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LncRNA-MIF, a c-Myc-activated long non-coding RNA, suppresses glycolysis via promoting FBW7-mediated c-Myc degradation

Pengfei Zhang, Limian Cao, Pingsheng Fan, Yide Mei and Mian Wu

Corresponding author: Mian Wu, USTC

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Revision received:	23 April 2016
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Editor: Esther Schnapp

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 March 2016

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting and novel. However, they also point out several missing controls and aspects of the study that require strengthening. I carefully read through all comments and think that all suggestions are useful and therefore should be addressed. The only exception is the before last point of referee 3 that is not clear, and does not need to be addressed experimentally (how the lncRNA-MIF regulates miR-586 expression).

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board.

REFEREE REPORTS

Referee #1:

Myc is one of the key oncogenic proteins in human cancer development. Zhang et al. identified a novel Myc regulated long noncoding RNA, named lncRNA-MIF (c-Myc inhibitory factor). The

authors provided strong evidence indicating that lncRNA-MIF may regulate the expression of Fbw7, an E3 ligase, via ceRNA mechanism mediated by the microRNA mir-586. A Myc/lncRNA-MIF/miR-586/Fbw7 regulatory loop was proposed based on this study. This manuscript provided interesting information on Myc biology. Few questions need to be addressed:

Major:
There are two major isoforms of lncRNA RP11-320M2.1.
http://useast.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000257135;r=2:10448694-10451327;redirect=no
Seems that the authors only studied isoform 001 (figure 1B), which is largely overlapped with isoform 002. Is 002 also detectable in those cell lines and regulated by Myc? Specific primers should be designed to separate these two isoforms. Even 002 is not regulated by Myc, it could also serve as a ceRNA for mir-586 since they share most exons. If using siRNA specifically knock down 002, what will happen in the same model system?

The Myc binding in the promoter region of RP11-320M2.1.of P493 cell should be carefully analyzed. Several P493 Myc ChiP-seq data sets have already been publically available. E.g. Cell. 2012 Sep 28;151(1):56-67; Nature 511, 483-487. The authors need to analyze Myc binding based on these ChiP seq data, but not only based on bioinformatics prediction (TargetScan). A detailed profile of Myc binding in the promoter region of RP11-320M2.1. of P493 cells (Myc on/off) needs to be shown.

Are lncRNA RP11-320M2.1. and miRNA mir-586 widely expressed in human cancers? Any clinical significance? Based on the TCGA dataset (lncRNA, mRNA and miRNA expression can be easily retrieved from: http://ibl.mdanderson.org/tanric/_design/basic/index.html), is there expressional correlations between lncRNA-MIF and Myc/Fbw7 in tumors?

Minor:
In the lncRNA overexpression experiments, an antisense control of lncRNA-MIF should be used.

Referee #2:

In this manuscript, Zhang et al. identify a novel regulatory loop controlling the degradation of the c-Myc oncoprotein. In this c-Myc-lncRNA-MIF-miR-586-Fbw7 axis, c-Myc induces lncRNA-MIF which then blocks miR-586 to unleash the E3 ligase Fbw7 to degrade c-Myc protein. The authors argue that lncRNA-MIF can inhibit aerobic glycolysis and tumorigenesis through this regulation of c-Myc expression and activity. In general, this is an interesting manuscript, which provides new insight into the regulation of c-Myc. However some key controls are noticeably missing and some data has been over-interpreted. Specific issues are listed below.

Major issues:
1. Results: The authors must reveal lncRNA microarray method and resulting dataset, as well as method of analysis used to identify lncRNA under study in this manuscript. As presently presented, the identity of lncRNA-MIF as a c-Myc induced gene simply appears. The experimental method and the results of that method need to be described in full, including the list of Myc-regulated target genes identified. Then lncRNA-MIF can be one hit the authors then further validate. This is essential.
2. shRNA targeting lncRNA-MIF. It is a shame that only one shRNA was used throughout this manuscript. The off-target effects of shRNAs are well established and the conventional approach to convince readers that the results are due to targeting the gene of interest, in this case lncRNA-MIF, is to show data for more than one shRNA throughout all experiments. This is important and needs to be rectified.
3. Fig 1: Is the down-regulation of c-Myc expression in response to ectopic lncRNA-MIF expression cause or consequence of lncRNA-MIF action. Does lncRNA-MIF inhibit c-Myc expression, which then leads to an anti-proliferative effect (cause), or does lncRNA-MIF inhibit cell proliferation, which then leads to the down-regulation of c-Myc (consequence). This needs to be investigated, shown and discussed within Figure 1 at the beginning of the manuscript as both an unknown and an important piece of the puzzle. By raising this issue at the start of the paper, the authors can then interrogate mechanism and fill this gap by showing lncRNA-MIF as a miR-586 sponge, which

regulates c-Myc degradation. It is incorrect to show c-Myc expression data without revealing the effect of lncRNA-MIF on the growth state of the cell. This needs to be conducted, shown and discussed.
4. Fig 4: Again the growth state of the cell needs to be shown (see #3 above for rationale).
5. Fbw7 regulates a number of substrates using a similar mechanism of action - what happens to all the other Fbw7 substrates? The biological regulatory effect of lncRNA-MIF cannot be exclusively ascribed as a Myc-only phenomenon. This effect on at least two other Fbw7 substrates needs to be shown and the text re-written to allow the real possibility that other Fbw7 substrates may also be playing a role in the biological effects of lncRNA-MIF, including the regulation of glycolysis and tumorigenesis. It is not surprising that regulators of c-Myc, such as lncRNA-MIF, are controlling an entire genetic program such as that regulated by Fbw7, which also includes c-Myc. This aspect of this manuscript will likely be of interest to an even broader readership and will further enhance the citation rate of this manuscript.

Minor issues:
1. The manuscript is inconsistent in its writing style and needs to be reviewed for scientific clarity and English grammar. For example, in the abstract "c-Myc, one of the most important proto-oncogenes" is too subjective and needs to be written with facts and figures. "However, it remains unidentified how the stability..." needs to be edited and written in a more conventional style, e.g. The regulatory mechanisms controlling c-Myc protein stability remain unclear. This editing is required throughout the manuscript to ensure clarity in communication. For example, "The reporter activity was noticeably suppressed by the presence of wild-type 3'UTR of Fbw7..."; noticeably suppressed is again subjective and should be replaced with a statistical descriptor.
2. References. In addition to the recent publications Lin et al. 2012 and Nie et al. 2012, the authors should include PMID:12695333, who were one of the first to show this phenomenon of Myc occupancy on chromatin.
3. Fbw7 is now called Fbxw7 by the new HUPO nomenclature.

Referee #3:

The manuscript by Zhang et al report that lncRNA-MIF (c-Myc inhibitory factor) is a direct target gene of c-Myc, and up-regulates c-Myc protein expression by working as a competing endogenous RNA for miR-586 and attenuating the inhibitory effect of Fbw7. The study is generally well-organized and the conclusions are generally sound. Specific comments:

Figure S1 showed regulation of c-Myc protein by lncRNA-MIF. The authors need to show that lncRNA-MIF does not regulate c-Myc mRNA in the experiment, before they can conlude that lncRNA-MIF regulates c-Myc through a translational mechanism.

Fig. 1F showed c-Myc protein half-life. Histograms should be added to show protein expression quantification.

Fig. 3A. there needs positive control experiments to show that the negative data in Fig. 3A is truly negative.

Fig. 3C showed that lncRNA-MIF regulated Fbw7 mRNA expression, and Fig. 4 demonstrated that miR-586 modulated Fbw7 mRNA translation to protein. How can the authors explain the discrepancy. Does miR-586 regulate Fbw7 mRNA stability?

Figure 4F showed that lncRNA-MIF regulated miR-586 expression. The authors please provide mechanistic explanation how lncRNA-MIF regulates miR-586 expression.

Fig. 6C-D. Please examine lncRNA-MIF, c-Myc and Fbw7 expression in tumour tissues from mice, to confirm that the mechanism works in vivo.

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1st Revision - authors' response 23 April 2016

Referee #1:

Major:

There are two major isoforms of lncRNA RP11-320M2.1.
http://useast.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000257135;r=2:10448694-10451327;redirect=no
Seems that the authors only studied isoform 001 (figure 1B), which is largely overlapped with isoform 002. Is 002 also detectable in those cell lines and regulated by Myc? Specific primers should be designed to separate these two isoforms. Even 002 is not regulated by Myc, it could also serve as a ceRNA for mir-586 since they share most exons. If using siRNA specifically knock down 002, what will happen in the same model system?

Indeed, there are two major isoforms of lncRNA RP11-320M2.1. According to referee #1's suggestion, we detected the level of isoform 002 (named as lncRNA-MIF-L, L for long) in HeLa cells expressing Flag-control, Flag-c-Myc, control shRNA or c-Myc shRNA respectively. We found that c-Myc did not regulate lncRNA-MIF-L (Fig S3A and S3B). Furthermore, the copy number of lncRNA-MIF-L is only 2% of that of lncRNA-MIF in MCF7, HeLa and H1299 cells (Fig S3C). These results indicate that lncRNA-MIF-L will have little, if any sponge effect on miR-586. We also knocked down lncRNA-MIF-L in HeLa cells, and found it did not affect the level of miR-586, Fbxw7 or c-Myc (Fig S3D and S3E). Based on these newly performed experiments, lncRNA-MIF-L is unlikely to serve as a ceRNA for miR-586. We therefore did not investigate the lncRNA-MIF-L further in this study. We have incorporated all these results into figure S3 of this revised manuscript.

The Myc binding in the promoter region of RP11-320M2.1.of P493 cell should be carefully analyzed. Several P493 Myc ChiP-seq data sets have already been publically available. E.g. Cell. 2012 Sep 28;151(1):56-67; Nature 511, 483-487. The authors need to analyze Myc binding based on these ChiP seq data, but not only based on bioinformatics prediction (TargetScan). A detailed profile of Myc binding in the promoter region of RP11-320M2.1. of P493 cells (Myc on/off) needs to be shown.

We appreciate very much for referee's comment. We analyzed Myc binding promoters based on ChIP seq data from two papers reviewer suggested. We found Myc-promoter-binding sequence was very conservative and closely matched to what we used. We again analyzed the c-Myc binding promoter region of lncRNA-MIF in P493-6 cell lines according to reviewer's advice. We found that three promoter regions including MIF1, MIF2 and MIF3 are all functional in P493-6 cells (Fig 2G). As a transcription factor, c-Myc heterodimerizes with its partner protein Max to bind to conserved E-box to transactive target genes**.** Since lncRNA-MIF is transactivated by c-Myc, we therefore examined whether Max is involved in c-Myc transcription. As seen in figure S2C, depletion of Max diminished the effect c-Myc on lncRNA-MIF transcription, implying that c-Myc transcriptionally activated lncRNA-MIF via conserved E-box. Moreover, since c-Myc was reported to transcribe approximately 10–15% of genes in the genome, some non-canonical binding sites cannot be excluded.

Are lncRNA RP11-320M2.1. and miRNA mir-586 widely expressed in human cancers? Any clinical significance? Based on the TCGA dataset (lncRNA, mRNA and miRNA expression can be easily retrieved from: http://ibl.mdanderson.org/tanric/_design/basic/index.html), is there expressional correlations between lncRNA-MIF and Myc/Fbw7 in tumors?

We compared the expression levels of lncRNA-MIF and miR-586 in patients' colorectal carcinoma and normal tissue (Fig. S5A). We found that both lncRNA-MIF and miR-586 are expressed in human cancers and their expression levels are higher in tumors than in normal tissues. In addition, based on the TCGA dataset, level of lncRNA-MIF was shown to be higher in head and neck squamous cell carcinoma (Fig. S5B). However, in TCGA data base, no data regarding expressional correlations between lncRNA-MIF and Myc/Fbw7 was currently shown.

Minor:

In the lncRNA overexpression experiments, an antisense control of lncRNA-MIF should be used.

According to reviewer #1's advice, antisense control of lncRNA-MIF was used in overexpression experiments (Fig 1H, 1I, 5A, 5B, 5C, 7A, 7C and 7E).

Referee #2:

Major issues:

1. Results: The authors must reveal lncRNA microarray method and resulting dataset, as well as method of analysis used to identify lncRNA under study in this manuscript. As presently presented, the identity of lncRNA-MIF as a c-Myc induced gene simply appears. The experimental method and the results of that method need to be described in full, including the list of Myc-regulated target genes identified. Then lncRNA-MIF can be one hit the authors then further validate. This is essential.

We show great appreciation for referee's careful review and sorry for not being able to state that clearly in our previous MS. In this revised MS, we have listed resulting dataset (Table S1). We also described how we chose lncRNA-MIF as a subject for investigation in this study (page 5, first paragraph in Results section; Fig S1A, S1B).

2. shRNA targeting lncRNA-MIF. It is a shame that only one shRNA was used throughout this manuscript. The off-target effects of shRNAs are well established and the conventional approach to convince readers that the results are due to targeting the gene of interest, in this case lncRNA-MIF, is to show data for more than one shRNA throughout all experiments. This is important and needs to be rectified.

We appreciate very much for referee's comment. Actually, we had used three lncRNA-MIF shRNAs in several key experiments (see Fig 1J, 1K, 5D, 5E, 7B, 7D and 7F) to exclude the possibility that lncRNA-MIF was an off-target. Furthermore, we performed a rescue experiment. Both MIF shRNA-1 and MIF shRNA-2 were shown to down-regulate c-Myc, and over-expression of shRNA resistant MIF-**1** or MIF-**2** indeed rescued the effect caused by MIF shRNA-1 or -2 (Figs. S1C, S1D). These experiments demonstrates that MIF shRNA-1 we used throughout in this study is not off-targeting.

3. Fig 1: Is the down-regulation of c-Myc expression in response to ectopic lncRNA-MIF expression cause or consequence of lncRNA-MIF action. Does lncRNA-MIF inhibit c-Myc expression, which then leads to an anti-proliferative effect (cause), or does lncRNA-MIF inhibit cell proliferation, which then leads to the down-regulation of c-Myc (consequence). This needs to be investigated, shown and discussed within Figure 1 at the beginning of the manuscript as both an unknown and an important piece of the puzzle. By raising this issue at the start of the paper, the authors can then interrogate mechanism and fill this gap by showing lncRNA-MIF as a miR-586 sponge, which regulates c-Myc degradation. It is incorrect to show c-Myc expression data without revealing the effect of lncRNA-MIF on the growth state of the cell. This needs to be conducted, shown and discussed.

We appreciate very much for referee's intriguing comment. In response to ectopic lncRNA-MIF expression, level of c-Myc was reduced, leading into decreased cell proliferation. Consistently, knockdown of lncRNA-MIF up-regulates c-Myc, which resulted in an increased cell proliferation. It is reasonable to believe that it is the lncRNA-MIF-regulated c-Myc that modulates cell growth. In this revised MS, we performed two more experiments to assess the effect of lncRNA-MIF on the growth state of the cells as seen in figure 7. In addition, our colony formation assays also proved that lncRNA-MIF affects cell proliferation through miR-586 (Fig 8A and 8B). We do not have direct evidence to show that lncRNA-MIF inhibits cell proliferation, which in turn leads to down-regulation of c-Myc. Yet, based on our current evidence, we believe that lncRNA-MIF regulates cell growth is c-Myc dependent, since in the absence of c-Myc, lncRNA MIF is unable to affect cell proliferation (Fig S4).

Fig 4: Again the growth state of the cell needs to be shown (see #3 above for rationale).

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We appreciate very much for referee's comment. According to reviewer #2's suggestion, we examined the growth state of the cell (Fig 7).

5. Fbw7 regulates a number of substrates using a similar mechanism of action - what happens to all the other Fbw7 substrates? The biological regulatory effect of lncRNA-MIF cannot be exclusively ascribed as a Myc-only phenomenon. This effect on at least two other Fbw7 substrates needs to be shown and the text re-written to allow the real possibility that other Fbw7 substrates may also be playing a role in the biological effects of lncRNA-MIF, including the regulation of glycolysis and tumorigenesis. It is not surprising that regulators of c-Myc, such as lncRNA-MIF, are controlling an entire genetic program such as that regulated by Fbw7, which also includes c-Myc. This aspect of this manuscript will likely be of interest to an even broader readership and will further enhance the citation rate of this manuscript.

According to reviewer #2's suggestion, except for c-Myc, we have tested another two substrates of Fbxw7 including c-Jun and Cyclin E1. We found that the shorter half-life protein c-Jun was regulated by lncRNA-MIF in the same way as c-Myc (Fig 5). However, LncRNA-MIF has little, if any effect on Cyclin E, this could be due to the high stability of this cell cycle regulator.

Minor issues:

1. The manuscript is inconsistent in its writing style and needs to be reviewed for scientific clarity and English grammar. For example, in the abstract "c-Myc, one of the most important proto-oncogenes" is too subjective and needs to be written with facts and figures. "However, it remains unidentified how the stability..." needs to be edited and written in a more conventional style, e.g. The regulatory mechanisms controlling c-Myc protein stability remain unclear. This editing is required throughout the manuscript to ensure clarity in communication. For example, "The reporter activity was noticeably suppressed by the presence of wild-type 3'UTR of Fbw7..."; noticeably suppressed is again subjective and should be replaced with a statistical descriptor.

According to the reviewer's instruction, we have tried our best to improve the English for this revised MS.

2. References. In addition to the recent publications Lin et al. 2012 and Nie et al. 2012, the authors should include PMID:12695333, who were one of the first to show this phenomenon of Myc occupancy on chromatin.

According to reviewer #2's advice, we added above reference (Ref 12) in this revised manuscript.

3. Fbw7 is now called Fbxw7 by the new HUPO nomenclature.

We changed Fbw7 to Fbxw7 throughout this MS.

Referee #3:

Figure S1 showed regulation of c-Myc protein by lncRNA-MIF. The authors need to show that lncRNA-MIF does not regulate c-Myc mRNA in the experiment, before they can conlude that lncRNA-MIF regulates c-Myc through a translational mechanism.

We appreciate very much for referee's comment. We showed the c-Myc mRNA level was not affected by lncRNA-MIF overexpression or knockdown in this revised MS (Fig 5C and 5E). Therefore lncRNA-MIF does not regulate c-Myc mRNA.

Fig. 1F showed c-Myc protein half-life. Histograms should be added to show protein expression quantification.

We appreciate very much for referee's comment. According to reviewer #3's suggestion, protein stability curves were shown in Fig 1I and 1K.

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Fig. 3A. there needs positive control experiments to show that the negative data in Fig. 3A is truly negative.

PTBP1, a RNA binding protein, was used in figure 3A as a positive control. This RNA binding protein was found to be associated with lncRNA-MIF in our mass spectrometry study.

Fig. 3C showed that lncRNA-MIF regulated Fbw7 mRNA expression, and Fig. 4 demonstrated that miR-586 modulated Fbw7 mRNA translation to protein. How can the authors explain the discrepancy. Does miR-586 regulate Fbw7 mRNA stability?

When microRNA targets 3'UTR of mRNA by a perfect match, mRNA will be degraded. We showed that miR-586 downregulated Fbxw7 mRNA in this MS (Fig 4E).

Figure 4F showed that lncRNA-MIF regulated miR-586 expression. The authors please provide mechanistic explanation how lncRNA-MIF regulates miR-586 expression.

lncRNA-MIF was shown to act as sponge to "absorb" miR-586 by Watson-Crick base pair rule. Level of free miR-586 will be affected by the amount of lncRNA-MIF. Details of how lncRNA-MIF regulates miR-586 expression remain unknown.

Fig. 6C-D. Please examine lncRNA-MIF, c-Myc and Fbw7 expression in tumour tissues from mice, to confirm that the mechanism works in vivo.

There is no mouse homolog of lncRNA-MIF and we are unable to examine their expressions in mice. However, we have compared lncRNA-MIF, c-Myc and miR-586 expressions in human tumor and normal tissues (Fig S5).

In summary, we believe we have improved this manuscript by adding substantial experimental results specified by the reviewers. We certainly hope this revision could meet the requirements of all reviewers. Last but not least, we are grateful to all reviewers and editor for their insightful and constructive comments, without which, this revised MS would have been impossible. We are looking forward to hearing from you soon.

2nd Editorial Decision 12 May 2016

Thank you for the submission of your revised manuscript to our journal. We have now received the comments from the referees that were asked to assess it, and I am happy to tell you that both referees support the publication of your revised study. Only some minor changes are required before we can proceed with the official accpetance of your manuscript.

The legend for Figure 1E does not specify the error bars, n, and the test used to calculate p-values. Please add this information.
Supplementary figures and tables are called "expanded view" (Figure EV1, 2, etc) now at EMBO press. Can you please change the names in the manusript text and figures? Table S1 should become Dataset EV1 (.doc or .xls), and table S2 should become table EV1. We will also need the figures at higher resolutions, ideally at 300 dpi.

Please explain the "randomization" and "blinding" statements in the author checklist. If randomization and blinding was performed, please include a full sentence, also in the manuscript methods section.

REFEREE REPORTS

Referee #1:

The authors have addressed most of my questions. The manuscript has been significantly improved and it is ready to be published in the journal.

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Referee #2:

The authors have addressed most of the issues raised by all three reviewers and is suitable for publication.

2nd Revision - authors' response 17 May 2016

Thank you very much for your favorable decision on our manuscript (EMBOR-2016-42067V2), and all the minor changes have been made as you suggested. Below are detailed descriptions of each point:

1. We specified the error bars, n, and the test used to calculate p-values in the legend for Figure 1E in this revised manuscript.
2. Figure EV1, 2, etc, Dataset EV1 and Table EV1 are now used in this revised text, figures and tables.
3. We explained the "randomization" and "blinding" statements in both the author checklist and the methods section in this revised manuscript.

3rd Editorial Decision 23 May 2016

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.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Mian Wu

Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-42067V3

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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).

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Based on published methords.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	In page 32, 35 and figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Randomization. We have used unbiased approach in choosing male athymic nude mice. Mice were used in the experiment at random. Page 22.
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding. During testing the tumors' weight, the experimentalists were blinded to the information and shape of tumor tissue masses. Page 22.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, we used two-tailed t-test.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes, in page 19.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes, in page 19.

* 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.	In page 22.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	Yes ,we confirm compliance. In page 22.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The protocol for the use of tissue samples from patients and follow-up study was approved by our Institutional Review Board,
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.	NA
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.	Yes, we confirm we have followed these guidelines.

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	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NCBI, UCSC, TargetScan and TCGA Research Network were used.
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	The results shown in figure S5B are in whole based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/.
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