THE
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JOURNAL

GSTZ1-1 Deficiency Activates NRF2/IGF1R Axis in HCC via Accumulation of Oncometabolite Succinylacetone

Fan Yang, Jingjing Li, Haijun Deng, Yihao Wang, Chong Lei, Qiujie Wang, Jin Xiang, Li Liang, Jie Xia, Xuanming Pan, Xiaosong Li, Quanxin Long, Lei Chang, Ping Xu, Ailong Huang, Kai Wang, Ni Tang

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Editor: Daniel Klimmeck

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 9 April 2019

Thank you for the submission of your manuscript (EMBOJ-2019-101964) to The EMBO Journal. Your manuscript has been sent to two referees, and we have received reports from both of them, which I enclose below.

As you will see, the referees acknowledge the potential high interest and quality of your work, and they are supportive of publication of your manuscript in The EMBO Journal, pending minor revision. In more detail, referee #2 requests clarification of GSTZ1's contribution to basal conditions. Further, referee #1 asks you to complement the discussion considering the GSTZ1 substrate DCA and its usage in cancer therapies.

I judge the comments of the referees to be generally reasonable and given their interest, we are happy to invite you to revise your manuscript to address the referees' comments.

REFEREE REPORTS

Referee #1:

GSTZ1-1 plays an essential role in the tyrosine and phenylalanine catabolism pathway. This work shows that in the absence of GSTZ1-1 the intermediate succinylacetone (SA) accumulates and triggers signalling through KEAP1/NRF2/IGF-1R to promote tumour growth. While it has previously been shown that GSTZ1-1 deficiency stimulates an antioxidant response through NRF2 this work significantly extends the earlier studies with more mechanistic detail. In addition this work reveals the involvement of IGF-1R and the tumour suppressor role of GSTZ1-1. This latter discovery is novel and of interest as it demonstrates that SA is one of a few known oncometabolites. The paper is well written and the experimentation appears sound.

There is one interesting deficiency in the discussion. It is recognized by the authors that genetically determined GSTZ1-1 deficiency is rare and it is unlikely that it would be a major cause of HCC.

However dichloroacetic acid (DCA) is a substrate and mechanism based inhibitor of GSTZ1-1 (see Tzeng et al Chem. Res. Toxicol. 2000, 13, 231-236). DCA also reverses the Warburg effect in cancer cells and there is a growing literature advocating its use as a cancer treatment. Consequently if DCA inhibits GSTZ1-1 it may release the oncogenic potential of SA. Given that in cancer therapy DCA is likely to be given in combination with cytotoxic drugs it may be wise for future studies to consider the potential tumourigenicity of such combinations.

A minor point. The proper nomenclature for the protein is GSTZ1-1 and the mouse gene is Gstz1 in italics. See Mannervik et al METHODS IN ENZYMOLOGY, VOL. 401 pp1-8 2005

Referee #2:

The manuscript by Yang et al. stems from the intriguing observation that the GSTZ1 enzyme of phenylalanine catabolism is down-regulated in liver cancer. The authors propose that Succinylacetone accumulation in GSTZ1 mutant tissues leads to KEAP1 alkylation, NRF2 activation and expression of IGF1R.
The experimental approach is sound and the great quality of the data supports the author conclusions. All the immunoblot analysis, proliferation/survival assays, Mass spectrometry and cancer biopsies data are convincing and properly quantified. The findings are relevant for cancer research, spanning from mechanistic studies, metabolomics and validation in in vivo cancer models. While additional experiments could be added (as always), this study is complete as is and will be of very broad interest. I would recommend clarifying one single issue:
1) The measurements of KEAP1 alkylation (Fig. 3G) and tumor formation (Fig. 7B) are in "stressed" conditions, either after phenylalanine treatment or DEN exposure. However, GSTZ1 modulation has dramatic effects on cell proliferation and survival in basal conditions (Fig. 2). How do the authors explain these effects? What are the Succinylacetone levels in basal conditions in vitro and in vivo? What are the rates of cell proliferation/survival in GSTZ1 mutant livers prior to DEN treatment?

1st Revision - authors' response 24 May 2019

REVIEWER 1

GSTZ1-1 plays an essential role in the tyrosine and phenylalanine catabolism pathway. This work shows that in the absence of GSTZ1-1 the intermediate succinylacetone (SA) accumulates and triggers signalling through KEAP1/NRF2/IGF-1R to promote tumour growth. While it has previously been shown that GSTZ1-1 deficiency stimulates an antioxidant response through NRF2 this work significantly extends the earlier studies with more mechanistic detail. In addition this work reveals the involvement of IGF-1R and the tumour suppressor role of GSTZ1-1. This latter discovery is novel and of interest as it demonstrates that SA is one of a few known oncometabolites.
The paper is well written and the experimentation appears sound.

There is one interesting deficiency in the discussion. It is recognized by the authors that genetically determined GSTZ1-1 deficiency is rare and it is unlikely that it would be a major cause of HCC. However dichloroacetic acid (DCA) is a substrate and mechanism based inhibitor of GSTZ1-1 (see Tzeng et al Chem. Res. Toxicol. 2000, 13, 231-236). DCA also reverses the Warburg effect in cancer cells and there is a growing literature advocating its use as a cancer treatment. Consequently if DCA inhibits GSTZ1-1 it may release the oncogenic potential of SA. Given that in cancer therapy DCA is likely to be given in combination with cytotoxic drugs it may be wise for future studies to consider the potential tumourigenicity of such combinations.

Response: We thank the reviewer for the positive and constructive comments. We completely agree that in the future, studies should consider the potential role of dichloroacetic acid (DCA) in tumourigenicity. As reported previously by the International Agency for Research on Cancer (IARC), DCA induces liver cancer in both mice and rats, although the evidence suggesting carcinogenicity of DCA in humans is inadequate (IARC, 2014). Based on the reviewer's suggestion, we have now discussed this in the revised version of our manuscript (on page 17) as follows:

"Interestingly, DCA, a substrate and mechanism-based inactivator of GSTZ1-1 (Tzeng et al, 2000), also reverses the Warburg effect in cancer cells and has been proposed for use in targeted therapy against cancer (Michelakis et al, 2008). However, as previously reported by the International Agency for Research on Cancer (IARC, 2014), DCA induces liver cancer in both mice and rats (DeAngelo et al, 1999; Bull et al, 2002). Although the evidence suggesting carcinogenicity of DCA in humans is inadequate, several studies indicate that DCA exposure could lead to hepatic toxicity (Ammini & Stacpoole, 2003). According to our study, DCA may release the oncogenic potential of SA by inhibiting GSTZ1-1. Given that in cancer therapy DCA is likely to be given in combination with cytotoxic drugs, future studies should consider the potential tumourigenicity of such combinations."

A minor point. The proper nomenclature for the protein is GSTZ1-1 and the mouse gene is Gstz1 in italics. See Mannervik et al METHODS IN ENZYMOLOGY, VOL. 401 pp1-8 2005.

Response: We thank the reviewer for pointing this out. We have made the necessary changes at all relevant instances.

REVIEWER 2

The manuscript by Yang et al. stems from the intriguing observation that the GSTZ1 enzyme of phenylalanine catabolism is down-regulated in liver cancer. The authors propose that Succinylacetone accumulation in GSTZ1 mutant tissues leads to KEAP1 alkylation, NRF2 activation and expression of IGF1R.
The experimental approach is sound and the great quality of the data supports the author conclusions. All the immunoblot analysis, proliferation/survival assays, Mass spectrometry and cancer biopsies data are convincing and properly quantified. The findings are relevant for cancer research, spanning from mechanistic studies, metabolomics and validation in vivo cancer models.

Response: We thank the reviewer for the positive comments.

While additional experiments could be added (as always), this study is complete as is and will be of very broad interest. I would recommend clarifying one single issue:
1) The measurements of KEAP1 alkylation (Fig. 3G) and tumor formation (Fig. 7B) are in "stressed" conditions, either after phenylalanine treatment or DEN exposure. However, GSTZ1 modulation has dramatic effects on cell proliferation and survival in basal conditions (Fig. 2). How do the authors explain these effects?

Response: We agree and thank the reviewer for this comment. In Figure 2, we illustrated the *in vitro* experiments performed under basal conditions to show the effects of GSTZ1 modulation on hepatoma cell proliferation and survival. In Figure 3G, we illustrated that Flag-tagged KEAP1 was transfected to GSTZ1-KO HepG2 cells to measure its alkylation.

First, as highly efficient enrichment is essential for mass spectrometry (MS) analysis of post-translational modification and since specific antibody for this novel modification is not available commercially, we intended to increase the alkylation stoichiometry of KEAP1 proteins and the sensitivity of detection approaches under "stressed" conditions in GSTZ1-KO cells.

Second, the concentration of intermediate metabolites such as succinylacetone is very low in HepG2 cells in basal conditions (please see also point 2), so we treated the cells with 2.0 mM phenylalanine (Phe) to increase the burden on the Phe catabolic pathway (such as succinylacetone accumulation). In another similar study of KEAP1 alkylation, the authors also detected the modification of KEAP1 by MS under "stressed" condition. They treated cells with 4-octyl itaconate, an itaconate derivative to exacerbate the accumulation of intracellular itaconate before analyzing KEAP1 using tandem MS (Mills *et al*, Nature, 2018). Similarly, HEK293T cells were treated with exogenous methylglyoxal (MGO, 5mM) to determine the MGO modification of KEAP1 by LC-MS/MS analyses (Bollong *et al*, Nature, 2018).

Third, as shown in Figure 7B, *Gstz1*$^{-/-}$ mice were treated with DEN/CCl_4 to induce hepatocellular carcinoma (HCC), as previous studies reported that *Gstz1*$^{-/-}$ mice exhibited focal hepatitis and hepatocyte necrosis, but showed no signs of HCC (Cindy *et al*, Am. J. Pathol., 2004; Fernández-Cañón *et al*, Mol. Cell. Biol., 2002). Diethylnitrosamine (DEN) is a carcinogen that has been previously used to induce HCC in mice (Nat Rev Gastroenterol Hepatol 2018; 15(9):536-554). In our study, the mice were also subjected to a Phe overloading treatment (0.25% Phe in the drinking water) in addition to DEN to examine the roles of GSTZ1 deficiency in HCC tumorigenesis.

Lastly, our *in vitro* functional studies were performed in HCC cell lines (Fig. 2), while *in vivo* experiments were performed in mice (Fig. 7B). The cell types used in the above two experiments were different, and we focused on the role of GSTZ1 in HCC tumorigenesis and progression. So we treated mice with DEN/CCl_4 to induce HCC and added 0.25% Phe to their drinking water to induce stress and consequently increase the burden on the Phe catabolic pathway and exacerbate the accumulation of succinylacetone.

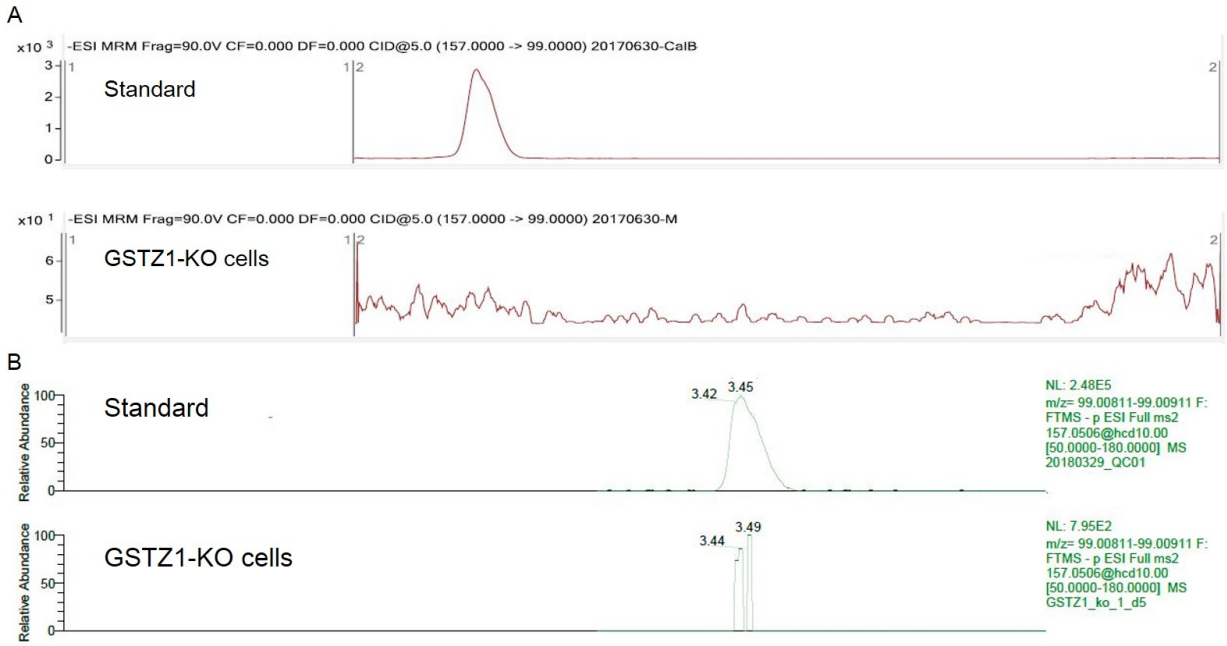
2) What are the Succinylacetone levels in basal conditions in vitro and in vivo?

Response: Thank you for this valuable comment. We attempted to determine the concentration of succinylacetone (SA) in GSTZ1-KO HepG2 cells by mass spectrometry, since we had hypothesized that endogenous SA in HepG2 cells may increase after *GSTZ1* knockout. However, the levels of SA are probably much lower in HepG2 cells, which is possibly why we failed to detect SA in these cells even after Phe overloading treatment. Additionally, SA detection in cell models was not reported as well. Since we opted not to show the original data in the paper, we have added the data here for the perusal of the reviewer (Rebuttal Fig. 1). We apologize that we were unable to determine the SA levels *in vitro* owing to technical limitations.

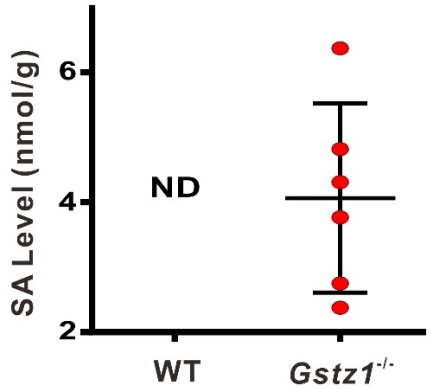
As requested, we detected SA levels in the liver tissues of wild-type (WT) and *Gstz1*$^{-/-}$ mice under basal conditions (without Phe and DEN treatment) by UHPLC-QqQ-MS. The results showed that the SA concentrations in the liver of *Gstz1*$^{-/-}$ mice was 4.07 nmol/g, whereas there was no detectable SA in the liver of WT mice (please see Rebuttal Fig. 2). These results were consistent with previously published data which stated that SA accumulated in the urine and serum of *Gstz1*$^{-/-}$ mice, as estimated by GC-MS or its capacity to inhibit δ-aminolevulinic acid dehydratase (Fernández-Cañón *et al*, Mol. Cell. Biol., 2002; Cindy *et al*, Am. J. Pathol., 2004).

3) What are the rates of cell proliferation/survival in GSTZ1 mutant livers prior to DEN treatment?

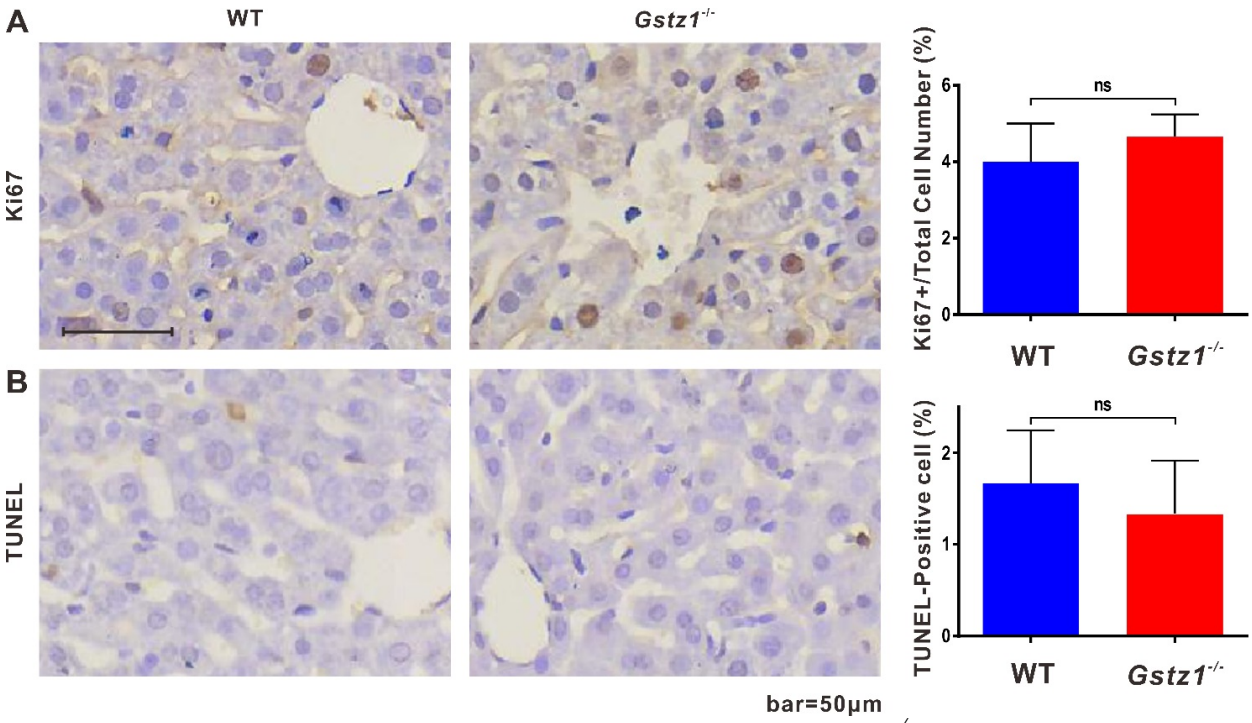
Response: We appreciate the reviewer's comment. In response to this question, we performed Ki67 proliferation assay and TUNEL apoptosis assay on the liver of WT and *Gstz1*$^{-/-}$ mice (2 weeks) without DEN treatment. Ki67-positive or TUNEL-positive cells were quantified respectively, however, no significant difference was found between WT and *Gstz1*$^{-/-}$ mice (please see Rebuttal Fig. 3). According to the results, the rates of cell proliferation/survival in *Gstz1*$^{-/-}$ mouse livers are consistent with WT mouse livers at the age of 14 days prior to DEN treatment.



Rebuttal Figure 1. Succinylacetone was not detected in GSTZ1-KO HepG2 cells by mass spectrometry. (A) UHPLC-QqQ-MS analysis and (B) UHPLC-MS/MS analysis of succinylacetone in standard solutions (top) and GSTZ1-KO HepG2 cell samples (treated with 2.0 mM phenylalanine) (bottom).



Rebuttal Figure 2. Succinylacetone concentrations in the liver tissues of wild-type (WT) and *Gstz1*$^{-/-}$ mice under basal conditions. Data are presented as mean ± SD (n=6).



Rebuttal Figure 3. Cell proliferation and survival in wild-type (WT) and *Gstz1*$^{-/-}$ mice livers prior to DEN treatment. (A) Ki67 proliferation assay. (B) TUNEL apoptosis assay. Quantification of Ki67-positive or TUNEL-positive cells are shown. Livers of WT and KO mice were isolated at 14 days of age without DEN treatment. The number of mice is 3 for each group. Data are expressed as mean ± SD. *ns*, no statistical significance.

2nd Editorial Decision enter date

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and also asked referee #2 to re-assess your revised study (please see his/her comment enclosed below). In light of all these information, I have now concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

REFEREE REPORT

Referee #2:

The authors addressed my minor issues.

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: Ailong Huang, Kai Wang, Ni Tang
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2019-101964

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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?	For in vitro cell experiments, each sample is performed in at least triplicate. The sample size of animal experiment was at least 6. See the "Materials and Methods" and "Appendix Supplementary Methods" sections for details.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See the "Materials and Methods" and "Appendix Supplementary Methods" sections for details.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	See the "Materials and Methods" and "Appendix Supplementary Methods" sections for details.
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.	All mice/samples and data collected were processed using the same procedures independently of their treatment received. Mice were age matched for the independent experiment and equally distributed for sex between the different treatment groups.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were randomly pooled according to sex from an established breeding colony.
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 randomly chosen from an established breeding colony. Researcher performing the immunostaining on mouse tumor tissues was blinded for sample staining and interpretation.
4.b. For animal studies, include a statement about blinding even if no blinding was done	All mice and data collected were processed using the same procedures independently of their treatment received.
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.	Statistical data meets the assumptions. Statistical methods are shown in the Figure legends.
Is there an estimate of variation within each group of data?	Yes, we included error bars for all graphs.
Is the variance similar between the groups that are being statistically compared?	Yes

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).	Antibody list and nucleotide sequences are provided in Appendix section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines used in this study were purchased from ATCC or stored in the laboratory. These cell lines have been tested for mycoplasma contamination and confirmed negative.

* 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.	Heterozygous 129-Gstz1tm1Jmfc/Cnbc mice (EM:04481) were purchased from the European Mouse Mutant Archive via Nanjing Biomedical Research Institute of Nanjing University, and were crossed to breed wild type (WT) and Gstz1-/- mice. All mice were maintained under specific pathogen-free conditions in the laboratory animal center of Chongqing Medical University.The male nude mice aged 4-6 weeks were obtained from Experimental Animal Center of Chongqing Medical University.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal procedures were performed according to protocols approved by the Rules for Animal Experiments published by the Chinese government. All procedures were also approved by the Research Ethics Committee of Chongqing Medical University (reference number: 2017010).
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.	Animal experimentations were done in compliance with ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Sampling was conducted with the approval of the Research Ethics Committee of Chongqing Medical University.
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.	Informed consent was obtained from participating patients.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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 RNA-seq data from this publication have been deposited to the GEO database [https://www.ncbi.nlm.nih.gov/geo] and assigned the identifier [GSE117822]
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).	N/A
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).	N/A
21. 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.	N/A

G- Dual use research of concern

22. 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.	N/A