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LRRK2 localizes to endosomes and interacts with clathrin-light chains to limit Rac1 activation

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Editor: Nonia Pariente

1st Editorial Decision 07 April 2014

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although the referees find the topic of interest, they all raise numerous serious concerns and find the study preliminary and insufficiently conclusive for publication.

As the reports are below, I will not detail them here. However, the study would clearly need to be substantially strengthened regarding its functional and physiological relevance (such as assaying the interaction between endogenous proteins and analyzing neurite outgrowth and retraction in relevant cell types), conclusiveness (referees 2 and 3 mention that knockdown studies are not appropriate to analyze epistasis) and cell biology of the LRRK2 interaction with CLC. In addition, several technical concerns, including the use of a fly stock collection that is problematic, would need to be addressed. In all, a substantial amount of new data would need to be included for further consideration in EMBO reports.

From the analysis of these comments, it is clear that we cannot consider the publication of your manuscript at this stage. On the other hand, given the potential interest of your study, I would like to give you the opportunity to address the reviewers concerns and would be willing to consider a revised manuscript, with the understanding that the referee concerns must be fully addressed. Please

note that it is our policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I appreciate that experimentally addressing all the referee concerns would involve extensive additional work of uncertain outcome and I would therefore also understand if you rather chose to seek rapid publication elsewhere. I could potentially extend our usual 3-month revision period, should you feel that time is the only limitation to a successful revision of the paper.

Do not hesitate to get in touch with me if I can be of any assistance.

REFEREE REPORTS:

Referee #1:

Schreij et al in this Ms provide evidence for complex formation between LRRK2, a leucine-rich repeat kinase mutated in dominant cases of PD with clathrin light chains, and clathrin light chains (CLCs). KD of either protein is shown to lead to elevated levels of Rac1 and corresponding cell morphological alterations. Moreover, the authors provide evidence using Drosophila for genetic interactions between LRRK2 and CLC in the control of Rac1 activation in vivo. These findings are interesting and novel, in principle. However, a number of points need to be worked out, in particular with respect to the question of whether and how LRRK2 regulates Rac1 and how this in turn is then modulated by CLCs.

Specific points:
1. Previous work by Chan et al has shown that LRRK2 activates Rac1, thereby regulating neurite length. Surprisingly, the data reported here suggest the opposite as loss of LRRK2 leads to increased active Rac1 levels. How can this be reconciled? Do CLCs and Rac1 compete for the same binding site on LRRK2? Conversely, do HIP1R and LRRK2 compete for the same site on CLCs?

2. All interaction data are based on the recombinant expression of at least one of the interacting proteins and binding assays with recombinant protein(s). It would thus be important to show that native LRRK2 and CLCs indeed interact in vivo as demonstrated for example by co-immunoprecipitation of endogenous proteins.

3. From the data contained in the paper it is impossible to distinguish whether phenotypes caused by CLC loss are mediated through HIP1R or LRRK2. This is an important issue that needs to be tackled as HIP1R is a well known actin regulator. Does KD of Rac1 occlude effects of CLC KD on cell morphology?

4. The actin-rich protrusions formed in LRRK2 or CLC KD cells remain poorly characterized. Are these indeed the result of elevated Rac1 activity? What is the effect of CLC KD and LRRK2 KD on actin dependent events, i.e. neurite outgrowth and retraction (as shown for LRRK2 and Rac1)?

5. The authors propose a model according to which LRRK2 localizes to CCPs. Can this be demonstrated i.e. by TIRF or spinning disc confocal imaging? How does this change in the absence of CLCs?

Minor:
6. From the images shown in fig. 3A it appears that loss of LRRK2 leads to increased recruitment of clathrin to the TGN. Can this be quantified? It would also be nice to know whether LRRK2 KD phenocopies CLC-KD with respect to MPR trafficking (though I don't regard this information as essential for the publication of the paper).

7. The Drosophila data lack evidence that Drosophila LRRK2 interacts with CLCs in the same way mammalian proteins do.

Referee #2:

In this manuscript the authors describe evidence for a direct physical interaction between LRRK2 and Clathrin Light Chains, initially using GST-fusion pull down studies.
Clathrin Light Chains CLC are not required for coated pits, but actin assembly and vesicular trafficking to lysosomes is defective. Although studies with GST fusions are complete and include structure/function analysis, additional data should be presented that antibodies to endogenous LRRK2 protein can bring down endogenous CLC or vice versa, in a controlled study.

Knockdown of LRRK2 led to activation of Rac1. Data are presented that suggest LRRK2 is downstream of the CLC effect on Rac1, but these are incomplete as they are not symmetrical - does CLC rescue a LRRK2 deficiency? And regardless, knockdown alleles are hypomorphic and can be misleading as to epistasis - thus this seems overstated.

The rough eye phenotype of a Rac1 overexpressor is worsened by LRRK2 knockdown, suggesting further an interaction - but additional studies with overexpression would increase the confidence here.

Overall the studies arevery interesting and of significance. Additional understanding of the cell biology implication of the interaction between LRRK2 and CLC on coated pits would greatly enhance the significance. Other comments are as above.

Referee #3:

In this article the authors show that LRRK2 binds to clathrin-light chain (CLC) with its ROC domain and they define the region in CLC that is important for this interaction. They go on to suggest that CLC could serve as a recruitment platform where the binding with LRRK2 is needed to coordinate cytoskeletal rearrangements in a rac dependent manner. I think the observations in this paper are a good basis for an interesting contribution but as it is, the data are spread thin, the conclusions are only weakly supported and there are numerous gaps that leave the reader with a unsatisfied feeling. I'm listing some issues below, but in my opinion for EMBO reps, this paper needs substantial revisions and additional work that are probably going beyond what could be achieved in a reasonable amount of time.

1) The argument that CLC and LRRK2 are in the same pathway because simultaneous knock down of clc and LRRK2 does not exacerbate the level of Rac1 activation makes only sense if (one of) the conditions used are (is) a complete loss of function condition. The knock down achieved here is only partial, hence, the inability to detect an additive effect may be a limitation of the assay (more activation is simply not detectable). I am not sure these data are interpreted in the most tight manner...
In relation to this point, I think the data in figure 4 fits better with the data in figure 2. I am not sure why the authors decided to split this up unless they felt not doing the interaction experiments in cells (see comment 2 below).

2) If the pathway of LRRK2 and CLC identified here indeed affects the actin cytoskeleton via rac1, it would have been useful to assess actin and the actin cytoskeleton and use the defects that are potentially found here as a read out of the different interactions tested (see also below). Somewhat related to this: what is the functional relevance of the LRRK2/CHC interaction? Is vesicle formation affected?

3) Fig3: If the cellular phenotypes are the result of Rac1 activation the authors could try rescuing the defects by using tools to reduce Rac1 activity. Idem for the interactions tested in fig 4...

4) The RNAi experiments in Drosophila are not sufficiently controlled. The stock collection used is known to cause false results through insertional mutagenesis and insertion into transcriptionally silent genomic regions (GD lines) and second site insertions leading to toxicity (KK lines) (Nature

Methods). There are 'clean' mutations in dLRRK available that could (should) be used in parallel. In addition, there is no data presented on the efficiency of dLRRK or clc knock down efficiency.

5) The interactions in the fly eye and in cells are both superficial. Does over expression of fly or human LRRK(2) rescue the phenotype in flies and cells respectively? Is this effect dependent on the GTPase domain? Does a kinase dead LRRK2 also yield rescue? Is the effect undone when a CLC mutant is expressed that lacks the LRRK2 binding domain? What is the nature of the disrupted eye morphology in fruit flies?

5) In the pull down experiment from rat brain lysate the authors identified clc to bind GST-LRRK2-ROC, but there is no information on how many proteins were identified or how specific this interaction was? A more complete description of this work would be useful.

6) In fig 1 it would be helpful to also add results concerning nCLCa as was done with nCLCb. Is there a conserved peptide in nCLCa similar to the one identified in nCLCb responsible for binding to the ROC domain of LRRK2?

7) The interaction data is not based on endogenous conditions. It would be more convincing to include Co-IP experiments using endogenous LRRK2 and endogenous CHC. Similarly, do fly LRRK and CHC also interact? This seems like a relevant experiment to include given the in vivo interactions presented in fruit flies.

8) In figure 3, do the authors have similar results in other cell lines?
The cell culture work was performed in a single cell line. Are the results consistent when performed in a different (maybe more neuronal-like) cell line? Where these experiments performed with synchronized cells to avoid the cell-cycle effects on cell morphology? How many cells/experiments were included.

9) In fig 2 quantification is lacking and information on the nr of repeats etc as well. There are not individually identifiable bands in the 5% starting material in Fig.2 B. This make difficult to follow the quantification process since it is rather difficult to confirm the amount of "input" as equal in all the treatments. Same concerns for Supplemental Fig. 2: it is difficult to identify single bands in the 10% starting material.

10) Concerning the binding of Rac1 to GST-PAK-CRIB, the authors should indicate in methods how much GST-PAK-CRIB they use for the pull down.

11) There is no negative control in fig 1F

12) The blots are not marked with the molecular weight-markers.

1st Revision - authors' response 10 September 2014

Reviewer 1

Reviewer 1, comment 1). *Previous work by Chan et al has shown that LRRK2 activates Rac1, thereby regulating neurite length. Surprisingly, the data reported here suggest the opposite as loss of LRRK2 leads to increased active Rac1 levels. How can this be reconciled? Do CLCs and Rac1 compete for the same binding site on LRRK2? Conversely, do HIP1R and LRRK2 compete for the same site on CLCs?*

Response to reviewer 1, comment 1) It is unlikely that CLCs and Rac1 compete for the same binding site on LRRK2. Chan et al. (*JBC*, 2011) demonstrate that Rac1 binds to the kinase domain and C-terminal of ROC (COR) domain of LRRK2 whereas we demonstrate that CLCs bind to the ROC domain. It is also unlikely that HIP1R and

LRRK2 compete for the same site on CLCs. We previously demonstrated that HIP1R binds to the conserved domain of CLCs (residues 20-41) (Legendre-Guillemin et al., *JBC*, 2005) whereas we now demonstrate that LRRK2 binds CLCs between residues 195 and 205.
Both Chan et al. (*JBC*, 2011) and Meixner et al. (*MCP*, 2011) report that LRRK2 knockdown decreases neurite outgrowth. In contrast, Parasiadou et al. (*J. Neurosci*, 2009) report that expression of the gain of function G2019S mutant of LRRK2 also decreases neurite outgrowth. Matta et al. (*Neuron*, 2012) demonstrate that both overexpression and knockdown of LRRK2 negatively affects synaptic vesicle endocytosis. Thus, it appears that a fine balance in the level of LRRK2 is required for normal function and that either too much or too little of the protein leads to LRRK2-related deficiencies. This could explain the apparent discrepancy between our study and that of Chan. This has been clarified in the text.

Reviewer 1, comment 2) *All interaction data are based on the recombinant expression of at least one of the interacting proteins and binding assays with recombinant protein(s). It would thus be important to show that native LRRK2 and CLCs indeed interact in vivo as demonstrated for example by co-immunoprecipitation of endogenous proteins.*

Response to reviewer 1, comment 2). Despite extensive effort, we have been unable to clearly demonstrate co-immunoprecipitation of endogenous LRRK2 with endogenous CLCs. A major complicating factor in these experiments is that LRRK2 is predominantly insoluble when generating lysates from cultured cells or tissue, even in the presence of detergents. For example, when we make a Triton X-100 soluble lysate from brain, approximately 90% of LRRK2 remains in the high-speed pellet (see reviewer Figure 1). This is not overly surprising as LRRK2 is a large, multi-domain protein and many such proteins are difficult to solubilize, as they are components of large protein complexes that pellet under high g spins. Additionally, CLCs are components of clathrin triskelia, composed of 3 linked clathrin-heavy chain (CHC) proteins and associated CLCs (Girard et al., *MCP*, 2005). Triskelia are found in the cytosol and as components of assembled clathrin coats on various membranes. Clathrin coats are also “insoluble” as they are massive protein complexes. Thus, if LRRK2 associates selectively with clathrin coats assembled on membranes it will not be possible to detect co-immunoprecipitation. These issues have been discussed in the revised manuscript. We do agree with the reviewer that the paper would be strengthened with additional evidence of physiological interaction between the two proteins. In response to reviewer 1, comment 5 we describe how we have now genome edited LRRK2 to allow for the localization of epitope-tagged protein driven off the endogenous promoter. This analysis reveals that a significant fraction of LRRK2 is localized to the endosomal system, particularly on the degradative pathway (revised Figure 2C). Importantly, there is partial co-localization of LRRK2 with CLCs (revised Figure 2D), likely reflecting the presence of CLCs on clathrin coats on endosomes. These clathrin coats are involved in the formation of multivesicular bodies during protein degradation.

Reviewer 1, comment 3). *From the data contained in the paper it is impossible to distinguish whether phenotypes caused by CLC loss are mediated through HIP1R or LRRK2. This is an important issue that needs to be tackled as HIP1R is a well known actin regulator. Does KD of Rac1 occlude effects of CLC KD on cell morphology?*

Response to reviewer 1, comment 3). The reviewer raises a valid point in that HIP1R is an actin regulator that also interacts with CLCs. In supplemental Figure 4A/B, we demonstrate that expression of LRRK2 rescues the activation of Rac1 resulting from CLC knockdown. Moreover, LRRK2 expression rescues changes in cell morphology resulting from CLC knockdown (supplemental Figure 4C/D). These data strongly support that the phenotypes resulting from CLC knockdown are mediated through LRRK2. Additionally, in supplemental Figure 5 of the revised manuscript, we demonstrate that treatment of cells with the Rac-specific inhibitor NSC-23766 blocks the changes in cell morphology resulting from LRRK2 knockdown. Our group was among the first to demonstrate that HIP1R is a component of the clathrin machinery and to describe and

characterize the CLC/HIP1R interaction (Metzler et al., *JBC*, 2001; Legendre-Guillemin et al., *JBC*, 2002; Legendre-Guillemin et al., *JBC*, 2005). To our knowledge there is no indication that HIP1R works through Rac1. In fact we feel this is an important aspect of the current story; CLCs interface with actin in two mechanistically independent ways.

Reviewer 1, comment 4). *The actin-rich protrusions formed in LRRK2 or CLC KD cells remain poorly characterized. Are these indeed the result of elevated Rac1 activity? What is the effect of CLC KD and LRRK2 KD on actin dependent events, i.e. neurite outgrowth and retraction (as shown for LRRK2 and Rac1)?*

Response to reviewer 1, comment 4). In supplemental Figure 5 we demonstrate that the changes in cell morphology, including the formation of protrusions, are blocked by the Rac inhibitor NSC-23766, strongly supporting that they are Rac1 dependent. However, we agree with the reviewer that the manuscript would be strengthened by the addition of data indicating alterations in a more physiological actin-dependent phenotype. Very recently, Parisiadou et al. (*Nat. Neurosci*., 2014) demonstrated that there is a decrease in the number of mature dendritic spines in neurons from LRRK2-/- mice. We thus used lentivirus to drive expression of shRNAmiRs targeting CLCs, a system that we have used and validated (Ritter et al., *PLoS Biology*, 2013). Knockdown of CLCa/b in cultured neurons was confirmed by blot (revised Figure 3G) and we observe a striking loss of mature dendritic spines (revised Figure 3H), similar to the phenotype reported for LRRK2 knockout.

Reviewer 1, comment 5) *The authors propose a model according to which LRRK2 localizes to CCPs. Can this be demonstrated i.e. by TIRF or spinning disc confocal imaging? How does this change in the absence of CLCs?*

Response to reviewer 1, comment 5) A systematic analysis of LRRK2 reagents has been recently published indicating that known LRRK2 antibodies are problematic for immunofluorescence (Davies et al., *Biochem. J*., 2013). Thus, it appears somewhat controversial as to whether any of the reported localizations of endogenous LRRK2 are accurate. Thus, we have now taken advantage of CRISPR/Cas9-based technology to genome edit LRRK2, adding an epitope tag to the protein driven off its endogenous promoter. We chose to edit LRRK2 in COS-7 cells since this line has been used for the cell-based studies in our manuscript. Cells were transfected with a plasmid encoding human optimized Cas9 and a guideRNA selective for LRRK2 flanking the start codon (described in Materials and Methods and based on the protocol in Petit et al., *JCB*, 2013). The cells were co-transfected with a large oligonucleotide encoding a triple-HA tag, flanked on either side by DNA sequence homologous to the LRRK2 gene (see revised Figure 2B). This protocol leads to cleavage of the genomic DNA of the LRRK2 gene with insertion of the oligonucleotide by homologous recombination, generating a triple HA tag between residues 1 and 2 of LRRK2, driven by the endogenous LRRK2 promoter. Recombinants were selected by immunofluorescence with an HA antibody and confirmation of the recombination was based on PCR (revised Figure 2A). We then used HA immunofluorescence, which revealed LRRK2 staining in punctate, endosomal-like structures that co-localize to a large part with internalized EGF (revised Figure 2C). Thus, it appears that a significant percentage of LRRK2 is localized to the endosomal system on the degradative pathway. In addition, there is partial co-localization of LRRK2 and CLCs (revised Figure 2D), likely reflecting clathrin coats on endosomes that are involved in the formation of multivesicular bodies during protein degradation. Thus, we feel that the CLC/LRRK2 interaction likely occurs on endosomes, a known site of Rac1 regulation (Palamidessi et al., *Cell*, 2008).

Reviewer 1, comment 6) *Minor: 6. From the images shown in fig. 3A it appears that loss of LRRK2 leads to increased recruitment of clathrin to the TGN. Can this be quantified? It would also be nice to know whether LRRK2 KD phenocopies CLC-KD with respect to MPR trafficking (though I don't regard this information as essential for the publication of the paper).*

Response to reviewer 1, comment 6) CLC staining on the TGN is variable from cell to cell. The original image resulted from such variability and not a change in the recruitment of CLC to the TGN. We have replaced the image (revised Figure 3E), such that the level of CLC staining on the TGN is similar in the control and LRRK2 knockdown. MacLeod et al (*Neuron*, 2013) demonstrate that expression of G2019S disrupts mannose phosphate receptor (MPR) trafficking similarly to CLC knockdown. We agree with the reviewer that it would be nice to directly compare knockdown of CLC and LRRK2 in terms of their influence on MPR trafficking. However, given the considerable effort placed on the CLC knockdown experiments in neurons, the CRISPR/Cas9 editing, and the extensive new work in *Drosophila*, we were not able to examine this issue within the allotted time for revisions.

Reviewer 1, comment 7) *The Drosophila data lack evidence that Drosophila LRRK2 interacts with CLCs in the same way mammalian proteins do.*

Response to reviewer 1, comment 7) We adopted *Drosophila* as a model system in order to definitely demonstrate that the LRRK2-CLC interaction is physiologically relevant and phylogenetically conserved. The extensive *Drosophila* data, including several new figures (see response to reviewer 2, comment 3 and response to reviewer 3, comment6). , have greatly enhanced our study. Biochemistry in *Drosophila* is difficult and we were not able to confirm biochemical interactions between Cls and dlrrk given the limited time constraints.

Reviewer 2

Reviewer 2, comment 1) *In this manuscript the authors describe evidence for a direct physical interaction between LRRK2 and Clathrin Light Chains, initially using GSTfusion pull down studies. Clathrin Light Chains CLC are not required for coated pits, but actin assembly and vesicular trafficking to lysosomes is defective. Although studies with GST fusions are complete and include structure/function analysis, additional data should be presented that antibodies to endogenous LRRK2 protein can bring down endogenous CLC or vice versa, in a controlled study.*

Response to reviewer 2, comment 1). The reviewer raises an important point and reviewer 1 had a similar comment. However, despite extensive effort, we have been unable to clearly demonstrate co-immunoprecipitation of endogenous LRRK2 with endogenous CLCs. An important complicating factor in these experiments is that LRRK2 is predominantly insoluble when generating tissue and cell lysates, even in the presence of detergents. For example, approximately 90% of LRRK2 remains in the high-speed pellet when we make a Triton X-100 soluble lysate from brain (see reviewer Figure 1). Perhaps this is not surprising as LRRK2 is a large (~280 kDa), multi-domain protein. Many such proteins are difficult to solubilize since they are components of large protein complexes. Additionally, CLCs are components of clathrin triskelia, composed of three copies of CHC and associated CLCs (Girard et al., *MCP*, 2005). Triskelia are found in the cytosol and as components of assembled clathrin coats on various membranes, including endosomes. Clathrin coats are also "insoluble" as they are large protein assemblies. Thus, if LRRK2 associates selectively with clathrin coats assembled on membranes it will not be possible to detect co-immunoprecipitation. These issues have been discussed in the revised manuscript. We do agree with the reviewer that the paper would be strengthened with additional evidence of interaction between the two proteins. In response to reviewer 1, comment 5 we describe how we have now genome edited LRRK2 to allow for the localization of epitope-tagged protein driven off the endogenous promoter. This analysis reveals that a significant fraction of LRRK2 is localized to the endosomal system on the degradative pathway (revised Figure 2C). We also detect partial co-localization of LRRK2 and CLCs (revised Figure 2D), likely reflecting clathrin coats on endosomes that are involved in the formation of multivesicular bodies during protein degradation.

Reviewer 2, comment 2) *Knockdown of LRRK2 led to activation of Rac1. Data are presented that suggest LRRK2 is downstream of the CLC effect on Rac1, but these are incomplete as they are not symmetrical - does CLC rescue a LRRK2 deficiency? And regardless, knockdown alleles are hypomorphic and can be misleading as to epistasis - thus this seems overstated.*

Response to reviewer 2, comment 2) As the reviewer points out, the epistasis experiments are not symmetrical since we did not demonstrate that CLC fails to rescue the LRRK2 knockdown phenotype on Rac1 activation. While we considered that specific experiment, we also agree that in general the knockdown alleles are misleading as to epistasis because of their hypomorphic nature. Thus, we have toned down our statements placing LRRK2 downstream of CLC. Most notably, we removed what was original Figure 4A, a model placing LRRK2 downstream of CLC.

Reviewer 2, comment 3*) The rough eye phenotype of a Rac1 overexpressor is worsened by LRRK2 knockdown, suggesting further an interaction - but additional studies with overexpression would increase the confidence here.*

Response to reviewer 2, comment 3) We agree with the reviewer and have performed the requested experiment. We obtained two new *Drosophila* lines, UAS-dlrrk wild-type (dlrrk is the *Drosophila* homologue of LRRK2) and UAS-dlrrk 3KD (kinase dead dlrrk), generated as described in Imai et al., (*EMBO J*, 2008). When we crossed these lines with the Rac1 overexpressing line, we saw a dramatic improvement in the rough eye phenotype resulting from Rac1 overexpression. Thus, dlrrk overexpression "rescues" the rough eye phenotype. The wild-type transgene displayed 100% penetrance (n=11) whereas the kinase dead line displayed 78.6% penetrance (n=14). We also obtained a Clc expressing line, which also rescues the Rac1 overexpression phenotype (84.6% penetrance, n=13). These data have been added to the revised manuscript as Figure 4C. We thank the reviewer for suggesting this experiment.

Reviewer 2, comment 4) *Overall the studies are very interesting and of significance. Additional understanding of the cell biology implication of the interaction between LRRK2 and CLC on coated pits would greatly enhance the significance. Other comments are as above.*

Response to reviewer 2, comment 4) We thank the reviewer for the positive assessment of the interest and significance of our study. Reviewer 1 also questioned the cell biological relationship/implication of the interaction between CLCs and LRRK2. As described in response to reviewer 1, comment 5, we have now taken advantage of CRISPR/Cas9-based technology to genome edit LRRK2, adding an epitope tag to the protein driven off its endogenous promoter. We chose to edit LRRK2 in COS-7 cells since this line has been used for the cell-based studies in our manuscript. Cells were transfected with a plasmid encoding human optimized Cas9 and a guideRNA selective for LRRK2 flanking the start codon (described in Materials and Methods and based on the protocol in Petit et al., *JCB*, 2014). The cells were co-transfected with a large oligonucleotide encoding a triple-HA tag, flanked on either side by DNA sequence homologous to the LRRK2 gene (see revised Figure 2B). This protocol led to cleavage of the genomic DNA of the LRRK2 gene with insertion of the oligonucleotide by homologous recombination, generating a triple HA tag between residues 1 and 2 of LRRK2, driven by the endogenous promoter. Recombinants were selected by immunofluorescence with an HA antibody and confirmation of the recombination was based on PCR (revised Figure 2A). We then used HA immunofluorescence, which revealed LRRK2 staining in large, endosomal-like structures (revised Figure 2C). Remarkably, there is co-localization of LRRK2 with internalized EGF (revised Figure 2C). Thus, it appears that a significant percentage of LRRK2 is localized to the endosomal system on the degradative pathway. Importantly, LRRK2 was found to localize in part with CLC (revised Figure 2D), likely representing bilayered clathrin coats on endosomes involved in the formation of multivesicular bodies involved in protein degradation.

Reviewer 3

Reviewer 3, comment 1) *The argument that CLC and LRRK2 are in the same pathway because simultaneous knock down of clc and LRRK2 does not exacerbate the level of Rac1 activation makes only sense if (one of) the conditions used are (is) a complete loss of function condition. The knock down achieved here is only partial,hence, the inability to detect an additive effect may be a limitation of the assay (more activation is simply not detectable). I am not sure these data are interpreted in the most tight manner...*

Response to reviewer 3, comment 1) We present multiple lines of evidence that CLCs and LRRK2 are in the same pathway; 1) the proteins directly interact (original Figure 1); 2) the proteins co-localize (new data, revised Figure 2D); 3) the lack of an additive effect on Rac1 activation with double knockdown (original Figure 2, revised Figure 3C/D) (although the reviewer is correct in that the assay could be a limiting factor in this experiment); 4) the lack of an additive effect of double knockdown for changes in cell morphology (original Figure 3, revised 3E/F); 5) LRRK2 expression rescues alterations in Rac1 activation resulting from CLC knockdown (original Figure 4, revised supplemental Figure 3A/B); 6) in *Drosophila*, both dlrrk and Clc knockdown enhance a rough eye phenotype resulting from Rac1 overexpression (revised Figure 4A); 7) in *Drosophila*, both dlrrk and Clc expression rescue the rough eye phenotype resulting from Rac1 overexpression (revised Figure 4C). While no single piece of data is conclusive that the two proteins function on the same pathway for regulation of Rac1 activity, the sum of the data strongly supports that conclusion.

Reviewer 3, comment 2) *In relation to this point, I think the data in figure 4 fits better with the data in figure 2. I am not sure why the authors decided to split this up unless they felt not doing the interaction experiments in cells (see comment 2 below).*

Response to reviewer 3, comment 2). In fact in the original manuscript we performed the Rac1 activation assays with CLC knockdown, LRRK2 knockdown, or double knockdown in Figure 2 and followed up with cell-based morphology changes in Figure 3. Then we performed rescue experiments on Rac1 activation in Figure 4 and followed up with cell based morphology changes with rescue in supplemental Figure 4. Thus, we did do the interaction experiments in cells. The order of the figures was simply set in a way that we felt best conveyed the message of the data. In the revised manuscript the data has been combined in two figures, Figure 3 for the Rac1 activation and morphological changes with single and double knockdown, supplemental Figure 2 for the Rac1 activation and morphological changes for the rescue experiments.

Reviewer 3, comment 3) *If the pathway of LRRK2 and CLC identified here indeed affects the actin cytoskeleton via rac1, it would have been useful to assess actin and the actin cytoskeleton and use the defects that are potentially found here as a read out of the different interactions tested (see also below).*

Response to reviewer 3, comment 3) While we agree with the reviewer that another phenotypic read out (actin changes) would be of value, we do not feel it would add greatly to the manuscript, which is focused predominantly on Rac1 activation. Thus, we focused our efforts on addressing other issues raised by this and other reviewers.

Reviewer 3, comment 4) *Somewhat related to this: what is the functional relevance of the LRRK2/CHC interaction? Is vesicle formation affected?*

Response to reviewer 3, comment 4) This manuscript is focused on an interaction between LRRK2 and CLCs. CHC, while part of the clathrin machinery, is distinct from CLC and does not interact directly with the ROC domain of LRRK2. Perhaps the reviewer meant to indicate CLCs. If so, the functional relevance is to regulate Rac1. We previously demonstrated that CLC regulates actin via interactions with HIP1R (Legendre-

Guillemin et al., *JBC*, 2005). This would add to the role of CLCs as physiological regulators of the actin regulatory machinery. We previously demonstrated that CLCs are not involved in vesicle formation (Poupon et al., *PNAS*, 2008). Thus, we have no reason to suspect that LRRK2 would play a role in this process.

Reviewer 3, comment 5) *Fig3: If the cellular phenotypes are the result of Rac1 activation the authors could try rescuing the defects by using tools to reduce Rac1 activity. Idem for the interactions tested in fig 4...*

Response to reviewer 3, comment 5) In supplemental Figure 5A/B we use the specific Rac inhibitor, NSC-23766 to block the cellular phenotypes resulting from LRRK2 knockdown. This demonstrates that the changes in cell morphology following LRRK2 knockdown are due to Rac1 activation. Original Figure 4 (now Figure 3) presents Rac1 activation assays, demonstrating that knockdown of either CLC or LRRK2 activates Rac1. Rac1 inhibition through knockdown or the Rac inhibitor would not be meaningful in this experiment.

Reviewer 3, comment 6) *The RNAi experiments in Drosophila are not sufficiently controlled. The stock collection used is known to cause false results through insertional mutagenesis and insertion into transcriptionally silent genomic regions (GD lines) and second site insertions leading to toxicity (KK lines) (Nature Methods). There are 'clean' mutations in dLRRK available that could (should) be used in parallel. In addition, there is no data presented on the efficiency of dLRRK or clc knock down efficiency*.

Response to reviewer 3, comment 6) In the original manuscript we used two independent RNAi lines for Clc and two independent RNAi lines for dlrrk. To further support the results obtained with these lines we have now performed the experiment requested by the reviewer. We obtained a dlrrk null allele line (lrrke03680, obtained from the Exelixis collection at Harvard). When crossed with the Rac1 overexpressing line, we observed a similar enhancement in the rough eye phenotype as that seen with dlrrk RNAi (revised Figure 4B).
Additionally, as described in response to reviewer 2, comment 3, we obtained several new *Drosophila* lines including a UAS-dlrrk wild-type allele, a UAS-dlrrk kinase dead line, and a UAS-Clc line. When we crossed these lines with the Rac1 overexpressing line, we saw a dramatic improvement in the rough eye phenotype resulting from Rac1 overexpression. Thus, dlrrk overexpression “rescues” the rough eye phenotype, as does a kinased dead mutant and Clc. The wild-type transgene displayed 100% penetrance (n=11), whereas the kinase dead line displayed 78.6% penetrance (n=14) and the Clc line displayed 84.6% penetrance (n=13). These data have been added to the revised manuscript as Figure 4C.
Finally, we followed the advice of the reviewer and examined the knockdown of Clc and dlrrk with the RNAi lines. In revised supplementary Figure 6 we demonstrate that the RNAi lines are efficient in knockdown of the respective proteins. Together, the data described here have strengthened the manuscript and we thank the reviewer for these suggestions.

Reviewer 3, comment 7) *The interactions in the fly eye and in cells are both superficial. Does over expression of fly or human LRRK(2) rescue the phenotype in flies and cells respectively? Is this effect dependent on the GTPase domain? Does a kinase dead LRRK2 also yield rescue? Is the effect undone when a CLC mutant is expressed that lacks the LRRK2 binding domain? What is the nature of the disrupted eye morphology in fruit flies?*

Response to reviewer 3, comment 7) In revised supplementary Figure 2 we demonstrate that expression of LRRK2 rescues Rac1 activation resulting from LRRK2 knockdown. Moreover, we demonstrate that LRRK2 expression rescues Rac1 activation resulting from CLC knockdown. As described in response to reviewer 3, comment 6 we have also added a new experiment demonstrating that expression of dlrrk in flies rescues the rough eye phenotype resulting from Rac1 overexpression (revised Figure 4C). In addition, we

have examined a kinase dead allele. We obtained a new *Drosophila* line UAS-dlrrk 3KD (kinase dead dlrrk), generated as described in Imai et al., (*EMBO J.*, 2008). When we crossed this line with the Rac1 overexpressing line, we saw a dramatic improvement in the rough eye phenotype resulting from Rac1 overexpression. Thus, kinase dead dlrrk overexpression "rescues" the rough eye phenotype. This demonstrates that the kinase activity is not required for the phenotype. This data has been added to the revised manuscript as (Figure 4C). We thank the reviewer for suggesting this experiment. In terms of the proposed experiments involving the GTPase domain of dlrrk or the dlrrkbinding domain of Clc, no such fly lines are available and it was not possible in the constrained time frame to create such lines. The precise mechanism by which Rac1 overexpression leads to a rough eye phenotype is not established but there is no question that it is a robust *in vivo* assay for Rac1 activity. Taken together, it seems highly likely that loss of Clc or dlrrk function enhances the rough eye phenotype by inducing activation of Rac1.

Reviewer 3, comment 8) *In the pull down experiment from rat brain lysate the authors identified clc to bind GST-LRRK2-ROC, but there is no information on how many proteins were identified or how specific this interaction was? A more complete description of this work would be useful*.

Response to reviewer 3, comment 8) It appears that many of the other proteins identified in this analysis were non-specific, that is they tend to appear in many such mass spec studies.

Reviewer 3, comment 9) *In fig 1 it would be helpful to also add results concerning nCLCa as was done with nCLCb. Is there a conserved peptide in nCLCa similar to the one identified in nCLCb responsible for binding to the ROC domain of LRRK2?*

Response to reviewer 3, comment 9) In the literature, nCLCa and b are considered functionally interchangeable (as are CLCa and b) (Acton et al., *JCB*, 1999). The amino acids between residues 195-205 in nCLCb, which are responsible for LRRK2 binding, are identical in CLCa and nCLCa. We have clarified this point in the revised manuscript.

Reviewer 3, comment 10). *The interaction data is not based on endogenous conditions. It would be more convincing to include Co-IP experiments using endogenous LRRK2 and endogenous CHC. Similarly, do fly LRRK and CHC also interact? This seems like a relevant experiment to include given the in vivo interactions presented in fruit flies.*

Response to reviewer 3, comment 10) As described in response to reviewer 1, comment 2 and reviewer 2, comment 1, we have been unable to clearly demonstrate coimmunoprecipitation of endogenous LRRK2 with endogenous CLCs despite extensive effort (we assume the reviewer meant CLC and not CHC). A major complicating factor in these experiments is that LRRK2 is predominantly insoluble when generating lysates from cultured cells or tissue, even in the presence of detergents. For example, when we make a Triton X-100 soluble lysate from brain, approximately 90% of LRRK2 remains in the high-speed pellet (see reviewer Figure 1). This is not overly surprising as LRRK2 is a large, multi-domain protein and many such proteins are weakly soluble as they are components of large protein complexes. Additionally, CLCs are components of clathrin triskelia, composed of clathrin-heavy chains and associated CLCs (Girard et al., *MCP*, 2005). Triskelia are found in the cytosol and as components of assembled clathrin coats on various membranes. Clathrin coats are also "insoluble" as they are massive protein complexes. Thus, if LRRK2 associates selectively with clathrin coats assembled on membranes it will not be possible to detect co-immunoprecipitation. These issues have been discussed in the revised manuscript. We do agree with the reviewer that the paper would be strengthened with additional evidence of interaction between the two proteins. In response to reviewer 1, comment 5 we describe how we have now genome edited LRRK2 to allow for the localization of epitope-tagged protein driven off the endogenous promoter. This analysis reveals that a significant fraction of LRRK2 is localized to membranes on the degradative pathway of the endosomal system (revised Figure 2C).

Importantly, there is partial co-localization of LRRK2 and CLCs (revised Figure 2D), likely reflecting clathrin coats on endosomes that are involved in the formation of multivesicular bodies during protein degradation.

Reviewer 3, comment 11) In figure 3, do the authors have similar results in other cell lines? The cell culture work was performed in a single cell line. Are the results consistent when performed in a different (maybe more neuronal-like) cell line? Where these experiments performed with synchronized cells to avoid the cell-cycle effects on cell morphology? How many cells/experiments were included.

Response to reviewer 3, comment 11) We performed all of our Rac1 activation assays in COS-7 cells for consistency. Recognizing the need to see similar results in different systems was a large part of what motivated us to examine the rough eye phenotype in *Drosophila*. The fact that we observe similar Rac1 activation phenotypes in both COS-7 cells and fly eyes suggests strongly that this is a conserved and general phenomenon. For the quantification of the experiments in Figure 3, we performed 3 independent experiments counting 30-37 cells per condition. We apologize for not including this information in the original submission. The experiments in Figure 3 were not performed in synchronized cells. While the reviewer is correct in that there are cell-cycle effects on cell morphology, at any given moment in cell culture the vast majority of cells are in interphase. Thus, given the quantification described above, it is highly unlikely that the results in cell morphology could be accounted for by cell cycle effects.

Reviewer 3, comment 12). *In fig 2 quantification is lacking and information on the nr of repeats etc as well. There are not individually identifiable bands in the 5% starting material in Fig.2 B. This make difficult to follow the quantification process since it is rather difficult to confirm the amount of "input" as equal in all the treatments. Same concerns for Supplemental Fig. 2: it is difficult to identify single bands in the 10% starting material.*

Response to reviewer 2, comment 12) For Figure 2A, we have now quantified the degree of knockdown and provide information regarding the number of repeats (revised Figure 3B). We apologize for this omission. For figure 2B (now revised Figure 3C), while the bands do touch each other, the inputs do not look different. We have carefully quantified and statistically analyzed this experiment. Even if there were minor differences in the amount of input, it could not account for the highly significant increase in Rac1 activity observed following knockdown of CLC or LRRK2.

Reviewer 3, comment 13) *Concerning the binding of Rac1 to GST-PAK-CRIB, the authors should indicate in methods how much GST-PAK-CRIB they use for the pull down*.

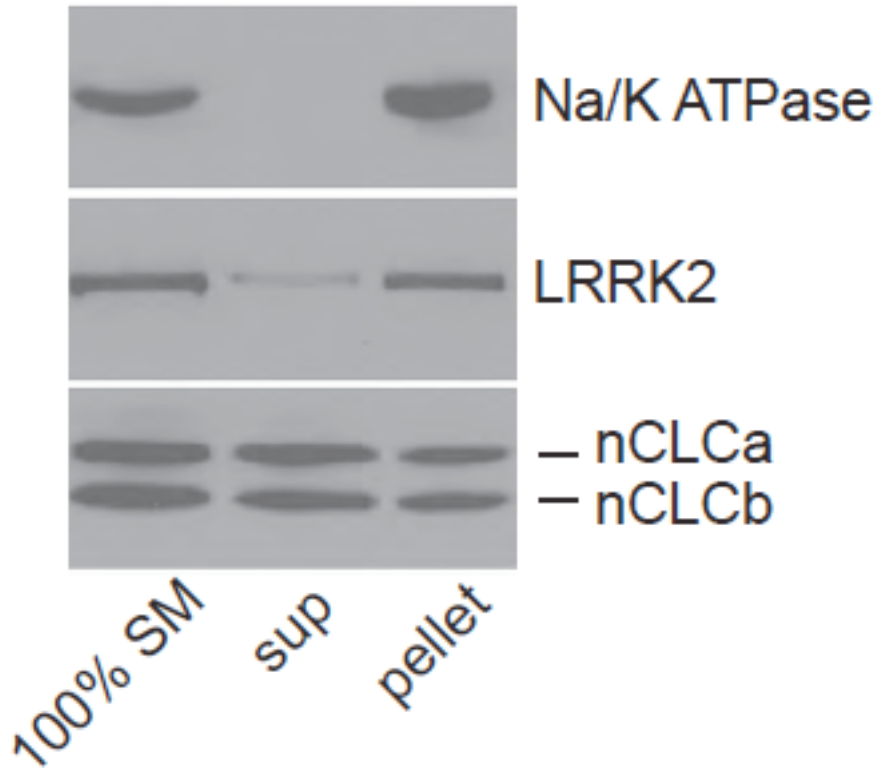
Response to reviewer 3, comment 14). We apologize for the omission and have added the information.

Reviewer 3, comment 15) *There is no negative control in fig 1F*

Response to reviewer 3, comment 15) In this experiment, we demonstrate that clathrin triskelia, purified from brain bind to the GST-ROC domain but not GST (the GST is a negative control). We cannot blot for something that does not bind GST-ROC because the starting material is purified triskelia, composed of CHC and CLC only.

Reviewer 3, comment 16) The blots are not marked with the molecular weight-markers.

Response to reviewer 3, comment 16) We apologize for this oversight and have added molecular weight markers to all blots.



Schreij et al., reviewer Figure 1

2nd Editorial Decision 25 September 2014

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the enclosed reports from the referees. As you will see, although both referees appreciate the extensive work done during revision, consider the study is novel and of interest, and are both supportive of its ultimate publication in EMBO reports, referee 2 still has some concerns. Upon further discussion with the referees, we have decided to open an exceptional second short round of revision for you to be able to respond to these issues before publication.

Several concerns can be addressed by providing additional details or modifying the text/title. However, we feel that an attempt should be made to provide additional data to address comments 1, 6 and 10. Showing a biochemical interaction between HA-LRRK2 and CLC (if possible due to HA-enhanced solubility), measuring the RNAi efficiency in Supp Fig 6 (and providing a better Supp. 6B image if possible), and co-staining of HA-LRRK2, Rac1 and an early endosomal marker, would add support to your findings.

To this end, we would be happy to allow a 2-3 week final revision period. Please let me know if this

is a feasible and reasonable option.

In going through your manuscript, I have realized that the Materials and methods section is rather succinct. Please note that basic Materials and Methods required for understanding the experiments performed must remain in the main text, although additional detailed information required to reproduce them can be supplementary. Please aslo include the statistical analysis subheading in the main text.

I look forward to receiving your final version as soon as possible.

REFEREE REPORTS:

Referee #1:

All my previous concerns have been addressed. In particular the new data using genome edited cells are an important addition to the Ms and I commend the authors for these excellent revisions.

Referee #3:

The authors have substantially addressed the comments and I have a few final clarifications that the authors should address:

Comment 1: generating HA-LRRK2 added valuable information. Co-localization of Lrrk2 and CLC is promising. However, it would be much more powerful to assess the biochemical interaction between HA-LRRK2 and CLC. Have the authors tried IP using their new HA-LRRK2 cells? Adding a tag may increase the solubility of proteins and there are exceptional antibodies to immunoprecipitate HA. Moreover, are the authors fully convinced their problems to observe the endogenous IP is due to solubility problems? Since in fig 1A and1B the authors show there is enough soluble nCLCa/nCLCb and LRKK2 to see a binding with GST-Roc and GST-nCLCb respectively.

Comment 3: If the authors consider actin cytoskeleton changes out of the scope of this article, they should change the title, as now it literally includes "control actin cytoskeleton dynamics" and this was not directly shown.

Comment 6: I really appreciate the effort of using a the dlrrk mutant and performing genetic interactions experiments with that mutant instead of the previous ones in the drosophila eye. I believe these data make the results more reliable.

While the genetics have become more solid, the RNAi efficiencies (Supp Fig.6) are still a problem and should be resolved. I still believe the data is not strong. Supp: Fig. 6A could be OK to demonstrate the efficiency because the GFP signal is strong enough to be detected and there is not a problem concerning antibody specificity. However, immunofluorescence pictures showed in Supp. Fig.6B are not convincing. Staining of dLRRK is too weak and this is a major problem considering the authors are using over-expression of LRRK (this problem may be likely due to the quality of the available antibodies...) and moreover, they do not provide data concerning the validation of the antibody. Also, DAPI staining seems different in all panels, suggesting different planes were used and this can be tricky. Because efficiency of the RNAi is essential to the conclusion of Fig 4, I strongly suggest the authors show quantitative data for the RNAi efficiency by using real time PCR (the authors can collect the eye imaginal disc or a retinal preparation/dissection as a source for RNA)

I believe there is a mistake in Fig.4-B-ii. The picture shows the legend GMR>UAS-dlrrke03680. I believe the authors are showing expression of Rac in the dlrrk mutant background.

Concerning the variability of the phenotypes: same genotype in figures Fig.4 Ai and Ci seem to give different phenotypes, being Ci more similar to Avi than Ai. Is the UAS-Rac1W phenotype so

variable or it is just a matter of imaging the Drosophila eye?

The legend is illegible in some pictures from fig 6: Av and Avii. Also I believe in figure C it would be necessary to show a wild type eye or an appropriate control in order to compare the rescued eyes. In addition, I assume the authors only show a scale bar in one picture of each panel because same settings are used. I am curious because the eye Ciii showing a rescued phenotype seems larger than Cvi.

Comment 10: data with endogenous proteins would make the data stronger

I really appreciate their effort is using genome editing to obtain HA-LRKK2 cells. I think some data is missing to link some conclusions in fig 2 and 3. A staining of HA-LRKK2 co-localizing with Rac1 and an early endosomal marker would strengthen the data.

Comment 11: I thank the authors to clarify the number of observed cells. I agree with the authors that most of cells are in interphase, but in figure 3E they show some cells that, based on cell morphology and/or CLC accumulation in what could be the spindle, may not be in interphase.

2nd Revision - authors' response 14 October 2014

Editor comment 1) In going through your manuscript, I have realized that the Materials and methods section is rather succinct. Please note that basic Materials and Methods required for understanding the experiments performed must remain in the main text, although additional detailed information required to reproduce them can be supplementary. Please aslo include the statistical analysis subheading in the main text.

Response to Editor comment 1) We have revised the Materials and methods section to retain information required for understanding the experiments performed. In addition, we have moved the statistical analysis section to the main text.

Referee #3, comment 1) generating HA-LRRK2 added valuable information. Colocalization of Lrrk2 and CLC is promising. However, it would be much more powerful to assess the biochemical interaction between HA-LRRK2 and CLC. Have the authors tried IP using their new HA-LRRK2 cells? Adding a tag may increase the solubility of proteins and there are exceptional antibodies to immunoprecipitate HA. Moreover, are the authors fully convinced their problems to observe the endogenous IP is due to solubility problems? Since in fig 1A and1B the authors show there is enough soluble nCLCa/nCLCb and LRKK2 to see a binding with GST-Roc and GST-nCLCb respectively.

Response to referee #3, comment 1) While we agree that co-immunoprecipitation of HALRRK2 with CLC would enhance the manuscript, the HA tag does not appear to increase LRRK2 solubility. This is not overly surprising because the triple HA-tag only adds 29 amino acids to a 280 kDa protein. The referee is correct in that in fig 1A there is sufficient soluble CLC to see interaction with GST-ROC and in fig 1B there is enough soluble LRRK2 to see interaction with GST-CLC. However, when combining the limited solubility of the two partners, due to the fact that they each appear to be components of large protein complexes, there does not appear to be enough of the soluble complex to allow co-immunoprecipitation. However, we cannot rule out that the lack of co-IP is due to a transient interaction and a statement to this effect has been added to the manuscript.

Referee #3, comment 2) If the authors consider actin cytoskeleton changes out of the scope of this article, they should change the title, as now it literally includes "control actin cytoskeleton dynamics" and this was not directly shown.

Response to referee #3, comment 2) The referee is absolutely correct. We have thus changed the title to “LRRK2 localizes to endosomes and functions in concert with clathrin-light chains to limit Rac1 activation”.

Referee #3, comment 3) I really appreciate the effort of using a the dlrrk mutant and performing genetic interactions experiments with that mutant instead of the previous ones in the drosophila eye. I believe these data make the results more reliable. While the genetics have become more solid, the RNAi efficiencies (Supp Fig.6) are still a problem and should be resolved. I still believe the data is not strong. Supp: Fig. 6A could be OK to demonstrate the efficiency because the GFP signal is strong enough to be detected and there is not a problem concerning antibody specificity. However, immunofluorescence pictures showed in Supp. Fig.6B are not convincing. Staining of dLRRK is too weak and this is a major problem considering the authors are using over-expression of LRRK (this problem may be likely due to the quality of the available antibodies...) and moreover, they do not provide data concerning the validation of the antibody. Also, DAPI staining seems different in all panels, suggesting different planes were used and this can be tricky. Because efficiency of the RNAi is essential to the conclusion of Fig 4, I strongly suggest the authors show quantitative data for the RNAi efficiency by using real time PCR (the authors can collect the eye imaginal disc or a retinal preparation/dissection as a source for RNA)

Response to referee #3, comment 3). First, we thank the referee for the appreciation of our effort to use a dlrrk mutant. We agree that this has improved the study. For Supp. Fig. 6B (demonstrating the efficiency of the dlrrk RNAi), the dlrrk antibody has been extensively characterized by the laboratory of Bingwei Lu and has been shown to be specific (*EMBO J*., 2008). It was an oversight on our part to not describe this antibody, and this has been corrected. The relatively weak staining of dlrrk in the immunofluorescence panels likely does reflect an inherent limitation of the antibody in immunofluorescence. To overcome this, we have now performed a new experiment very much along the lines of that suggested by the referee. We have crossed dlrrk expressing lines with a control RNAi line or 3 different dlrrk RNAi lines and have made lysates from heads for Western blot. These data clearly demonstrate that the RNAi lines cause reduced expression of dlrrk protein. These data have been added as revised Supplemental Figure 6C.

Referee #3, comment 4) I believe there is a mistake in Fig.4-B-ii. The picture shows the legend GMR>UAS-dlrrke03680. I believe the authors are showing expression of Rac in the dlrrk mutant background.

Response to referee #3, comment 4) We thank the referee for pointing out this oversight, the legend has been corrected.

Referee #3, comment 5) Concerning the variability of the phenotypes: same genotype in figures Fig.4 Ai and Ci seem to give different phenotypes, being Ci more similar to Avi than Ai. Is the UAS-Rac1W phenotype so variable or it is just a matter of imaging the Drosophila eye?

Response to referee #3, comment 5) The UAS-Rac1W phenotype is in fact quite consistent. As the referee indicated, the apparent variability if Figure 4Ci was a reflection of imaging the *Drosophila* eye, which can be technically challenging. We have now provided a more representative image.

Referee #3, comment 6) The legend is illegible in some pictures from fig 6: Av and Avii.

Response to referee #3, comment 6) We thank the referee for pointing this out. We have changed several labels to white to make them more legible.

Referee #3, comment 7) Also I believe in figure C it would be necessary to show a wild type eye or an appropriate control in order to compare the rescued eyes.

Response to referee #3, comment 7) We have added a wild-type eye as recommended by the referee.

Referee #3, comment 8) In addition, I assume the authors only show a scale bar in one picture of each panel because same settings are used. I am curious because the eye Ciii showing a rescued phenotype seems larger than Cvi.

Response to referee #3, comment 8) The referee is correct in that the same settings were used. The image used for Figure 4Ciii was not scaled properly. This was a mistake on our part and has been corrected.

Referee #3, comment 9) I really appreciate their effort is using genome editing to obtain HA-LRKK2 cells. I think some data is missing to link some conclusions in fig 2 and 3. A staining of HA-LRKK2 co-localizing with Rac1 and an early endosomal marker would strengthen the data.

Response to referee #3, comment 9) We thank the referee for the appreciation of the effort to use genome edited cells as this involved a great deal of work. As suggested by the referee, we have now performed co-staining of HA-LRRK2 with the early endosomal marker EEA1. This reveals a partial co-localization of LRRK2 to early endosomes, consistent with the partial co-localization with CLCs. This has been added as revised figure 2E.

Referee #3, comment 10) I thank the authors to clarify the number of observed cells. I agree with the authors that most of cells are in interphase, but in figure 3E they show some cells that, based on cell morphology and/or CLC accumulation in what could be the spindle, may not be in interphase.

Response top referee #3, comment 10) The staining pattern for CLC in Figure 3E is the classical pattern for an interphase cell with the perinuclear CLC staining corresponding to clathrin-coats on membranes of the trans-Golgi network. In fact, when cells leave interphase, the Golgi disperses and this classic pattern is lost. This supports that these cells are in interphase.

3rd Editorial Decision 17 October 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication.

