target sites in cells (Denzler et al., Mol. Cell 2014; Thomson and Dinger, Nat. Rev. Genetics 2016). What is lacking in this paper is the demonstration that natural targets of miR-133b and miR-511 are globally deregulated by lncRNA-

PAGBC. The authors should show that, stoichiometrically, the quantity of endogenous lncRNA-PAGBC target sites could significantly impact on the overall number of miR-133b and miR-511 target sites in the cell. The measurement of lncRNA-PAGBC, miR-133b and miR-511 copies/cell does not address this issue, as the miR-133b and miR-511 binding sites in the cell transcriptome were not estimated. Furthermore, although both SOX4 and PI3KR3 were apparently validated as miR-133b and miR-511 targets, their upregulation after lncRNA-PAGBC over-expression may well be indirect.

2. The authors study the activity of miR-511, which is the old designation for the miRNA now referred to as miR-511-5p. Squadrito et al. (Cell Rep 2012) have shown that miR-511-5p is the passenger strand of the precursor miR-511, and does not have miRNA activity. miR-511-5p (the miRNA strand studied by the authors) is indeed undetected according to sequencing studies tracked by miRBase (http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0003127). The authors should also see the work of Chang et al. (BMC Genomics201213(Suppl 7):S18) on miR-511 isoforms. The authors have neglected this important information in the current study. In order to demonstrate that miR-511-5p is indeed expressed in gallbladder cancer cells to meaningful levels, further studies should be performed, including RNA sequencing of NOZ cells, with and without lncRNA-PAGBC over-expression or knock-down.

3. Related to point 2 above, it should be noted that miR-511 is an intronic miRNA that lacks an internal promoter, and its expression is therefore dependent on that of the hosting gene, MRC1. The MRC1 gene is expressed by myeloid cells, including tumor-infiltrating macrophages and dendritic cells. Although the authors show that miR-511-5p is detectable in gallbladder cancer specimens (Figure 4G), they do not speculate on the possibility that macrophages, and not cancer cells, are the source of this miRNA. The authors should look at the expression of MRC1 in their samples, and make efforts to resolve the cellular source of miR-511-5p in the tumor samples.

4. The authors state that, based on clinical and pathological data, higher levels of lncRNA-PAGBC are associated with more advanced tumor stages (Supplemental Table 3). However, they subsequently state that multivariate analyses indicate that lncRNA-PAGBC is an independent prognostic factor for overall patient survival (with P = 0.002). One wonders whether lncRNA-PAGBC predicts survival independent of tumor stage.

1st Revision - authors' response 01 June 2017

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "LncRNA-PAGBC Promotes Tumorigenesis by Binding to miRNA133b and miRNA511 in Gallbladder Cancer Cells" (ID: EMBOR-2017-44147-T). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made modification which we hope meet with approval. Revised portion are marked in red in the paper. The main modification in the paper and the responds to the reviewer's comments are in rebuttal letter. We appreciate for Editors/ Reviewers' warm work earnestly, and hope that the correction will meet with approval.
Once again, thank you very much for your comments and suggestions.

POINT-BY-POINT RESPONSE

Thank you for your comments concerning our paper. Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made modification which we hope meet with approval.

Referee #1:

The manuscript by Liu and colleagues entitled "lncRNA-PAGBC promotes tumorigenesis by binding to miR-133b and miR-511 in gallbladder cancer cells" is timely, original and provides interesting insights into the role of a lncRNA in GBC. However, multiple issues need to be experimentally addressed prior to publication.

Major issues:

1) Microarray analysis – Specificity: It seems impossible that more than 16000 genes are significantly deregulated and relevant between the tumor and normal samples analyzed. Since not enough details are provided on the selection of "differentially expressed" genes, this needs to be specified and the analysis should be repeated with more stringent criteria. Most of the hits - also the ones chosen to be verified by qPCR - seem to be moderately differentially expressed around twofold. Within the 9153 lncRNAs differentially expressed, how did linc01133 rank?

The referee mentioned his/her concern about the possibility of the 16000 differentially expressed genes identified by the microarray. Actually, we performed the microarray on 9 pairs of human gallbladder cancer (GBC) samples and over 170,000 genes (140,886 lncRNAs and 35,020 mRNAs) were rated. Most of the published papers [1-3] identified thousands of genes by microarray because the number of rated genes were around 30,000 ~ 40,000. However, in our study, we determined about 170,000 genes. Moreover, the threshold set for up- and down-regulated genes was a fold change >= 2.0 and a P value <= 0.05. This detail was added in the supplemental information and highlighted by red color. In addition, as mentioned in the manuscript, we randomly selected four lncRNAs and lncRNA-MALAT1 (an lncRNA reported to be highly expressed in GBC by our previous work) to validate microarray analysis findings. In microarray, during the cDNA formation we amplify the available cDNA, so in microarray you cannot measure the exact quantity of starting mRNA. The microarray results can only give us a clue for UP and DOWN expression. Real-time PCR is often referred to as the "gold standard" for gene expression measurements, due to its advantages in detection sensitivity, sequence specificity, large dynamic range as well as its high precision and reproducible quantitation compared to other techniques. Although the lncRNAs seem to be moderately differentially expressed around two folds, the results confirmed that BC010117, NR_038835 and MALAT1 were highly expressed in the GBC samples, whereas AC240664.3 and ENST00000415656 were deregulated in non-tumour samples ($P < 0.05$ for all). Thus, our data indicate that a set of lncRNAs is frequently aberrantly expressed in GBC tissues. Meanwhile, linc01133 rank the 10th in the up-regulated lncRNAs.

2) Prior studies on linc01133: The authors claim that linc01133 has never been studied before (p. 5) - however, there are four studies in PubMed on this lncRNA which should be added to the discussion. Also, the authors should compare their proposed mechanisms to the two other mechanisms published (SRSF6, EZH2) and experimentally test whether these may also explain the detected phenotypes.

LINC01133 have been reported in NSCLC [4, 5], colorectal cancer[6] and osteosarcoma[7]. It could promote NSCLS cells' proliferation, migration and invasion through binding to EZH2 and LSD1 to repress KLF2, P21 and E-cadherin transcription[5]. As in colorectal cancer, LINC01133 inhibits epithelial-mesenchymal transition and metastasis by directly binding to SRSF63. LINC01133 could also sponge miR-422a to aggravate the tumorigenesis of human osteosarcoma [6]. So LINC01133 served as an oncogene in NSCLS and osteosarcoma, which is the same function as our study in gallbladder cancer, but the underlying mechanisms are different. While it served as a tumor suppressor in colorectal cancer. From all these studies, we could conclude that the functions and molecular mechanisms of LINC01133 are complicated and differ in different cancers, so we investigated the unique underlying functional mechanism of LINC01133 in gallbladder cancer. The relevant detail was added in the Discussion and highlighted by red color.

3) Regulation of linc01133: The median expression of linc01133 seems to be similar in normal and tumor tissue (figure 1G). Hence, linc01133 seems to be upregulated only in a subgroup of patients. This needs to be analyzed and defined better. Did the authors perform also pairwise analysis and compared tumors with and without upregulation? Also, the figure 1G should be moved after 1B.

The referee mentioned whether PAGBC was upregulated in a subgroup of patients.
To address this issue, we performed a pairwise analysis on the fold change of each pair of cancer and corresponding non-cancerous tissue (Figure 1D in the revised edition). The results indicated PAGBC was upregulated in 46 out of 60 GBC tissues. Moreover, in our manuscript, we have analyzed the relationship between the pathological information and PAGBC expression levels (Supplemental Table 3). The results demonstrated that the more advanced tumor stage is associated with a higher expression level of PAGBC. In addition, the referee advised that figure 1G should be

moved after 1B. Thanks for the advice. After consideration, we moved 1G after 1C to make the whole story more logical.

4) RNA Affinity Purification: For the RAP experiment, one or two additional RNA controls should be added. Has Ago2 been found in the RAP?

Thanks for your suggestion, but we think antisense RNA is enough as control for LINC01133. This method is also adopted by many papers [3, 8]. As to ago2, we did western blot and find its existence in the sense band (Supplemental Figure 6B).

Minor issues:

5) Multivariate regression analysis: It is not explicitly stated which parameters were included in the multivariate regression analysis. Since linc01133 was associated with stage (p. 5), was it an independent prognostic marker even if corrected for stage, so within tumors of the same stage?

In the multivariate regression analysis, we included the following parameters: tumor TNM stage, PAGBC expression level, R0 dissection, CA19-9 levels, pre-operative serum total bilirubin, tumor differentiation, age and gender. The relevant information were added in the MEM section and highlighted by red. In addition, to determine whether lnc01133 was an independent prognostic marker even if corrected for stage, we performed a survival analysis stratified by TNM stage (Figure 1E in the revised edition). Even corrected for TNM stage, patients with a low expression level of PABGC had significantly better OS than those with a high expression level of PABGC. The relevant results were added in Results Section and highlighted by red.

6) Migration assays: The migration assays were executed in the absence of mitomycin C although the genes tested had detectable effects on proliferation. One of these experiments should be repeated to ensure that

We totally agree with the reviewer that proliferation might influence the results of migration in the absence of mitomycin C. However, we performed migration assays within 24 h. During this period, lncRNA-PAGBC had no effect on the viability of gallbladder cancer cells as demonstrated in Figure 2A. We thus believed that our migration assays could actually represent the migration ability even in the absence of mitomycin C.

7) MicroRNA target sites – Specificity: The authors have identified 1000 potential microRNA binding sites on a 1000 nt transcript. This argues strongly in favor of a lack of specificity. How did the authors select the six microRNAs then?

After identifying the possible target sites, we first narrowed down the results by the instructions of the Segal Lab program (https://genie.weizmann.ac.il/ pubs/mir07/mir07_notes.html, FAQ #3 and #4). Then we predicted the target genes of these possible binding miRNAs by the Targetscan Program (http://www.targetscan.org/vert_71/) and overlapped these target genes with upregulated mRNAs in our previous microarray results, which have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE90001. According to the ceRNA theory, the target genes of the miRNAs could be positively associated with lncRNAs and thus these target genes should be upregulated in GBC tissues. Moreover, we performed literature research to identify the miRNAs working as a tumor-suppressor in cancer. After all these means, we narrowed our results to miR-133b, miR-150, miR-511, miR-625, miR-765 and miR-1258 as the most likely candidates to bind lncRNA-PAGBC.

8) MicroRNA targets – Specificity: The authors have predicted again hundreds of potential targets for the two selected miRNAs. How did the authors select the two targets then?

After predicting the possible targets of miR-511-5p and miR-133b by the Targetscan Program, we overlapped these target mRNAs with the upregulated mRNAs in our previous microarray results. Then we further narrowed the targets by the presence of 8mer or 7mer and been reported as oncogenes by previous studies. Finally, we performed the dual luciferase assays to confirm the targets of these two miRNAs.

9) shRNAs: The use of only two shRNAs is critical given the prevalence of off-target effects. Why was the shRNA 1 not included in any additional experiments?

Indeed, two shRNAs should be adopted in the experiments to exclude off-target effects. Once this problem being solved, any one of the two shRNAs could knockdown LINC01133, so we used one shRNA in the following experiments. This strategy is also adopted in many articles [8, 9].

10) Quantification & statistics: Quantifications and statistical analysis of the Western blot replicates as well as the mouse luciferase (figure 3C) should be provided.

We thank the reviewer for suggestion of improving our manuscript. We have quantified all the western blot results by band density and the value shown in the revised figures was normalized to the internal control GAPDH.

11) Overexpression & ceRNA: It should be discussed that the overexpression is 10000fold while the regulation in tumor is about twofold - and even 10000fold overexpression of the lincRNA only downregulates the microRNAs by twofold. While most of the experiments are well-controlled and the overall mechanism is plausible, it should be more critically discussed that these findings as well as others leave some open questions about the simple ceRNA competition hypothesis.

The referee noticed that 10000fold overexpression of the lincRNA only downregulates the microRNAs by twofold. About these abovementioned results, we proposed the following explanation. First, the MREs on ceRNA are not equal[10]. Although several miRNAs are predicted to bind the same ceRNA, the nucleotide components of their MREs may be different. lnRNA-PAGBC can possibly bind to various miRNAs and have been proved to interact with miRNAs, such as miR-133b, miR-511 and miR-422a [7]. Different miRNAs were detected with different binding affinities due to the binding strength rather than target site frequency [11]. Second, it has been observed that expression of a reporter at higher than physiological levels may itself contribute to saturating miRNA activity [11]. Maybe it can also happen to lncRNAs when they work as ceRNAs in cytoplasm. The relevant discussion has been added in the revised version and marked by red color.

Referee #2:

The paper by Wu et al describes a piece of lncRNA which correlates with poor prognosis of GBC. It stimulates growth and metastasis by sequestering two microRNAs and by that activate AKT/mTOR pathway. Interaction with PABPC1 is required for its stability. Generally, the authors provide compelling evidence and very well controlled experiments to substantiate the interpretation. However, before acceptance I would recommend the authors to edit the manuscript by professionals to take out the many typo's and to improve much the English language and grammar.
1.Further, in Fig.1 D and F: I miss a control lane with siRNA to show that the signals are specific to PAGBC, as they assume.

We greatly appreciate the suggestion of this reviewer. We have performed FISH experiments after knockdown of lncRNA-PAGBC. The results are now illustrated in Figure 1F in the revised version. The results showed that the signals in the FISH experiment are specific to PAGBC.

2.In Fig. 2A I was expecting to see also a rescue experiment with an shRNA-resistant lncRNA. In Fig 5C and D, I miss control lanes of mutant lncRNA+microRNA for each condition.

Thanks for the meaningful suggestion. We have added mutant lncRNA+microRNA lane in each condition and presented the data in Figure 5C and 5D in the revised edition.

3.Figs 6A, D, G: It is difficult to spot the relevant differences for each panel. I suggest to highlight the relevant blots - but also to take care to have the full ones as PDFs in supplementary. Also quantification of multiple repeats in panels B and E is required.

We appreciate the Reviewer's point and have added the relative qualification of western blot results by band density and normalized to GAPDH, and presented the data in Figure 6 in the revised edition. The quantification of multiple repeats in panels B and E is actually shown in sup. Fig. 5.

4.Fig.7 which describes the interaction of lnc-PAGBC with PABPC1 is somewhat out of the focus of the story, and the results are not very convincing. Panel A, judging from the picture clearly the pointed bands appear also in the control lane, and other bands appear in the control but less in the sense. Therefore, this result should either be improved or taken out. Panel C shows over exposed bands. The authors need to show a less exposed blots and also quantify several experiments to reach the conclusion that there is indeed no difference. Panel F should include a rescue experiment with siRNA-resistant PABPC1 to be convincing that it is not a result of off target effects.

We appreciate the Reviewer's suggestion. We tried to find the potential interacting partner by performing RNA pulldown assay and we found two bands were enriched after lncRNA-PAGBC pulldown compared with antisense control. Although the pointed band seemed to be also observed in the antisense lane, it is possible that two bands of equal molecular weight contain different proteins. So we identified the bands by mass spectrometry and further confirmed the interaction of lncRNA-PAGBC with PABPC1 by western blot and RIP data (Fig 7B). The reviewer pointed out that other bands appear in the control but less in the sense. It should be noted that anti-sense lncRNA-PAGBC was used as the control in the RNA pulldown assay. it is rational that some proteins may interact with anti-sense sequence but not sense sequence. Thus, some bands may appear in the control but less in the sense. The previous images in Figure 7C are indeed overexposed. We have shown a less exposed blots and quantified each panel as illustrated in Fig 7C in the revised version.

Referee #3:

In this manuscript, Wu et al. identify a long non-coding RNA (lncRNA), termed lncRNA-PAGBC, which is upregulated in human gallbladder cancer patients compared to the normal organ. Over-expression of lncRNA-PAGBC in the NOZ human gallbladder cancer cell line enhanced tumor growth in immunodeficient mice. Conversely, silencing lncRNA-PAGBC by shRNA reduced tumor growth and experimental liver metastasis. By using bioinformatics predictions and cell assays, the authors show that lncRNA-PAGBC binds two miRNAs, miR-133b and miR-511. In particular, they observed that lncRNA-PAGBC reduces the activity of miR-133b and miR-511 through a competing endogenous RNA (ceRNA)-like effect. In the absence of lncRNA-PAGBC, heightened miR-133b and miR-511 levels suppress the AKT/mTOR pathway by targeting SOX4 and PIK3R3, respectively. This ceRNA cross talk between miR-133b/miR-511 and lncRNA-PAGBC may regulate cancer cell proliferation and invasion.

Main criticisms:

1. The authors propose that lncRNA-PAGBC may limit miR-133b and miR-511 activity by competing with endogenous target mRNAs. However, at least according to recent studies, lncRNA target sites are much less abundant than other miRNA target sites in cells (Denzler et al., Mol. Cell 2014; Thomson and Dinger, Nat. Rev. Genetics 2016). What is lacking in this paper is the demonstration that natural targets of miR-133b and miR-511 are globally deregulated by lncRNA-PAGBC. The authors should show that, stoichiometrically, the quantity of endogenous lncRNA-PAGBC target sites could significantly impact on the overall number of miR-133b and miR-511 target sites in the cell. The measurement of lncRNA-PAGBC, miR-133b and miR-511 copies/cell does not address this issue, as the miR-133b and miR-511 binding sites in the cell transcriptome were not estimated. Furthermore, although both SOX4 and PI3KR3 were apparently validated as miR-133b and miR-511 targets, their upregulation after lncRNA-PAGBC over-expression may well be indirect.

The referee's advice is much appreciated. To further identify the relationship between lncRNA-PAGBC and the targets of miR-133b and miR-511, we have now examined the mRNA and protein levels of several natural potential targets of miR-133b and miR-511 after depletion of PAGBC. As shown in Figure R1, the results showed that LASP1 and FAM117B, which are the predicted targets of miR-133b and miR-511 respectively, were not affected by lncRNA-PAGBC knockdown at both

mRNA and protein levels. However, other targets of miR-133b and miR-511, including MET, CXCR4, TRIB2 and LIF, showed a significant reduction at both mRNA and protein levels. It should be noted that FSCN1 and CLDN11 only showed a decrease in protein levels but not in mRNA levels. Moreover, we did not perform further experiments to demonstrate the relationship between the PAGBC target sites and the overall number of miR-133b and miR-511 target sites for the following reasons.

Firstly, in the original version, the mutations of the binding sites on PAGBC have already shown their effect on the mRNA and protein level of SOX4 and PIK3R3 (Figure 5A and 5B). Secondly, in the study by Denzler (Denzler et al., Mol. Cell 2014), they considered all 6, 7, and 8 nt sites as the apparent TA. This TA obviously overestimated the effective number of binding sites of miRNAs and led to false positive results, because only a proportion of the predicted target sites by bioinformatics algorithms (such as Targetscan and miRanda) can be supported by experimental evidence. Thirdly, it is widely known that miRNAs can cause their target mRNAs destabilization or less efficient translation. In the last scenario, no significant change would happen at the mRNA expression level. This is also what we found in Figure R1. Last but not least, the ceRNA theory between lncRNAs and miRNAs has been supported by other studies [8, 9] even without demonstrating the change of binding sites in the cell transcriptome.

As mentioned above, our study can support the ceRNA relationship between PAGBC and miR-133b/511 even without demonstration the relationship between the PAGBC target sites and the overall number of miR-133b and miR-511 target sites. About the concerns of indirect relationship between PAGBC and SOX4/PIK3R3. In our study, we have validated miR-133b and miR-511 work as PAGBC's targets (Figure 4). Luciferase reporter assays and MS2-RIP indicated miR-133b and miR-511 binds to PAGBC. qRT-PCR demonstrated that miR-133b and miR-511 were negatively regulated by PAGBC in GBC cells. In human GBC samples, lncRNA-PAGBC transcript level was significantly negatively correlated with miR-133b and miR-511 transcript levels. In addition, SOX4 and PIK3R3 were validated as miR-133b and miR-511 targets (Figure S3C-S3G). Therefore, PAGBC regulates SOX4 and PIK3R3 directly.

Figure R1 (below): The figure has been removed upon the authors' request.

2. The authors study the activity of miR-511, which is the old designation for the miRNA now referred to as miR-511-5p. Squadrito et al. (Cell Rep 2012) have shown that miR-511-5p is the passenger strand of the precursor miR-511, and does not have miRNA activity. miR-511-5p (the miRNA strand studied by the authors) is indeed undetected according to sequencing studies tracked by miRBase (http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0003127). The authors should also see the work of Chang et al. (BMC Genomics201213 (Suppl 7):S18) on miR-511 isoforms. The authors have neglected this important information in the current study. In order to demonstrate that miR-511-5p is indeed expressed in gallbladder cancer cells to meaningful levels, further studies should be performed, including RNA sequencing of NOZ cells, with and without lncRNA-PAGBC over-expression or knock-down.

We greatly appreciate the scholarship of this reviewer. According to the Reviewer's suggestions, we have performed qRT-PCR analysis in NOZ and EH-GB-1 cells using miR-511-5p primers, then products were subcloned into TA vector and sequenced. We found the products were indeed miR-511-5p, which demonstrate miR-511-5p is indeed expressed in gallbladder cancer cells. Furthermore, as presented in the source data of Fig 4C and G, the mean delta CT value of miR-511-5p in NOZ and EH-GB-1 cells was 16.7, 15.6 while the mean delta CT value of miR-133b was 13.8 and 11.4. In addition, the mean delta CT value of miR-511-5p ranged from 5.94 to 20.67 in GBC clinical samples, which further support that miR-511-5p was expressed at a meaningful level in GBC cells and clinical samples.

3. Related to point 2 above, it should be noted that miR-511 is an intronic miRNA that lacks an internal promoter, and its expression is therefore dependent on that of the hosting gene, MRC1. The MRC1 gene is expressed by myeloid cells, including tumor-infiltrating macrophages and dendritic cells. Although the authors show that miR-511-5p is detectable in gallbladder cancer specimens (Figure 4G), they do not speculate on the possibility that macrophages, and not cancer cells, are the

source of this miRNA. The authors should look at the expression of MRC1 in their samples, and make efforts to resolve the cellular source of miR-511-5p in the tumor samples.

We thank the Reviewer for the insightful comment and suggestions. We have done a series of experiments according to the Reviewer's suggestions. First, we examined the expression of MRC1 in GBC clinical samples. Our IHC assay showed that MRC1 was indeed expressed in gallbladder cancer specimens(Figure S3E). Furthermore, we examined the MRC1 protein levels in GBC cell lines by western blot assays (Figure S3D). The result revealed that MRC1 was indeed expressed in GBC cells. The relevant results were added in Results Section and highlighted by red.

4. The authors state that, based on clinical and pathological data, higher levels of lncRNA-PAGBC are associated with more advanced tumor stages (Supplemental Table 3). However, they subsequently state that multivariate analyses indicate that lncRNA-PAGBC is an independent prognostic factor for overall patient survival (with P = 0.002). One wonders whether lncRNA-PAGBC predicts survival independent of tumor stage.

To address this issue, we performed a survival analysis stratified by TNM stage (Figure 1E in the revised edition). Even corrected for TNM stage, patients with a low expression level of PABGC had significantly better OS than those with a high expression level of PABGC. The relevant results were added in Results Section and highlighted by red.

References:

1. Huang FT, Chen WY, Gu ZQ, et al. The novel long intergenic noncoding RNA UCC promotes colorectal cancer progression by sponging miR-143. Cell Death Dis. 2017;8(5):e2778.
2. Peter S, Borkowska E, Drayton RM, et al. Identification of differentially expressed long non-coding RNAs in bladder cancer. Clin Cancer Res. 2014;20(20):5311-21.
3. Cao C, Sun J, Zhang D, et al. The long intergenic noncoding RNA UFC1, a target of MicroRNA 34a, interacts with the mRNA stabilizing protein HuR to increase levels of β-catenin in HCC `cells. Gastroenterology. 2015;148(2):415-26.e18
4. Zhang J, Zhu N, Chen X. A novel long noncoding RNA LINC01133 is upregulated in lung squamous cell cancer and predicts survival. Tumour Biol. 2015 Sep; 36(10):7465-71.
5. Zang C, Nie FQ, Wang Q et al. Long non-coding RNA LINC01133 represses KLF2, P21 and E-cadherin transcription through binding with EZH2, LSD1 in non small cell lung cancer. Oncotarget. 2016 Mar 8; 7(10):11696-707.
6. Kong J, Sun W, Li C et al. Long non-coding RNA LINC01133 inhibits epithelial-mesenchymal transition and metastasis in colorectal cancer by interacting with SRSF6. Cancer Lett. 2016 Oct 1; 380(2):476-84.
7. Zeng HF, Qiu HY, Feng FB. Long Noncoding RNA LINC01133 Sponges miR-422a to Aggravate the Tumorigenesis of Human Osteosarcoma. Oncol Res. 2017 Mar 28.
8. Le Qu, Jin Ding, Cheng Chen, Zhen-Jie Wu, Bing Liu, Yi Gao, et al. Exosome-Transmitted lncARSR Promotes Sunitinib Resistance in Renal Cancer by Acting as a Competing Endogenous RNA. 2016, Cancer Cell 29, 653–668 May 9, 2016.
9. Yuan et al. A Long Noncoding RNA Activated by TGF-b Promotes the Invasion-Metastasis Cascade in Hepatocellular Carcinoma. Cancer Cell 25, 666–681, May 12, 2014.
10. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell 2011; 146: 353–358.
11. Thomson DW, Dinger ME. Endogenous microRNA sponges: evidence and controversy. Nat Rev Genet. 2016;17(5):272-83.

2nd Editorial Decision 04 July 2017

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the referees that were asked to re-evaluate your study (you will find enclosed below).

As you will see, all three referees support the publication of your study in EMBO reports. However, referees #1 and #2 have some further concerns and suggestions, we ask you to address in a final revised version. The single point by referee #1 and the first point of referee #2 need to be addressed

with further data and additions to the manuscript text. Nevertheless, after cross-commenting with the other referees, we do not require that further experiments regarding the published mechanisms of linc01133 to be performed (if you have such data, though, we ask you to add it to the manuscript), and we also do not require additional controls for the RAP assay (point 4 of referee #2). Further, I have the following editorial requests that also need to be addressed in a final revised version of the manuscript.

The manuscript is currently very long, in particular the methods part. Could you shorten the text to below 60,000 characters with spaces (including the references)?

Please provide the abstract written in present tense.

Table EV1 is too large to be displayed in the online version of the manuscript. Please call this item Dataset EV1 and update the callouts in the manuscript file. Please remove the legend for Table EV1 (now Dataset EV1) from the manuscript text file and add it directly to the table/data file.

Please move Table EV2 to the Appendix. There is no need that this information is shown online. Please remove the legends for Table EV2 from the manuscript text file and add it to the table in the Appendix. Please call this table then Appendix Table S5 and change the callouts in the manuscript file.

Please remove the text on methods and patient information to be found in the supplemental information (pages 27/28). Please be sure that all methods related information is provided in the main text. For the patient info, just add callouts to the respective Appendix tables to the text.

Please format the references in the Appendix according to EMBO reports style and call these only References. See: http://embor.embopress.org/authorguide#referencesformat

Please provide a ToC and page numbers for the Appendix.

As the Western blot panels show significantly cropped images, we would like to ask you to provide the original source data for these that will then be published together with the paper (with the aim of making primary data more accessible and transparent to the reader). The source data will be published in a separate source data files online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of the entire gels or blots) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

Please also provide source data for Fig. 5F. It seems this panel contains images from different sources.

Please add clearly visible scale bars of the same style to ALL microscopic images, without any writing on them. Please put the information on the size oft he bars in the figure legend.

Some of the fonts in the figures are very small and hard to read (specifically Fig 1, 2C, 3D, 4E, EV1). Please change this to bigger fonts.

In panel 6E (bottom left) the writing LV-Control has two fonts in the same word. Please use the same font here.

Finally, we need the ORCIDs for Lei Zheng and Shu-Han Sunto be linked to their profiles on our website. This can only be done by the authors themselves. They need to log in and in their profile there should be a button to link the IDs. If you have problems regarding this (or any other questions), please contact our editorial assistant: elizabeth.corrao@embl.de

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

In this version of the manuscript the authors have addressed some of my criticisms and suggestions. However the authors do not provide the results of the RNA sequencing. They should provide the detailed protocol, the sequences that were generated and the number of clones.

Referee #2:

While the authors have considerably revised their manuscript, unfortunately they have not adequately responded to a number of my previous concerns, which needs to be done in a second revision prior to publication.

Previous 1) Microarray analysis: Still, to find 16000 genes as being regulated is unusually high and likely indicates a lack of stringency. Was the analysis corrected for multiple testing? If their hit linc01133 indeed ranked 10th among the upregulated lncRNAs, the authors should easily be able to heighten the stringency of their analysis without losing this hit - and in the end provide a much more relevant table of specifically regulated genes.

Previous 2) Other functions of linc01133: In my previous review, I had specifically asked for experiments to dissect whether the published mechanisms of linc01133 could also be at play in GBC or whether they could be excluded. None of these experiments was carried out, e.g. to rescue the linc01133 phenotype with an overexpression or knockdown of the published mediators of its function.

Previous 4) Control for RAP experiments: I maintain my disagreement that the antisense would be a sufficient control, but leave the decision about this point to the editor.

Referee #3:

The authors have answered my comments. As far as I am concerned, the manuscript is ready for publication.

2nd Revision - authors' response 12 July 2017

Again, thank you for your positive comments concerning our paper. We have studied comments carefully and have made modification, which we hope meet with approval.

Referee #1:

The referee mentioned the authors do not provide the results of the RNA sequencing.

In the revised manuscript, we have added the detailed protocol of miR-511-5p RNA sequencing in "Materials and Methods", page 23. Actually, we selected 4 clones in NOZ cells and 2 clones in EHGB-1 cells for subsequent sequencing. The sequences can be found in Appendix Figure S1D and Source data for Appendix Figure S1D.

Referee #2:

Previous 1) Microarray analysis: Still, to find 16000 genes as being regulated is unusually high and likely indicates a lack of stringency. Was the analysis corrected for multiple testing. If their hit linc01133 indeed ranked 10th among the upregulated lncRNAs, the authors should easily be able to heighten the stringency of their analysis without losing this hit - and in the end provide a much more relevant table of specifically regulated genes.

To address the issue about stringency, P values were corrected using a false discovery rate (FDR) <0.05. The results indicated that 7798 lncRNAs and 7290 protein-coding genes were differentially expressed between 9 pairs of GBC and non-tumour samples. With consideration of the results of PCR validation with randomly selected lncRNAs and MALAT1 (Figure 1B), we believe the results

of microarray was reliable. In addition, to heighten the stringency to identify relevant genes, we constructed gene-coexpression networks based on the genes with fold change >= 10.0 and a P value <= 0.05. The results were shown in Figure EV1C and D. lncRNA-PAGBC was associated with 17 lncRNAs and 30 protein-coding genes in the coexpression network (Figure 1C). 28 of the 30 protein-coding genes have been identified to be involved in tumour growth and metastasis (Appendix Table S1).

Editor

The manuscript is currently very long, in particular the methods part. Could you shorten the text to below 60,000 characters with spaces (including the references)?

Due to the large amount of our data, although we have tried our best, the manuscript has been barely shortened to about 75,000 characters with spaces (including the references and figure legends). If you have further questions about this character limits, please do not hesitate to contact us.

Please provide the abstract written in present tense.

The abstract has been modified in present tense in the revised version.

Table EV1 is too large to be displayed in the online version of the manuscript. Please call this item Dataset EV1 and update the callouts in the manuscript file. Please remove the legend for Table EV1 (now Dataset EV1) from the manuscript text file and add it directly to the table/data file.

Table EV1 has been called as Dataset EV1 in the revised version (Page 4).

Please move Table EV2 to the Appendix. There is no need that this information is shown online. Please remove the legends for Table EV2 from the manuscript text file and add it to the table in the Appendix. Please call this table then Appendix Table S5 and change the callouts in the manuscript file.

Table EV2 has been moved to the Appendix as Appendix Table S6 in the revised version.

Please remove the text on methods and patient information to be found in the supplemental information (pages 27/28). Please be sure that all methods related information is provided in the main text. For the patient info, just add callouts to the respective Appendix tables to the text.

The relevant information has been moved to the main text in the revised version.

Please format the references in the Appendix according to EMBO reports style and call these only References. See:
http://embor.embopress.org/authorguide#referencesformat

In the revised version, the format of references in the Appendix have been modified according to the requests of your website.

Please provide a ToC and page numbers for the Appendix.

In the revised version, a Toc and page numbers have been added in the Appendix.

As the Western blot panels show significantly cropped images, we would like to ask you to provide the original source data for these that will then be published together with the paper (with the aim of making primary data more accessible and transparent to the reader). The source data will be published in a separate source data files online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of the entire gels or blots) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

As request, the relevant source data have been uploaded as PDF files in the revised version.

Please also provide source data for Fig. 5F. It seems this panel contains images from different sources.

As request, the relevant source data have been uploaded as an excel file in the revised version.

Please add clearly visible scale bars of the same style to ALL microscopic images, without any writing on them. Please put the information on the size of the bars in the figure legend.

All the scale bars have been modified clear in the revised version.

Some of the fonts in the figures are very small and hard to read (specifically Fig 1, 2C, 3D, 4E, EV1). Please change this to bigger fonts.

The fonts have been changed to bigger ones in the revised version.

In panel 6E (bottom left) the writing LV-Control has two fonts in the same word. Please use the same font here.

The same font has been used in the revised version.

Finally, we need the ORCIDs for Lei Zheng and Shu-Han Sun to be linked to their profiles on our website.

Dr. Zheng and Dr. Sun have linked their profiles to their ORICDs as requested.

3rd Editorial Decision 19 July 2017

Thank you for the submission of your revised manuscript to our editorial offices. We think that the points made by the referees are now all sufficiently addressed. However, there are some final editorial issues that need to be addressed.

I would suggest a shorter, more concise title. How about: “LncRNA-PAGBC acts as a microRNA sponge and promotes gallbladder tumorigenesis?”

Some of the fonts in the figures are still too small for publication. In particular the writing in panels 1A-E, 2C and E (the small diagrams), 4D-G, 5C-F, 6C and F, 7E (here also numbers on the y-axis overlap), EV1A-D, G and H, and EV2C. Please increase the size and re-arrange the figures.

Please refer to our guidelines for figure preparation:

http://embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

Please check again that all your figure files are in line with these!

Please also provide also source data for Fig. EV1E. It seems this panel contains images from different sources.

Please add scale bars to the microscopic images in Fig. EV2.

Please remove the text marked in red in the Appendix, and upload this file only named Appendix.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

3rd Revision - authors' response 23 July 2017

Again, thank you for your positive comments concerning our paper. We have studied comments carefully and have made modifications, which we hope meet with approval.

About the suggestion to make the title shorter and more concise.

We really appreciate your suggestion. After discussion with our co-corresponding authors, we all agree with that "LncRNA-PAGBC acts as a microRNA sponge and promotes gallbladder tumorigenesis" as the title of our manuscript.

Some of the fonts in the figures are still too small for publication. In particular the writing in panels 1A-E, 2C and E (the small diagrams), 4D-G, 5C-F, 6C and F, 7E (here also numbers on the y-axis overlap), EV1A-D, G and H, and EV2C. Please increase the size and re-arrange the figures.

All the above-mentioned figures have been modified in the revised version as request by the editor. The fonts of Figure EV1C and D are still relatively small because of the large size of these co-expression network. Therefore, we have uploaded the original pictures as the Source data for Figure EV1C and EV1D just in case that it is needed.

Please also provide also source data for Fig. EV1E. It seems this panel contains images from different sources.

The source data has been uploaded in the revised version and it has been named as Source data for Figure EV2B because the relevant figure EV1in the last submission has been re-arranged as figure EV1 and EV2.

Please add scale bars to the microscopic images in Fig. EV2.

Scale bars have been added in Figure EV3 because the Figure EV2 in the last submission was named as EV3 in the revised version.

Please remove the text marked in red in the Appendix, and upload this file only named Appendix.

The text marked in red has been removed and the file has been named as Appendix.

4th Editorial Decision 27 July 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ying-Bin Liu
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44147-T

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
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http://www.consort-statement.org	CONSORT Flow Diagram
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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	In general, we balance the sample size to achieve power to detect statistically significant effects. For in vitro experiments, eg involving protein measurements, our long term experience on the number of biological repeats needed to ensure statistical power, additionally statistical guidelines for each individual test is followed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We have ensured that the sample size is large enough to detect the effects of interest and only Sample size was chosen based on the minimum number of animals needed to obtain statistical power, all in accordance with the ethical regulations in Institutional Animal Care and Use Committee of Shanghai Jiao Tong University, Shanghai and in line with the Principles of the 3R`s (to refine, reduce and replace number of experimental animals used).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Prior to experiments, end point was set to be 4 weeks after subcutaneous injection and 8 weeks after intrasplenic injection. Thus, the mice were sacrificed at the end point.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Yes, when randomizing the treatment groups two researchers were involved in dividing the individuals in to the experimental groups by flipping coins, to prevent any selection bias.
For animal studies, include a statement about randomization even if no randomization was used.	Mice at the same age were randomly divided into two groups to take xenagraft or metastatic experiments.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Mice were monitored for symptoms by two researchers as well as technical staff at the animal facility daily/weekly throughout the experiments. Mice were monitored by multiple people and subsequent analysis was in part done by additional investigator not involved in the actual in vivo work.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	All statistical analyses were performed using SPSS for Windows, Version 19.0. For statistical comparisons, one-way analysis of variance, chi-square tests, Fisher's exact test and two-tailed Student's t tests were performed as appropriate. The survival curves were calculated using the Kaplan-Meier method, and the differences were assessed with a log-rank test. The Cox proportional hazards model was used to determine independent factors, which were based on the variables selected through univariate analysis.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The raw values of the biological samples follow a normal distribution when plotted.
Is there an estimate of variation within each group of data?	For various in vitro experiments variation are expressed as standard deviation. For animal experiments Kaplan-Meier survival analysis was performed and analyzed as described in materials and methods.
Is the variance similar between the groups that are being statistically compared?	Because the experiments were done in a very controlled manner, comparing parameters within the same experimental testing objects, we expect similiar data variance.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	P22
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	P21

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mus musculus, 4-6 week old athymic Nude - BALB/C-nu/nu mice were maintained at approved facilities in Xinhua Hospital, Shanghai using green-line cage systems with paper enrichment and food and water ad libitum.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The animal experiments were performed in strict accordance with international ethical guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (SYXK[Shanghai] 2013-0106).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Compliance confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Institutional Ethical Board of Xinhua Hospital
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	P16
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	P18,The microarray data discussed in this article have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE76633.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	P18,The microarray data discussed in this article have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE76633.
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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