EMBO
Molecular Medicine

Mitochondrial MsrB2 serves as a switch and transducer for mitophagy

Seung Hee Lee, Suho Lee, Jing Du, Kanika Jain, Min Ding, Anis J Kadado, Gourg Atteya, Zainab Jaji, Tarun Tyagi, Won-ho Kim, Raimund I Herzog, Amar Patel, Costin N. Ionescu, Kathleen A Martin and John Hwa.

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Transaction Report:

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

1st Editorial Decision 25th March 2019

Thank you for the submission of your manuscript to EMBO Molecular Medicine. I apologise for the delay in reaching a decision. Although I was hoping to obtain a third evaluation, this referee is very late and never replied to our chasers. I am now proceeding based on the two consistent evaluations obtained so far as further delays cannot be justified.

You will see that the evaluations are positive and both referees have minor comments only that still need to be addressed in the next version of your article. Please note that depending on the nature of the revisions, this may be sent back to the referees for another round of review

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REFEREE REPORTS

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

Lee et al. dissect the role of the mitochondria matrix protein MsrB2 in regulating platelet mitophagy in high oxidant tone conditions such as diabetes. They identify MrsB2 by directional co-immunoprecipitation with LC3, a central protein in autophagosome formation, and colocalization studies in platelets from diabetic patients. These platelets had elevated levels of MrsB2 compared to healthy controls. Using multiple complimentary knockdown and knockout strategies in vitro and in vivo, the authors demonstrate that MrsB2 interacting with LC3 plays a functional role in mitophagy. They show that the E3 ubiquitin ligase parkin is an MsrB2 target for reduction of oxidized methionine in platelet mitochondria. They go on to demonstrate that MsrB2 in turn is ubiquinated by parkin allowing it to interact with LC3. Importantly, they show that platelet mitochondria damage induced by oxidant stress in diabetes, results in MsrB2 release from the mitochondrial matrix to the cytosol, where it interacts with parkin and LC3 to induce mitophagy as a protective mechanism. Finally, they use platelets from Parkinson patients to illustrate the reverse case, the impact of reduction of MsrB2 function.

Referee #1 (Remarks for Author):

The manuscript is excellently written and provides important and novel insights into how mitophagy

protects platelets in high oxidant stress conditions, such as diabetes. The work shows very elegantly that MrsB2 acts as a switch to activate parkin and plays a direct role in mediating mitophagy. An intriguing translational implication is that MsrB2 may be targeted therapeutically to enhance protective mitophagy in oxidative stress diseases such as diabetes and or neurodegenrative diseases. I have no major criticism. The technical quality is outstanding.

Minor points:
1) Typo: first sentence of results: "...of cristae..."
2) Should it say "knockdown" instead of "knockout" in the results section? "....(Fig. 2B). Given our ex vivo immunoprecipitation results demonstrating an interaction between MsrB2 and LC3, and our in vitro knockout results supporting a role for MsrB2 in mitophagy and thus preventing apoptosis, we then assessed for platelet apoptosis, in vivo."
3) The mass spectrum of MetO (shown in figure 5E) is mislabeled as 4E in the results section.
4) Please explain the annotation of parent peptide M/Z and highlighted peaks in the mass spectra in the figure legend. Consider showing carbamidomethylated forms of the parent peptide and annotated b2 ions for all three conditions.

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

A new interaction is shown in multiple ways using different approaches, including platelets from healthy donors and patients as well as genetically modified mouse studies.

Referee #2 (Remarks for Author):

Autophagy contributes to the maintenance of intracellular homeostasis in a range of vascular cells including cardiomyocytes, endothelial cells, and arterial smooth muscle cells. Mitophagy is an autophagic response that specifically targets damaged cytotoxic mitochondria. In a high oxidative stress environment such as found in diabetes, how selective removal of a damaged mitochondria is achieved remains unclear. This clearly written manuscript from a group of researchers with expertise in this area describes the release of the enzyme methionine sulfoxide reductase (Msr) B2 from damaged mitochondria, initiating autophagosome formation. The overall conclusion is that MsrB2 can act on Parkin, reducing the oxidized (inactive) form of Parkin.
An enormous amount of data is presented in the MS and supplementary files. The major strengths of the work are the novel findings and identification of MsrB2 as a molecular link between mitochondrial damage and induction of mitophagy and description of the interaction between MsrB2 and LC3 in platelets from patients with diabetes mellitus. This was shown in experiments using either MsrB2 or LC3 antibodies for IP followed by mass spectrometry; a role of MsrB2 to prevent aggregate formation of Parkin protein in the outer mitochondrial membrane is supported by data gained from both genetically modified mice and in vitro experiments. Authors have used platelets from both DM patients and patients with Parkinson's disease to demonstrate the importance of MsrB2 and that this pathway is important in pathophysiological contexts.

Specific points
How prevalent is the LC3 interacting motif in other proteins and across across biology? For example, the autophagy adaptor p62 (SQSTM1) also carries an LC3 binding domain. Do levels of p62 increase in DM platelets?

It would be of interest to evaluate MsrB2, LC3 and Parkin localization in DM platelets treated with mitoquinone or similar.

Given that levels of MsrB2 are elevated in diabetic platelets, can the authors be sure that the interaction is induced as a consequence of these altered levels. This might confound interpretation of experiments using confocal co-localization imaging.

The data is consistent with a novel regulatory mechanism for oxidative stress-induced mitophagy. Do other types of cellular stress also trigger the same pathway?
What happens to the level of Parkin-mediated/mitochondrial ubiquitinylation in diabetic platelets? While multiple apoptotic markers and an apoptosis array were used, the authors have used only one

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mitophagy marker LC3 to measure mitophagy triggered by changes in MsrB2 expression in DM platelets. Was mitochondrial clustering or then the role of MsrB2 in mitophagy would be more convincing.

The Authors have described the various statistical approaches that have been used in the study but it is not clear which statistical test has been used on which data set. It would be helpful to add this information to respective figure legends.

Other comments
The abstract contains a number of undefined abbreviations eg LO, ROS (an ROS?) Parkin MetO
Final sentence of introduction, "not only to be confined" is very confusing. Suggest replace with "This mechanism appears to occur in other nucleated cells".
The quality of the western blot in Fig 1B is not as good as later blots with this antibody. The authors could provide a densitometry analysis of the Figure 1B western blot to better differentiate between levels of LC3 in DM samples versus IgG control

Bar graphs showing the mean data of several replicates should always display the individual points e.g. 3E, 3A, 3C, 4D , 6B etc

We thank the Editor and the Reviewer's for the thorough and insightful review of the manuscript. We are particularly pleased that the mechanistic as well as the clinical relevance to disease processes were appreciated. We would like to present a point-by-point response to the remaining concerns raised by the Reviewers. All changes have been highlighted in the manuscript.

Referee #1

The manuscript is excellently written and provides important and novel insights into how mitophagy protects platelets in high oxidant stress conditions, such as diabetes. The work shows very elegantly that MrsB2 acts as a switch to activate parkin and plays a direct role in mediating mitophagy. An intriguing translational implication is that MsrB2 may be targeted therapeutically to enhance protective mitophagy in oxidative stress diseases such as diabetes and or neurodegenerative diseases. I have no major criticism. The technical quality is outstanding.

We thank the Reviewer for the insightful evaluation and for identifying important remaining concerns.

Minor points:
1) Typo: first sentence of results: "...of cristae..."
This has now been corrected

2) Should it say "knockdown" instead of "knockout" in the results section? "....(Fig. 2B). Given our ex vivo immunoprecipitation results demonstrating an interaction between MsrB2 and LC3, and our in vitro knockout results supporting a role for MsrB2 in mitophagy and thus preventing apoptosis, we then assessed for platelet apoptosis, in vivo."
We have replaced "knockout" with "knockdown".

3) The mass spectrum of MetO (shown in figure 5E) is mislabeled as 4E in the results section.
This has now been corrected.

4) Please explain the annotation of parent peptide M/Z and highlighted peaks in the mass spectra in the figure legend. Consider showing carbamidomethylated forms of the parent peptide and annotated b2 ions for all three conditions.
Thanks for highlighting this oversight on our part. The explanation and forms have now been provided. Representative mass spectrometry analysis demonstrating a healthy control (HC) Parkin peptide (containing Met192) of 769 (M/Z, mass/charge), and oxidative stressed peptide of 735 (M/Z) and DM peptide of 734 (M/Z). Fragmentation identified an a(2) of 191 in healthy control (HC) subjects. The b(2) fragment identified (in H_2O_2 treated and DM patients) has the a(2) fragment (mw 191) plus C=O (mw 12+16) plus the O on Met192 (mw 16) giving a final molecular weight of 235. This has now been added to the figure legend.

Referee #2:

Autophagy contributes to the maintenance of intracellular homeostasis in a range of vascular cells including cardiomyocytes, endothelial cells, and arterial smooth muscle cells. Mitophagy is an autophagic response that specifically targets damaged cytotoxic mitochondria. In a high oxidative stress environment such as found in diabetes, how selective removal of a damaged mitochondria is achieved remains unclear. This clearly written manuscript from a group of researchers with expertise in this area describes the release of the enzyme methionine sulfoxide reductase (Msr) B2 from damaged mitochondria, initiating autophagosome formation. The overall conclusion is that MsrB2 can act on Parkin, reducing the oxidized (inactive) form of Parkin. An enormous amount of data is presented in the MS and supplementary files. The major strengths of the work are the novel findings and identification of MsrB2 as a molecular link between mitochondrial damage and induction of mitophagy and description of the interaction between MsrB2 and LC3 in platelets from patients with diabetes mellitus. This was shown in experiments using either MsrB2 or LC3 antibodies for IP followed by mass spectrometry; a role of MsrB2 to prevent aggregate formation of Parkin protein in the outer mitochondrial membrane is supported by data gained from both genetically modified mice and in vitro experiments. Authors have used platelets from both DM patients and patients with Parkinson's disease to demonstrate the importance of MsrB2 and that this pathway is important in pathophysiological contexts.

We thank the Reviewer for the thorough review and the important remaining concerns.

Specific points.

1) How prevalent is the LC3 interacting motif in other proteins and across across biology? For example, the autophagy adaptor p62 (SQSTM1) also carries an LC3 binding domain. Do levels of p62 increase in DM platelets?

This is an important point as highlighted by the Reviewer. LC3 interacting motifs (LIRs) have a number of configurations.

LC3 interacting motifs (LIFs)		
W Y F	X1X2	L V I

LIRs can be found in diverse proteins[1-7]. Meticulous evaluation is needed to confirm the motif to be relevant and real. We have checked p62 in DM platelets and observed no significant change. The explanation may be differences in platelet autophagy response to stress compared to nucleated cells. We have previously observed differences in the mitophagy machinery in platelets[8].

2) It would be of interest to evaluate MsrB2, LC3 and Parkin localization in DM platelets treated with mitoquinone or similar.

This is a great suggestion. We have used N-acetylcysteine (NAC) for our experiments and demonstrated a reduction in oxidative stress induced mitophagy (**Fig. 2A, Appendix Fig. 4A, 4B and 4C**). We now further demonstrate that Parkin, LC3 and MsrB2 colocalization **(Reviewer's response Figure 1A &1B)** as well as ubiquitin, Parkin and MsrB2 colocalization **(Reviewer's response Figure 1C &1D)** are reduced in NAC treated platelets.

3) Given that levels of MsrB2 are elevated in diabetic platelets, can the authors be sure that the interaction is induced as a consequence of these altered levels. This might confound interpretation of experiments using confocal co-localization imaging.

The Reviewer has raised an excellent point. We provide evidence for MsrB2 induction in diabetic platelets and confirm increased colocalization among MsrB2, Parkin and LC3 (**Fig 4C and D**). Moreover, we confirmed that interaction between Parkin and MsrB2 through IP experiments (**Fig 4A and B**). For the healthy control where MsrB2 is not induced, reduction of MsrB2 with shMsrB2 (**Figure 2**), platelet selective MsrB2 knockout mice (**Figure 3**), and Parkinson's disease patients (**Figure 8**) there appears to be reduced mitophagy and enhanced apoptosis. Multiple approaches were needed to answer this important concern which we also had.

4) The data is consistent with a novel regulatory mechanism for oxidative stress-induced mitophagy. Do other types of cellular stress also trigger the same pathway?

We have been exploring other cells including cardiomyocytes, particularly myoblast (H9C2 cells) (**Appendix Fig 5**). Active LC3 (LC3II) is increased in MsrB2 overexpression in H9C2 cells **(Reviewer's response figure 2A)**. We additionally confirm an interaction between MsrB2, Parkin and LC3 using HEK293 cells **(Reviewer's response figure 2B and 2C).** Moreover, endothelin-1 (ET-1, cardiac damage inducer) increased MsrB2 expression and LC3 activation **(Reviewer's response figure 2D)**. Functional studies are currently being performed on cardiomyocytes and H9C2 cells. Our data for Parkinson's disease (**Figure 8**) also suggests that such a process (MsrB2 regulation of Parkin mediated mitophagy) may also be important for the central nervous system. We therefore believe that this process is likely to be found in many cells.

5) What happens to the level of Parkin-mediated/mitochondrial ubiquitinylation in diabetic platelets?

This was also an important question that we had to address. Parkin mediated ubiquitination of MsrB2 is increased in diabetic platelets following MsrB2 induction (**Figure 6A**). Reduction of oxidized Parkin recovers Parkin's function as a ubiquitin E3 ligase.

6) While multiple apoptotic markers and an apoptosis array were used, the authors have used only one mitophagy marker LC3 to measure mitophagy triggered by changes in MsrB2 expression in DM platelets. Was mitochondrial clustering or then the role of MsrB2 in mitophagy would be more convincing.

We have confirmed the induction of several autophagy components in a previous reports [8]. Beclin1, ATG3, ATG7 and ATG12-5 complex were all increased in DM patients (**Reviewer's response Figure 3** [8]). Mitophagy related protein (PINK1 and Parkin) were also increased in DM platelets **(Reviewer's response Figure 3)**. With MsrB2 overexpression, there is increased LC3 II (active form of LC3, lipidated form).

7) The Authors have described the various statistical approaches that have been used in the study but it is not clear which statistical test has been used on which data set. It would be helpful to add this information to respective figure legends.

We have now added the statistical tests to the figures.

Other comments

1) The abstract contains a number of undefined abbreviations eg LO, ROS (an ROS?) Parkin MetO

We have now defined the abbreviations in the abstract.

2) Final sentence of introduction, "not only to be confined" is very confusing. Suggest replace with "This mechanism appears to occur in other nucleated cells".

The sentence has now been changed.

3) The quality of the western blot in Fig 1B is not as good as later blots with this antibody. The authors could provide a densitometry analysis of the Figure 1B western blot to better differentiate between levels of LC3 in DM samples versus IgG control.

We have now provided an improved figure. This is an immunoprecipitation for a native protein interaction in platelets, and thus the signal was relatively weak compared to overexpression IPs. Multiple other experiments were thus necessary to confirm this interaction (**Fig 1C and Fig 1D**).

4) Bar graphs showing the mean data of several replicates should always display the individual points e.g. 3E, 3A, 3C, 4, 6B etc.

We have now changed the figure formats.

REFERENCES

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2nd Editorial Decision 6th June 2019

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referee who was asked to re-assess it. As you will see the reviewer is now supportive and I am pleased to inform you that we will be able to accept your manuscript pending editorial final amendments.

REFEREE REPORTS.

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

stated in previous review

Referee #2 (Remarks for Author):

Thank you for the revision and discussion of my points. Highly interesting paper.

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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: John Hwa, Seung Hee Lee

Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2019-10409

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

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

USEFUL LINKS FOR COMPLETING THIS FORM

Link	Resource
http://www.antibodypedia.com	Antibodypedia
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http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
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http://biomodels.net/miriam/	MIRIAM Guidelines
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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Power analysis was performed under the supervision of an expert statistician. For the human sample study, we used individual healthy donor platelets (more n=3), and compared them with patient platelets(more n=11) using Western blotting (Fig 5). Reduction of mitochondria membrane potential, apoptosis and autophagy generated in almost all DM patient platelet, so we used 3 to 5 samples for EM, immunostaing, FACS experiments (Fig1-4). Such sample sizes were adequate to determine significant differences between the means. Further corroboration was performed with alternative techniques (i.e. flow cytometry and confocal microscopy) in addition to chemical and genetic inhibition. Each of these experiments were also adequately powered.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Venous blood was drawn from healthy and diseased patients at Yale University School of Medicine (HIC#1005006865) from multiple outpatient clinics including the cardiovascular, diabetes, and neurology clinics.Power studies were also performed for the animal studies. Blood was drawn from each group (WT, MsrB2 fl/fl, MsrB2 Ko (wholebody and heart specific mice). In fig3A-F, Blood was drawn from MsrB2 fl/fl (n=5), MsrB2 Ko(n=6). In fig3G and H, we drawed blood from 3 mice in each group. We often repeated experiments more than 2 times in addition to confirming our results with multiple approaches as described with the human studies.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	The mouse studies were consecutive bred mice. Only mice that were extremely sick (unable to handle procedures) were excluded from the studies. The human patients were also consecutive recruited patients. Those patients on medications or with concurrent diseases (e.g. inflammatory disease, sepsis) were excluded as this could affect platelet function. The criterias were all preestablished.
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.	Human subject and mice used for the studies were not preselected. The human subjects were consecutive recruitments from the diabetic clinic. The mice were randomly assigned to induction of diabetes mellitus. The experimentor was blinded to the level of blood glucose. Moreover as described, further validation of experiments were performed using different approaches i.e. chemical inhibition, chemical activation, and genetic knockout. The combination of multiple randomized groups using multiple approaches reduced the bias.
For animal studies, include a statement about randomization even if no randomization was used.	For the animal studies the mice were randomly selected for induction of diabetes mellitus and experiments performed without the knowledge of blood glucose levels.
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.	The investigator was blinded to the induction of diabetes mellitus and the blood glucose. Mice were randomly selected for experimentation.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The investigator was blinded to the blood glucose levels indicative of whether the mouse was diabetic or not.
5. For every figure, are statistical tests justified as appropriate?	Each experiment was carefuly designed and analyzed with standard and accepted statistical analysis used for such studies. We further validated the results using independent complementary experiments also rigorously statistically anlyzed.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	To confirm of assumption, we did repetitive experiment (three times) or increased sample size as needed for statistical power. All data were expressed as mean±SD. The nonparametric t test was performed for comparisons of 2 groups. Analysis was performed with Prism software (GraphPad Software, Inc, La Jolla, CA).A difference of *P<0.05 was considered significant. we mwntioned all p values in main documents
Is there an estimate of variation within each group of data?	The mice were from the same genetic background and were often siblings and thus there was no significant variance within the groups. Any differences would therefore be directly related to treatment. The experiment were corroborated using other groups of mice.
Is the variance similar between the groups that are being statistically compared?	yes, there are similar varication between in each group.

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).	We have developed a table for all the antibodies described (Appendix Table 1 and 2)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We used Human megakaryocytes (Meg-01, ATCC: CRL-2021) in this study. These lines have been cheacked for mycoplasma contamination and are 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.	C57BL/6, male and female, WT, MsrB2 fl/fl, MsrB2 heart specific KO and wholebody KO. We ordered the WT C57Bl/6 from Jackson laboratory and drew blood from the heart after HFD (feed High fat diet for 12 wks protocol #11413)or not. The animals (C57BL/6, male and female, WT, MsrB2 fl/fl, MsrB2 heart specific KO)were housed at Yale Animal facility 300 George St. New Haven, CT under the supervision of YARC and Rita Weber (Animal facility manager Yale CVRC). The animals (wholebody MsrB2 KO) were generated at TGIM
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We have an approved animal protocol #11413 (Yale IACUC) . As per approved protocol the mice were exanguinated under deep anesthesia with high dose ketamine/xylazine or isoflurane, and followed by cervical dislocation to assure death. This method is consistent with AVMA guidelines.
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.	We have followed all guidelines as rigorously set by Yale IACUC.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Yale Human Investigation Committee (protocol# 1005006865)
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 each subject and conform to the rpinciples set oout in the WMA Declaration of Helsinki and the Belmont Report. These are requirement for the Yale HIC.
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.	All data and sample use were specifically consented for by each subject. No studies were performed outside of what was consented for.
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	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).	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