THE EMBO JOURNAL

Manuscript EMBO-2012-84290

Ret rescues mitochondrial morphology and muscle degeneration of Drosophila Pink1 mutants

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Review timeline:

Submission date:	20 December 2012
Editorial Decision:	11 February 2013
Revision received:	01 July 2013
Editorial Decision:	07 August 2013
Revision received:	08 November 2013
Accepted:	29 November 2013

Editor: Karin Dumstrei

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 11 February 2013

Thank you for submitting your manuscript to the EMBO Journal. Please find enclosed the two reports on the paper. I am still waiting for a third report, but given the present comments I can take a decision at this time.

As you can see below, both referees appreciate that the analysis provides new insight, but they also find the analysis at present too preliminary. Significant revisions are needed for publication here. In particular the findings need to be extended to dopaminergic neurons, the rescue effects need to be better characterized and we need more mechanistic insight into how Ret1 affect complex1 activity. Should you be able to extensively revise the manuscript and address the concerns raised by the referees then we would be interested in considering a revised manuscript. I should add that it is EMBO Journal policy to allow only a single round of revision, and that it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

I will forward you referee #3's report as soon as I receive it.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

Manuscript # EMBOJ-2012-84290
Ret rescues mitochondrial complex I deficiency and muscle degeneration of Drosophila Pink1 mutants

In this paper the Klein lab investigates the effect of Ret signaling on pink1 mutant phenotypes in fruit flies and (superficially) in mammalian cells. They find that mitochondrial defects (morphological phenotypes) in muscles of pink1 mutant flies, but not those in parkin mutant flies are rescued by expressing an active form of Ret. The study also provides a potential target underlying Ret-mediated cell protection as they find that in pink1 mutants the enzymatic defect in complex1 of the electron transport chain is significantly rescued by expression of active Ret. The authors conclude that Ret-mediated rescue of the complex1 defect in pink1 mutants rescues the mitochondrial defects seen in these animals. Importantly, given that Ret does not rescue parkin mutants, the data also independently highlight that some of the functions of Pink1 and Parkin in the regulation of mitochondrial biology are divergent.

I find this a very timely paper that is placed in the context of the current literature. Overall the findings are of interest, but the work lacks in several instances completeness, and several very obvious experiments are not performed. I believe these will be necessary if the work is to stand the test of time. Hence, while I believe this work can make an important contribution, it is currently too preliminary, and I hope that the comments that I list below can be addressed in a revised version of the paper.

Major concerns:

1) The authors analyze some of the reported pink1 phenotypes but are far from presenting a complete picture as to the extend by which Ret rescues the pink1 mutants
a)The authors should assess if RetMEN2B rescues sterility of pink1 mutants
b)Particularly important to their study, the authors should test if the mitochondrial membrane potential defects of the pink1 mutants are rescued by Ret and if so, if in neurons this rescue is sufficient to also rescue the synaptic function deficits that have been reported in these mutants.
c)Given the effect of Ret on dopaminergic neuron survival, and given that pink1 mutants show defects at the level of their dopaminergic neurons (notably at the level of mitochondrial morphology), the authors should determine if Ret can rescue these defects as well. This is not necessarily a 'copy' of what they already did in muscle cells as the mitochondrial defects in different cell types may be influenced by cell-specific factors. Furthermore, assessing the phenotype in these cells may be more relevant to understand pink1 function in neurons.
d)In line with this last comment, they should also assess if wild type Ret can rescue the dopaminerginc neuron defect; maybe in contrast to muscles, there is enough ligand available at these cells?

2) Rotenone treatment or loss of complex1 function by genetic means seems to result in weak (if any) mitochondrial morphological defects in muscles (previous studies), while loss of pink1 results in much stronger defects (this study and many others). As alluded to by the authors (and also in previous publications referred to by the authors) Pink1 may thus be controlling mitochondrial morphology both by acting with Parkin in remodelling/mitophagy and on the other hand by regulating complex1 activity. Apparently, rescuing either pathway independently seems sufficient to alleviate the mitochondrial morphological defects (in muscle cells). Can the authors discuss this more directly and clearly in their discussion?

3) In relation to the previous point, it is critical to test if Ret can rescue the mitochondrial membrane potential defects and complexI defects in rotenone treated animals and in animals where complexI was reduced by genetic means (e.g. RNAi). This would address if Ret acts on a Pink1 specific pathway or is capable to generally improve complex1 activity in situations where complex1 function is impaired.

4) The mechanism by which Ret activates complex1 function in pink1 mutants was not elucidated, and in the discussion a number of possibilities are listed. The authors could check in a very straightforward way at least one of these: it should be easy to assess if complex1 levels are altered (using blue native gels or western blotting).

5) I believe the authors, in conjunction with previous literature, start to make a compelling case that Pink1 can regulate mitochondrial function in part independently from Parkin, and the fact that pink1 mutants show complex1 defects and parkin mutants do not are in good support of this. Nonetheless, the data do not formally allow the authors to exclude the possibility that Ret were to act at the level of Parkin recruitment to defective mitochondria. It would therefore be interesting and critical to test if expression of active Ret can rescue the defect to recruit Parkin to depolarized mitochondria in pink1 mutant cells. If their model is correct, then expression of active Ret in pink1 mutant cells will rescue the defect in mitochondrial membrane potential, but not the defect in Parkin recruitment (when mitochondria are artificially depolarized). This experiment may solidify their model significantly.

6) I have a few questions related to the genetics: I found it a bit curious that the authors have used heterozygous pink1 or parkin females to control for the 'mutant condition' that -at least for pink1 must have been hemizigous males. First, what is the nature of the '+' chromosome? I cannot find information on this in the manuscript. Second, how are the authors sure that none of the alleles harbored on this '+' genetically interact with pink1 and finally, better would be to also include a wild type male control that harbors an X chromosome of the same genetic background as the pink1 allele they used. In addition, -and importantly- did the authors control for X-chromosome non-dysjunction in their mutant stocks (ie did they verify that the pink1 mutation is really present in the 'rescued' flies?)

Minor comment:
-the introduction is unnecessarily long

Referee #2

In this manuscript the authors tested the ability of a constitutively active Ret to rescue Drosophila PINK1 mutant phenotypes in the muscle. There results suggest that constitutive but not wild type Ret can rescue the muscle disorganization, mitochondrial morphological abnormality, and ATP deficit in the pink1 but not parkin mutant flies. The authors propose that Ret signaling may regulate mitochondrial complex-I to influence pink1 mutant effects. Overall, the study appears to be rather preliminary at this stage.

Major comments:

1. The fact that only the overexpression of constitutive active Ret (but not wild type Ret) can rescue pink1 mutant raised concern that the reported effects might be simply due to overexpression artifact. For example overexpression of constitutive active Ret may inadvertently activates other signaling pathways to cause the observed effects. To prove the physiological relevance, the authors need to demonstrate the effects of inhibiting the endogenous Ret and its ligand in pink1 background.

2. The assays used in the study are limited to muscle. As the PINK1 model is a PD model, the effects of Ret loss and gain-of-function on pink1 mutant phenotypes in dopaminergic neurons should be analyzed.

3. The fact that constitutive Ret rescues pink1 but not parkin mutant phenotypes does not necessarily mean that Ret and Parkin are not functionally related. It could simply be that Parkin acts downstream of Ret in a genetic pathway, i.e., Ret may act in between PINK1 and Parkin. Some genetic interaction analysis should be done to address this point.

4. Ret has previously been shown to also rescue neurodegeneration in the DJ-1 model. Without some mechanistic insight, the novelty of this study is relatively low due to their previous study. Some data on how Ret signaling affects mitochondrial complex-I should be presented.

Additional Corrrespondence 25 February 2013

I have still not heard back from the last referee and at this stage I don't suspect that I will. We will therefore go ahead with the two reports that we have on hand.

Looking forward to seeing the revised version.

1st Revision - authors' response 01 July 2013

Answers to referee's comments

Referee #1

Major concerns:

1) The authors analyze some of the reported pink1 phenotypes but are far from presenting a complete picture as to the extend by which Ret rescues the pink1 mutants
a)The authors should assess if RetMEN2B rescues sterility of pink1 mutants
b)Particularly important to their study, the authors should test if the mitochondrial membrane potential defects of the pink1 mutants are rescued by Ret and if so, if in neurons this rescue is sufficient to also rescue the synaptic function deficits that have been reported in these mutants.
c)Given the effect of Ret on dopaminergic neuron survival, and given that pink1 mutants show defects at the level of their dopaminergic neurons (notably at the level of mitochondrial morphology), the authors should determine if Ret can rescue these defects as well. This is not necessarily a 'copy' of what they already did in muscle cells as the mitochondrial defects in different cell types may be influenced by cell-specific factors. Furthermore, assessing the phenotype in these cells may be more relevant to understand pink1 function in neurons.
d)In line with this last comment, they should also assess if wild type Ret can rescue the dopaminergic neuron defect; maybe in contrast to muscles, there is enough ligand available at these cells?

a) Although sterility indeed is another prominent phenotype of Pink1 mutants, we think that this is outside the scope of this study. The aim of this study is to provide novel mechanistic insights in the way Ret signaling can provide cell protection in situations relevant for human PD. Sterility does not seem relevant for human PD.

b) We fully agree that this would be an interesting experiment. However, measuring membrane potential and synaptic activity in flies are technically challenging experiments for which we do not currently have sufficient knowledge and manpower to perform within the designated time frame. We therefore focused on other aspects that needed additional experimental data.

c) This is also a valid point which we have addressed experimentally (new figure 4). We tested whether Ret^{MEN2B} can also modify defects in dopaminergic neurons, specifically mitochondrial morphology and neuronal loss. In line with the muscle analysis, we find that Ret^{MEN2B} markedly rescues mitochondrial morphology in dopaminergic neurons in vivo. In accordance with the situation in muscle, Ret^{MEN2B} rescues mitochondrial morphology in Pink, but not Parkin mutant neurons. On a technical note, we used isosurface rendering in the software Imaris on image stacks with high z resolution, which allowed an unbiased quantification of mitochondrial volume.

With regards to dopaminergic neuron numbers, we did not observe any loss of PPL1 TH+ neurons in aged Pink1 mutants (up to 30 days old). Hence, we were not able to investigate whether Ret^{MEN2B} also has a survival function. This agrees with the more recent literature on dopaminergic cell loss in Pink1 mutant flies (Imai et al, 2010; Liu & Lu, 2010).

d) This is also a valid point which we have addressed, but as in the case of the muscles, Ret^{WT} was unable to rescue mitochondrial morphology in dopamine neurons (data not shown).

2) Rotenone treatment or loss of complex1 function by genetic means seems to result in weak (if any) mitochondrial morphological defects in muscles (previous studies), while loss of pink1 results in

much stronger defects (this study and many others). As alluded to by the authors (and also in previous publications referred to by the authors) Pink1 may thus be controlling mitochondrial morphology both by acting with Parkin in remodelling/mitophagy and on the other hand by regulating complex1 activity. Apparently, rescuing either pathway independently seems sufficient to alleviate the mitochondrial morphological defects (in muscle cells). Can the authors discuss this more directly and clearly in their discussion?

This is a very interesting problem, as it appears somewhat paradoxical. While complex-I inhibition or reduction indeed causes mild morphological phenotypes in fly muscle cells, function and morphology are still connected entities, and complex I inhibition is sufficient to for example cause disintegrating cristae and swelling in S2 cells or fragmentation in HeLa cells. Another complicating factor in this topic is that morphology can mean different things, which makes comparing different studies using different methods difficult. While our analysis of the muscle mitochondria evaluated ultrastructure where disintegrating cristae was central, other studies are focused on size and remodeling. However, one hypothesis of why Pink1 mutants develop such a severe phenotype is that it is due to synergism of both pathways – impaired mitophagy together with decreased OXPHOS function. In the current study, Ret signaling only produces a partial rescue, which would fit with this model. It should also be noted that we are not claiming that Ret acts directly on complex I. Following this, another hypothesis is that Ret signaling activates a broader pathway that affects both complex-I activity and morphology. We thank the reviewer for noticing this issue and have added a section in the discussion on the subject.

3) In relation to the previous point, it is critical to test if Ret can rescue the mitochondrial membrane potential defects and complex I defects in rotenone treated animals and in animals where complex I was reduced by genetic means (e.g. RNAi). This would address if Ret acts on a Pink1 specific pathway or is capable to generally improve complex1 activity in situations where complex1 function is impaired.

We agree with the reviewer that this is an interesting question, which we have attempted to address experimentally. Unfortunately, due to large variations in the data, we could not conclude anything clear from these experiments and due to the large amounts of animals required for measuring complex I activity, we were not able to repeat the experiment in a reasonable time.

4) The mechanism by which Ret activates complex1 function in pink1 mutants was not elucidated, and in the discussion a number of possibilities are listed. The authors could check in a very straightforward way at least one of these: it should be easy to assess if complex1 levels are altered (using blue native gels or western blotting).

We have measured the levels of the nuclear-encoded subunit NDUFS3, which has recently been identified to be reduced in Pink1 mutants, by western blot (new supplementary figure S5). We can confirm the reduction in the mutants, but we did not see an upregulation by Ret^{MEN2B} suggesting that Ret targets complex I activity by other means.

5) I believe the authors, in conjunction with previous literature, start to make a compelling case that Pink1 can regulate mitochondrial function in part independently from Parkin, and the fact that pink1 mutants show complex1 defects and parkin mutants do not are in good support of this. Nonetheless, the data do not formally allow the authors to exclude the possibility that Ret were to act at the level of Parkin recruitment to defective mitochondria. It would therefore be interesting and critical to test if expression of active Ret can rescue the defect to recruit Parkin to depolarized mitochondria in pink1 mutant cells. If their model is correct, then expression of active Ret in pink1 mutant cells will rescue the defect in mitochondrial membrane potential, but not the defect in Parkin recruitment (when mitochondria are artificially depolarized). This experiment may solidify their model significantly.

We fully agree with the reviewer, this is an important concern. We have addressed this in cultured cells, where we have tested whether Ret affects Parkin translocation to mitochondria (new figure S4). We treated SH-SY5Y cells with CCCP to depolarize mitochondria. Endogenous Parkin was not sufficient to induce mitophagy; however, overexpressed Parkin was first recruited to mitochondria and then induced mitophagy, so that after 24h half of the cells had lost their mitochondria. This effect required the presence of Pink1, but not Ret. More importantly, overexpressed active Ret did

not induce Parkin translocation or mitophagy under any condition, including Pink1 knock-down in combination with Parkin overexpression. Similar results were obtained by GDNF treatment of wild type Ret (data not shown). Furthermore, GDNF treatment also rescued mitochondrial fragmentation in Ret-overexpressing HeLa cells, a cell type with little or no Parkin expression (Denison et al, 2003; Pawlyk et al, 2003), further indicating that Ret signaling rescues PINK1 loss-of-function phenotypes independently of Parkin (Figure S3F-J).

6) I have a few questions related to the genetics: I found it a bit curious that the authors have used heterozygous pink1 or parkin females to control for the 'mutant condition' that -at least for pink1 must have been hemizigous males. First, what is the nature of the '+' chromosome? I cannot find information on this in the manuscript. Second, how are the authors sure that none of the alleles harbored on this '+' genetically interact with pink1 and finally, better would be to also include a wild type male control that harbors an X chromosome of the same genetic background as the pink1 allele they used. In addition, -and importantly- did the authors control for X-chromosome non-dysjunction in their mutant stocks (ie did they verify that the pink1 mutation is really present in the 'rescued' flies?)

We agree with the reviewer that these are important points that require clarifications. While we agree that the revertant alleles in some regards are better controls, heterozygous animals are good controls in the sense that all analyzed offspring originate from the same maternal stock and have a similar genetic background, in particular to the maternally inherited mitochondrial genome. This approach has also been used in other recent papers (Zhang et al, 2013). We don't believe that sex difference is a major factor in experiments of mitochondrial biochemistry and histology. The "+" chromosome is the W^{1118} X-chromosome which is extensively characterized, and the nature of "+" has now been clarified in the materials and methods section. In the original publication of the $Pink1^{B9}$ mutant line (Park et al, 2006), the $Pink1^{RV}$ allele was compared to W^{1118} and no differences were found in any of several experiments. Regarding the second question, while this is formally possible, all Pink1 phenotypes in this study have been described previously, our manuscript is primarily concerned with differences between $Pink1^{B9/Y}$ and $Pink1^{B9/Y}$; Ret^{MEN2B} males. Since $Pink1^{B9}$ for the analysis was crossed to W^{1118} and thus carries 2nd and third 3rd chromosomes from this stock, or to Ret^{MEN2B}, which is kept in a W^{1118} background, we would by some certainty claim that the only significant genetic difference that could confound the rescue is the UAS-Ret^{MEN2B} transgene. X-chromosome non-disjunction would in the Mef2-Gal4 and Mhc-Gal4 experiments be detected by weaker eye-color and in the Mhc-Gal4 experiment, in addition by y^- background. However, due to some uncertainty in eye-color discrimination, and to generally control correct genotypes, flies for all histological experiments have been genotyped by PCR, including primers for both WT-Pink1 and $Pink1^{B9}$. This routine procedure was previously omitted in the materials and methods but has now been added.

Minor comment:
-the introduction is unnecessarily long

The introduction was shortened.

Referee #2:

Major comments:

1. The fact that only the overexpression of constitutive active Ret (but not wild type Ret) can rescue pink1 mutant raised concern that the reported effects might be simply due to overexpression artifact. For example overexpression of constitutive active Ret may inadvertently activate other signaling pathways to cause the observed effects. To prove the physiological relevance, the authors need to demonstrate the effects of inhibiting the endogenous Ret and its ligand in pink1 background.

We do not wish to suggest that endogenous Ret in Drosophila muscle or dopaminergic neurons plays a significant role in maintaining mitochondrial integrity or in preventing parkinsonian phenotypes, and we apologize if this was not clear. Analogous to infusion of GDNF in mammalian PD models, we are in fact investigating the effect of excessive amounts of Ret signaling, and we propose that it has beneficial effects on mitochondrial damage caused by loss of Pink1 in flies. The

expression level of endogenous Ret is very low, and the Ret^{WT} experiment suggests that the elusive Ret ligand is not expressed in sufficient amounts. Ret loss of function was tested in a screen for modifiers of muscle development (Schnorrer et al, 2010) and did not cause dysfunction in flight ability, suggesting that Ret does not have an important endogenous function in muscle maintenance.

2. The assays used in the study are limited to muscle. As the PINK1 model is a PD model, the effects of Ret loss and gain-of-function on pink1 mutant phenotypes in dopaminergic neurons should be analyzed.

We fully agree with the reviewer that this is an important point, which we have addressed experimentally presented in the new figure 4 (see also answer to reviewer #1, 1d). In brief, we tested whether Ret^{MEN2B} can also modify defects in dopaminergic neurons, specifically mitochondrial morphology and neuronal loss. In line with the muscle analysis, we find that Ret^{MEN2B} markedly rescues mitochondrial morphology in dopaminergic neurons in vivo. In accordance with the situation in muscle, Ret^{MEN2B} rescues mitochondrial morphology in Pink, but not Parkin mutant neurons. On a technical note, we used isosurface rendering in the software Imaris on image stacks with high z resolution, which allowed an unbiased quantification of mitochondrial volume.

With regards to dopaminergic neuron numbers, we did not observe any loss of PPL1 TH+ neurons in aged Pink1 mutants (up to 30 days old). Hence, we were not able to investigate whether Ret^{MEN2B} also has a survival function. This agrees with the more recent literature on dopaminergic cell loss in Pink1 mutant flies (Imai et al, 2010; Liu & Lu, 2010).

3. The fact that constitutive Ret rescues pink1 but not parkin mutant phenotypes does not necessarily mean that Ret and Parkin are not functionally related. It could simply be that Parkin acts downstream of Ret in a genetic pathway, i.e., Ret may act in between PINK1 and Parkin. Some genetic interaction analysis should be done to address this point.

We thank the reviewer for noting this interesting point, also alluded to by reviewer #1. We have addressed this in cultured cells, where we have tested whether Ret affects Parkin translocation to mitochondria (new figure S4). We treated SH-SY5Y cells with CCCP to depolarize mitochondria. Endogenous Parkin was not sufficient to induce mitophagy; however, overexpressed Parkin was first recruited to mitochondria and then induced mitophagy, so that after 24h half of the cells had lost their mitochondria. This effect required the presence of Pink1, but not Ret. More importantly, overexpressed active Ret did not induce Parkin translocation or mitophagy under any condition, including Pink1 knock-down in combination with Parkin overexpression. Similar results were obtained by GDNF treatment of wild type Ret (data not shown). Furthermore, GDNF treatment also rescued mitochondrial fragmentation in Ret-overexpressing HeLa cells, a cell type with little or no Parkin expression (Denison et al, 2003; Pawlyk et al, 2003), further indicating that Ret signaling rescues PINK1 loss-of-function phenotypes independently of Parkin (Figure S3F-J).

4. Ret has previously been shown to also rescue neurodegeneration in the DJ-1 model. Without some mechanistic insight, the novelty of this study is relatively low due to their previous study. Some data on how Ret signaling affects mitochondrial complex-I should be presented.

We disagree. In our previous paper (Aron et al, 2010), we had not shown that Ret rescues neurodegeneration in the DJ-1 model (in fact, it is controversial whether there is neurodegeneration in the DJ-1 KO mouse). We had reported that Ret and DJ-1 double loss-of-function causes an increased phenotype in mice indicating that DJ-1 can have survival promoting activity in situations of trophic deprivation. In the same study, we had shown that DJ-1 loss of function rescues $Ret^{MEN2A/B}$ overexpression phenotypes in the Drosophila eye, indicating genetic interaction of these two proteins. In difference to that study, we can now, for the first time, demonstrate that active Ret can rescue phenotypes of a genetic PD model; in other words Ret promotes cell protection in a situation relevant for human PD. In addition to establishing a new link between signaling downstream of Ret and Pink1 and mitochondria, we believe that this is interesting because GDNF/Ret has previously failed to rescue alpha-synuclein models, and this study suggests that Ret, as a therapeutic target, may only be relevant for certain types of PD. Furthermore, the results reveal a striking difference in how Pink1 and Park mutants respond, which underscore the few but interesting previous studies suggesting that these proteins may not only act together (i.e. in mitophagy), but also independently.

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Zhang L, Karsten P, Hamm S, Pogson JH, Muller-Rischart AK, Exner N, Haass C, Whitworth AJ, Winklhofer KF, Schulz JB, Voigt A (2013) TRAP1 rescues PINK1 loss-of-function phenotypes. *Human molecular genetics*

2nd Editorial Decision 07 August 2013

Thank you for submitting your revised manuscript to The EMBO Journal. Referee #1 has now seen your revision and the comments are provided below.

Referee #1 appreciates the introduced changes and finds the data set improved. However, significant concerns remain in particular pertaining to the mechanism for how Ret1 affects complex1 activity., which we still gain limited insight into. This issue was raised upfront and I had indicated in my previous decision letter that this was an important concern to address in a revised version. Our normal policy is to allow for one round of revision only. I can offer a second one in this case, but only if you are able to add mechanistic insight into how Ret1 affect mitochondrial function and address the other remaining concerns raised by referee #1. Referee #1 offers a number of suggestions for how to extend the analysis, but their might be other possibilities as well. Please note that I can't guarantee the outcome of another round of revision as that depends upon what data you can include. I would like to ask you to consider your options carefully and to let me know if you wish to extend the analysis and submit a revised version or if you would prefer to take the manuscript elsewhere at this stage.

REFEREE REPORTS

Referee #1

1-This revised version of their manuscript is already much improved over the previous version. The authors were now able to more convincingly show that Ret can rescue Pink1 independently of Parkin. They performed these experiments in cells and used typical mitophagy assays to back their claim. I think these are important data that might be too much hidden in the current version of the manuscript and I would present them in the main figures. I cannot think of a sensible Drosophila genetics experiment that could be performed alongside these cell-based experiments to further strengthen this (important and controversial) point.

2- I had asked to look at mitochondrial membrane potential and synaptic activity in my previous review. The request to look at synaptic activity stemmed from the fact that only data on mitochondria in muscles was shown, but by showing data in dopaminergic neurons, I can live without the synaptic activity experiments. However, assessment of mitochondrial membrane potential is essential in the context of the current work. It is an independent measure that may, in an important way, further support their claims. Many groups have performed these experiments before and I see little reason not to perform them.
In addition, I also come back on my request to test if Ret can alleviate defects upon reduced complex I activity (using rotenone or RNAi). Again, measuring mitochondrial membrane potential and measuring complex I activity are needed. These experiments are critical to further define how specific the rescue of pink1 mitochondrial defects by Ret are and they are required in the context of this work. Obtaining enough flies should really not be an issue...

3- The final issue that I believe ought to be resolved is to obtain insight in the mechanism by which Ret acts on mitochondria. The authors looked at the expression of one single complex I subunit upon ret expression, but this presented experiment in the rebuttal is incomplete. I agree that S3 would be a good candidate to test but not seeing an increase in S3 does not exclude that other subunits are upregulated. It is really not difficult to assess the expression level of all complex I subunits using RT-PCR. In addition, many groups have also performed blue native gel analyses of complex I integrity.

2nd Revision - authors' response 08 November 2013

Answers to referee's comments

Referee #1

1-This revised version of their manuscript is already much improved over the previous version. The authors were now able to more convincingly show that Ret can rescue Pink1 independently of Parkin. They performed these experiments in cells and used typical mitophagy assays to back their claim. I think these are important data that might be too much hidden in the current version of the manuscript and I would present them in the main figures. I cannot think of a sensible Drosophila genetics experiment that could be performed alongside these cell-based experiments to further strengthen this (important and controversial) point.
We thank the reviewer for these comments. We agree that the cell culture part is more important and have therefore moved it to new Figure 4. The mitophagy experiments have been completed with a dataset for GDNF treated cells. (Before we had only preliminary data for GDNF and "data not shown")

2- I had asked to look at mitochondrial membrane potential and synaptic activity in my previous review. The request to look at synaptic activity stemmed from the fact that only data on mitochondria in muscles was shown, but by showing data in dopaminergic neurons, I can live without the synaptic activity experiments. However, assessment of mitochondrial membrane potential is essential in the context of the current work. It is an independent measure that may, in an important way, further support their claims. Many groups have performed these experiments before and I see little reason not to perform them.
In addition, I also come back on my request to test if Ret can alleviate defects upon reduced complex I activity (using rotenone or RNAi). Again, measuring mitochondrial membrane potential and measuring complex I activity are needed. These experiments are critical to further define how specific the rescue of pink1 mitochondrial defects by Ret are and they are required in the context of this work. Obtaining enough flies should really not be an issue...

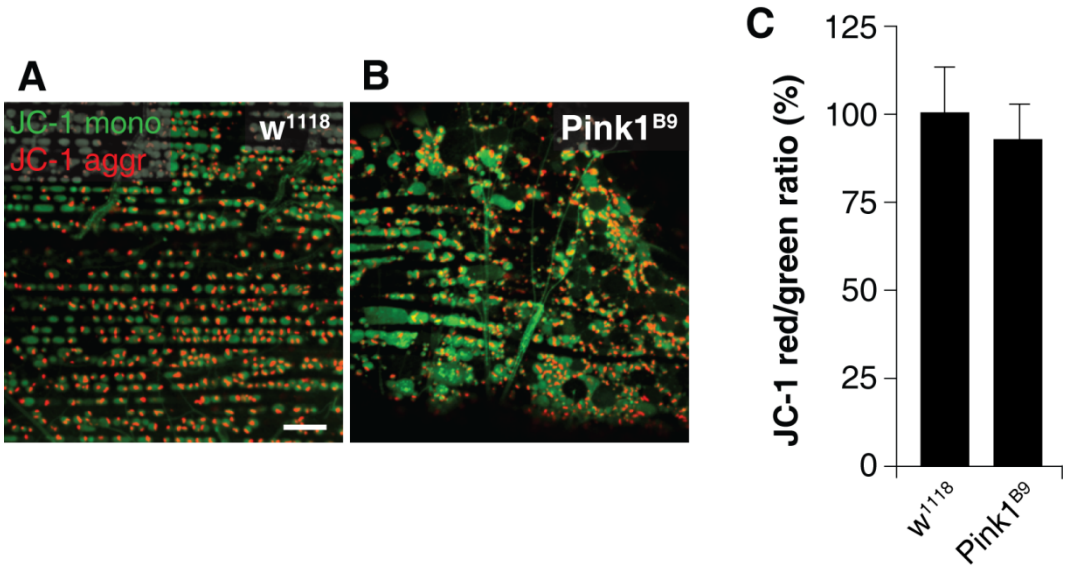
We agree with the reviewer that mitochondrial membrane potential is an important aspect, central in oxidative phosphorylation and related to Pink1 function. Unfortunately, the methods are limited. We chose the potentiometric dye JC-1, since it was used in similar situations in (Morais et al, 2009; Vilain et al, 2012; Vos et al, 2012). In these studies it was used to investigate larval neuromuscular junction boutons, genetically targeted by *da-GAL4*. Unfortunately Ret^{MEN2B} causes early lethality when expressed with broad drivers such as *da-GAL4,* and to establish a new driver and verify that Ret can modify a *Pink1* phenotype using this driver would require significant time. For these

reasons, we decided to perform the experiments in adult indirect flight muscles (IFMs). However, despite significant optimization of the method, we were not able to detect a decrease in the *Pink1* mutants To our knowledge, it has not previously been shown that adult IFMs of *Pink1* mutants (although expected) have decreased membrane potential. If our result is due to compensatory changes in adult mitochondria, or technical issues of the method (Brand & Nicholls, 2011) is unclear. These results are now presented in (Rebuttal figure 1).

As suggested, we used RNAi against the complex I subunit CG11455, chosen as it was previously reported not to cause lethality (Vilain et al, 2012), and measured complex I activity. Knocking down this subunit almost fully abolished complex I activity, and Ret^{MEN2B} did not rescue this defect, demonstrating that Ret signaling cannot compensate for a deficient or incomplete complex I, but more likely enhances the activity of a functional complex I. These data are now presented in new Figure 5F.

3- The final issue that I believe ought to be resolved is to obtain insight in the mechanism by which Ret acts on mitochondria. The authors looked at the expression of one single complex I subunit upon ret expression, but this presented experiment in the rebuttal is incomplete. I agree that S3 would be a good candidate to test but not seeing an increase in S3 does not exclude that other subunits are upregulated. It is really not difficult to assess the expression level of all complex I subunits using RT-PCR. In addition, many groups have also performed blue native gel analyses of complex I integrity.

We have performed RT-PCR for most of the remaining subunits, 45 out of 48; 3 had to be excluded from the analysis due to technical problems. The general message is that there was very little change, suggesting that Ret perhaps does not increase transcription of these genes. Interestingly, one subunit, CG6485, was decreased in *Pink1* mutants, and significantly increased by Ret^{MEN2B}. This effect may in part be responsible for the Ret-mediated rescue of Pink1 deficiency. However, we do not exclude the possibility that Ret signaling targets complex I, and perhaps other metabolic components, by different means. The data are presented in Figure 5H and S4.
Since PINK1 deficiency is known to impair mitochondrial respiration, we also investigated whether activation of Ret signaling via GDNF/GFRα-1 treatment could influence this phenotype. We measured mitochondrial function under basal and stress conditions in SH-SY5Y cells silenced for PINK1 expression by using an extracellular oxygen flux analyzer. In comparison to control siRNA-treated cells, PINK1-deficient cells were characterized by a decreased oxygen consumption rate even under basal conditions (Figure 5A). Remarkably, GDNF/GFRα-1 treatment fully rescued basal respiration in PINK1-deficient cells (Figure 5A), indicating that the beneficial effect of increased Ret signaling in PINK1-deficient models can be explained by influencing the bioenergetic capacity of mitochondria rather than mitophagy (as shown in Figure 4).



Rebuttal figure 1: No difference in mitochondrial membrane potential in *Pink1* mutant indirect flight muscles.

(A, B) IFMs of (A) w^{1118} controls and (B) $Pink1^{B9}$ mutants stained with potentiometric dye JC-1 (5 μM) in Schneider's medium. Green monomeric dye labels primarily mitochondria and to a lesser extent cytosol. Increasing mitochondrial membrane potential generates higher mitochondrial JC-1 concentration and subsequent aggregation and shift to red fluorescence. (C) Quantification of JC-1 red/green fluorescence intensity indicating no measured difference between controls and Pink1 mutants, n=17 animals per genotype. Scale bar: 10 μm

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Accepted 29 November 2013

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referee #1 and the comments are provided below.

As you can see, the referee appreciates the introduced changes and support publication here. The referee points out that one issue has not been dealt with (assessing mitochondrial membrane potential), but also finds that given the added data that the paper is ready to go. I am therefore pleased to accept the paper as is.

REFEREE REPORT

Referee #1:

Most of my comments were dealt with adequately. The single issue that was not addressed was assessing mitochondrial membrane potential that appeared to be difficult due to early lethality. One option would indeed be to use another driver, but I believe that the other new data that was added nicely complements the main point the authors wanted to make, making the need to these mitochondrial membrane potential measurements somewhat less critical.

