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Parkin and PINK1 Function in a Vesicular Trafficking Pathway Regulating Mitochondrial Quality Control

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Editor: Andrea Leibfried

1st Editorial Decision 02 July 2013

Thank you for submitting your manuscript entitled 'Parkin and PINK1 Function in a Vesicular Trafficking Pathway Regulating Mitochondrial Quality Control'. I have now received the three reports on your paper.

As you can see below, all three referees appreciate your data and conclusions very much. However, further amendments and additional experiments are needed to support your data and claims.

Given the comments provided, I would like to invite you to submit a revised version of the manuscript, addressing the concerns of the referees.

I should also add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address the concerns raised at this stage.

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

REFEREE COMMENTS

Referee #1

In this manuscript authors present the novel and exciting data on the involvement of Pink1/Parkin in the generation of mitochondria-derived vesicles, a new yet substantiated pathway for mitochondrial quality control.
Using a combination of imaging and genetics, the authors identify Pink1 and Parkin as key components of the generation of MDVs in response to antimycin A and cargoed to the lysosome, where they are degraded. They provide genetic evidence that the pathway is
1. Mitochondrial fission-independent
2. Autophagy independent
3. Selective for the inner mitochondrial membrane
4. Selective for mitochondria-derived ROS
5. Impaired by Parkinson's disease (PD) associated Parkin mutations
I believe that this manuscript represents a leap forward of our understanding of the molecular mechanisms of PD. The identification of MDVs as a potential mechanism is exciting and will pave the way for future research on this hot topic. This paper will for sure represent the start for a new field of research
I only have a few questions.
1. Did the authors identify parkin mutations that dissociate formation of MDVs from mitophagy? I understand that all the experiments here are performed in conditions where mitophagy is inhibited (using the Drp1 dominant negative or the models of deficient autophagy) but such a mutant would say a definitive word on the role of this pathway in PD.
2. Are MDVs happening together with mitophagy? In other words, if the authors treat cells with AA and oligomycin to cause the simultaneous ROS formation and depolarization, do they see the two processes happening together, or are MDVs overwhelmed by mitophagy? Similarly, what is the ratio between mitophagy and MDVs in cells that for example harbor a mtDNA mutation or are rho0? These experiments would clarify the relationship between these two processes.
3. Some editing would be required to eliminate jargon. For example, I found funny to read "Our HeLa cells".

Referee #2

The manuscript continues an interesting series of papers from this laboratory that describe a mechanism for mitochondrial quality control through the formation of mitochondrial-derived vesicles (MDVs) that can turn over damaged proteins by conveying them to lysosomes. Unlike the standard mitophagy pathway in which an entire mitochondrion is targeted for removal, the MDV pathway can selectively eliminate damaged components. The present manuscript extends their earlier description of the pathway by demonstrating the involvement of PINK1 and Parkin, two proteins previously associated only with the mitophagy branch of mitochondrial quality control. The relative importance of the two pathways for the etiology of Parkinsonism in general or in the particular case of hereditary mutations in these two proteins remains to be seen, but the expansion of PINK1/Parkin function to MDVs is clearly an important advance. The work is straightforward and generally well executed and clear. That said, there are some concerns that should be addressed prior to publication in EMBO J. or elsewhere.

Major issues:
1. Does the pathway require Parkin or is it merely enhanced or accelerated by overexpression of Parkin? In discussing Figure 2A they say MDV production is dependent on Parkin and elsewhere they describe it as required, but they have only shown it is increased by Parkin expression, not that it is required. Although they state that endogenous levels of Parkin are low in these cells, there is still MDV production in the absence of Parkin expression. I was surprised, upon coming to the end of the manuscript to find that, although they knock down PINK1by RNAi and many other proteins, they never knock down Parkin or use Parkin -/- MEFs and so, although they demonstrate that Parkin overexpression enhances MDV production, we never find out if it is absolutely required for either the baseline of MDVs in cells or the MDVs that are stimulated by antimycin.

2. I am not convinced by the argument in the Discussion that their treatments induce ROS but not global depolarization. Much of their Discussion is based on contrasting global depolarization with local ROS production, so it is a significant issue for understanding the pathway. They should show, with TMRM for instance, that antimycin A does not globally depolarize mitochondria at their

concentrations, in these cells, and with the Drp mutations they employ. Moreover, the argument that MDVs are the first line of defense could be made stronger if they examined the time course of their formation or an antimycin A dose/response relationship to show that parkin-positive MDVs become less abundant with increasing mitophagy.

3. TOM20 degradation issue: the manuscript rejects the possibility that the MDVs are TOM20-negative because TOM20 is a Parkin substrate and TOM20 has therefore been cleared from the MDVs. The conclusion derives from the use of proteasome inhibitors and the demonstration that total TOM20 levels are unchanged. Not so fast! If TOM20 is removed from the surface of the mitochondrion by p97 after its ubiquitination by Parkin, it will continue to give a TOM20 negative vesicle even when MG132 blocks the proteasome. P97 has already been implicated in the Parkin pathway and mitophagy. The authors should use dominant negative p97 constructs to see if they affect the composition of the MDVs. Also, because the MDVs are only a very small percentage of the total mitochondrial volume, it is not possible to exclude that the MDVs are TOM20 negative due to Parkin-dependent ubiquitination of TOM20 and its subsequent removal from the membrane.

4. The authors don't really address what the parkin-negative population of MDVs are, especially since they are also induced by antimycin. They presume that they are MDVs that were once Parkin-positive but have lost their Parkin, but there is no time-course or experimental data to support this precursor hypothesis. Along the same lines, why does Parkin R42P lower total MDVs but not the parkin positive ones?
5. In Figure S2D, the TOM20 blot looks pretty bad and there is no quantification of the bands.

6. Because all the experiments were done with inhibition of Drp1, it is not clear that the MDV pathway would involve Parkin if normal mitophagy were permitted. I appreciate that it is valuable to prevent mitophagy to make it easier to distinguish MDVs from mitochondrial fragments, but their model predicts that 1) MDVs may be produced under conditions where there is little mitophagy and 2) that even with Drp1 present and active, some TOM20-negative, PDH-positive, Oct-dsRED-positive vesicles will appear and be Parkin-GFP positive as well.

Some more minor points:

1. Figure 1B. It appears that some Parkin-GFP is mitochondrial even before CCCP. This is unusual and they should comment. Is it due to very high levels of Parkin -GFP expression?
2. Expression of Parkin GFP seems much lower in Figure 1C - there is very little on mitos compared to 1B.
3. Figure 2A: the statistical comparisons for the Parkin mutants are not clear. Are they being compared only to themselves +/- Antimycin or are they being compared to the GFP control? All the mutants appear to increase the number of MDVs relative to GFP alone, though they lack sensitivity to further increases from antimycin.
4. Figure 2 - the images suggest very different levels of GFP in each of the mutant Parkins, though the blot indicates very similar expression levels. Were very different settings used for the imaging?

Very minor points:
p.9 The sentence describing the effects of RNAi against components of the autophagy pathway (Fig 4D,E) should be reworded- it could be taken to mean there was no effect of GFP-Parkin, when in fact they mean that the effect of antimycin was not affected by the RNAi.

In figure 1c it would be nice to trace the cell boundaries.

For my taste, the Discussion wanders a little far from the actual content of the manuscript when it discusses synuclein and LRRK2 and their possible role in vesicular pathways.

Referee #3

Recent work indicates that damaged mitochondria are turned over by mitophagy in a process that requires PINK1, Parkin and the mitochondrial dynamin GTPase (Drp1) that mediates fission.

However, some mitochondrial respiratory chain proteins that have undergone oxidative damage are also turned over by a process that is much more selective and leaves the bulk of the mitochondrion intact. Mitochondrial derived vesicles (MDVs) that select oxidized cargo and bud off the organelle in a fission independent mechanism are implicated in this selective degradation. In this manuscript, McLelland et al. provide new and important information about this latter class of MDVs. Specifically, they demonstrate that mitochondrial stress generated by ROS inside the organelle triggers formation of MDVs with selective cargo in a manner that is Pink1- and parkin-dependent but Drp1-independent. In addition, they provide evidence that turnover of this class of MDVs depends on lysosomal function and is autophagy-independent. Based on these new findings, the authors suggest that parkin-dependent MDV formation functions as an early form of mitochondrial quality control that aims to preserve mitochondrial function. Only after damage becomes very severe, does the less selective parkin-dependent mitophagy pathway come into play. This model is attractive and nicely synthesizes a number of different findings by researchers in the field. The data are excellent and the manuscript is clearly written. Minor comments that the authors should consider are listed below.

-In some experiments the authors use CFP-Drp1K38E to dominantly inhibit fission in their experiments. However, it does not appear that CFP signal was monitored in the data shown. This should be clarified in the text for the reader.

-Fig. 4A, a bar graph quantifying this data should be included.

-The discussion of Fig. 4D and E is confusing. Are the conclusions based solely on the data indicated by the white bars (total MDVs)? If so, the lack of significant change after knockdown of autophagy components makes sense. However, it looks like there is a significant change in the GFP-positive MDVs upon siBcn1 knockdown. The authors should comment on this and clarify whether the statistics refer to the white bars, grey bars or both in 4E.

-Discussion, page 13, please define AR-JP and SNpc for the reader.

-Fig S2D, the arrow at the left of the image (near the 82kDa marker) does not have a label. Is this GFP-Parkin? If so, it would help to move the label down so it is just above the arrow.

1st Revision - authors' response 24 October 2013

Response to reviewers' comments

Re: EMBOJ-2013-85902
Revised manuscript submitted to the EMBO Journal, entitled "Parkin and PINK1 function in a vesicular mitochondrial quality control pathway"

Reviewer 1

We are pleased that the reviewer considers our findings to be a "*leap forward of our understanding of the molecular mechanisms of PD*". We have addressed their comments below.

Did the authors identify parkin mutations that dissociate formation of MDVs from mitophagy? I understand that all the experiments here are performed in conditions where mitophagy is inhibited (using the Drp1 dominant negative or the models of deficient autophagy) but such a mutant would say a definitive word on the role of this pathway in PD.

The reviewer raises an interesting point concerning parkin mutations that could specifically disrupt MDV formation and not mitophagy. Such a mutant would indeed provide a useful molecular tool with which to tease apart these two parkin-dependent, mitochondrial quality control pathways. However, the PD-linked mutations we have tested so far (R42P, K211N and C431F) appear to impact both pathways to a similar extent (i.e. K211N and C431F abolish them whereas R42P has a partial effect). We also briefly surveyed several other parkin mutants that are not defective in

mitophagy (M192V, D280N and G328E) and so far do not see any that dissociate the two pathways (not shown). In the Discussion, we present the hypothesis that local vs. global PINK1 accumulation may regulate initiation of vesicle formation and mitophagy, respectively. However, the mechanisms by which parkin is recruited to mitochondria may be the same for both processes and it does not appear obvious to find mutations that dissociate the two pathways. Nonetheless, we believe that the data demonstrating pathway defects in several PD-linked parkin mutants in Fig. 2 imply that this pathway may be dysregulated in PD.

Are MDVs happening together with mitophagy? In other words, if the authors treat cells with AA and oligomycin to cause the simultaneous ROS formation and depolarization, do they see the two processes happening together, or are MDVs overwhelmed by mitophagy?

We have addressed this important point with kinetic experiments using CCCP, antimycin A and antimycin A with oligomycin to induce, respectively, depolarization, ROS, and both depolarization and ROS simultaneously. We looked at both MDV formation (new Fig. 6) and mitophagy (new Fig. 7 and S7). We note that, while depolarization alone tirggers mitophagy, ROS induces strictly MDVs. ROS and depolarization together induce both mitophagy and MDVs. Under this latter condition, the peak of the MDV response (2 to 4 hours) occurs prior to mitophagy (after 4 hours), indicating that vesicles are indeed a "rapid response" to stress. We thank the reviewer for suggesting this informative experiment.

Similarly, what is the ratio between mitophagy and MDVs in cells that for example harbor a mtDNA mutation or are rho0?

Throughout our study, we have used antimycin A to acutely generate ROS and, accordingly, MDVs. The issue with using a cybrid cell line harbouring mtDNA mutations or rho^0 cells is that these lines generate very little to no electron transport, and would thus be relatively unresponsive to antimycin A or other ETC poisons. A steady-state study concerning parkin-dependent mitophagy in cybrid cell lines by Richard Youle's group demonstrated that parkin overexpression could only clear defective mitochondria over a period of weeks in the case of a line harbouring a particularly deleterious mtDNA mutation (Suen et al, 2010). Thus, even parkin-mediated mitophagy remains largely uninvestigated in these types of cell lines, and we believe that a quantitative assessment of the relative contributions of mitophagy and MDV formation in these lines lies beyond the scope of our current study.

Some editing would be required to eliminate jargon. For example, I found funny to read "Our HeLa cells".

At the request of the reviewer we have removed jargon.

Reviewer 2

We thank the reviewer for considering our study an "*important advance*", and appreciate them deeming our methodology "*straightforward and generally well executed and clear*". We have responded to their concerns point-by-point below.

R2: *Does the pathway require Parkin or is it merely enhanced or accelerated by overexpression of Parkin? In discussing Figure 2A they say MDV production is dependent on Parkin and elsewhere they describe it as required, but they have only shown it is increased by Parkin expression, not that it is required. Although they state that endogenous levels of Parkin are low in these cells, there is still MDV production in the absence of Parkin expression. I was surprised, upon coming to the end of the manuscript to find that, although they knock down PINK1by RNAi and many other proteins, they never knock down Parkin or use Parkin -/- MEFs and so, although they demonstrate that Parkin overexpression enhances MDV production, we never find out if it is absolutely required for either the baseline of MDVs in cells or the MDVs that are stimulated by antimycin*.

We appreciate the reviewer's concern about whether this pathway truly requires parkin, or is merely enhanced by parkin. HeLa cells do not contain any endogenous parkin; it lies in a fragile site on chromosome 6 that is deleted in this cell type (Denison et al, 2003) and thus has been considered by many as a *bona fide* parkin null cell model (Narendra et al, 2008). Therefore, only upon transfection

of functional, wild-type GFP-Parkin do we observe matrix-positive MDVs (Fig. 2). Nonetheless, at the reviewer's request, we have knocked down endogenous parkin in COS7 cells (new Fig. S3), a cell line that exhibits an antimycin A-dependent increase in the same MDV population that is parkin-dependent in HeLa cells (Soubannier et al, 2012). We show that the antimycin A-induced increase in MDVs is abrogated in parkin knockdown cells.

R2: *I am not convinced by the argument in the Discussion that their treatments induce ROS but not global depolarization. Much of their Discussion is based on contrasting global depolarization with local ROS production, so it is a significant issue for understanding the pathway. They should show, with TMRM for instance, that antimycin A does not globally depolarize mitochondria at their concentrations, in these cells, and with the Drp mutations they employ. Moreover, the argument that MDVs are the first line of defense could be made stronger if they examined the time course of their formation or an antimycin A dose/response relationship to show that parkin-positive MDVs become less abundant with increasing mitophagy.*

We thank the reviewer for raising these important points. Firstly, we performed time-course experiments in U2OS cells stably expressing GFP-parkin (U2OS:GFP-parkin). By analyzing both MDV formation (new Fig. 6) and mitophagy (new Fig. 7 and S7) in these cells, we show that the peak of the MDV response (2 to 4 hours) occurs prior to mitophagy (after 4 hours). Importantly, both responses can be triggered independently of one another, using either ROS or depolarization alone. Secondly, we show, using TMRM, that antimycin A alone does not induce global depolarization under the conditions used for our kinetic experiments (Fig. 6A).

R2: *TOM20 degradation issue: the manuscript rejects the possibility that the MDVs are TOM20-negative because TOM20 is a Parkin substrate and TOM20 has therefore been cleared from the MDVs. The conclusion derives from the use of proteasome inhibitors and the demonstration that total TOM20 levels are unchanged. Not so fast! If TOM20 is removed from the surface of the mitochondrion by p97 after its ubiquitination by Parkin, it will continue to give a TOM20 negative vesicle even when MG132 blocks the proteasome. P97 has already been implicated in the Parkin pathway and mitophagy. The authors should use dominant negative p97 constructs to see if they affect the composition of the MDVs. Also, because the MDVs are only a very small percentage of the total mitochondrial volume, it is not possible to exclude that the MDVs are TOM20 negative due to Parkin-dependent ubiquitination of TOM20 and its subsequent removal from the membrane.*

As the reviewer suggests, we have inhibited p97/VCP function (we used siRNA-mediated knockdown of p97/VCP) and observed no difference in the amount of vesicles induced by antimycin A (new Fig. S4G). Moreover, in three independent studies concerning parkin-dependent mitophagy, proteasomal inhibition or p97 suppression was sufficient to prevent TOM20 degradation from mitochondrial fractions (Chan et al, 2011; Tanaka et al, 2010; Yoshii et al, 2011). In our assay, under either of these conditions, we do not observe a change in the number of vesicles generated by antimycin A. Thus, we conclude that TOM20 is not degraded from the surface of these MDVs.

R2: *The authors don't really address what the parkin-negative population of MDVs are, especially since they are also induced by antimycin. They presume that they are MDVs that were once Parkin-positive but have lost their Parkin, but there is no time-course or experimental data to support this precursor hypothesis.*

This is an excellent point. Although we do not see a straightforward way to address this, one approach would be to quantify the mean distance from the closest mitochondrial tubule for GFP-parkin-positive and -negative MDVs. Indeed, we find that MDVs colocalizing with parkin are ~3 times as close to the nearest mitochondrion compared to those that are parkin-negative (new Fig. 3E). This new data is in line with our hypothesis that, as vesicles move away from the tubule, they lose their GFP-parkin colocalization. In addition, the loss of parkin inhibits all matrix-positive MDVs, even though GFP-parkin is localized to a few of them at any given time. This further indicates that parkin acts upstream in the MDV pathway as otherwise it's loss should have a minor effect on the total matrix-MDV population.

Along the same lines, why does Parkin R42P lower total MDVs but not the parkin positive ones?

We have previously shown that the ubiquitin-like (Ubl) domain of parkin, comprising residues 1 to

76, is a critical protein-protein interaction interface (Fallon et al, 2006; Trempe et al, 2009). Thus, there are two possible interpretations of the phenotype concerning GFP-parkin^{R42P}, which harbours an unfolded Ubl domain (Safadi & Shaw, 2007). The first is that GFP-parkin^{R42P} can form vesicles but cannot interact with the necessary downstream trafficking factors, thus leading to a buildup of parkin-positive vesicles, as, for example, Ubl-mediated interactions may be crucial for parkin's removal from MDVs and recycling back into the cytosolic parkin pool. The second interpretation is that, kinetically, GFP-parkin^{R42P} is slower to recruit to mitochondria than the wild-type. Thus, at the time-point of observation in Fig. 2, GFP-parkin^{R42P} has only just formed vesicles while the vesicles formed by GFP-parkin^{WT} are predominantly at downstream steps in the pathway. There is some evidence for this latter idea, as CCCP-induced recruitment of GFP-parkin^{R42P} has been shown to only be partially defective (Matsuda et al, 2010).

5. In Figure S2D, the TOM20 blot looks pretty bad and there is no quantification of the bands.

We have include new blots (new Fig. S3D), and quantified the bands from three independent experiments (Fig. S3E).

R2: *Because all the experiments were done with inhibition of Drp1, it is not clear that the MDV pathway would involve Parkin if normal mitophagy were permitted. I appreciate that it is valuable to prevent mitophagy to make it easier to distinguish MDVs from mitochondrial fragments, but their model predicts that 1) MDVs may be produced under conditions where there is little mitophagy and 2) that even with Drp1 present and active, some TOM20-negative, PDH-positive, Oct-dsRED-positive vesicles will appear and be Parkin-GFP positive as well.*

We agree with the reviewer in that we may be biasing our system to observe strictly MDVs. We have addressed this, predominantly in our time-course experiments where we have not silenced Drp1, and have demonstrated that a) simultaneously inducing depolarization and ROS triggers both MDVs and mitophagy, albeit with significantly different kinetics (new Fig. 6, 7 and S7), b) MDVs occur in the presence of Drp1 expression (new Fig. 3C), and c) MDVs and mitophagy can be selectively triggered (i.e. without the other) by ROS (MDVs) and depolarization (mitophagy) in Drp1-expressing cells (Fig. 6, 7 and S7).

R2: *Figure 1B. It appears that some Parkin-GFP is mitochondrial even before CCCP. This is unusual and they should comment. Is it due to very high levels of Parkin -GFP expression?*

Although some endogenous parkin is mitochondrially-associated at the steady-state (Narendra et al, 2008), GFP-parkin is generally cytosolic in unstimulated cells, as is indicated in all of our immunofluorescence panels showing untreated or DMSO-treated cells.

R2: *Expression of Parkin GFP seems much lower in Figure 1C - there is very little on mitos compared to 1B.*

Due to the limitations of a) confocal microscopic techniques (stemming from resolution and saturation issues), and b) transient transfection of GFP-parkin, is it difficult to control for expression levels on a cell-to-cell basis, especially given the fact that, in contrast to concentrating on puncta upon stimulation, GFP-parkin has a diffuse, cytoplasmic localization in control conditions. We also overcome this by quantitative analysis of the number of vesicles in a given population of cells over several experiments. In our revision experiments, we have addressed this problem at least partially by using a U2OS cell line stably expressing GFP-parkin (for example, new Fig. 3F and Fig. 6).

R2: *Figure 2A: the statistical comparisons for the Parkin mutants are not clear. Are they being compared only to themselves +/- Antimycin or are they being compared to the GFP control?*

The reviewer raises an important point of concern. We have clarified the mutant quantification (Fig. 2A), indicating that we are comparing the mutants +/- antimycin A to themselves.

All the mutants appear to increase the number of MDVs relative to GFP alone, though they lack sensitivity to further increases from antimycin.

The reviewer is right in this observation. Early assays using pOCT-DsRed2 as a positive MDV

marker suffered from high variability between assays, likely owing to the overexpression of several constructs within the same cell (i.e. GFP-parkin, pOCT-DsRed2, CFP-Drp1^{K38E}). To address these overexpression issues, we a) ablated Drp1 function by siRNA-mediated knockdown, and b) used endogenous cargo (mainly PDH E2/E3bp) as positive markers. This markedly cut down on experimental noise, and is exemplified in Fig. 4B, where cells transfected with either GFP or GFP-parkin have relatively similar baseline levels of MDVs.

R2: *Figure 2 - the images suggest very different levels of GFP in each of the mutant Parkins, though the blot indicates very similar expression levels. Were very different settings used for the imaging?*

For every experiment, we were sure to use the same imaging parameters to acquire each image for each condition. As explained above, transient transfection of the GFP-parkin constructs results in some variation of expression between cells. We would like to emphasize that the importance lies in the quantification, resulting from the analysis of up to almost 70 cells per condition.

R2: *p.9 The sentence describing the effects of RNAi against components of the autophagy pathway (Fig 4D,E) should be reworded- it could be taken to mean there was no effect of GFP-Parkin, when in fact they mean that the effect of antimycin was not affected by the RNAi.*

We have reworded this part of the text. It now reads "When GFP-parkin was expressed in these autophagy-deficient HeLa cells, we again observed an antimycin A-dependent increase in the number of PDH E2/E3bp-positive/TOM20-negative vesicles (Fig. 4C and D). As these numbers remained unchanged between autophagy-deficient and control cells, it is unlikely that these autophagy-related proteins play a role in parkin-dependent MDV biogenesis or degradation, as, in these instances, the number of vesicles would be expected to decrease or increase, respectively".

R2: *In figure 1c it would be nice to trace the cell boundaries.*

We have traced the cell boundaries in this figure at the request of the reviewer.

R2: *For my taste, the Discussion wanders a little far from the actual content of the manuscript when it discusses synuclein and LRRK2 and their possible role in vesicular pathways.*

We have removed the text concerning the putative trafficking roles of synuclein and LRRK2.

Reviewer 3

We thank the reviewer for their positive comments on our findings, indicating that our "*data are excellent and the manuscript is clearly written*". We have addressed their points below.

R3: *In some experiments the authors use CFP-Drp1K38E to dominantly inhibit fission in their experiments. However, it does not appear that CFP signal was monitored in the data shown. This should be clarified in the text for the reader.*

We have included an image demonstrating that transduction of the dominant-negative Drp1 was successful in almost all cells (Fig. S1).

R3: *Fig. 4A, a bar graph quantifying this data should be included.*

In our experience, both MEF genotypes (WT and Atg5$^{-/-}$) proved difficult to transfect with both GFP-parkin and the positive MDV marker pOCT-DsRed2 – i.e. finding a cell transfected with high enough amounts of both plasmids was rare. Although we attempted new assays using PDH E2/E3bp as a positive MDV marker, the transfections and resulting quantifications sill proved highly variable. Our initial findings from these cells were merely included as qualitative data, and our more in-depth analysis and quantifications were subsequently performed in HeLa cells with siRNA (new Fig. 4C and D), as these results were less variable. As such, we have now moved the Atg5 MEF data into the supplemental material as a qualitative piece of data.

R3: *The discussion of Fig. 4D and E is confusing. Are the conclusions based solely on the data indicated by the white bars (total MDVs)? If so, the lack of significant change after knockdown of*

autophagy components makes sense. However, it looks like there is a significant change in the GFP-positive MDVs upon siBcn1 knockdown. The authors should comment on this and clarify whether the statistics refer to the white bars, grey bars or both in 4E.

The reviewer is right in raising this point. The statistics reflected the white bars (total MDVs) only, and this has been made clearer in the present version of the manuscript. There is indeed a change in the number of GFP-positive MDVs in the siBcn1 condition. Beclin-1 is a component of the PI3K complex that regulates the induction of autophagy, as well as many other functions, and therefore its knockdown likely has more far-reaching, cell-wide effects than knockdown of Atg5, which acts downstream. For example, beclin binds Bcl-2, so it may have secondary effects on mitochondrial behavior that result in kinetic changes in parkin recruitment. Although this decrease in parkin-positive MDVs in the siBcn1 HeLa cells is interesting and worth exploring further in the future, we do not observe a change in the total number of vesicles, contributing further supporting evidence indicating that MDVs are autophagy-independent.

R3: *Discussion, page 13, please define AR-JP and SNpc for the reader.*

In an effort to keep the Discussion concise, we have replaced "AR-JP" with "PD patients harbouring defects in parkin or PINK1" and "SNpc" with "substantia nigra".

R3: *Fig S2D, the arrow at the left of the image (near the 82kDa marker) does not have a label. Is this GFP-Parkin? If so, it would help to move the label down so it is just above the arrow.*

The arrow in Fig. S2D of the previous version of the manuscript was indicating the TOM20 band. We have replaced this immunoblot with one that is more clear (new Fig. S3D), and thus have removed the arrow.

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2nd Editorial Decision 19 November 2013

I have now received reports from two of the original referees who reviewed your paper. As you can see below, both referees strongly support publication now and I am very happy to be able to accept your paper for publication here! Before formal acceptance, however, a few issues should be addressed:

1) Referee #2 still suggests some minor amendments to the text and to one figure panel, and I would appreciate it very much if you could address at least the textural point.
2) Also, while re-reading your manuscript I realized that some of the figures contain bar charts with standard error indications on "2 to 3" experiments. Could you please clarify how the standard error (I assume it's the standard error, it is not always indicated in the legends) was based on? For low numbers of independent experiments performed, I suggest to not display the error bar or to display the averages + error bars of the independent experiments separately next to each other (please see also our author guidelines).

I would also be grateful at this stage if you were to provide original source data (uncropped/-processed electrophoretic blots, spread sheets for the graphs) for the main figures of your manuscript. This is in accord with our policy to make original results better accessible for the community and thus increase reliability of published data. We would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files.

Congratulations on this great work! I am looking forward to hearing back from you.

REFEREE COMMENTS

Referee #2

The present manuscript represents a thoughtful and thorough revision of what was already a very interesting and important manuscript. The gaps in the story and the portions that were unclear have all been remedied. The manuscript is a significant advance over the authors' earlier studies of mitochondria-derived vesicles and a valuable addition to the literature on PINK1 and Parkin.

In preparing this revision, the authors have done a really exemplary job of

plugging the holes in the story and building an airtight case for ROS-triggered MDV production and the absolute requirement for Parkin (my previous points 1 and 2). They've also made a very serious attempt to address some of the trickier issues such as why there are some Parkin-negative MDVs and I think they have taken that analysis as far as technically possible at present. I very much appreciate the attention they gave the issue and the clarity with which the matter is now discussed.

One extremely minor point: I don't understand why Figure 3G is arranged with a y axis that goes all the way to 12 puncta/cell when the largest number in the graphs is just 5. But I wouldn't make them redo the figure if they don't want to; it just seems strange to squash the data down into the x-axis.

Also, I re-read the manuscript to see why I had suspected that there were Parkin-independent MDVs in the HeLa cells. I think the misimpression arose because the text refers to not seeing an increase caused by the mutated Parkins rather than expressly stating that they were never seen without Parkin. Similarly they refer to a 5-fold increase caused by expression of Parkin and antimycin treatment, which I took to mean that there were already some MDVs without Parkin. I would suggest that they say explicitly in the text that MDVs were absent when Parkin was not expressed in the cells. The current text is clearer than the earlier, but could still be made more explicit.

Referee #3

The revised version of the McLelland et al. manuscript addresses all of this reviewer's concerns/queries raised in the initial review. The experiments included in response to the comments of other reviewers further strengthen the manuscript. This interesting and important advance should be published without further delay.

Additional correspondence (author) 02 December 2013

We are very pleased that the reviewers have recommended our study for publication in the *EMBO Journal*, and would like to thank them for their technical and conceptual comments that ultimately enhanced our study concerning parkin- and PINK1-dependent MDV formation. We have addressed the final points raised by yourself and Reviewer 2 (R2) – as well as some other very minor changes – in the manuscript and figures, and have outlined these changes below.

1. R2 raises a good point concerning Figure 3G ("I don't understand why Figure 3G is arranged with a y-axis that goes all the way to 12 puncta/cell when the largest number in the graphs is just 5"). The scale of the y-axis was the same as Figure 3C, which would have allowed the reader to compare the number of puncta for any given marker with the number of PDH E2/E3bp-positive vesicles. However, we understand that, taken by itself, Figure 3G does look odd, and have adjusted the y-axes of these histograms accordingly.
2. We appreciate R2's concern about language, as the text does become a little convoluted when simultaneously discussing the increase in MDVs that we see with antimycin A in conjunction with expressing parkin in parkin-deficient HeLa cells. We have clarified the text on p.6 which, instead of "We observed a ~5-fold, parkin- and antimycin A-dependent increase in the number of these MDVs (Fig. 2A)", is now combined with the next sentence to read "Although we only observed these antimycin A-induced, matrix-positive/TOM20-negative MDVs in HeLa cells expressing GFP-parkin and not GFP alone (Fig. 2A), only 20% to 40% of these structures colocalized with parkin, often at the periphery of mitochondria

on what appeared to be nascent, budding vesicles (Fig. 1C, arrowheads), as well as ones that had dissociated completely from mitochondria (Fig. 1C, arrows)". With this sentence, we unambiguously state that MDVs require both parkin and antimycin A. We also modified the text on p.7 to read "In contrast, both GFPparkinK211N and GFP-parkinC431F failed to generate mitochondrial vesicles".

3. With regards to the point about standard error and the number of independent experiments, we have indicated, for every histogram, that it is the mean±SEM that is being represented. Moreover, although we routinely have indicated the number of independent experiments that have been performed, we have also included the range of n-values (i.e. the number of cells counted). It is these n-values that we have used in generating the SEM and our statistical analyses. We have clarified this latter point in the Materials & Methods section, under the title *Statistical analyses*.

Minor text corrections:

4. In the second paragraph of p.12, we have removed the citation to Trempe *et al*., *Science*, 2013 in reference to the U2OS:GFP-parkin cell line undergoing mitophagy in response to CCCP. Only Lefebvre *et al*., *Autophagy*, 2013 showed this for this line. Please note that Trempe *et al*., *Science*, 2013 remains in the References, as we cite this study when mentioning the catalytic site of parkin (p. 7).

5. In the Materials & Methods section, on p.20, we changed the phrase "parkin overexpression" to "ectopic parkin", and also put in the correct catalog number of the PDH E1a antibody.

6. In the Acknowledgements section on p. 22, we have reorganized the first sentence to read "We thank Miguel A. Aguileta and Marty Loignon (Montreal Neurological Institute) for the generation of the pEGFP-parkin mutants and technical assistance with the collection and analysis of FACS data, respectively."

7. In the legend for Figure 3F (p.32), we have included the sentence "Arrowheads show lack of colocalization between GFP-parkin and the indicated mitochondrial marker", as the nature of the arrowheads in the figure had not been indicated.

Minor figure corrections

8. In Figure 7A, we have changed "hrs" to "hours", and re-aligned the text for the DMSO immunblot.

9. The histogram in Figure 4D erroneously left out one of the replicates. We have included a new histogram that contains the complete data (this can be crossreferenced with the source data). All the statistical analyses were performed on the complete data, and thus the inferences in the text remain the same.

