EMBO Molecular Medicine

CRISPR/Cas9 mediated somatic correction of a novel coagulator factor IX gene mutation ameliorates hemophilia in mouse

Yuting Guan, Yanlin Ma, Qi Li, Zhenliang Sun, Lie Ma, Lijuan Wu, Liren Wang, Li Zeng1, Yanjiao Shao, Yuting Chen, Ning Ma, Wenqing Lu, Kewen Hu, Honghui Han4, Yanhong Yu,Yuanhua Huang, Mingyao Liu and Dali Li

Corresponding authors: Dali Li, Mingyao Liu (East China Normal University) and Yanlin Ma (Hainan Medical University)

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Transaction Report:

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

Editor: Roberto Buccione

1st Editorial Decision 09 December 2015

Thank you for the submission of your manuscript to EMBO Molecular Medicine. I am sorry for not having been able to get back to you sooner

In this case, notwithstanding the current interest and timeliness of the topic, we experienced unusual difficulties in securing three willing and appropriate reviewers. As a further delay cannot be justified I have decided to proceed based on the two available consistent evaluations.

Both Reviewers are quite positive on your manuscript although they raise some issues that require your action. I will not dwell into much detail as their comments are detailed. I would like, however, to highlight a few main points.

As you will see the Reviewers agree on a few basic issues, which require your action. These include the request that actual FIX levels, activity and stability be assayed. Both also suggest that a tail-bleeding assay should be performed to measure correction of the phenotypes. I agree that the above needs to be carried out.

Reviewer 1 has additional comments. S/he notes that the genome editing efficiency of the different CRISPR-Cas9-based approaches should be compared using the same technique (deep sequencing of the targeted locus). However, after Reviewer cross-commenting, it was agreed that this will not be

necessary if the above points are dealt with satisfactorily. You will need, however, to tone down statements on comparing single-stranded donor oligonucleotides and dsDNA efficiencies.

In conclusion, while publication of the paper cannot be considered at this stage, we would be pleased to consider a revised submission, with the understanding that the Reviewers' concerns must be addressed as outlined above with additional experimental data where appropriate and that acceptance of the manuscript will entail a second round of review. I will endeavour to reach a final decision as quickly as possible.

Please note that it is EMBO Molecular Medicine policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

As you might know, EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. However, I do ask you to get in touch with us after three months if you have not completed your revision, to update us on the status. Please also contact us as soon as possible if similar work is published elsewhere.

Please note that EMBO Molecular Medicine now requires a complete author checklist (http://embomolmed.embopress.org/authorguide#editorial3) to be submitted with all revised manuscripts. Provision of the author checklist is mandatory at revision stage; The checklist is designed to enhance and standardize reporting of key information in research papers and to support reanalysis and repetition of experiments by the community. The list covers key information for figure panels and captions and focuses on statistics, the reporting of reagents, animal models and human subject-derived data, as well as guidance to optimise data accessibility.

I also suggest that you carefully adhere to our guidelines for publication in your next version, including presentation of statistical analyses and our new requirements for supplemental data (see also below) to speed up the pre-acceptance process.

I look forward to seeing a revised form of your manuscript as soon as possible.

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

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

In this paper, Guan et al. characterized a novel mutation of F9 gene in a patient with Hemophilia B and generated an appropriate mouse model harboring this mutation, reproducing the hemophilic phenotype observed in the patient. Moreover, they tested different genome editing strategies to restore hemostasis in this mouse model of hemophilia. Some experiments should be performed to confirm these findings and to improve the technical quality of the paper as detailed in the " Remarks to be sent to the author".

Referee #1 (Remarks):

In this paper, Guan et al. characterized a novel mutation of F9 gene in a patient with Hemophilia B and generated an appropriate mouse model harboring this mutation, reproducing the hemophilic phenotype observed in the patient. Moreover, they tested different CRISPR-Cas9-based genome editing strategies to restore hemostasis in this mouse model of hemophilia. A plasmid-based platform is shown to be less toxic am more effective compared to the adenoviral delivery of the CRISPR-Cas9 system. Some experiments should be performed to confirm these findings and to improve the technical quality of the paper.

Major comments:
1. The Authors have to better characterize the effects of the novel mutation in terms of protein concentration and stability in plasma. They should also evaluate the potential impairment of FIX secretion and of its protease activity. We believe that these factors contribute to the extent of correction that is necessary to ameliorate the hemophilic phenotype.

2. The authors should evaluate the long-term correction of the hemophilic phenotype and the persistence of the correction after induced liver regeneration (e.g.: after partial hepatectomy).
3. The Authors have to compare the genome editing efficiency of the different CRISPR-Cas9-based approaches using the same technique (preferably deep sequencing of the targeted locus).

Minor comments:
1. The Authors should collect the blood for the aPTT test using tail bleeding. Retro-orbital bleeding may alter the results of this test because of the potential contamination of the blood samples with Tissue Factor.
2. The Authors should also perform PT assay testing different sample dilutions to better evaluate possible differences among mice groups that might go unnoticed with such short clotting times.

3. Did the Authors notice any difference in the extent of liver damage and in the increase of the inflammation markers between the mice receiving a low dose and a high dose of adenoviral vectors?
4. The Authors should comment on the possibility of using a lower dose of adenoviral vectors to restore hemostasis without any liver damage and inflammation response.
5. The Authors should explain better the rational of the use of ssODN harboring silent mutations (to avoid the re-cutting of the Cas9-gRNA complex, to better detect the genome editing efficiency) and the different frequency of gene conversion observed in Figure 3C.

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

The current manuscript deals with correction through homologous recombination of a defect in the factor IX gene through CRISPR-CAS9. This is one of the first reports that shows that an disease phenotype can be corrected in vivo through homologous recombination. As such the impact of the current study is very high.

The medical impact is high since this paper provides proof-of-concept for gene therapy through homologous recombination in a small animal model.

Referee #2 (Remarks):

This a very interesting manuscript that shows phenotypic correction of hemophilia B through homologous recombination.

The approach taken is very novel and highly relevant for curative approaches for mono-genetic disorders. As such the potential impact of this study is very high.

Technically the experiments are well-performed. The molecular genetic analyses are of the highest possible level. The phenotypic correction of the factor IX gene is monitored by the APTT; this a global coagulation test. Actual levels of factor IX in plasma of the "corrected" mice are not available. Measuring factor IX levels in plasma following gene-correction would be very informative and would provide an independent means to demonstrate that a considerable percentage of the mutated FIX gene has indeed been corrected. An ELISA-based assay that estimate the amount of factor IX protein in plasma would be most appropriate.

Lack of tail-vein bleeding following an incision is commonly used to demonstrate correction of a bleeding phenotype in mouse models of hemophilia. It would be nice if this information was also included in the current manuscript.

Minor comments.

Figure 1 describes a novel mutation in the FIX gene. A large number of mutations in the FIX gene have already been described. This information can also be presented as part of the Supplementary information.

1st Revision - authors' response 02 February 2016

Referee #1 (Remarks):

In this paper, Guan et al. characterized a novel mutation of F9 gene in a patient with Hemophilia B and generated an appropriate mouse model harboring this mutation, reproducing the hemophilic phenotype observed in the patient. Moreover, they tested different CRISPR-Cas9-based genome editing strategies to restore hemostasis in this mouse model of hemophilia. A plasmid-based platform is shown to be less toxic am more effective compared to the adenoviral delivery of the CRISPR-Cas9 system. Some experiments should be performed to confirm these findings and to improve the technical quality of the paper.

Major comments:
***Comment 1**. The Authors have to better characterize the effects of the novel mutation in terms of protein concentration and stability in plasma. They should also evaluate the potential impairment of FIX secretion and of its protease activity. We believe that these factors contribute to the extent of correction that is necessary to ameliorate the hemophilic phenotype.*
Response: Thanks for the reviewer's comment. We tried our best to use all commercially available ELISA kits against mouse FIX and 2 kits against human FIX, but none of them can detect secreted mouse FIX. For this reason, we could not determine the actual FIX level in mice. Alternatively, we detected the mRNA and protein level of FIX in mouse hepatic tissue. After testing 3 antibodies claimed to be against mouse FIX, we found one of them worked. Since the novel mutation is a point mutation, we found it did not affect the stability of F9 mRNA or protein but the mutation harboring a premature stop codon greatly impaired F9 mRNA and protein level. These data have been presented in revised Figure 2C. Unfortunately, since the commercial available commercial FIX activity assay kit is also for human FIX, we were unable to evaluate mouse FIX activity. In order to evaluate the extent of correction, we used tail bleeding assays which we explain in detail to answer the comment below.

***Comment 2**. The authors should evaluate the long-term correction of the hemophilic phenotype and the persistence of the correction after induced liver regeneration (e.g.: after partial hepatectomy).*
Response: To evaluate the long-term correction effect, we employed a tail-bleeding assay beginning 12 weeks after the mice had received tail vein injection. As shown in revised Figure 3C, it suggested that the correction of F9 mutation ameliorated the HB phenotype for a prolonged period. Although it will be interesting to induce liver regeneration in our model to test the correction, we used naked DNA but not viral system to deliver Cas9/sgRNA and donor template which were very unstable in mice. In addition, the donor template was not a full-length F9 cDNA which could not produce any protein. For these two reasons, we think the therapeutic effect is from corrected endogenous FIX rather than expression of exogenous FIX DNA.

***Comment 3**. The Authors have to compare the genome editing efficiency of the different CRISPR-Cas9-based approaches using the same technique (preferably deep sequencing of the targeted locus).*
Response: We used TA-clone sequencing for Adv, ssODN and plasmid treated groups (over 120 clones/mouse for each group). To increase the accuracy, we employed deep sequencing and showed the data for plasmid DNA injected group. We found that the correction efficiency obtained by these two strategies were comparable, so we did not use deep sequencing for the other two groups. The editors also suggested that deep sequencing is not necessary, so we compared the correction efficiency of the two groups either using dsDNA or ssODN as donor templates.

Minor comments:
***Comment 4.** The Authors should collect the blood for the aPTT test using tail bleeding. Retro-orbital bleeding may alter the results of this test because of the potential contamination of the blood samples with Tissue Factor.*
Response: We used both aPTT and tail bleeding assays to evaluate the HB phenotype in the revised manuscript as shown in Figures 2G and 3C.

***Comment 5.** The Authors should also perform PT assay testing different sample dilutions to better*

evaluate possible differences among mice groups that might go unnoticed with such short clotting times.
Response: Originally, we did perform PT assays when testing aPTT but did not present the data. In revision, we showed the PT assays alone with aPTT.

Comment 6. *Did the Authors notice any difference in the extent of liver damage and in the increase of the inflammation markers between the mice receiving a low dose and a high dose of adenoviral vectors?*
Response: We noticed that the mice receiving a low dose Adv did not exhibit very severe liver damage, in contrast to what was observed in high dose Adv treated mice. These data are presented in supplementary figure S5 in the revised version.

Comment 7. *The Authors should comment on the possibility of using a lower dose of adenoviral vectors to restore hemostasis without any liver damage and inflammation response.*
Response: In revised manuscript, we explore this issue as the reviewer suggested in the *Discussion*.

Comment 8. *The Authors should explain better the rational of the use of ssODN harboring silent mutations (to avoid the re-cutting of the Cas9-gRNA complex, to better detect the genome editing efficiency) and the different frequency of gene conversion observed in Figure 3C.*
Response: We explained the issue as the reviewer suggested in page 8 in the revised manuscript.

Referee #2 (Remarks):

This a very interesting manuscript that shows phenotypic correction of hemophilia B through homologous recombination.

The approach taken is very novel and highly relevant for curative approaches for mono-genetic disorders. As such the potential impact of this study is very high.

Comment 1*: Technically the experiments are well-performed. The molecular genetic analyses are of the highest possible level. The phenotypic correction of the factor IX gene is monitored by the APTT; this a global coagulation test. Actual levels of factor IX in plasma of the "corrected" mice are not available. Measuring factor IX levels in plasma following gene-correction would be very informative and would provide an independent means to demonstrate that a considerable percentage of the mutated FIX gene has indeed been corrected. An ELISA-based assay that estimate the amount of factor IX protein in plasma would be most appropriate.*
Response: As we answered in Comment 1 of reviewer 1, we attempted ELISA-based assays to measure the actual FIX level in mutant mice or corrected mice, but all the kits we tried did not work for mouse. As an alternative approach, we examined the mRNA and protein level of FIX in the mouse liver and employed tail bleeding assays as an independent means to show the hemophilic phenotype.

Comment 2: *Lack of tail-vein bleeding following an incision is commonly used to demonstrate correction of a bleeding phenotype in mouse models of hemophilia. It would be nice if this information was also included in the current manuscript.*
Response: As the reviewer suggested, we performed the tail bleeding assays and the data were presented in revised Figure 2G and Figure 3C.

Minor comments.

Comment 3*: Figure 1 describes a novel mutation in the FIX gene. A large number of mutations in the FIX gene have already been described. This information can also be presented as part of the Supplementary information.*
Response: Thanks for the reviewer's comment. Since there are about one thousand mutations has been recorded, we have cited two main database (Hemophilia B Mutation Database (http://www.factorix.org/) or the Human Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php)) which will help the readers to find more information. In addition, the manuscript has 5 supplementary figures which is the limitation of EMM. Since the

main topic of the manuscript is Cas9-mediated gene therapy, we think that it is better not to substitute any original supplementary figure.

2nd Editorial Decision 15 February 2016

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

1) Please note and respond to Reviewer 2's comment on Fig. 2c concerning the molecular weight of the truncated FIX.

2) We are now encouraging the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or at least the key gels used in the manuscript? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation may be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this just contact me.

3) You are welcome to suggest a striking image or visual abstract to illustrate your article. If you do please provide a jpeg file 550 px-wide x 400-px high.

4) Please note that we now mandate that all corresponding authors list an ORCID digital identifier. You may do so though our web platform upon submission and the procedure takes <90 seconds to complete. We encourage all authors to supply an ORCID identifier, which will be linked to their name for unambiguous name identification.

5) I have introduced some minor changes in the manuscript (Title, Abstract and "The Paper Explained"). Please check and approve (or not) based on the attached manuscript file.

6) Please provide 5 keywords in the title page.

7) Please remove the appendix figure legends from the manuscript file and place them instead under each figure in the appendix data file.

I look forward to seeing a revised final form of your manuscript as soon as possible.

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

Referee #1 (Remarks):

The Authors improved the technical quality of the paper by answering to most of the reviewer's comments. The paper is now suitable for publication.

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

The technologies used in this manuscript are a very high level. correction in hemophilia B; the approach outlined in this paper provides important novel information on the application of CRISPR/Cas9 system for correction of monogenetic disorders. Therefore the medical impact is

high.

Referee #2 (Remarks):

The revised manuscript has been improved. Gene correction of the F9 Y371S mutation is achieved for 0.56% of the F9 alleles. This results in shortening of the APTT (Figure 3B) and increased survival following tail-clip (Figure 3B). The amount of FIX activity and antigen in plasma has not been measured. Despite the clear hemostatic effect as measured by APTT and tail vein bleeding, it is not clear how much FIX is present in plasma of mice receiving Cas9/donor-plasmids.

Additional comment: the immunoblot displayed in Figure 2C shows expression of F9-Y381D and F9-383Stop. The mouse FIX protein is 421 amino acids long. Introduction of a stop-codon at amino acid position 383 is expected to result in a truncated FIX which is expected to migrate at a lower apparent molecular weight when compared to wild type FIX. This is not observed in Figure 2C. Please provide an explanation for the unexpected size of F9383Stop; please add a molecular weight marker to the Figure. What is the evidence that the indicated band corresponds to FIX? Inclusion of a sample of a hemophilia B (lacking FIX) mice would be highly informative.

2nd Revision - authors' response 17 February 2016

Referee #2 (Remarks):

Comment 1*: The revised manuscript has been improved. Gene correction of the F9 Y371S mutation is achieved for 0.56% of the F9 alleles. This results in shortening of the APTT (Figure 3B) and increased survival following tail-clip (Figure 3B). The amount of FIX activity and antigen in plasma has not been measured. Despite the clear hemostatic effect as measured by APTT and tail vein bleeding, it is not clear how much FIX is present in plasma of mice receiving Cas9/donor-plasmids.*

Response: As the Y381S mutation did not prolong the aPTT, we did not do any gene correction experiments on this strain of mice. Since the Elisa kit and FIX activity kit are not suitable for measurement of mouse FIX level, we could not know the exact amount of FIX in plasma of mice receiving Cas9/donor-plasmids.

Comment 2*: Additional comment: the immunoblot displayed in Figure 2C shows expression of F9-Y381D and F9-383Stop. The mouse FIX protein is 421 amino acids long. Introduction of a stop-codon at amino acid position 383 is expected to result in a truncated FIX which is expected to migrate at a lower apparent molecular weight when compared to wild type FIX. This is not observed in Figure 2C. Please provide an explanation for the unexpected size of F9383Stop; please add a molecular weight marker to the Figure. What is the evidence that the indicated band corresponds to FIX? Inclusion of a sample of a hemophilia B (lacking FIX) mice would be highly informative.*

Response: The reviewer raised a very good point. In our WB image of figure 2c, the bands of FIX in $F9^{383STOP}$ lanes are in very low condense with small molecular weight smears. We think that the faint small molecular weight bands could be the truncated FIX in $F9^{383STOP}$ strain. Since the premature stop codon greatly affect mRNA stability and protein translation, the band of FIX in $F9^{383STOP}$ strain is almost invisible. In the revision, we added a note in the figure legend of Figure 2C for explanation of the issue. We included the molecular maker and labeled the molecular weight of the blot in the revision. It is a very good suggestion to use hemophilia B (lacking FIX) mice as a control, but it is not available for us at this moment.

We sincerely hope that these revisions will satisfy the requirements requested by the reviewers, and thank you for consideration to publish our story in *EMBO Molecular Medicine*.

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: Dali Li
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2015-06039

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 $\chi 2$ 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)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We used always more than 5 mice per group for each experiments in the manuscript.The information can be find in the figure legends in page 27-30.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical methods were used to predetermine sample sizes, but our sample size is comparable to gene therapy studies for HB model in the field. The sample size is listed in figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No sample was excluded from the analysis.
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.	To minimize the effects of subjective bias, we increased the sample size of Cas9 treated mice. The information can be find in the figure legends in page 27-30.
For animal studies, include a statement about randomization even if no randomization was used.	The mice were randomly grouped for therapy. The information can be found in page 17.
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.	In aPTT test, the samples were sent to core facility for the assay.This was a double-blinding test.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For generation of heamophilia mouse model, One researcher do the microinjection and extracted the genomic DNA of F0 animals. Another group did PCR amplification and sent the fragments to the company for sequencing. For in vivo gene editing, one researcher do the aPTT assay specially without knowing the genotyping of experimental mice. Another one group raised the mice and treat them with adenovirus or naked DNA.For the precise correct efficiency studies, the samples were analyzed by the company through a double-blinding strategies.
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.The distribution of the variables in each experimental group was approximately normal.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Statistical comparisons were performed with the two-tailed unpaired Student's t-test. See methods "Statistic analysis" section,page 20.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	anti-GFP antibody: GFP(B-2), santa cruze, sc-9996 anti-actin antibody: sigma,A5441 anti-F9 antibody: proteintech, 21481-1-AP
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	293A cell lines used in this study were directly bought from ATCC. 293A have not been tested for mycoplasma contamination before use.

* 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.	Animal models used in this study is 8-10 week old mice. The strain is C57BL/6J. Mice are from Slaccas company in Shanghai.Mice were housed in standard cages in a specific pathogen-free facility on a 12 h light/dark cycle with ad libitum access to food and water. See in page 20.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments conformed to the regulations drafted by the Association for Assessment and Accreditation of Laboratory Animal Care in Shanghai and were approved by East China Normal University Center for Animal Research.See page 16.
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.	Compliant.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	All participating individuals were informed and gave documented consent prior to participation, and the studies were approved by the Human Ethics Committee of the Affiliated Hospital of Hainan Medical University.See page 16.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	The statement can be found in page 16.
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	The high throughput sequence data from this publication have been submitted to the Sequence Read Archive database and the accession number is PRJNA299277.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. 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