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# Miro proteins prime mitochondria for Parkin translocation and mitophagy

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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 17th April 2018

Thank you for submitting your manuscript on a role for Miro 1 in PINK1-independet Parkin recruitment to The EMBO Journal. We have now received three referee reports on your study, which are enclosed below for your information.

As you can see, while the referees consider the findings interesting, they also raise several key critical points that need to be addressed before they can support publication here.
In particular, referee #1 is concerned that conflicting results are presented in the manuscript and requests you to solve them. In addition, referee #2 points out that the biological relevance of the Miro 1-Parkin interaction remains unclear.

Addressing these issues through decisive additional data as suggested by the referees would be essential to warrant publication in The EMBO Journal. Given the overall interest of your study, I would thus like to invite you to revise the manuscript in response to the referee reports.

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REFEREE REPORTS

Referee #1:

Recent studies have converged on the potential role of Miro/RHOT in mitophagy because these proteins have been shown to be substrates in the PINK1/Parkin pathway. Based on some recent publications the Authors speculated that Miro1 is not only a substrate for PINK1/Parkin dependent degradation but might also have additional roles in the regulation of mitophagy. Considerable evidence is presented in the manuscript to suggest that Miro1 can support Parkin recruitment independent of PINK1 and that Miro1's EF hands are somehow involved. Moreover, the initial Parkin-Miro interaction seems to play a critical role in the response to mitochondrial damage

 

because knockdown of Miro proteins reduces Parkin translocation to mitochondria and suppresses mitophagic removal of mitochondria. Unfortunately, this potentially important work is described in a manuscript with several pitfalls
The Authors main conclusion is that "Miro1 initiates Parkin recruitment, which is amplified by PINK1 upon mitochondrial depolarization". However, Fig1C shows that the initial phase of Parkin recruitment induced by antimycin+oligomycin is unaffected by targeting Miro, rather the slow phase is suppressed.
Furthermore, the other time courses for Miro dependence of Parkin recruitment are different (Fig6A: both the initial and maximal responses are suppressed by MiroEF overexpression; Fig6E: only the initial kinetics is slow) but the source of these discrepancies remains unexplained.
Indeed, it is a recurrent problem with this manuscript that apparently conflicting results are shown in different figures, or relevant conditions are not shown or inducers are switched from one experiment to the next (eg antimycin+oligomycin to FCCP) without explanation. This creates uncertainty about the findings.
Some examples for this problem and other specific shortcomings are listed below:
- The Miro silencing condition without mitophagy inducers is not shown in Fig1B,D,E. The Authors note in the text that expression of the reporter was low in the silenced cells but this does not prevent them to show the results for the PINK1 condition. The baseline (without PINK1) should also be shown at least in Fig1E to validate whether the effect of PINK1 was less in the Miro1 deficient condition.
- Next, the Authors claim that Miro1 rescue restored the effect of PINK1 but rather Miro1 overexpression ALONE resulted in a Parkin response as large as the effect of PINK1 in mock silenced cells (Fig1F).
- The 2 assays used for Parkin translocation give conflicting results for the different stimuli. When the cells with Parkin translocation are counted, Miro overexpression alone is as effective as PINK1 (Fig1F). However, when the heterogeneity in Parkin fluorescence is measured Miro overexpression is barely above the baseline, whereas PINK1 exerts a robust effect (Fig2B). Please explain. Also, please state what time point was evaluated in Fig2B and in the other panels?
- Fig2A shows that Miro1 overexpression alone can stimulate Parkin recruitment to the mitochondria and this is claimed to be independent of the EF-hand. However, Fig6A shows the lack of increased Parkin recruitment in the Miro1EF expressing cells inconsistently with the Authors' model.
- Is Parkin interaction with Miro specific of Miro1 isoform? Could Parkin interact with Miro2? Miro2 silencing seems to give and even stronger phenotype than Miro1 silencing (Fig1) but the rest of the studies are confined to Miro1. Did the authors try to overexpress Miro2 and measure the percentage of cells with Parkin translocation?
- Is Miro1 and Miro2 shRNA specific to each isoform? The reference used to validate the experimental model (Cagalined et al, 2013) doesn't show a western blot quantification of the protein amount of both Miro isoforms when one of them is silenced. Moreover, the antibody used for recognizing Miro1 in this paper (Atlas, HPA010687) is known to recognize both Miro isoforms.
- The Authors concluded in the legend of Figure 2I that: "Overexpression of Miro1 alone increased the fraction of motile mitochondria moving in both directions as well as the relative mitochondrial velocity." However, the time in motion analysis doesn't provide the information of the fraction of motile mitochondria. This analysis only shows a histogram with the percentage of mitochondria that spent a certain time moving in the retrograde or anterograde direction. Is the fraction of mobile mitochondria different after Miro1 overexpression?
- Is the EF hand motif of the Miro1 K572R functional? The authors state that "Note also that Miro1 K572R was otherwise fully functional as it was able to restore mitochondrial motility in Miro shRNA expressing cells similar to Miro1 WT (Fig. 4e)". However, Fig. 4e doesn't show if the Ca2+ binding capacity of Miro1 is intact in Miro1 K572R. At resting cytoplasmic [Ca2+], mitochondrial movement is enhanced by Miro overexpression independently of the presence of the EF-hands, as it was shown in Saotome et al, 2008. In order to check if Miro1 K572R is fully functional the authors should determine if the Ca2+-induced arrest of mitochondrial motility is still intact.
- In Fig. 6 the Authors showed that Ca2+-insensitive Miro1 inhibits Parkin translocation to mitochondria. Is the Parkin translocation to mitochondria altered in cells expressing Miro1 K572R? Are the Ca2+ sensing EF-hands required for Parkin translocation to mitochondria as the Authors state or is the ubiquination of Miro1 (which is abolished in the Ca2+-insensitive Miro1) required for Parkin translocation to mitochondria?
- Is the glutamate-induced Ca2+ rise in cells expressing Miro1 EF or Miro1 WT similar to control cells? It would be important to show that the Glutamate-induced Ca2+ entry is not affected by the

 

overexpression of Miro1 EF or Miro1 WT since the authors have shown that the cytosolic Ca2+ levels affect Parkin translocation to mitochondria.
- Is the parkin-Miro1 interaction observed physiological or is only due to the overexpression of Miro1?
- It remains untested whether the change in mitochondrial motility associated with Miro loss has a primary role in the change in Parkin recruitment.
- Fig1F and Fig3A seem to evaluate Parkin translocation by the same method but the effect of Miro overexpression is 20 vs 90 in the 2 graphs. What is the reason for the difference?

Minor concerns
- Figs1&2 used antimycin+oligomycin to perturb mitochondria. Why is FCCP used in Fig3?
- The Authors state: "Results depicted in Fig. 1a,b demonstrate that loss of Miro proteins suppressed significantly the number of PC6 cells showing PINK1-induced Parkin translocation to mitochondria. This cannot be explained by altered PINK1 levels because the latter was not affected in the Miro shRNA-treated groups." How was the PINK1 level determined?
- Images of Fig.1A are dark and the colors are not defined in the figure and also in the legend.
- In the manuscript and legends both YFP-Parkin and EYFP-Parkin are referred. Have you used both type of sensors or it is a misspelling?
- Fig 2A, C, D and E are missing their respective controls to compare Parkin distribution.
- From Fig. 2E cannot be concluded that "Miro1 overexpression did not induce mitochondrial depolarisation but instead it led to slight hyperpolarization" because TMRE quantification or comparison to control is missing.
- Mitochondrial accumulation of the dye used for mitochondrial membrane potential measurement is affected by many factors. Thus, it is essential that the uncoupler sensitivity is validated (Fig2F).
- More detailed explanation of how "Time in motion (%)" was measured should be provided in material and methods section.
- Fig. 2J missing the duration of the A&O treatment
- In Fig. 7I, is the control after glutamate treatment the same in the right and left panels? Left panel, control present around 45-50% of cells with Parkin punctae after glutamate treatment, while in the right panel the value is around 35%. Could be the results on control merged? In the affirmative case, would the expression of Miro1 EF still induce a significant decrease in the percentage of cells with Parkin punctae?
- Missing controls in Fig. 7J and 7K. Was the number of autophagosomes colocalising with mitochondria in neuron body (Fig. 7J) and the mitochondrial density in axons (Fig. 7K) altered by Miro1 EF and Miro1 wt overexpression at baseline?
- Correction at page 11: "Transient glutamate exposure induces Ca2+ influx into the cytosol leading to mitochondrial Ca2+ uptake and depolarisation of the inner mitochondrial membrane (Schinder et al, 1996; White & Reynolds, 1996)."

The functional relevance of the findings on modifying Parkin translocation to mitochondria could be tested by cell viability using cells overexpressing Miro1 EF (suppressed Parkin translocation) and Miro1 WT (enhanced Parkin translocation) compared to control cells. Another possibility would be to check the O&A induced degradation of mitochondrial proteins (such as COXIV or TIM23) in these conditions.

Referee #2:

Miro proteins are critical components of mitochondrial transport, which is in part regulated by Ca2+ binding to Miro's EF-hands. Successful mitophagy likely requires termination of mitochondrial transport but details remain enigmatic. Previously, it has been suggested that ubiquitination of Miro1 may be a trigger for mitochondrial arrest prior to mitophagy instead of its (ubiquitin-mediated) degradation. Consistently, Miro1 exhibits multi-monoubiquitination and an atypical lysine-27-mediated ubiquitin chain indicative of ubiquitination-mediated roles beyond degradation. Moreover, Miro1 may also actively contribute to mitophagy as it enhances the catalytic activity of Parkin.

The study by Safiulina et al. further dissects the mechanism underlying the Miro1 enhancement of Parkin-mediated mitophagy. Specifically, the study shows that Miro1 is required for Parkin translocation to mitochondria, which is independent of PINK1 and Parkin's E3 ligase activity. The

 

study also provides evidence that Miro1-mediated Parkin recruitment is a two-step process and requires Ca2+ binding to Miro's EF-hands. Interestingly, the study provides evidence for a small pool of Parkin that interacts with Miro1 before mitochondrial damage occurs, which may be a critical factor of fast and efficient mitophagy of damaged mitochondria. Taken together, the study suggests that Miro1 may function as a Ca2+-dependent mitochondrial association site for Parkin recruitment in the absence of a trigger for mitophagy.

The study is conceptually and technically well executed and the provided data support the main conclusion. Its findings provide a significant advance in our mechanistic understanding of mitophagy. However, I have a number of concerns.

1) Are both Miro1 and 2 synergistically required for the inhibition of antimycin/oligomycin-induced Parkin translocation, or can each alone also mediate the translocation? In other words, I am asking whether knock-down (KD) of only Miro1 or 2 inhibits antimycin/oligomycin-induced Parkin translocation?

2) Does Miro2 overexpression have similar effects as Miro1 overexpression, or is the recruitment of Parkin by Miro1 overexpression specific to Miro1?

3) Show the data that indicate that PINK1 levels were not affected in the Miro shRNA-treated groups after PINK1-induced Parkin translocation.

4) Fig. 1C-D. The text mentions that scrambled shRNAs were used but the data are not explicitly shown in the figure next to untreated control. The preferred control for the KD is co-expression of resistant Miro1 to truly control for off-target sites.

5) Fig 1F. A control for basal mitophagy in untreated control is required to evaluate the effect of "scr shRNA", which I assume stands for scrambled shRNA. Please define this in figure legend.

6) Fig. 1G. According to the shown rescue data using co-expression of shRNA-resistant Miro1, it seems that either Miro2 is not required for the PINK1-induced Parkin translocation, or Miro1 and Miro2 act redundantly. It is critical to clarify which possibility is the case. Expression of resistant Miro2 to rescue the Miro1/2 double KD should resolve this ambiguity.

7) page 5, 1st paragraph: "When performing the rescue experiments, we observed, to our surprise, that Miro1 overexpression alone changed the Parkin localisation in the cytosol (Fig. 1g)".
Fig 1G does not really show these data; it shows co-expression of shRNA-resistent Miro1 with Miro1/2 shRNAs. Accordingly, one expects that Miro2 is still knocked down, while Miro 1 levels may be slightly reduced or elevated. Because of this ambiguity, I suggest to show data for Miro1 overexpression alone.
8) Fig. 3B. Is there a difference between Miro1 overexpression in PINK1 +/+ and -/- cells? In addition, is FCCP treatment of PINK1 +/+ cells indeed not significant to non-treated control and treated PINK1 -/- cells, as indicated in the figure?

9) Fig. 5A. Co-IPed Parkin is barely visible. Please provide a better image and a quantification for both conditions.

10) Fig. 5B: Co-IPed Miro1 and especially Miro-EF are barely visible. In the case of Miro-EF, its very unclear whether there is no higher-molecular weight band, especially for the basal condition. Please provide a better image and a quantification.

minor
1) Fig. 1A-B. Please provide a short explanation of the shown experiment in the text, where it is currently a very hard jump into the data.

2) I highly suggest to show the rescue of the KD upfront by combining Figs 1B and 1G.

3) page 5, 2nd paragraph. "Miro1 overexpression did not induce mitochondrial depolarisation but instead
it led to slight hyperpolarisation (Fig. 2e,f) consistently with previous report in

 

Drosophila (25855186)". Please explain the meaning of "25855186".

Referee #3:

In the current manuscript, Dr. Kaasik and colleagues suggest that Miro1, an atypical GTPase involved in mitochondrial trafficking and one of the Parkin substrates, also acts as a Ca2+ dependent docking site and safety switch for Parkin recruitment with additional roles in the regulation of mitophagy. The authors provide evidence that a small pool of Parkin interacts with Miro1 before mitochondrial damage occurs. This initial weak interaction does not require PINK1 (or Parkin ubiquitin ligase activity), does not involve ubiquitination of Miro1 and also does not disturb Miro1 functions. However, following mitochondrial damage and PINK1 accumulation, this initial pool of Parkin becomes activated leading to robust Parkin translocation, ubiquitination and degradation of Miro1. Authors further show that the EF hand domains control Parkin recruitment to damaged mitochondria and mutations disturb Ca2+ provoked mitophagy.

Overall this is a well written manuscript with interesting findings and for the most part good quality results, though we don't know the underlying raw data. Such docking mechanisms have been proposed earlier for several mitochondrial Parkin substrates, and indeed would have implications for its activation and future therapeutic strategies. However, given that only overexpression conditions have been investigated herein, the biological relevance remains unfortunately unclear.

Major concerns:
The main concern is that the study is entirely based on a potentially artefactual overexpression system. Experimental evidence on the endogenous levels would significantly strengthen the biological relevance of the presented findings. So would more functional follow up along the sequence of the mitophagy pathway. At this point it is unclear if the observed effects are simply an overexpression phenomenon. While it might be technically challenging, it will be essential to demonstrate the initial weak interaction of Parkin and Miro1 on the endogenous level and to ascertain that defects translate into a biological effect.

Most experiments are transient double/triple transfections (combinations of co-overexpression and shRNA knockdown), but transfection efficiencies are unclear across conditions and for each and every cell (of the same conditions). As authors noted there was reduced overall mtKeima signal when combined with Miro shRNA. Knockdown and overexpression efficiencies are not documented, but must be shown relative to endogenous levels of PINK1, Parkin and Miros (with and without the respective treatment). Ideally on a single cell level for imaging purposes. In general, authors should include representative IF images alongside the quantifications.

When comparing data across figures, a certain variation is evident in the context of Miro overexpression or A/O treatment; the percentage of cells with Parkin translocation ranges between 30 and 80% (compare e.g. Fig. 1D/G, 3A/C and 6B). Similarly for the spatial heterogeneity over time (Fig. 1C and 6A), the slope and max values are quite different in the respective conditions. Is this reduction or delay of Parkin recruitment? Is this the normal variation in these types of experiments?
It would be best to show distribution of cells, rather than means (e.g. Fig 2B, 4D, etc.).

Authors need to determine not only membrane potential at base line, but also response to A/O or FCCP treatment along with stabilization of PINK1 protein in all cases to further exclude confounding effects. For instance there is no stabilization of endogenous PINK1 upon A/O treatment in Fig. 5C.
The present manuscript seems at odds with a previously published study (PMID 22396657) suggesting that loss of Miros rather stimulates mitophagy and results in faster turnover of damaged mitochondria. Perhaps even more important, this study also notes that PINK1 protein levels are altered in Miro1/2 knockdown cells.

Towards a better understanding of the initial Miro1-Parkin interaction, authors should test if Parkin has some additional function besides docking to and traveling alongside with Miro1. Or is Parkin merely waiting for mitochondrial damage and to amplify its recruitment then? E.g. With constant

 

Parkin levels, but gradually increasing Miro1 expression, does that result in more initial recruitment? Does overexpression of Parkin mutations increase retrograde mitochondrial transport similar to WT? What is the role of the mono-ubiquitination?

Though Miro2 knockdown is shown early on and seemed more effective in suppressing Parkin translocation (Fig. 1B) than Miro1, the study focusses on the latter. Are there relevant differences between the two homologs with respect to the findings made? At least this should be discussed in the discussions section.

For most of the overexpression studies it is not entirely clear what the actual control is: untransfected, empty vector transfected? In some instances, Miro1 WT would be an appropriate control (e.g. in Fig 6). Or Miro2.?

Other comments:
Fig. 2G shows YFP bands without Parkin overexpression.
Fig. 2I: Transport effects of Parkin alone are missing.
Fig. 4E: Is this total motility, retrograde or anterograde? Why does Miro1 overexpression not increase movement similar to Fig. 2I?
Fig. 5B: why is there no Parkin co-IP in non-treated conditions?

Molecular weight labels are missing on most western blots.
Few typos and unformatted references.

1st Revision - authors' response — 10th September 2018

**Referee #1:**

**We thank Reviewer 1 for a very detailed review and for their helpful comments and suggestions. We appreciate that the Reviewer acknowledged the importance of our work and pointed out the pitfalls, the majority of which we have addressed.**

The Authors main conclusion is that "Miro1 initiates Parkin recruitment, which is amplified by PINK1 upon mitochondrial depolarization". However, Fig1C shows that the initial phase of Parkin recruitment induced by antimycin+oligomycin is unaffected by targeting Miro, rather the slow phase is suppressed. Furthermore, the other time courses for Miro dependence of Parkin recruitment are different (Fig6A: both the initial and maximal responses are suppressed by MiroEF overexpression; Fig6E: only the initial kinetics is slow) but the source of these discrepancies remains unexplained.

We repeated the A&O-induced Parkin recruitment kinetics in PC6 cells (Fig **1**C - Miro shRNAs and Fig **6**A- Miro1 EF) under similar experimental conditions (A&O-induced Parkin translocation varies depending on the passage number of culture, plating density and number of days in culture) and included also curves for untreated conditions for Fig **1**C. Repeated experiments show more homogenous kinetics and show clearly that both the slopes and maximal responses of the curves are similarly suppressed in Miro shRNAs and Miro1 EF-expressing cells.

Fig **6**E shows the laser-induced Parkin recruitment kinetics in primary neurons. Different induction (A&O versus laser), cell types (PC6 cells versus neurons) and analysis method (heterogeneity analysis versus manual scoring) could underlie why the shapes of the curves are different.

Indeed, it is a recurrent problem with this manuscript that apparently conflicting results are shown in different figures, or relevant conditions are not shown or inducers are switched from one experiment to the next (eg antimycin+oligomycin to FCCP) without explanation. This creates uncertainty about the findings.

We have done our best to minimise the number of discrepancies (FCCP experiments were repeated with A&O for example) to improve our manuscript (see below).

 

**Some examples for this problem and other specific shortcomings are listed below:**

- The Miro silencing condition without mitophagy inducers is not shown in Fig1B,D,E. The Authors note in the text that expression of the reporter was low in the silenced cells but this does not prevent them to show the results for the PINK1 condition. The baseline (without PINK1) should also be shown at least in Fig1E to validate whether the effect of PINK1 was less in the Miro1 deficient condition.

We included the requested control groups for Fig **1**B and D. Note that we repeated the experiment depicted in Fig **1**D to include the additional Miro1 or Miro2 shRNA groups as requested by Referee 2 (comment 1).

We also repeated the experiment shown in Fig **1**E to include a Miro shRNAs baseline group (without PINK1). The effect of PINK1 was considerably stronger in the repeated experiment but Miro shRNAs were similarly protective. We also performed a two-way ANOVA, which showed a clear interaction between the PINK1 and Miro shRNAs groups ($P < 0.0001$ for interaction) suggesting that Miro shRNAs suppress specifically PINK1-induced mitophagy.

- Next, the Authors claim that Miro1 rescue restored the effect of PINK1 but rather Miro1 overexpression ALONE resulted in a Parkin response as large as the effect of PINK1 in mock silenced cells (Fig1F).

To avoid misunderstanding, we excluded the panel showing this experiment. We followed the suggestion of Referee 2 (comment 7) and replaced Fig 1F with new data (see new Fig **2**B), showing that overexpression of myc-Miro 1 or untagged Miro 2 alone induces a Parkin response.

- The 2 assays used for Parkin translocation give conflicting results for the different stimuli. When the cells with Parkin translocation are counted, Miro overexpression alone is as effective as PINK1 (Fig1F). However, when the heterogeneity in Parkin fluorescence is measured Miro overexpression is barely above the baseline, whereas PINK1 exerts a robust effect (Fig2B). Please explain. Also, please state what time point was evaluated in Fig2B and in the other panels?

The results depicted previously in Fig **1**G (now Fig 2**B**) show the percentage of cells in which Parkin translocated to mitochondria, i.e., a population-based analysis. This assay does not show the *strength* of Parkin translocation, i.e., the amount lost from the diffuse cytosol pool. On the other hand, the heterogeneity assay depicted previously in Fig **2**B (now Fig **2**C) does show the strength of Parkin translocation from cytosol to mitochondria within single cells. Miro1 or PINK1 overexpression induce Parkin translocation in a similar proportion of cells but the translocation is significantly stronger in individual PINK1-overexpressing cells (compare panels in Fig 2A). We did our best to explain the difference in revised manuscript (**RESULTS: Miro1 recruits Parkin to polarized mitochondria**, first paragraph*)*.

The time point evaluated in (previous) Fig **2**B (now Fig **2**C) was 3h after A&O treatment. It is now mentioned in all legends.

- Fig2A shows that Miro1 overexpression alone can stimulate Parkin recruitment to the mitochondria and this is claimed to be independent of the EF-hand. However, Fig6A shows the lack of increased Parkin recruitment in the Miro1EF expressing cells inconsistently with the Authors' model.

Fig **2**A indeed shows that Miro1 WT overexpression alone recruits Parkin to mitochondria but we make no claim that it is independent of the EF-hands. In contrast, in Fig **6** we show that Miro1 with mutated EF hands inhibits EYFP-Parkin translocation induced by several different stimuli (A&O, PINK1 overexpression, laser irradiation). We claim that the Miro1 EF-hand domain plays a relevant role in Parkin recruitment to damaged mitochondria.

- Is Parkin interaction with Miro specific of Miro1 isoform? Could Parkin interact with Miro2? Miro2 silencing seems to give and even stronger phenotype than Miro1 silencing (Fig1) but the rest

 

of the studies are confined to Miro1. Did the authors try to overexpress Miro2 and measure the percentage of cells with Parkin translocation?

When we overexpressed myc-Miro1 and myc-Miro2 (Addgene plasmids 47888 and 47891, Fransson et al, J Biol Chem. 2003: 278:6495-502), myc-Miro1 but not myc-Miro2 induced Parkin translocation. However, immunohistochemistry experiments demonstrated that while overexpressed myc-Miro1 co-localised perfectly with a mitochondrial marker, overexpressed myc-Miro2 remained largely cytosolic (**Appendix Fig S**2). As we believed that this could be due to Miro2's myc tag, we performed similar experiments using untagged Miro2 (Origene SC127208). In contrast to myc-Miro2, the untagged-Miro2 localised perfectly to mitochondria and also induced Parkin translocation. Parkin also co-immunoprecipitated with untagged Miro2 (**Appendix Fig S4**).

These data enable us to conclude that Miro1 and Miro2 act redundantly in these circumstances. We included these data in the revised manuscript version (see Fig **2**B and **Appendix Fig S4**).

- Is Miro1 and Miro2 shRNA specific to each isoform? The reference used to validate the experimental model (Cagalined et al, 2013) doesn't show a western blot quantification of the protein amount of both Miro isoforms when one of them is silenced. Moreover, the antibody used for recognizing Miro1 in this paper (Atlas, HPA010687) is known to recognize both Miro isoforms.

We revalidated our Miro1 and Miro2 shRNAs by PCR. Fig **EV**1A in the revised version shows that each shRNA specifically suppresses its specific isoform without suppressing the other. We observed an increase in Miro2 expression when we suppressed Miro1 that could be compensatory and only a very slight decrease in Miro1 expression when we suppressed Miro2.

- The Authors concluded in the legend of Figure 2I that: "Overexpression of Miro1 alone increased the fraction of motile mitochondria moving in both directions as well as the relative mitochondrial velocity." However, the time in motion analysis doesn't provide the information of the fraction of motile mitochondria. This analysis only shows a histogram with the percentage of mitochondria that spent a certain time moving in the retrograde or anterograde direction. Is the fraction of mobile mitochondria different after Miro1 overexpression?

Miro1 overexpression increased the fraction of mobile mitochondria (**Appendix Fig S3** in the revised version).

Furthermore, we repeated the experiment previously shown in Fig **2**I **(**now Fig **2**F) to include also Parkin only group to as requested by Reviewer 3. Note that we also corrected the legend to Fig 2F.

- Is the EF hand motif of the Miro1 K572R functional? The authors state that "Note also that Miro1 K572R was otherwise fully functional as it was able to restore mitochondrial motility in Miro shRNA expressing cells similar to Miro1 WT (Fig. 4e)". However, Fig. 4e doesn't show if the Ca2+ binding capacity of Miro1 is intact in Miro1 K572R. At resting cytoplasmic [Ca2+], mitochondrial movement is enhanced by Miro overexpression independently of the presence of the EF-hands, as it was shown in Saotome et al, 2008. In order to check if Miro1 K572R is fully functional the authors should determine if the Ca2+-induced arrest of mitochondrial motility is still intact.

The referee raised an important question, and we performed additional experiments to check whether mitochondrial motility in neurons expressing Miro1 K572R is arrested in response to increased cytosolic $Ca^{2+}$. Indeed, we found that the $Ca^{2+}$-induced arrest of mitochondrial motility in Miro1 K572R-expressing neurons was similar to wt Miro1-expressing neurons (see Fig **EV**3B,C) suggesting that the EF hands in Miro1 K572R are functional.

- In Fig. 6 the Authors showed that Ca2+-insensitive Miro1 inhibits Parkin translocation to mitochondria. Is the Parkin translocation to mitochondria altered in cells expressing Miro1 K572R?

Miro1 K572R expression alone recruited Parkin to mitochondria similarly to Miro1 WT (Fig **4**C). However, the A&O induced Parkin recruitment in the Miro1 K572-expressing cells was not different from control (Fig **4**D). Thus, we concluded that K572 is not involved in the initial step of Parkin recruitment but rather is relevant for the second step of massive ubiquitination.

 

Are the Ca2+ sensing EF-hands required for Parkin translocation to mitochondria as the Authors state or is the ubiquination of Miro1 (which is abolished in the Ca2+-insensitive Miro1) required for Parkin translocation to mitochondria?

The referee raises a very interesting point for discussion. We have several lines of evidence suggesting that in context of the Miro-Parkin interaction, the initial docking step of Parkin recruitment does not involve ubiquitination of Miro but involves the $Ca^{2+}$ sensing EF-hands of Miro:

- WT Miro1 also recruits ubiquitin ligase-dead Parkin (i.e., ubiquitination by Parkin is not required; Fig **3**C)
- Miro1 EF overexpression does not recruit Parkin to mitochondria (Fig **6**C) but both Miro1 WT and Miro1 K572R do (Fig **4**C,D).
- The Miro1 EF interaction with Parkin tends to be weaker than between Miro1 WT and Parkin (Fig **5**A)

We therefore suggest that the $Ca^{2+}$-sensing EF hands are required for the first step of Parkin recruitment. Ubiquitination of Miro seems to not be necessary for docking but is required for the second step of Parkin recruitment.

- Is the glutamate-induced Ca2+ rise in cells expressing Miro1 EF or Miro1 WT similar to control cells? It would be important to show that the Glutamate-induced Ca2+ entry is not affected by the overexpression of Miro1 EF or Miro1 WT since the authors have shown that the cytosolic Ca2+ levels affect Parkin translocation to mitochondria.

We performed an additional experiment (now shown as Fig **7**C,D) showing that glutamate-induced $Ca^{2+}$ entry is not different in Miro1 EF- or Miro1 WT-overexpressing neurons when compared with control neurons.

- Is the parkin-Miro1 interaction observed physiological or is only due to the overexpression of Miro1?

We absolutely agree that this point is critical to address (it was also raised by the other referees). We have now included new data showing that endogenous Parkin and Miro interact (see new Fig **2**I). We also observed that endogenous Parkin co-immunoprecipitated with overexpressed Miro1 (see Fig 5A).

- It remains untested whether the change in mitochondrial motility associated with Miro loss has a primary role in the change in Parkin recruitment.

We thank the reviewer for raising this question. We performed additional experiments showing that while overexpression of syntaphilin inhibited mitochondrial motility, it had no effect on Parkin translocation either alone or in presence of inducers (Fig **EV**2).

- Fig1F and Fig3A seem to evaluate Parkin translocation by the same method but the effect of Miro overexpression is 20 vs 90 in the 2 graphs. What is the reason for the difference?

The amplitude of the effect of A&O treatment or Pink1 and Miro1 overexpression on Parkin translocation varies depending on the passage number of culture, plating density and number of days in culture. We best saw the effects of co-treatments when the dynamic range was between 20-40%. We repeated the experiments depicted in (previous) Fig**3**A and B using A&O rather than FCCP (see next comment, see new Fig 3A, B). Thus, the data are now comparable.

*Minor concerns*

- Figs1&2 used antimycin+oligomycin to perturb mitochondria. Why is FCCP used in Fig3?

We performed new experiments with A&O to replace the mentioned FCCP experiments (Fig**3**A and B). We note that FCCP is a traditional depolarising agent, and use of antimycin & oligomycin for Parkin translocation has only recently become widely accepted.

 

- The Authors state: "Results depicted in Fig. 1a,b demonstrate that loss of Miro proteins suppressed significantly the number of PC6 cells showing PINK1-induced Parkin translocation to mitochondria. This cannot be explained by altered PINK1 levels because the latter was not affected in the Miro shRNA-treated groups." How was the PINK1 level determined?

PINK1 levels were determined by Western blotting and are now shown in Fig **EV**1B,C.

- Images of Fig.1A are dark and the colors are not defined in the figure and also in the legend.

We increased the contrast of Fig**1**A and defined the colours in the legend.

- In the manuscript and legends both YFP-Parkin and EYFP-Parkin are referred. Have you used both type of sensors or it is a misspelling?

We used only EYFP-Parkin and have now corrected all iterations in the revised manuscript.

- Fig 2A, C, D and E are missing their respective controls to compare Parkin distribution.

We have now included the requested control images. Note that the previous Fig **2**C is now Appendix Fig **S**1A, previous Fig **2**D is now Appendix Fig **S**1B and previous Fig 2E is now Fig **2**D.

- From Fig. 2E cannot be concluded that "Miro1 overexpression did not induce mitochondrial depolarisation but instead it led to slight hyperpolarization" because TMRE quantification or comparison to control is missing.

We now show control images in Fig **2**D and the quantification (including statistical comparison to control) in Fig **2**E demonstrating conclusively that Miro1 overexpression leads to slight mitochondrial hyperpolarisation.

- Mitochondrial accumulation of the dye used for mitochondrial membrane potential measurement is affected by many factors. Thus, it is essential that the uncoupler sensitivity is validated (Fig2F).

We repeated the experiment and performed the requested treatment using an uncoupler and present these data in Fig **2**E. We also include the FCCP controls for membrane potential experiment depicted now in Fig **EV**4C.

- More detailed explanation of how "Time in motion (%)" was measured should be provided in material and methods section.

In the revised version, we explain the calculation of motility parameters in detail in **MATERIALS AND METHODS: Mitochondrial trafficking and mitochondrial density** (at the end of first paragraph).

- Fig. 2J missing the duration of the A&O treatment

We have now included the treatment duration (3 h) in the legend (see legend to new Fig **2**H)

- In Fig. 7I, is the control after glutamate treatment the same in the right and left panels? Left panel, control present around 45-50% of cells with Parkin punctae after glutamate treatment, while in the right panel the value is around 35%. Could be the results on control merged? In the affirmative case, would the expression of Miro1 EF still induce a significant decrease in the percentage of cells with Parkin punctae?

We merged the right and left panels of Fig 7F (earlier Fig 7I) as suggested. The difference between the glutamate-treated control and glutamate-treated Miro-EF groups remains statistically different (***P*** = 0.0028, one-way ANOVA).

- Missing controls in Fig. 7J and 7K. Was the number of autophagosomes colocalising with mitochondria in neuron body (Fig. 7J) and the mitochondrial density in axons (Fig. 7K) altered by Miro1 EF and Miro1 wt overexpression at baseline?

 

We now show the number of autophagosomes colocalising with mitochondria, mitochondrial density, ATP levels and neuronal viability at baseline in Fig **EV**5. Note that the number of autophagosomes colocalising with mitochondria and the mitochondrial density were not altered by Miro1 EF and Miro1 WT overexpression at baseline.

- Correction at page 11: "Transient glutamate exposure induces Ca2+ influx into the cytosol leading to mitochondrial Ca2+ uptake and depolarisation of the inner mitochondrial membrane (Schinder et al, 1996; White & Reynolds, 1996)."

We have now amended the format of the ionic charge.

The functional relevance of the findings on modifying Parkin translocation to mitochondria could be tested by cell viability using cells overexpressing Miro1 EF (suppressed Parkin translocation) and Miro1 WT (enhanced Parkin translocation) compared to control cells. Another possibility would be to check the O&A induced degradation of mitochondrial proteins (such as COXIV or TIM23) in these conditions.

We performed ATP measurements in single neurons and valuable excitotoxicity experiments to show that our findings have physiologic relevance. Glutamate treatment induced a clear and significant decrease in ATP levels that was reversed by the Miro1 EF and augmented by the Miro1 WT (see new Fig **7**I). Miro1 EF but not Miro1 WT also improved viability after glutamate excitotoxicity (Fig **7**J).

**Referee #2:**

**We thank Reviewer #2 for their helpful comments and suggestions, which have improved our manuscript and clarified the presentation of our data.**

1) Are both Miro1 and 2 synergistically required for the inhibition of antimycin/oligomycin-induced Parkin translocation, or can each alone also mediate the translocation? In other words, I am asking whether knock-down (KD) of only Miro1 or 2 inhibits antimycin/oligomycin-induced Parkin translocation?

We repeated the experiment to include the requested groups. Results (new Fig **1**D) show that both Miro1 shRNA and Miro2 shRNA inhibit antimycin & oligomycin-induced Parkin translocation.

2) Does Miro2 overexpression have similar effects as Miro1 overexpression, or is the recruitment of Parkin by Miro1 overexpression specific to Miro1?

Miro2 overexpression induces Parkin recruitment similarly to Miro1 (new Fig **2**B). Note that a myc-tagged Miro2 construct fails to localise properly to mitochondria. We can see the translocation only when overexpressing untagged Miro2.

3) Show the data that indicate that PINK1 levels were not affected in the Miro shRNA-treated groups after PINK1-induced Parkin translocation.

PINK1 levels were determined by Western blotting and are now shown in Fig **EV**1B,C.

4) Fig. 1C-D. The text mentions that scrambled shRNAs were used but the data are not explicitly shown in the figure next to untreated control. The preferred control for the KD is co-expression of resistant Miro1 to truly control for off-target sites.

We used scrambled shRNA for all control groups to compensate for the Miro shRNAs. We included this explanation under the **MATERIALS AND METHODS/Transfection** (at the end of the section). We agree that shRNA-resistant Miro1 or Miro2 co-expressions with shRNAs would have been the perfect controls but as both Miro isoforms induced Parkin translocation itself we were not able to perform these experiments.

 

5) Fig 1F. A control for basal mitophagy in untreated control is required to evaluate the effect of "scr shRNA", which I assume stands for scrambled shRNA. Please define this in figure legend.

We defined scr shRNA in the legend of Fig **1B**.

6) Fig. 1G. According to the shown rescue data using co-expression of shRNA-resistant Miro1, it seems that either Miro2 is not required for the PINK1-induced Parkin translocation, or Miro1 and Miro2 act redundantly. It is critical to clarify which possibility is the case. Expression of resistant Miro2 to rescue the Miro1/2 double KD should resolve this ambiguity.

When we overexpressed myc-Miro1 and myc-Miro2 (Addgene plasmids 47888 and 47891, Fransson *et al*, J Biol Chem. 2003: 278:6495-502), only myc-Miro1 but not myc-Miro2 induced Parkin translocation. However, immunohistochemistry experiments demonstrated that while overexpressed myc-Miro1 was perfectly co-localised to a mitochondrial marker, overexpressed myc-Miro2 remained largely cytosolic (Appendix Fig **S**2). As we believed that this could be an artefact of the myc tag, we performed similar experiments using untagged Miro2 (Origene SC127208). In contrast to myc-Miro2, the untagged-Miro2 localised perfectly to mitochondria and also induced strong Parkin translocation. These data suggest that Miro1 and Miro2 act redundantly in these circumstances. We have now included these data in the revised manuscript (see new Fig **2**B).

7) page 5, 1st paragraph: "When performing the rescue experiments, we observed, to our surprise, that Miro1 overexpression alone changed the Parkin localisation in the cytosol (Fig. 1g)".
Fig 1G does not really show these data; it shows co-expression of shRNA-resistent Miro1 with Miro1/2 shRNAs. Accordingly, one expects that Miro2 is still knocked down, while Miro 1 levels may be slightly reduced or elevated. Because of this ambiguity, I suggest to show data for Miro1 overexpression alone.

We now show data for Miro1 and Miro2 overexpression alone (see new Fig **2**B).

8) Fig. 3B. Is there a difference between Miro1 overexpression in PINK1 +/+ and -/- cells?

We tested that and overexpressed Miro levels are higher in PINK1-/- MEFs (Appendix Fig **S**5). Our data shows that overexpression of GFP-Miro1 yields a brighter signal in PINK1 -/- MEFs suggesting that a lack of PINK1 inhibits Miro1 ubiquitination and removal. Previously, Liu *et al*, 2012 published contradictory results showing that PINK1 negatively regulates Miro level in Drosophila while in mammalian cells endogenous Miro1 and Miro2 levels were reduced in PINK1-depleted cells.

In addition, is FCCP treatment of PINK1 +/+ cells indeed not significant to non-treated control and treated PINK1 -/- cells, as indicated in the figure?

The FCCP treatments were significant but we omitted the asterisks. In the revised manuscript, we have replaced the FCCP data with A&O treatment to make the experiment comparable to other experiments as suggested by other Reviewers (see new Fig **3**B).

9) Fig. 5A. Co-IPed Parkin is barely visible. Please provide a better image and a quantification for both conditions.

We include a high-contrast grayscale image for Parkin to show the differences more clearly and also include the quantification (see new Fig **5**A).

10) Fig. 5B: Co-IPed Miro1 and especially Miro-EF are barely visible. In the case of Miro-EF, its very unclear whether there is no higher-molecular weight band, especially for the basal condition. Please provide a better image and a quantification.

We now include a high-contrast grayscale image for Miro1 to show the differences more clearly and also include the quantification (see new Fig **5**B).

**minor**

 

1) Fig. 1A-B. Please provide a short explanation of the shown experiment in the text, where it is currently a very hard jump into the data.

We now provide a short explanation to make it easier to follow.

2) I highly suggest to show the rescue of the KD upfront by combining Figs 1B and 1G.

We followed the suggestion of Referee 1 and omitted the rescue experiments depicted in (previous) Fig **1**G. Since overexpression of both Miro1 alone and Miro2 alone resulted in a Parkin response as large as the effect of A&O or PINK1, a rescue experiment will not provide any conclusive results. We show the data for overexpression of Miro1 or Miro2 in (new) Fig **2**B as requested by Referee (major concern 7).

3) page 5, 2nd paragraph. "Miro1 overexpression did not induce mitochondrial depolarisation but instead it led to slight hyperpolarisation (Fig. 2e,f) consistently with previous report in Drosophila (25855186)". Please explain the meaning of "25855186".

We have now inserted the correct citation.

**Referee #3:**

**We thank Reviewer #3 for their helpful comments and suggestions. We have now included the source data, included the data showing interaction of endogenous Parkin and Miro1 and provided additional data to show the physiological relevance of our findings. Below, we provide a point-by-point reply to the Reviewer's comments.**

**Major concerns:**

The main concern is that the study is entirely based on a potentially artefactual overexpression system. Experimental evidence on the endogenous levels would significantly strengthen the biological relevance of the presented findings. So would more functional follow up along the sequence of the mitophagy pathway. At this point it is unclear if the observed effects are simply an overexpression phenomenon. While it might be technically challenging, it will be essential to demonstrate the initial weak interaction of Parkin and Miro1 on the endogenous level and to ascertain that defects translate into a biological effect.

We agree with the reviewer that a lack of endogenous interaction was one of the weakest points of our manuscript. In the revised manuscript, we have included new data showing that endogenous Parkin and Miro also interact (see new Fig **2**I).

With regard to our data's physiological relevance, we have also performed ATP measurements in single neurons and excitotoxicity experiments. Glutamate treatment induced a clear and significant decrease in ATP levels that was augmented by Miro1 WT and reversed by the Miro1 EF construct (see new Fig **7**I). Additionally, Miro1 EF, but not Miro1 WT, also improved viability after glutamate excitotoxicity (see new Fig **7**J).

Most experiments are transient double/triple transfections (combinations of co-overexpression and shRNA knockdown), but transfection efficiencies are unclear across conditions and for each and every cell (of the same conditions). As authors noted there was reduced overall mtKeima signal when combined with Miro shRNA.

We have earlier validated that, independently of the transfection efficiency, in the case of double/triple transfections with Lipofectamine 2000, transfected plasmids are mostly localised together to the same cells (Cagalinec *et al.* 2016 (https://doi.org/10.1371/journal.pbio.1002511). For example, when neurons were transfected with plasmids encoding mitochondrial CFP mitochondrial YFP, and mitochondrial mKate2, 93±1% transfected cells expressed all three markers, 5±1% expressed two markers, and 2±0.4% expressed one marker).

 

Knockdown and overexpression efficiencies are not documented, but must be shown relative to endogenous levels of PINK1, Parkin and Miros (with and without the respective treatment). Ideally on a single cell level for imaging purposes. In general, authors should include representative IF images alongside the quantifications.

We validated the expression of PINK1, Parkin and Miro levels by immunofluorescence as suggested. Appendix Fig **S**2 shows that overexpression of PINK1, Parkin, Miro1 or Miro2 significantly increase the expression of the respective proteins in PC6 cells expressing the transfection marker mito-Kate2. There was also a clear decrease in expression of Miro proteins in Miro shRNA-expressing cells. We are very thankful for this suggestion as it helped us find out that the product of the myc-Miro2 plasmid did not localise to mitochondria as we were expecting (Appendix Fig **S**2, lowest panel).

Unfortunately it is technically very demanding, if not impossible, to perform immunofluorescence in the same cells as used for live cell imaging. For that, the imaged cells should be somehow marked, fixed and subjected to immunocytochemistry, after which the same cells should be re-identified and analysed. Although theoretically possible, this is not compatible with routine live-cell imaging experiments.

When comparing data across figures, a certain variation is evident in the context of Miro overexpression or A/O treatment; the percentage of cells with Parkin translocation ranges between 30 and 80% (compare e.g. Fig. 1D/G, 3A/C and 6B). Similarly for the spatial heterogeneity over time (Fig. 1C and 6A), the slope and max values are quite different in the respective conditions. Is this reduction or delay of Parkin recruitment? Is this the normal variation in these types of experiments?

The amplitude of the effect of A&O treatment or Pink1 and Miro1 overexpression varies depending on the passage number of culture, plating density and number of days in culture. A dynamic range of translocation of between 20-40% was where we could best see the effects of co-treatments. We repeated the experiments depicted in Fig **3**A and B (see next comment) and these are more comparable with other experiments.

We repeated the A&O-induced Parkin recruitment kinetics in PC6 cells (Fig **1**C - Miro shRNAs and Fig **6**A- Miro1 EF) under similar experimental conditions and included also curves for untreated conditions for Fig **1**C. These repeated experiments show more homogenous kinetics and show clearly that both the slopes and maximal responses of the curves are similarly suppressed in Miro shRNAs and Miro1 EF expressing cells.

It would be best to show distribution of cells, rather than means (e.g. Fig 2B, 4D, etc.).

In the revised manuscript, we show the distribution of cells in (previous) Fig **2**B (see now Fig **2**C). However, for the sake of clarity, we prefer to show the means in Fig **4**D as the number of data points is too high (152-172 per group) for a scatter dot blot. We have included the source data for Fig **4**D for interested readers.

Authors need to determine not only membrane potential at base line, but also response to A/O or FCCP treatment along with stabilization of PINK1 protein in all cases to further exclude confounding effects.
For instance there is no stabilization of endogenous PINK1 upon A/O treatment in Fig. 5C.

We performed additional experiments and now show also membrane potential after FCCP treatment (new Fig **2**E and new Fig **EV**4C). The results demonstrate that the FCCP-insensitive fraction of membrane potential is very small.

We now also include a blot (new Fig **EV**1D) showing that PINK1 accumulates after A&O treatment.

In Fig 5C, we show overexpressed PINK1-V5 using anti-V5 antibody. The weak bands in the PINK1 panel at 72 kDa are nonspecific bands and not endogenous PINK1. We mention this in the revised manuscript (new Fig 5 legend, last sentence) to avoid misunderstanding.

 

The present manuscript seems at odds with a previously published study (PMID 22396657) suggesting that loss of Miros rather stimulates mitophagy and results in faster turnover of damaged mitochondria. Perhaps even more important, this study also notes that PINK1 protein levels are altered in Miro1/2 knockdown cells.

We include in the revised manuscript quantitative Western blotting data showing that the level of overexpressed PINK1 in PC6 cells is not significantly altered when both Miro1 and Miro2 are knocked down (Fig **EV**1B,C).

In Fig 6A of Liu et al, 2012 (PMID 22396657), CCCP-induced PINK1 levels are reduced in Miro1/2 knockdown cells. However, authors themselves do not mention whether PINK1 levels are altered in their study. Moreover, there appears to be is no visible difference in the basal (CCCP untreated) levels of endogenous PINK1 in control and Miro RNAi-treated HeLa cells. Importantly, this study depicts WB and immunofluorescence images only without quantitative data.

Towards a better understanding of the initial Miro1-Parkin interaction, authors should test if Parkin has some additional function besides docking to and traveling alongside with Miro1. Or is Parkin merely waiting for mitochondrial damage and to amplify its recruitment then? E.g. With constant Parkin levels, but gradually increasing Miro1 expression, does that result in more initial recruitment?

We speculate that Miro1 could serve as a mitochondrial "docking station" or receptor for inactive Parkin that facilitates its recruitment to depolarised mitochondria. Indeed, a gradual increase in plasmid DNA concentration of Miro also leads to increased initial Parkin recruitment (for example: Miro2 DNA per dish 0µg: 1.5±0.6%; 0.1µg: 10.7±3.2%; 0.2µg: 19.5±1.7%; 0.3µg: 34.0±1.1%; n = 5 dishes).

It is likely not to be its only function (Parkin also has substrates other than in the mitochondrial membrane, *PMID: 28399880*) but it is likely to be one of its major functions.

Does overexpression of Parkin mutations increase retrograde mitochondrial transport similar to WT?

In our experiments, overexpression of T240R Parkin in cortical neurons had no significant effect on retrograde ($p = 0.118$, Mann-Whitney test, 399 mitochondria analysed from the control group and 426 from the Miro-T240R expressing group) or anterograde ($p = 0.387$) velocities. Thus, our data suggests that ligase-dead Parkin has no effect on mitochondrial motility. However, we do not include these data in the manuscript as they are not directly related to our main research questions.

What is the role of the mono-ubiquitination?

We do not have a clear-cut answer to that question. Fig **4**B and Fig **5**B suggest that Miro1 WT, Miro1-EF and Miro1-K572R (where the primary ubiquitination site is mutated) are monoubiquitinated already at basal conditions but we do not know which other lysine residue is monoubiquitinated (in vitro ubiquitination assay results published by Kazlauskaite et al, 2014a and Klosowiak et al, 2016 suggests that Miro1 is multi-monoubiquitinated) or whether that residue is ubiquitinated by Parkin or by other ubiquitin ligases. In the latter case, it could be speculated that the monoubiquitination might be not related to mitophagy and may regulate some other function of the protein. The K572R mutation does not affect known functions of Miro1: it is still capable of mediating mitochondrial transport and responds to glutamate (Fig **EV**3)

Though Miro2 knockdown is shown early on and seemed more effective in suppressing Parkin translocation (Fig. 1B) than Miro1, the study focusses on the latter. Are there relevant differences between the two homologs with respect to the findings made? At least this should be discussed in the discussions section.

Miro1 and Miro2 knockdown suppressed Parkin translocation more or less similarly (see new Fig **1**B and D). Also, when overexpressed, then Miro1 and untagged Miro2 (but not the myc-Miro2 construct that remained largely cytosolic when overexpressed) both recruited Parkin alone (see new Fig **2**B). This suggests that Miro1 and Miro2 act redundantly in these circumstances.

 

For most of the overexpression studies it is not entirely clear what the actual control is: untransfected, empty vector transfected? In some instances, Miro1 WT would be an appropriate control (e.g. in Fig 6). Or Miro2.?

In the case of Miro shRNA experiments, we always used scrambled shRNA as a control. In the case of overexpression, we always used either an empty vector, EGFP/YFP or firefly luciferase for compensation. We have included this explanation in the **MATERIALS AND METHODS/Transfection** (at the end of the section) and have also corrected the legend of Fig **1**.

We were not able to use Miro1 or Miro2 as a negative control in most of the Parkin translocation experiments as each induced weak Parkin translocation themselves.

**Other comments:**

Fig. 2G shows YFP bands without Parkin overexpression.

For the first two lanes in Fig **2**G, we used YFP as a control (compensatory plasmid) in groups not expressing Parkin. This is now mentioned in the legend.

Fig. 2I: Transport effects of Parkin alone are missing.

We have now included a Parkin-only group (see new Fig **2**F) and repeated this experiment altogether four times. The summarised results show that overexpression of Parkin alone had no any effect on mitochondrial motility. Overexpression of Parkin together with Miro1 had a tendency to increase the mitochondrial motility when compared with Miro1 but this difference did not reach statistical significance in new combined dataset.

Fig. 4E: Is this total motility, retrograde or anterograde? Why does Miro1 overexpression not increase movement similar to Fig. 2I?

The right panel of (previous) Fig. **4**E (see now Figure **EV**3A) represents total motility. It shows that both Miro1 wt and Miro1 K572R can restore mitochondrial motility when endogenous Miro1 and Miro2 are suppressed by their shRNAs. Endogenous Miro2 remains silenced and this is the likely reason why the values are below the control level. In (previous) Fig **2**I (see now Fig **2**F), we overexpressed Miro1 without suppressing endogenous Miros. We have rephrased the legend for Fig **EV**3A and relabelled the figure to prevent misunderstanding.

Fig. 5B: why is there no Parkin co-IP in non-treated conditions?

We immunoprecipitated ubiquitin in this experiment and Parkin should not co-IP with ubiquitin under control conditions. Parkin should co-IP with ubiquitin only after A&O, when it becomes autoubiquitinated.

Molecular weight labels are missing on most western blots.

We have now included molecular weight labels for all blots.

Few typos and unformatted references.

We did our best to correct all typos and references.

 

2nd Editorial Decision 9th October 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by the original referees whose comments are shown below.

As you will see they find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can officially accept your manuscript there are a few editorial issues concerning text and figures that I would ask you to address in a final revised version.

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REFEREE REPORTS

Referee #1:

The Authors carefully addressed each point. I support publication of the revised ms.

Referee #2:

The revised manuscript has been markedly improved and the authors have addressed all of my previous concerns. I have no further concerns.

Referee #3:

In the new version the authors have addressed the majority of my concerns. They now provide considerable evidence that supports a role of Miro as a calcium sensitive docking site for Parkin on mitochondria and thereby reveal a new and important step for its recruitment. Overall the revised manuscript has significantly improved and includes now additional data such as the endogenous interaction, important controls and repeated experiments that show less variation. The findings are novel and significant and the conclusions are supported by the presented data. I recommend publication of this study.

 

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

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

## A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

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

### 2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple 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? | No pre-specified effect size was measured. The sample size was chosen based on experiment type according our previous experience. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | NA |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | No samples were excluded from the analysis |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | Culture dishes for transfection and treatment were taken randomly |
| For animal studies, include a statement about randomization even if no randomization was used. | NA |
| 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. | All microscopy image analysis were performed blindly: dishes were coded after transfection and codes revealed after analysis. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | NA |
| 5. For every figure, are statistical tests justified as appropriate? | Yes. Student t test or one-way ANOVA and repeated measures ANOVA followed by Sidak posthoc test where used in case of normal distribution. t-test with Welch's correction or Welch's ANOVA followed by Games-Howell test were used when the variances were different. Mann Whitney U test or Kruskal-Wallis tests followed by the Dunn test were used when the data did not pass the normality test. Two-way ANOVAs were used to analyse interactions between two factors. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | Yes, D'Agostino and Pearsons omnibus test was applied |
| Is there an estimate of variation within each group of data? | Yes , standard error of the mean or 1.5 IQR (Tukey boxplot) were used |
| Is the variance similar between the groups that are being statistically compared? | Yes, to test equality of variances, we used the F test for two conditions or Brown and Forsythe test for more than two conditions. |

## C- Reagents

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| http://www.selectagents.gov/ | List of Select Agents |

| | |
|---|---|
| 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). | Immunoprecipitation: mouse anti-MYC (Invitrogen, 46-0603), rabbit anti-HA (Abcam, ab9110), mouse anti-RHOT1 (Atlas, AMAb90852), rabbit anti-RHOT2 (11237-1-AP, Proteintech). Immunoblotting: rabbit anti-RHOT1 (Atlas, HPA010687), rabbit anti-PINK1 (Atlas, HPA001931), rabbit anti-PARK2 (Abcam, ab15954), rabbit anti-MYC (Abcam, ab9106), mouse anti-MYC (Invitrogen, 46-0603), mouse anti-β actin (Sigma-Aldrich, A2228), goat anti-Ubiquitin (Abcam, ab9134), rabbit anti-S65-Ubiquitin (Biotechne, A-110), mouse anti-GFP (Abcam, ab1218), rabbit anti-GFP (Abcam, ab290). IRDye® Secondary Antibodies for Western blot immunodetection: goat anti-rabbit IRDye 800CW (926-32211), goat anti-rabbit680LT (926-68021), goat anti-mouse IRDye 800CW (926-32210), goat anti-mouse 680LT (926-68020), donkey anti-goat IRDye 680RD (926-68074; all from LI-COR Bioscience). Immunofluorescence: rabbit anti-RHOT1 (Atlas, HPA010687), rabbit anti-PINK1 (Atlas, HPA001931), rabbit anti-PARK2 (Abcam, ab15954), rabbit anti-RHOT2 (11237-1-AP, Proteintech), Goat anti-Rabbit IgG (H+L) Secondary Antibody Alexa Fluor 488 (ThermoFisher Scientific, A-11034). |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | Primary culture of (Wistar) rat cortex (generated for this study according to doi: 10.1242/jcs.118844); 293 H Cells (Thermo Fisher Scientific, Cat#11631017); PC6.3 Cells (from https://doi.org/10.1007/s00018-010-0305-y); Mouse embryonic fibroblasts (generated from PINK1-deficient mice B6;129-PINK1tm1Aub/J (Jackson Laboratories RRID:IMSR_JAX:013050) and wild type C57Bl/6J mice (Choubey et al. Autophagy. 2014;10:1105-19)). We haven't tested for mycoplasma contamination. |

* for all hyperlinks, please see the table at the top right of the document

## D- Animal Models

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| 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. | NA |
| 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. | National and ARRIVA guidelines were followed |

## E- Human Subjects

| | |
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| 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

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| 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'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>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. 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

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| 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. | No |