Inducing mitophagy in diabetic platelets protects against severe oxidative stress

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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: Céline Carret

1st Editorial Decision 06 December 2016

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript. Although the referees find the study to be of potential interest, they also raise a number of concerns that need to be addressed in the next final version of your article.

You will see that both referees are rather positive about the study while still highlighting some shortcomings that if satisfactorily addressed would improve the conclusiveness of the findings and provide additional understanding of the process. In addition, both referees spotted some text inconsistencies, and these should be corrected.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can address the issues that have been raised within the 3-months constraints of a revision time. Please note that it is EMBO Molecular Medicine policy to allow only a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

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. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published we may not be able to extend the revision period beyond three months.

I look forward to receiving your revised manuscript.

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

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

The authors utilize human subjects as well as a mouse model. These models are adequate, although there are some unanswered questions regarding the phenotype of the mouse model, which I raise in my review.

Referee #1 (Remarks):

The manuscript by Lee et al, describes a series of studies in platelets isolated from human subjects, demonstrating a higher basal level of both ROS and autophagy/mitophagy in diabetic compared to healthy individuals. The authors provide substantial evidence for increased levels of mitochondrial damage in DM (diabetic mellitus) platelets and convincing evidence for increased mitophagy in these cells. The data presented are consistent with the model proposed in which platelets have an autonomous program for activating autophagy which is mediated by increased JNK activation in response to ROS, to recycle damaged mitochondria and ameliorate ROS in the cell. Further corroborating evidence in support of this model is obtained in mice where ablation of key mitophagy genes in mice (Parkin and PINK1) results in dramatic decrease in the time to occlusion of blood vessels in response to chemical challenge. The authors provide substantial evidence for the induction of mitophagy (including EM, confocal microscopy, biochemical analyses of autophagy/mitophagy markers) and have included key experimental controls throughout the manuscript. The data presented in general support the conclusions; however, there are some issues with data interpretation and unanswered questions, especially in terms of the pathways leading to mitophagy in response to ROS in platelets, which detract from overall impact.

Major issues:

1. Although a large number of autophagic markers are analyzed in healthy and DM platelets, the key upstream initiator of autophagy (Atg1/Ulk1) is not studied. Phosphorylation of Ulk1 needs to be analyzed.
2. If, according to the authors' model and as explained in the text referring to Figure 7, platelets from DM patients have higher levels of ROS and mitophagy, why are there higher levels of p-p53 (as shown in Figure 1), since mitophagy should conceivably degrade the mt-localized p53 ?
3. In experiments attempting to link increased ROS with mitophagy and p-JNK, the authors use normal (healthy) platelets, treat them with H2O2 and then reverse the observed changes with NAC. However, they also need to demonstrate that in DM platelets which have elevated ROS, treatment with NAC reverses the observed increases in these ROS markers.
4. The best readout for increased mitophagy is a decrease in overall mitochondrial DNA content. This assay needs to be incorporated in the analysis of both human and mouse platelets.
5. 3MA is a rather crude inhibitor of autophagy and has toxic effects in many cells. Additional, more specific inhibitors such as Spautin-1 or chloroquine (a fusion inhibitor) should be used in at least a few of these studies.
6. A key question form the mouse data is why do the platelets from the PINK-/- mice (and presumably also from the Parkin -/- mice) show such a dramatic propensity to aggregate and cause vessel occlusion in such a short time (1-2 minutes) following chemical challenge. Some additional biochemical data is needed to demonstrate which property of these platelets is compromised. If these platelets are more apoptotically sensitive (compared to those form WT animals) upon FeCl2 challenge, this needs to be demonstrated. Moreover, can this sensitivity be reversed by treating mice with NAC (which can be administered in vivo)?

Minor points:

1. The results section can be shortened by moving some portions to the discussion, which is rather

short. For instance, there is a whole introductory paragraph in the results section before Fig.1 which is not necessary.
2. In the immunoblots showing patient samples, a legend is needed indicating the patient number (or code).
3. In Fig. 1E, the different numbers in each quadrant of the flow image is too small to discern.
4. There's a reference in the results section (top pg. 7) which is in a number format (instead of author name format).
5. The sentence "As a control, and CCCP is recognized...." on pg. 11 does not make sense, needs reformatting.

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

This paper provides convincing data, authors have performed substantial work of quality, from observations in patients to molecular mechanistic insights with also the use of murine models, thus deserving publication in EMBO Molecular Medicine. However, some important drawbacks that I raised should be addressed for improvement before publication. Therefore, I recommend acceptance following revision.

Referee #2 (Remarks):

This paper by Lee and coll. entitled "The induction of mitophagy in diabetic platelets is protective against severe oxidative stress" investigates the importance of mitophagy in removal of damaged mitochondria in platelets in diabetic conditions (high glycemia) to prevent impaired thrombosis. This issue is crucial from a clinical point of view, and authors provide convincing evidence for underlying mechanisms. However, several points should be addressed.

- The most important point in my opinion is that authors claim that elevated ROS during DM is the cause of mitochondrial dysfunction promoting mitophagy, but they do not address the possibility that ROS increase in DM platelets could be the consequence of mitochondrial dysfunction. Could high glucose rate directly affect mitochondria?
- The paragraph at the beginning of the Results section is more a summary than an introductive paragraph thus useless.
- Results Paragraph 1 and Figure 1. The protein p53 is activated through post-translational modifications including phosphorylation on different sites: why authors do test only phosphorylation of serine 15? Fig.1I: show also % of healthy and damaged mitochondria in the HC cohort. Fig. S1: for HC n=4 in text but n= 5 in the legend of Fig.S1. Mitotracker: please precise which probe was used because some are not washed out of the cells when the mitochondrion's membrane potential is lost. Page 6: precise what is EM. ROS level is measured using dyes (which ones?) from Invitrogen, but kit from Enzo is mentioned in Methods section: please indicate precisely what was used.
- Results Paragraph 2 and Figure 2. Fig.2A&B: assess also BNIP3 and Nix in WB blots experiments. Fig.2C&D: is there autophagic vacuoles in HC?
- Results Paragraph 3 and Figure 3. The mTOR pathway actually inhibits autophagy, thus to completely get rid of its impact in platelets, other molecules should be evaluated such as S6K (reduction of its phosphorylation is expected) or use Rapamycin as autophagy inducer for example in the context of experiments shown in Figure 4.
- Results Paragraph 5 and Figures 5 and 6. The transition towards murine model is poor. Why experiments done with PINK1 KO mice (Figure 6) are different from experiments shown in Figure 5 using PARKIN KO mice? Also the experiments shown in Figure 6 are specific to the platelets research field thus not enough explained for a broad audience. What are PINK -/- DM mice?
- The manuscript is overall well written. However, some inaccuracies in the text bother somewhere the reading. In the reference to the Figures: page 7 bottom "(Supplementary Figure 3D)" instead of "(Supplementary Figure 3A)", page 11 "(Figure 5C-5D)" instead of "(Figure 4C-4D)". Page 9 "...3 fold change in H2O2 vs. HC..." instead of "...3 fold change in DM vs. HC ...". "the amount of energy required for mitophagy into the platelet is considerable ....." "prepackaged in the absence of a nucleus in platelets" the authors point to these notions at several locations in the Results section text (twice in Page 10) which is redundant and somehow useless.

- References: some references are incomplete, or in double (Kaneto 2010), or cited in the text as a number ("11" page 7), or without the year, or page 6 reference Michelle L. Boland 2013 not in the References list.... Please be more careful.

1st Revision - authors' response 10 March 2016

Response to Reviewer's

We thank the Reviewer's and the Editor for giving us the opportunity to revise our manuscript. We are grateful for the insightful and helpful comments provided by the Reviewer's that have significantly strengthened the manuscript. In response to the Reviewer's concerns we have reorganized the manuscript to improve clarity. Of particular importance, we performed many additional experiments in response to the Reviewer's concerns. The changes have been highlighted in the manuscript.

Referee #1:

We thank the Reviewer for the excellent comments and suggestions. We now provide additional experiments as requested by the Reviewer.

1. Although a large number of autophagic markers are analyzed in healthy and DM platelets, the key upstream initiator of autophagy (Atg1/Ulk1) is not studied. Phosphorylation of Ulk1 needs to be analyzed.
*We thank the Reviewer for this excellent suggestion. Western blot analysis using Ulk and pUlk1(Ser757) antibodies demonstrated no significant change in diabetic human platelets (**Appendix Figure Supplement 6A**). Previous studies have suggested that phosphorylation of Ulk at Ser757 is induced by mTOR (Russell, Tian et al. 2013). Our results support that activation of mTOR is unchanged in DM platelets. As further support downstream targets of mTOR, p70S6K and S6 were also not activated in DM platelets (**Appendix Figure Supplement 6B**). These additional studies provide support that mTOR is not significantly involved in this DM platelet induced mitophagy pathway.*

2. If, according to the authors' model and as explained in the text referring to Figure 7, platelets from DM patients have higher levels of ROS and mitophagy, why are there higher levels of p-p53 (as shown in Figure 1), since mitophagy should conceivably degrade the mt-localized p53 ?
*The Reviewer highlights a very insightful and important concern. We were also initially perplexed by the high pp53 in the DM patients and mice in spite of the active mitophagy process. We believe that the key is acute versus chronic oxidative stress. Acute oxidative stress in normal platelets can be substantially reversed by mitophagy whereas DM platelets undergo continuing substantial stress arising from poorly controlled sugars, lipids, e.t.c. Thus mitophagy is only able to degrade and protect from pp53 to a degree, due to this high continuing oxidative stress associated with DM. In support of this, the absence of mitophagy (chemical or genetic inhibition) in DM, as also demonstrated with our mitophagy knockout studies, demonstrated further elevations of pp53 (**Figure 4C-4F** and **Figure 6A**). Many of the DM patients are very poorly controlled thus in spite of enhanced mitophagy there is still substantial pp53 and apoptosis occurring.*

3. In experiments attempting to link increased ROS with mitophagy and p-JNK, the authors use normal (healthy) platelets, treat them with H_2O_2 and then reverse the observed changes with NAC. However, they also need to demonstrate that in DM platelets which have elevated ROS, treatment with NAC reverses the observed increases in these ROS markers.
*In response to the Reviewer's comments we have now treated DM mice in vivo with NAC (15mg/kg intraperitoneally for 1 hour). This demonstrates a clear reduction in ROS and a reduction in platelet apoptosis (**Appendix Figure Supplement 12A and 12B**). The reduced ROS and JNK activation leads to no significant induction of mitophagy (**Appendix Figure Supplement 12C**). As now described, this demonstrates the key importance of high levels of ROS in the induction of the protective mitophagy process (**Figure 7**).*

4. The best readout for increased mitophagy is a decrease in overall mitochondrial DNA content. This assay needs to be incorporated in the analysis of both human and mouse platelets.

*This is an excellent suggestion from the Reviewer. To assess mitochondrial DNA content, we quantitated the human mitochondrial DNA for ND1 (NADH dehydrogenase subunit 1) and 16sRNA expression using specific primers in the human sample (Suliman, Carraway et al. 2007, N Dasgupta 2015) (**Appendix Figure Supplement 5**). We additionally quantified the mouse mitochondrial DNA for ND1 (**Appendix Figure Supplement 5**). All data was normalized to 18s RNA.*

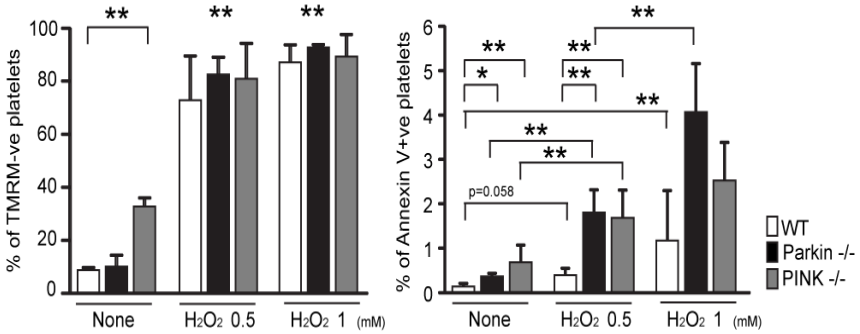
There were significant reductions with both human and mice platelet DM mitochondria contents when compared to healthy controls consistent with increased mitophagy.

5. 3MA is a rather crude inhibitor of autophagy and has toxic effects in many cells. Additional, more specific inhibitors such as Spautin-1 or chloroquine (a fusion inhibitor) should be used in at least a few of these studies.

*This is an excellent suggestion from the Reviewer. Platelets from three DM patients were treated with 20μM Spautin-1 for 2h (**Appendix Figure Supplement 9**). Consistent with our previous results the inhibition of autophagy as observed with reduced LC3II resulted in increased pp53.*

6. A key question form the mouse data is why do the platelets from the PINK1-/- mice (and presumably also from the Parkin -/- mice) show such a dramatic propensity to aggregate and cause vessel occlusion in such a short time (1-2 minutes) following chemical challenge. Some additional biochemical data is needed to demonstrate which property of these platelets is compromised. If these platelets are more apoptotically sensitive (compared to those form WT animals) upon FeCl2 challenge, this needs to be demonstrated. Moreover, can this sensitivity be reversed by treating mice with NAC (which can be administered in vivo)?

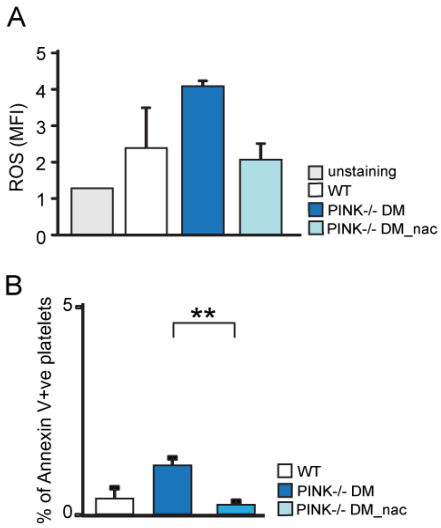
*We agree with the Reviewer and were also surprised with the dramatic nature of the vessel occlusion. It is recognized that apoptotic platelets induce clotting 50 to 100 times faster, the presence of phosphatidylserine on their surface (as detected by annexin V) acts as a catalytic site for clotting enzymes assembly and thrombin generation (Sinauridze, Kireev et al. 2007, Lannan, Phipps et al. 2014). Thus apoptosis of platelets is key to enhanced thrombosis associated with DM in the absence of mitophagy. As outlined by the Reviewer it is important to demonstrate whether these platelets from the knockout mice (mitophagy deficient) are more apoptotically sensitive and can potentially be reversed with NAC. When challenged with H_2O_2 (0.5 mM) the PINK1-/- mice show markedly enhanced annexin V staining (**Appendix Figure Supplement 11C**). This increased apoptosis sensitivity is also observed in the Parkin -/- mouse (**Figure 5I**). To confirm the increased sensitivity to apoptosis we have repeated these experiments using H_2O_2 0.5mM and 1mM with subsequent significant increases in annexin V staining (**Response Figure 1**).*



Response Figure 1. Absence of mitophagy during oxidative stress increases mitochondrial damage and apoptosis.

Significantly decreased mitochondria membrane potential and increased apoptosis in Parkin-/- and PINK1 -/- mice platelets treated with H_2O_2.

*As suggested by the Reviewer we also treated PINK1 -/- DM mice in vivo with NAC (intraperitoneal injection of 150mg/kg NAC for 1hr). NAC decreased ROS levels and decreased the % of Annexin-V positive platelets (**Response Figure 2**)*



Response Figure 2. In vivo injection of NAC in PINK1-/- DM mice. *Reduced ROS and apoptosis is observed with treatment of PINK1-/-DM mice with NAC.*

Platelet ROS levels are decreased by NAC injection in PINK1-/- DM mice leading to decreased apoptotic platelets (annexin V staining). This arises from the reduced oxidative stress providing a similar result to ***Appendix Figure Supplement 12****. As described in* ***Figure 7,*** *ROS plays a key role in triggering the protective mitophagy response. By reducing ROS, mitophagy is no longer induced (****Appendix Figure Supplement 12C****).*

Minor points:

1. The results section can be shortened by moving some portions to the discussion, which is rather short. For instance, there is a whole introductory paragraph in the results section before Fig.1 which is not necessary.
Thank you. We have shortened the results section as suggested.

2. In the immunoblots showing patient samples, a legend is needed indicating the patient number (or code).
Patient numbers according to when they were recruited have now been added as suggested.

3. In Fig. 1E, the different numbers in each quadrant of the flow image is too small to discern.
We have now presented a larger flow image size

4. There's a reference in the results section (top pg. 7) which is in a number format (instead of author name format).
Thank you. It has been changed

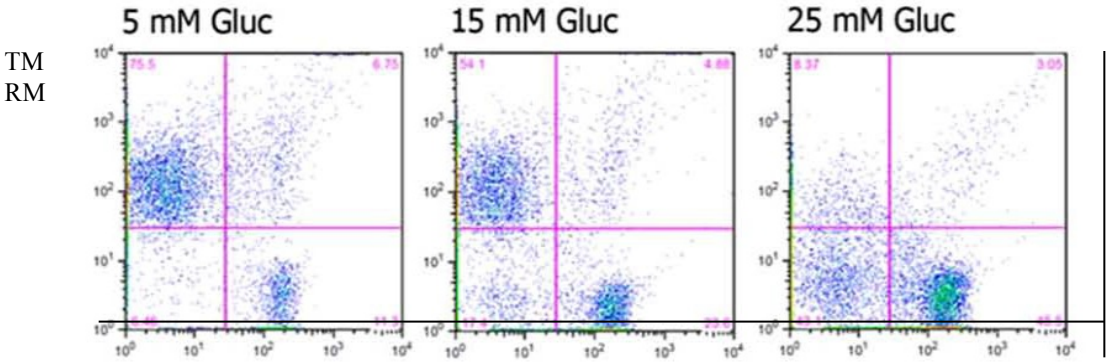
5. The sentence "As a control, and CCCP is recognized...." on pg. 11 does not make sense, needs reformatting.
Thank you. We have changed the sentence.

Referee #2:

We appreciate the very insightful comments presented by the Reviewer. In response to the concerns we have performed additional experiments and substantially reorganized the manuscript.

1. The most important point in my opinion is that authors claim that elevated ROS during DM is the cause of mitochondrial dysfunction promoting mitophagy, but they do not address the possibility that ROS increase in DM platelets could be the consequence of mitochondrial dysfunction. Could high glucose rate directly affect mitochondria?

*This is an excellent point raised by the Reviewer. We have previously demonstrated that glucose leads to increased ROS and mitochondrial dysfunction through an aldose reductase dependent pathway (Tang, Stitham et al. 2014) (**Response Figure 3**). What we have been most excited about in this present study is the presence of a robust mitophagy pathway in the short lived and anuclear platelet that serves to protect against such ROS inducing glucose stressors.*



Response Figure 3.
Treatment of platelets with increasing glucose concentrations demonstrates progressive decreased mitochondrial function and increased apoptosis (adapted from Tang, Stitham et al 2014).

2. The paragraph at the beginning of the Results section is more a summary than an introductive paragraph thus useless.
Thank you for this suggestion. We have removed the paragraph as suggested.

3. Results Paragraph 1 and Figure 1. The protein p53 is activated through post-translational modifications including phosphorylation on different sites: why authors do test only phosphorylation of serine 15?
The Reviewer is correct in pointing out that many sites can be phosphorylated on p53. We have performed extensive phosphoproteomic arrays in assessing the DM and apoptotic platelets and confirmed that Ser15 is the key phosphorylation site in platelets leading to mitochondrial dysfunction and apoptosis. No significant changes were observed with Ser46 and Ser 392.

4. Fig.1I: show also % of healthy and damaged mitochondria in the HC cohort.
*Thank you. We have now added % of healthy and damaged mitochondria in the HC in **Figure1J**. Approximately 85% of mitochondria are healthy and 14 % are unhealthy in HC platelets, likely reflecting basal mitochondrial turnover.*

5. Fig. S1: for HC n=4 in text but n= 5 in the legend of Fig.S1.
We apologize for this error. We used 4 HC platelets for these experiments. The error has been corrected.

6. Mitotracker: please precise which probe was used because some are not washed out of the cells when the mitochondrion's membrane potential is lost.
As suggested by the Reviewer the precise probes have now been added to the manuscript. Washed platelets ($1x10^6$ cells/ml) were allowed to settle on glass-bottom dishes for 1h and stained mitochondria using Mitotracker (Lifescience, MitoTracker Red M7512, or deep Red FM, M22426) for 15 min prior to fixing with 4% paraformaldehyde solution (Santa cruz Biotechnology).

7. Page 6: precise what is EM.
Thanks. EM means Electron Microscopy.

8. ROS level is measured using dyes (which ones?) from Invitrogen, but kit from Enzo is mentioned in Methods section: please indicate precisely what was used.
*We used a number of different assays (most suitable to the experimental samples) to confirm and validate our results. We used CellROX green (Molecular Probes, C10444) for **Figure1A** experiments, and we used ROS detection kit from Enzo for the experiments in **Figure 3C**. A new section has been added to Methods describing the assays used.*

9. Results Paragraph 2 and Figure 2. Fig.2A&B: assess also BNIP3 and Nix in WB blots experiments.
*We thank the Reviewer for this suggestion. We have recruited additional DM patients and assessed for BNIP3L/NIX (**Appendix Figure Supplement 4**). There are increases in BNIP3L/NIX in these platelets supporting the presence of an enhanced mitophagy process.*

10. Fig.2C&D: is there autophagic vacuoles in HC?
There are the occasional autophagy vacuoles in HC platelets however very few were detected compared to the DM platelets. This is reflected by the occasional LC3 staining even in HC which represents a degree of basal autophagy.

11. Results Paragraph 3 and Figure 3. The mTOR pathway actually inhibits autophagy, thus to completely get rid of its impact in platelets, other molecules should be evaluated such as S6K (reduction of its phosphorylation is expected) or use Rapamycin as autophagy inducer for example in the context of experiments shown in Figure 4.
*Thank you for these excellent suggestions. We have now assessed pp70S6K and pS6 in DM platelets (**Appendix Figure Supplement 6B**). Consistent with the absence of significant changes in mTOR there were no significant changes, supporting that mTOR likely does not play an important role in the DM induced mitophagy process.*

12. Results Paragraph 5 and Figures 5 and 6. The transition towards murine model is poor. Why experiments done with PINK1 KO mice (Figure 6) are different from experiments shown in Figure 5 using PARKIN KO mice? Also the experiments shown in Figure 6 are specific to the platelets research field thus not enough explained for a broad audience. What are PINK1 -/- DM mice?
*We apologize for the lack of clarity and hope that the revised manuscript is much improved. Both the PINK1 and Parkin knockout mice represent two different mouse lines where mitophagy has been knocked out. Rather than repeating everything in exactly the same fashion (and presenting redundant data) we wanted to present complementary data which support the importance of mitophagy in DM platelets using different techniques. In response to the Reviewer's concerns we have now repeated some of the Parkin-/- experiments in the PINK1-/- mice and demonstrated that there is lack of induction of mitophagy upon increased oxidative stress associated with increased platelet apoptosis (**Appendix Figure Supplement 11**). Such data was consistent with, and is supported by, the human data.*

13. The manuscript is overall well written. However, some inaccuracies in the text bother somewhere the reading. In the reference to the Figures: page 7 bottom "(Supplementary Figure 3D)" instead of "(Supplementary Figure 3A)", page 11 "(Figure 5C-5D)" instead of "(Figure 4C-4D)". Page 9 "...3 fold change in H_2O_2 vs. HC..." instead of "...3 fold change in DM vs. HC ...". "the amount of energy required for mitophagy into the platelet is considerable ....." "prepackaged in the absence of a nucleus in platelets" the authors point to these notions at several locations in the Results section text (twice in Page 10) which is redundant and somehow useless.
We thank the Reviewer for the comment that the manuscript is "overall well written". We apologize for the inconsistencies and redundancies, and have corrected them as suggested.

14. References: some references are incomplete, or in double (Kaneto 2010), or cited in the text as a number ("11" page 7), or without the year, or page 6 reference Michelle L. Boland 2013 not in the References list.... Please be more careful.
We apologize for these errors. They have been corrected.

2nd Editorial Decision 04 April 2016

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

1) Please address the minor text changes commented by referee 2. We would like to encourage you to also add the figures to referee 1 points 1 and 2 as Appendix figures. Please provide a letter INCLUDING the reviewer's reports and your detailed responses to their comments (as Word file).

Please submit your revised manuscript within two weeks. 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):

I believe that on technical grounds as well as innovation, this is a high-caliber manuscript. The medical impact is not readily apparent; however, it lays the foundations for potential therapeutic intervention in the future if agents which increase basal autophagy levels are developed.

Referee #1 (Remarks):

The manuscript by Lee et al has been substantially improved. Additional data confirming mitophagy are presented. Moreover, a mechanistic explanation supported by additional data is provided for the rapid induction of aggregation in autophagy/mitophagy-deficient platelets (the data presented in "Response Figures 1 and 2" should be included in the manuscript, even as supplemental data). Overall, the work described in this manuscript provides a novel and potentially clinically significant mechanism for the role of mitophagy in aggregation of platelets in diabetic conditions.

Referee #2 (Remarks):

The authors have satisfactorily addressed the concerns that I raised, and greatly improved the publication.
Minor suggestions to further ameliorate:
Figure 1. MitoTracker probe can also be used to assess mitochondria number, which is decreased in DM platelets as shown by EM (Fig.1I). Thus reduced Mitotracker fluorescence in DM platelets (Fig.1D) could also result from reduced mitochondria number, additionally (or not) to MMP decrease. Authors should discuss this point.
Page 9 and Figure 3C: "High glucose alone induced only a small increase in ROS (1.5 fold change)": it seems less than 1.5 on Figure 3C.
This point is also important regarding the first issue that I raised. HG seems to directly induce mitochondrial damage and not ROS increase which is thus likely the consequence of mitochondrial stress.
Page 13 "When stressed with DM levels of oxidative stress...": this part of the sentence is weird.
Page 13: DM mice: it is not clear what are these DM mice in both Results and Methods sections.
Figure S4B: error: "Bnip2" instead of "Bnip3L"
Figure S12B: error: "unstaining" not shown

2nd Revision - authors' response 15 April 2016

Referee #1:

We thank the Referee for the comments regarding our manuscript being a "high-caliber manuscript".

The manuscript by Lee et al has been substantially improved. Additional data confirming mitophagy are presented. Moreover, a mechanistic explanation supported by additional data is provided for the rapid induction of aggregation in autophagy/mitophagy-deficient platelets

(the data presented in "Response Figures 1 and 2" should be included in the manuscript, even as supplemental data).
As requested, we have now moved Response Figure 1 and 2 to the Appendix Figure S11D/E and Appendix Figure S12D/E, respectively.

Referee #1:

We thank the Referee for the comment that the manuscript is greatly improved. We now address the remaining minor suggestions.

1. Figure 1. MitoTracker probe can also be used to assess mitochondria number, which is decreased in DM platelets as shown by EM (Fig.1I). Thus reduced Mitotracker fluorescence in DM platelets (Fig.1D) could also result from reduced mitochondria number, additionally (or not) to MMP decrease. Authors should discuss this point.
That is an excellent point raised by the Referee regarding the reduced MitoTracker probe reflecting reduced mitochondrial numbers. This has now been added to the Results as suggested.

2. Page 9 and Figure 3C: "High glucose alone induced only a small increase in ROS (1.5 fold change)": it seems less than 1.5 on Figure 3C.
This point is also important regarding the first issue that I raised. HG seems to directly induce mitochondrial damage and not ROS increase which is thus likely the consequence of mitochondrial stress.
We thank the Referee for highlighting this important point. High glucose application to normal healthy platelets induces mitochondrial stress with a small increase in ROS. There is no substantial mitochondrial damage in comparison to the exposure to severe oxidative stressors.

3. Page 13 "When stressed with DM levels of oxidative stress...": this part of the sentence is weird.
Thanks. This has now been clarified.

4. Page 13: DM mice: it is not clear what are these DM mice in both Results and Methods sections.
The DM mice were produced using 5 days STZ (50mg/kg in each day) and 12 weeks high fat diet. This has now been highlighted in Material and Methods.

5. Figure S4B: error: "Bnip2" instead of "Bnip3L"
Thanks. The error has been corrected.

6. Figure S12B: error: "unstaining" not shown
Thanks. The error has been corrected.

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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
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2015-06046

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?	Power analysis was performed under the supervision of an expert statistician. For the human sample study, we used individual healthy donor platelets (n=5), and compared them with patient platelets(n=18) using Western blotting (Fig 1B, 2A, 3A). Reduction of mitochondria membrane potential, apoptosis and autophagy was generated in DM patient platelet. 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.	Power studies were also performed for the animal studies. Blood was drawn from a minimum of 3 mice in each group (WT, PINK and Parkin Ko mice). We drew blood from 3 more 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 parametric t test was performed for comparisons of 2 groups. Comparisons of the group means were made with a Student's t test or one-way ANOVA with a Bonferroni post-hoc test (Stat view V5.0, SAS). Analysis was performed with Prism software (GraphPad Software, Inc, La Jolla, CA).A difference of P<0.05 was considered significant.

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/

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?	There are similar variation between in each group as they were matched sibling controls.

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 presented a table for all the antibodies described (Appendix Table supplement 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 and Parkin or Pink knock out mice. We ordered the WT C57Bl/6 from Jackson laboratory and drew blood from the heart after STZ Injection/HFD (50mg/kg for 5 days and then feed High fat diet for 12 wks protocol #11413)or not. Parkin and PINK knock out mice were obtained from collaborators at Yale university. The animals 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).
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