EMBO reports

Optogenetic control of mitochondrial protonmotive force to impact cellular stress resistance

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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 4th Oct 2019

Thank you for the submission of your research manuscript to our journal, which was now seen by three referees, whose reports are copied below.

I apologize for the delay in getting back to you, it took longer than anticipated to receive the full set of referee reports.

As you can see, the referees express interest in the presented optogenetic tool to manipulate mitochondrial proton motive force. However, they also raise a number of concerns that need to be addressed to consider publication here. In particular, whereas referee #1 is overall positive in his/her evaluation, both referees #2 and 3 find that the tool should be verified by using alternative models such as different ETC mutant strains. I have further discussed this issue with the referees, and we came to the conclusion that we could not see taking this forward without addressing this issue. Moreover, technical concerns raised by referees #1 and 2 should be addressed to strengthen the conclusions.

Should you be able to address all referee criticisms in full, we could consider a revised manuscript. I do realize that addressing all the referees' criticisms will require a lot of additional time and effort and be technically challenging. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know so we can withdraw it from our system.

If you decide to thoroughly revise the manuscript for EMBO Reports, please
revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS:

Referee #1:

This paper reports the development of a genetically encoded photo-activatable tool for elevating the protonmotive force across the mitochondrial inner membrane. Tools for direct manipulation of this bioenergetic parameter represent a major need in the field of mitochondrial biology and bioenergetics, especially since many of the existing tools for elevating mitochondrial membrane potential are indirect and have major caveats (e.g. using oligomycin). The new technology reported here is elegant, thoroughly characterized, and will be extremely useful to the field of mitochondrial biology. Another strength of this paper is the application of mtON to living worms and physiologic adaptations to hypoxia, demonstrating the importance of PMF in hypoxic adaptation. I think this is a very exciting manuscript and I strongly support publication. I only have a few minor comments.

1. Are the mtON effects titratable by light intensity? Some characterization of the relationship between intensity and activation in each model system would be useful.
2. ATP measurements were made using the luminescence method, which is amenable to subsequent determination of ADP. It would be useful to determine and report whether the relative changes in ATP levels are attributable to specific changes in the ATP/ADP ratio.
3. mtON activation inhibits AMPK phosphorylation in worms, presumably because light-induced membrane potential generation is supporting ATP synthesis. This correlated with decreased movement, interpreted as decreased foraging behavior. This suggests that mtON activated worms may survive longer in nutrient depleted conditions compared to WT. This would be interesting to know one way or another.
4. The PC results are very interesting. Did the authors try mtON activation during hypoxia instead of during PC? One might expect that in this context it may be protective if it supports ATP/ADP levels to a level that would mitigate RET ROS in early reoxygenation

Referee #2:

Berry et al present a manuscript in which a modified variant of the light-activated proton pump derived from Leptosphaeria maculans, termed "MtON", is targeted to mitochondria. The study characterizes the MtON as a means of establishing a mitochondrial proton motive force independently of the canonical mitochondrial electron transport chain and associated substrate oxidation. Using MtON in a C. elegans model the authors demonstrate MtON as a tool to investigate physiological energy sensing pathways and the pathophysiological mechanisms involved with hypoxia/reoxygenation.
Overall, the study is a commendable attempt to create a tool of broad biological significance and utility to the field of mitochondrial science, enabling investigators to isolate the role of proton motive force. However, there are drawbacks. Firstly, the study requires stronger evidence to support the conclusions drawn. Secondly, the study misses the opportunity to use the vast genetic resources available in the C. elegans, there are many ETC mutants that would represent excellent (patho)physiological models. Thirdly, the novelty of the study may be compromised: Hara et al., Nature Scientific Reports 2013, demonstrated a mitochondrial rhodopsin to increase PMF, this should be referenced and discussed. Lastly, the study contains some surprising data that need further explanation.
Specific Points
Validation of proper targeting of MtON to the mitochondrial inner membrane is insufficient. Fig1. Fig and EV1 supply some evidence that MtON targets to the mitochondria. However, no attempt is made to demonstrate that MtON is correctly inserted into the inner mitochondrial membrane. The referenced study, Tkatch et al., demonstrates more completely the targeting of the probe. Protease cleavage and carbonate extraction assays are examples of techniques that would provide more compelling evidence.
Fig2 While Waschuck et al. indicate that the proton pump has a broad optical absorbance, Fig 2C and EV3 demonstrate that TMRE may be imaged using light below the activation threshold of MtON. If true, then Fig2A would benefit from a protocol with a stable baseline.

Same y scale should be shown for Fig2A & B. Same y scale should be shown for Fig2C & D.
I didn't find information on whether SE or SD is shown in the figures e.g. Fig2.
Fig2 A&C differ in light exposure/FCCP addition. It would be useful to see multiple pulses of light to inform on/off kinetics and light+FCCP measurements would be useful to demonstrate the proton specificity of MtON.
Fig2 A-F The generation of PMF using light alone should be demonstrated with classical ETC inhibitors rotenone, antimycin etc. Please show $\Delta\psi m$/pH change induced by MtON during inhibition of CI, CIII & CIV.
Fig3 (FigEV5) Fig EV5 mentions WT animals, but I find no information on the genetic status of animals in Fig3?
Fig3 To demonstrate the role of MtON H+ pumping, a toxin acting downstream of the PMF, e.g. oligomycin would serve as a desirable negative control.
Fig3 A broad range of C. elegans mutants are available, in addition to cells lacking MtDNA.
Fig EV3 indicates that TMRE fluorescence can be measured at low light intensity (0.016 J/cm2) and short-term exposure ~ 1min. Such conditions would allow vital baseline measurements to be made e.g. Fig2A
FigEV3 A&B Data on TMRE response to light intensity/duration likely operate as a dose/response relationship, as such a dose-response curve would be appropriate.
Fig4 A The MtON suppression of AMPK phosphorylation should have been checked with + light, + ATR, +food to ensure specificity.
Fig4 C, Control condition of AICAR alone should be included.
Fig5 D, The effectiveness of light alone is very surprising. This should be investigated since the effect of MtON is only significant compared to this anomalous result. It is tempting to suspect a role for illumination-induced redox effects that might operate as a preconditioning stress.
A non-functioning MtON mutant would serve as an excellent additional negative control to investigate the role of light and the antioxidant effects of ATR.
The use of ATR for all-trans retinal is confusing with respect to the field of mitochondrial biology that routinely assigns ATR to atractyloside.

Referee #3:

These authors generated an interesting new tool: a light-driven proton pump targeted to mitochondria in C. elegans. They showed that it increases membrane potential and was able to suppress the effects of several ETC inhibitors. The authors provide 2 proof of principle physiological applications of this pump, namely reducing the effects of AMPK on locomotion when food is withheld and inhibition of hypoxia adaptation by maintaining a relatively high membrane potential.

The approach is interesting and it was nice to see that it worked, but the examples of applications shown here were rather limited. It is also unclear how helpful this approach will be for other studies. In sum, I think the paper is fine technically, but it may belong in a more specialized journal.

1st Revision - authors' response 26th Nov 2019

Please see next page.

Manuscript ID: EMBOR-2019-49113V1
Title: "Controlling the Mitochondrial Protonmotive Force with Light to Impact Cellular Stress Resistance."

We thank the editor and reviewers for their enthusiasm and critiques, as well as for the opportunity to revise our manuscript. We have reproduced the reviewer comments below in bold and provided responses to individual points in regular typeface. Articles referenced in our response are listed using PubMed IDs (PMID). We are hopeful that the reviewers will recognize our sincere attempt to address their comments and that the manuscript is stronger as a result and suitable for publication in EMBO Reports.

Referee #1:

This paper reports the development of a genetically encoded photo-activatable tool for elevating the protonmotive force across the mitochondrial inner membrane. Tools for direct manipulation of this bioenergetic parameter represent a major need in the field of mitochondrial biology and bioenergetics, especially since many of the existing tools for elevating mitochondrial membrane potential are indirect and have major caveats (e.g. using oligomycin). The new technology reported here is elegant, thoroughly characterized, and will be extremely useful to the field of mitochondrial biology. Another strength of this paper is the application of mtON to living worms and physiologic adaptations to hypoxia, demonstrating the importance of PMF in hypoxic adaptation. I think this is a very exciting manuscript and I strongly support publication. I only have a few minor comments.

1. Are the mtON effects titratable by light intensity? Some characterization of the relationship between intensity and activation in each model system would be useful.

The activity of proton pumps are light dose dependent (PMID: 20054397). We have now provided a new figure presenting pump activity vs. light dose (i.e. fluence; units=joules/area, where a watt=joule/s) (Figure EV3A; also see Referee #2, comment #11).

2. ATP measurements were made using the luminescence method, which is amenable to subsequent determination of ADP. It would be useful to determine and report whether the relative changes in ATP levels are attributable to specific changes in the ATP/ADP ratio.

ATP was measured in isolated mitochondria with known, given amounts of ADP, explicitly to avoid confounding effects from changes in the ATP/ADP ratio *in vivo*. ATP levels are highly regulated *in vivo,* and we simply sought to directly assess the effects of acute mtON activation on ATP production. We have now clarified this approach in the methods section (lines 439-440). In other words, in the isolated mitochondrial system as measured, the changes in ATP cannot have been due to a bulk change in the level of adenine nucleotide pool.

3. mtON activation inhibits AMPK phosphorylation in worms, presumably because light-induced membrane potential generation is supporting ATP synthesis. This correlated with decreased movement, interpreted as decreased foraging behavior. This suggests that mtON activated worms may survive longer in nutrient depleted conditions compared to WT. This would be interesting to know one way or another.

To address this comment, we starved animals beginning on day 1 of adulthood and scored survival. We found that light has a detrimental effect on starved lifespan (2 tailed 2 sample unpaired t test, p = 0.006), as expected (PMID: 29500338), and that median lifespan (number of days to 50% survival) was no different between +/- ATR conditions within no light exposure (-light, -ATR: average 3.67 days, -light +ATR average 3.5 days, p = 0.78) and within light exposure (-ATR +light: average 2.33 days, +ATR +light: average 2 days, p = 0.37). n = 3 plates each condition with 108-142 total animals.

This suggests that the maintenance of the PMF in conditions without a food/carbon source is not sufficient to extend the starved lifespan.

4. The PC results are very interesting. Did the authors try mtON activation during hypoxia instead of during PC? One might expect that in this context it may be protective if it supports ATP/ADP levels to a level that would mitigate RET ROS in early reoxygenation

As suggested, we tested the effect of mtON activation during the hypoxic insult. We found a detrimental effect of light during hypoxia that was exacerbated by mtON activation. Rather than preserving ATP production, maintaining PMF during hypoxia may drive detrimental cation accumulation (i.e. calcium overload) and contribute to ROS production and damage at reoxygenation. ROS production at reoxygenation is extremely sensitive to PMF changes (PMIDs: 15175007, 9369223), where lower PMF would mitigate damage, and increased PMF would increase damage.

We have now discussed these findings (lines 265-274) and included the data in a new Figure 5E.

Referee #2:

Berry et al present a manuscript in which a modified variant of the light-activated proton pump derived from Leptosphaeria maculans, termed "MtON", is targeted to mitochondria. The study characterizes the MtON as a means of establishing a mitochondrial proton motive force independently of the canonical mitochondrial electron transport chain and associated substrate oxidation. Using MtON in a C. elegans model the authors demonstrate MtON as a tool to investigate physiological energy sensing pathways and the pathophysiological mechanisms involved with hypoxia/reoxygenation.
Overall, the study is a commendable attempt to create a tool of broad biological significance and utility to the field of mitochondrial science, enabling investigators to isolate the role of proton motive force. However, there are drawbacks. Firstly, the study requires stronger evidence to support the conclusions drawn. Secondly, the study misses the opportunity to use the vast genetic resources available in the C. elegans, there are many ETC mutants that would represent excellent (patho)physiological models. Thirdly, the novelty of the study may be compromised: Hara et al., Nature Scientific Reports 2013, demonstrated a mitochondrial rhodopsin to increase PMF, this should be referenced and discussed. Lastly, the study contains some surprising data that need further explanation.

Thank you for the thorough review, we appreciate the opportunity to add clarity to our manuscript. We have numbered the following specific points for ease of referencing.

We now cite and discuss Hara *et al.* (PMID: 23567447) in the context of this work (lines 175-177), as well as a new manuscript (no PMID available; https://doi.org/10.1038/s42003-019-0674-1)

from the same group that was published during the resubmission process. Importantly, these reports do not diminish the impact of our manuscript, but rather it further solidifies our findings. The following sentences were added in the discussion section (lines 200-204):

While this work was under revision, a similar technique using an alternative light-activated proton pump was characterized in a Drosophila model (Imai, Inoshita et al., 2019), supporting our findings, and demonstrating the broad application of these optogenetic tools in neurodegeneration. Our results with mtON compliment these findings, and expand them in other areas including the following energy-sensing and hypoxia adaptation studies.

Specific Points

1) Validation of proper targeting of MtON to the mitochondrial inner membrane is insufficient. Fig1. Fig and EV1 supply some evidence that MtON targets to the mitochondria. However, no attempt is made to demonstrate that MtON is correctly inserted into the inner mitochondrial membrane. The referenced study, Tkatch et al., demonstrates more completely the targeting of the probe. Protease cleavage and carbonate extraction assays are examples of techniques that would provide more compelling evidence.

We and others have demonstrated that the IMMT-1 mitochondrial inner membrane targeting sequence (PMIDs: 21248201, 21078595, 30573525) is a validated targeting sequence.

As suggested, we performed a proteinase K protection experiment in swollen mitoplasts to demonstrate the insertion of mtON in the inner membrane. We found that the mtON signal (GFP) coincided with an inner membrane associated protein (ATP5a), located on the matrix side of the membrane, as expected (Figure EV 1F; lines 106-109).

In addition, we validated the position of the GFP tagged mtON compared to mCherry in the intermembrane space, and MitoTracker Red in the matrix. We (PMID: 30887829, see supplementary Figure S2) and others (PMID: 31427612) have used this approach to determine protein localization in mitochondrial compartments. The fluorescence intensity line scans determine the degree of overlap in red and green signal (PMID: 30887829). For these experiments we created a new CRISPR strain expressing mCherry in the intermembrane space. The results demonstrate that mtON is localized in the inner membrane and is inserted as expected. This new localization data is presented in the new, expanded Figure EV1A-E, and discussed in lines 103-106, and the methods are described in (lines 317-328, 336-343,and 391-400).

Moreover, our PMF functional measurements in isolated mitochondria support that mtON is correctly inserted and functions in the inner membrane, as mtON elsewhere could not result in the PMF changes observed.

2) Fig2 While Waschuck et al. indicate that the proton pump has a broad optical absorbance, Fig 2C and EV3 demonstrate that TMRE may be imaged using light below the activation threshold of MtON. If true, then Fig2A would benefit from a protocol with a stable baseline.

The excitation wavelength of TMRE in our protocol activates mtON at the lowest light dose tested. Under these conditions, it is not possible to acquire an initial flat trace, as the light dose always increases the protonmotive force and subsequent TMRE accumulation.

As an alternative to TMRE, we also used BCECF to measure pH changes (shown in Figure 2C) and due to the different excitation/emission wavelengths of BCECF the measurement did not activate mtON. In this pH experiment, we can clearly observe baseline matrix pH and the response to mtON activation separately.

However, it is important to note that the baseline is not part of the TMRE measurement, rather the change in fluorescence upon addition of FCCP is the measurement – i.e. a stable baseline provides no experimental value. TMRE accumulation can be kinetically slow and researchers usually allow it to accumulate (PMID: 21486251). Only the FCCP-mediated fluorescence change yields the experimental data, and represents the accumulation of TMRE in response to a membrane potential.

3) Same y scale should be shown for Fig2A & B. Same y scale should be shown for Fig2C & D.

Figures 2A and B have different units, where 2A is raw fluorescence, and 2B is the normalized fluorescence change relative to maximum, substrate-driven membrane potential.

However, this comment also helped us to recognize an oversight in Figure 2C, which was incorrectly indicated as ΔBCECF ratio, but is actually raw BCECF ratio. Figure 2D is the normalized ΔBCECF. We thank the reviewer for the opportunity to correct this axis label.

Given that these measurements are in different units, the scales cannot be matched.

4) I didn't find information on whether SE or SD is shown in the figures e.g. Fig2.

We apologize for this oversight. The error bars represent SEM in figure 2, and we have now added this description to all the figure legends.

5) Fig2 A&C differ in light exposure/FCCP addition. It would be useful to see multiple pulses of light to inform on/off kinetics and light+FCCP measurements would be useful to demonstrate the proton specificity of MtON.

The “on” kinetics can now be inferred from the dose response relationship with TMRE signal in the new Figure EV3A (see Comment #11). We show that mtON requires ~60 seconds, or 0.1 J/cm^3 of illumination for saturation. The measurements in these experiments, however, are the result of fluorescent probes and are not truly kinetics of the proton pump.

The “off” kinetics in this system are not a feature of the pump, but would rather be a measure of how “leaky” the mitochondrial inner membrane is to protons

The Mac proton pump in mtON has been fully characterized as proton specific using FCCP (PMID: 15860584, 20054397; as well as in our system, Figure 2A illumination occurs throughout FCCP treatment).

We have now expanded the discussion to further clarify that our data are consistent with the published proton specificity of Mac (lines 128-129, 135-137).

6) Fig2 A-F The generation of PMF using light alone should be demonstrated with classical ETC inhibitors rotenone, antimycin etc. Please show Δψm/pH change induced by MtON during inhibition of CI, CIII & CIV.

All the experiments in Figure 2 included rotenone; we have now made this clear (line 123-126). As the reviewer suggests, the addition of inhibitors result in mitochondria unable to generate a PMF in the presence of substrates. Indeed, we have tested if mtON activation can generate a PMF in isolated, inactived mitochondria directly in our experiments. We used isolated mitochondria in the absence of substrates (- succinate, Figure 2B, 2D, 2E), therefore no electron entry to the ETC occurs in our experiments. Furthermore, FCCP completely collapses the PMF, which is an appropriate control for baseline.

Additionally, we demonstrate that mtON improves survival in the presence of the classical ETC inhibitors (Figure 3).

Overall, the evidence we present (+/-ATR, +/- light conditions, +/- substrates in Figures 2B, 2D, 2E; survival data in the presence of classical ETC inhibitors in Figure 3) supports our claims that mtON energizes mitochondria in the absence of ETC (CI, CIII & CIV) activity.

7) Fig3 (FigEV5) Fig EV5 mentions WT animals, but I find no information on the genetic status of animals in Fig3?

The animals were the transgenic mtON animals and the genetic status is now included in the figure legend.

8) Fig3 To demonstrate the role of MtON H+ pumping, a toxin acting downstream of the PMF, e.g. oligomycin would serve as a desirable negative control.

Thank you for this suggestion, we have performed the experiment using oligomycin A and have included the data in Figure 3D (discussed in lines 172-175).

We found that mtON activation had no effect on the survival of animals exposed to oligomycin A. As the reviewer predicted, these data serve as a negative control, as PMF rescue is not expected to rescue ATP synthesis inhibition. This has strengthened our conclusions and provided more evidence for mtON's ability to rescue inhibited proton pumping by the electron transport chain, and that rescue requires ATP synthesis driven by a PMF.

9) Fig3 A broad range of C. elegans mutants are available, in addition to cells lacking MtDNA.

We thank the reviewer for this suggestion. For the limited format of the Scientific Report, we chose two specific strains to bolster our characterization of mtON. Since mitochondrial mutant worms have often adapted to mitochondrial stress by upregulating hormetic pathways (PMID: 16137850, 17914900), we decided to test mitochondrial mutant strains in two ways, acutely and adaptively (i.e. short- versus long-term intervention).

First, we used the *gas-1* mutant strain harboring a mutation in complex I of the electron transport chain. We show these animals have decreased locomotion compared to wild type (Figure 3E).

We hypothesized that acute mtON activation would rescue locomotion to a wild type level, as *gas-1* animals have a decreased baseline PMF (PMID 19900588). We generated a new strain expressing mtON in the *gas-1* background. Applying each of our control conditions, we found that mtON could partially rescue *gas-1* locomotion (Figure 3E, discussed in lines 178-183).

To further address this point, we used a mtDNA mutant strain harboring the uaDf5 deletion (PMID: 12440704). These animals have a heteroplasmic mtDNA population where ~60% of mtDNA contains a large deletion, detectable by PCR (see methods, lines 492-501). Animals harboring uaDf5 require mitochondrial stress responses to survive in the presence of the deleterious uaDf5 DNA (PMID: 27135930). Specifically, responses that originate from dysfunctional mitochondria and decreased PMF (hormetic responses) are required to maintain viability (PMID: 22700657, 31412237). Therefore, we generated a strain expressing mtON and carrying uaDf5 mtDNA (Figure EV 6A). We hypothesized that the activation of mtON in animals carrying uaDf5 would signal "healthy" mitochondria despite the deleterious uaDf5 deletion. Therefore, adaptive signaling required to keep the mtDNA mutant worms alive would be turned off. The resulting progeny would carry a heavy uaDf5 load resulting in death. Indeed, we found mtON activation in the uaDF5 background resulted in very low or no progeny (Figure EV 6B, discussed in lines 184-199, 502-506).

These experiments have expanded our characterization of mtON, and so, we have renamed the section discussing these results (line 158).

10) Fig EV3 indicates that TMRE fluorescence can be measured at low light intensity (0.016 J/cm2) and short-term exposure ~ 1min. Such conditions would allow vital baseline measurements to be made e.g. Fig2A

Please refer to our response to comments #2 and #5 addressing this concern.

11) FigEV3 A&B Data on TMRE response to light intensity/duration likely operate as a dose/response relationship, as such a dose-response curve would be appropriate.

Thank you for this suggestion, we have made a new figure and reanalyzed the data as a dose response curve (Figure EV 3A) as described above.

12) Fig4 A The MtON suppression of AMPK phosphorylation should have been checked with + light, + ATR, +food to ensure specificity.

For this experiment, our question was to address the change in phosphorylation status of AMPK in animals without food. AMPK is highly characterized to be responsive to fed and starved states (PMID: 27642785, 31411562, 29107506). Under fed conditions, AMPK is not phosphorylated (Figure 4A) and therefore mtON activation could not suppress an already suppressed condition. We included fed controls for completeness, and to show the dynamic range.

In order to address the specificity for AMPK, we demonstrated mtON activity using both pharmacological (AICAR) and genetic (AMPK knockout) approaches to control AMPK activity in the locomotion behavior (Figures 4 C & B, respectively).

13) Fig4 C, Control condition of AICAR alone should be included.

We performed the experiment and, as expected, AICAR alone did not have a confounding role. We found AICAR supplementation did not affect locomotion off of food. This data is now included in Figure 4C.

14) Fig5 D, The effectiveness of light alone is very surprising. This should be investigated since the effect of MtON is only significant compared to this anomalous result. It is tempting to suspect a role for illumination-induced redox effects that might operate as a preconditioning stress.

We agree the result was surprising, although we consider a full characterization of the effect is beyond the scope of the manuscript, and would exceed the space restrictions in an EMBO *Scientific Report* format. Importantly, our extensive controls have independently controlled for potentially confounding factors to investigate the effect of the PMF. We controlled for light exposure and ATR, which allowed us to show light or ATR on its own is protective. These effects could be predicted to be additive however, they were not, leading to our conclusion that mtON is mediating the effects observed.

We have provided new data and have added new discussion on the effect of light during hypoxia (Referee 1, comment #4, lines 265-274).

15) A non-functioning MtON mutant would serve as an excellent additional negative control to investigate the role of light and the antioxidant effects of ATR.

We agree this would serve as a useful additional control, however, currently a non-functional mutant of the *Leptosphaeria maculans* proton pump (pump component of mtON) is not available. Instead, the pump in the absence of ATR serves as such a control.

Importantly, the goal of this study was to investigate the role of the mtON-mediated protonmotive force generation, not the role of light or to study the antioxidant effect of ATR. We extensively controlled for these potentially confounding variables (Figure EV 2A-D) and our results isolate the effects of mtON activation.

16) The use of ATR for all-trans retinal is confusing with respect to the field of mitochondrial biology that routinely assigns ATR to atractyloside.

We acknowledge that our approach spans many disciplines. We define each of the acronyms in our manuscript, and ATR is commonly used in optogenetic literature (PMID: 27642785). To serve the broad readership, we maintain our use of ATR for clarity and brevity. Also, we do not use atractyloside in this manuscript, which avoids internal confusion throughout the text.

Referee #3:

These authors generated an interesting new tool: a light-driven proton pump targeted to mitochondria in C. elegans. They showed that it increases membrane potential and was able to suppress the effects of several ETC inhibitors. The authors provide 2 proof of

principle physiological applications of this pump, namely reducing the effects of AMPK on locomotion when food is withheld and inhibition of hypoxia adaptation by maintaining a relatively high membrane potential.

The approach is interesting and it was nice to see that it worked, but the examples of applications shown here were rather limited. It is also unclear how helpful this approach will be for other studies. In sum, I think the paper is fine technically, but it may belong in a more specialized journal.

Our rigorously controlled and precise approach is useful in many contexts, a view shared by Referee #1 and Referee #2. We have now expanded our findings to further demonstrate the physiologic applications of our approach (see above comments, added text and Figures 3E, 5E, EV6A&B). Specifically, we demonstrate the applicability of mtON to study mtDNA mutations, lifespan extension, hypoxic biology, mitochondrial signaling, and energy-sensing.

2nd Editorial Decision 15th Jan 2020

Thank you for submitting your revised manuscript, which was now seen by all of the original referees, whose comments I copied below. As you can see, they find that their concerns are satisfactorily addressed and recommend publication here. From the editorial side, I have carefully looked at everything and all looks fine with the manuscript. Therefore I am very pleased to accept it for publication in EMBO Reports.

REFEREE REPORTS:

Referee #1:

The authors have addressed all of my questions. In my opinion, this manuscript will be an excellent addition to EMBO reports. I strongly support publication.

Referee #2:

The revised manuscript is much improved. It's both more convincing and interesting. Most of our points are addressed, and the clarifications are acceptable. The Authors have missed opportunities to use MtON to generate a membrane potential with a completely inhibited ETC, or in Rho0 cells which were specifically requested but we don't want to hold back the publication because of this weakness.

Referee #3:

The authors did a good job addressing the other two reviewers' comments. My comments expressed an opinion about the overall value and were not so easily addressed. Given the other reviewers' enthusiasm for this work, I am happy change my opinion and acknowledge that this work will have a lasting impact.

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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: Andrew Wojtovich
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-49113V1

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 iustified
- ➔ 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.

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?	Power analysis was used to determine most sample sizes. Post-hoc power analysis was also used in certain experiments to ensure power of statistical conclusions.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Power analysis was used to determine most sample sizes. Post-hoc power analysis was also used in certain experiments to ensure power of statistical conclusions.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals or data were excluded from analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	When possible, the investigator was blinded to the experimetal conditions and animals were randomly assigned to experimetnal conditions each day.
For animal studies, include a statement about randomization even if no randomization was used.	Sample conditions were randomized day-to-day to minimize temporal confounding effects.
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.	When possible, the investigator was blinded to the experimetal conditions and animals were randomly assigned to experimetnal conditions each day.
4.b. For animal studies, include a statement about blinding even if no blinding was done	When possible, the investigator was blinded to the experimetal conditions.
5. For every figure, are statistical tests justified as appropriate?	Each statistical test is justified for the question being asked, and each post hoc test is appropriate for the relevant comparisons.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Shaprio-wilkes normality tests were used to determine further statistical tests.
Is there an estimate of variation within each group of data?	Standard deviations or standard errors of the mean are given for each group of data.

USEFUL LINKS FOR COMPLETING THIS FORM

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

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-tumo

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 the variance similar between the groups that are being statistically compared?	Yes, variances were similar across groups being compared in each experiment.

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).	anti-GFP (ClonTech Living Colours #ab632375), anti-ATP5a (Abcam, #ab14748), anti-HSP60 (Department of Biology, Iowa City, IA 52242. Developmental Studies Hybridoma Bank, University of Iowa Department of Biology, Iowa City, IA 52242), (Cell Signaling, #4188), anti-Actin (Abcam #ab14128), anti-phospho-AMPKα (Cell Signaling, #2535).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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.	C. elegans hermaphrodites were used exclusively. Transgenic animals were generated by transgene overexpression by DNA plasmid microinjection and by CRISPR-Cas9 custom technology. All strains were maintained on standard Nematode Growth Medium on OP%) E. coli food at 20 degrees celsius. Some strains provided by the C. elegans Genetics Center.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Our study complies with ARRIVE guidelines and NIH guidelines of animal research.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

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	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).	Raw data will be available for graphs and statistics upon request, and this is clearly stated in our manuscript for readers.
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. 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

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.	NA