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Title: Leucine-rich Repeat Kinase 2 regulates Sec16A at ER exit sites to allow ER-Golgi export

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Editor: Andrea Leibfried

1st Editorial Decision 21 January 2014

Thank you for submitting your manuscript entitled 'Leucine-rich Repeat Kinase 2 acts upstream of Sec16A at ER exit sites in regulating ER-Golgi export'. I have now received reports from three referees, which are enclosed below.

As you will see, all referees find your study interesting and referee #3 supports publication of a revised version of your manuscript here. However, referee 1 and 2 think that further insight to reveal the role of LRRK2 at ERES is required as well as some experiments and amendments to better support your conclusions.
Given the comments provided, I would like to invite you to submit a revised version of the manuscript, addressing all concerns of the referees. More specifically, referee #1 gives very constructive comments and an analysis along the lines suggested by this referee would be very important for further proceedings here. We would also appreciate if you could address minor point 1 of referee #2. Please do not hesitate to contact me in case of further questions.

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

REFEREE COMMENTS

Referee #1:

This is an interesting paper that reports a role for LRRK2 in the control of ER exit site function through binding to Sec16A.
The work in the paper is generally of high quality and the findings are certainly novel and of interest to the field. My enthusiasm is somewhat dampened as I think that some of the conclusions are not well supported by the data and there is a lack of mechanistic insight into what LRRK2 actually does at ERES - it is unclear after reading this manuscript as to how many of the phenotypic effects shown are down to the interaction with Sec16 rather than the many other functional interactions of LRRK2 that have been described. As such, one is left with the sense that this is a little under-developed for publication in EMBO Journal.

Given that LRRK2 regulates both the actin and microtubule networks, the authors should show that the outcomes shown here are not down to such indirect effects. Similar arguments could be made for its interaction and regulation of and by ArfGAP1 - highly relevant in the context of the current work.

Specifically it seems to me that one could explain the findings not as a loss of ERES function but as a change in their organization. If LRRK2 is so critical for Sec16 function then I would be very surprised since the knockout cells are seemingly viable and have minimal perturbation of secretory pathway function. Therefore I find I cannot agree with the conclusion that "These results suggest a critical role of LRRK2 in anchoring Sec16 at ERES". An alternative explanation is that the number, size and organization of ERES is perturbed. Higher resolution data are required to reconcile these alternatives.

Some assay for COPII assembly is essential for one to properly interpret this work. Wither this should take the form of an in vitro budding assay in the presence and absence of LRRK2 or by exploiting FRAP of COPII subunits, including Sec16, inner and outer coat components, to define the role of LRRK2 in the assembly of COPII. The knockout and knock-in cells provide an exceptional opportunity to do this in a very clean background.
In terms of conclusions, I find that the BFA assay does not provide an accurate reflection of increased cargo load. That the ERES do not enlarge further in the presence of BFA suggests a size limit (as has been proposed by Glick previously) such that no further increase is seen. This is not the same as there being no effect. Farhan 2008 is also not the first time that the effect of BFA on ERES was reported.

The VSV-G assay, although widely used, requires further support from the use of an endogenous cargo e.g. BFA washout and repopulation of the Golgi with glycosyltransferases. An EndoH assay for VSV-G traffic would be a possible alternative. I also do not understand the term cytoplasmic membrane in the context of these assays (the absence of page and line numbers is frustrating in terms of providing a reference point).

A similar point arises from the neuronal trafficking work which while very nicely executed is similarly open to other interpretations. The labeling for Sec16A for example is suggestive of a cytoplasmic pool, not a bulk ER pool (Figure 7D). On what basis do the authors conclude that this is ER associated?
Similarly, I find that the data showing localization of Sec16 are unconvincing in many panels. The Sec16 labeling in Figure 2 looks dramatically different to that of Sec31 in Figure 3 and especially Figure 4. Some ultrastructural characterization would be of enormous benefit here.

In terms of the biochemistry, if the important interaction is with the CCD of Sec16, a critical question is whether this affects the association of Sec13 which binds tightly to this region. This could have profound mechanistic implication if Sec13 is displaced by LRRK2.

Specific examples within the discussion where I do not find that the data support the conclusion are the headings:
"LRRK2 regulates COPII vesicle trafficking" - LRRK2 regulates the distribution of Sec16A is supported but not that this is reflected in a change in COPII vesicle trafficking.

Similarly "LRRK2 regulates ERES in response to load" is similarly not supported by the limited data shown here.

In conclusion, I find this an interesting story that might be published in EMBO Journal but the conclusions require substantial support from other experiments.

Referee #2:

In this manuscript Cho et reported a new molecular interaction between LRRK2 and components of the ER exit sites (ERES). Their finding suggest that one of the normal functions of LRRK2 is to regulate ER-Golgi transport, and that defects in this process may underlie the pathogenic effects of at least the R1441C mutation. Overall, the biochemical and cell biological experiments were carried out adequately to support the LRRK2-Sec16A interaction. However, it is not clear at this stage the contribution of this newly identified molecular interaction to the normal function of LRRK2 or to LRRK2 pathogenesis, or its general relevance to Parkinson's disease.

Main comments:

Pathogenic forms of LRRK2, including mutations at the R1441 site, have previously been shown to cause neurotoxicity in cultured neurons or Tg animals, reducing neurite length and branching. It will be important to test whether impairment of ERES alone is sufficient to cause such toxic effects, and whether genetic manipulation of ERES-related genes would modify LRRK2 toxicity in neurons. It would also be important to test whether the altered glutamate receptor trafficking documented in the cell biological studies in cultured neurons has electrophysical consequence on neurotransmission.

Minor comments:

1. Although other pathogenic mutation do not seem to show altered binding to Sec16A, it would be important to test whether ERES structure or function is affected by these mutations. This will test whether ERES dysfunction is more generally involved in LRRK2 pathogenesis.

2. In the VSVG-GFP trafficking experiments, it is not clear whether the different time points were derived from the same cell. Based on the outlines, they are different cells. It would be important to follow the reporter trafficking in the same cell over time.

3. In Figure 4, it was shown that ERES and Golgi structures are abnormal in LRRK(-/-) cells. To demonstrate specificity of LRRK2 effect on ER and Golgi, other membrane structures such as endosomes, lysosomes, etc. should be shown.

4. In Figure 5A, myc-LRRK2 WB panel, why the control lane has a drastically different background?

5. In Figure 6, the specificity of the antibodies should be shown.

6. In Figure 7, the GFP-Sec16A signals are weaker in LRRK(+/+) than that in LRRK2(-/-). This may contribute to the weaker spine signal in the LRRK(+/+) cells. To make a convincing argument, images with equal GFP-Sec16A signals should be shown.

7) In Figures 6 & 7, Some kind of data quantification would be helpful.

Referee #3:

<I>LRRK2</I> is a most important gene linked to Parkinson's disease (PD). It encodes a large gene product that contains GTPase and kinase enzymatic domains in a characteristic configuration. The physiological role(s) of LRRK2 are largely unknown. It has been noted previously that a portion of LRRK2 is associated with intracellular membrane structures. Here Cho et al. discover that LRRK2 binds to Sec16A, a protein regulating ER to Golgi traffic at endoplasmatic reticulum exit sites (ERES). The functional interaction is perfectly validated by co-immunoprecipitation and co-

fractionation experiments, and co-localization is documented. Using RNAi technology as well as their battery of genetically engineered LRRK2 mice and cells derived from these animals, that authors prove that LRRK2 positively influences the localization of Sec16A at ERES. Excitingly, among a number of PD mutations, R1441C within the GTPase domain stands out as having lost the functional interactions with Sec16A. Indeed, the GTPase domain, but not the phosphotransferase domain of LRRK2 mediates Sec16A interactions. Therefore, this fantastic paper not only provides novel information about the regulation of ER-Golgi trafficking through ERES, but provides also important novel information about the specific molecular genetics of PARK8/LRRK2. Thus, this work provides a very large advance in knowledge. The experiments are well done and convincing, just a few details:

1) Reference to Fig. S3I should be made already in the results section, not only late in the discussion.

2) Fig. S4 is comparatively less convincing. Total NR2B tends to increase after TTX. Clear effects as for NR1 and NR2A as shown in Fig. 8A should be shown to prove this particular point.

3) It would be interesting to discuss the present work with reference to LRRK2 interactions with Rab7L1 recently published by MacLeod et al. (2013) Neuron 77:425. This also was reported to influence ER-Golgi sorting. Could this point to a more comprehensive GTPase network?

4) Although I find this manuscript should be published as soon as possible, the authors might find the time to check if LRRK2 regulates ERES trafficking also in immune cells that express LRRK2 in abundance.

1st Revision - authors' response 07 July 2014

Referee #1:
This is an interesting paper that reports a role for LRRK2 in the control of ER exit site function through binding to Sec16A.
The work in the paper is generally of high quality and the findings are certainly novel and of interest to the field. My enthusiasm is somewhat dampened as I think that some of the conclusions are not well supported by the data and there is a lack of mechanistic insight into what LRRK2 actually does at ERES - it is unclear after reading this manuscript as to how many of the phenotypic effects shown are down to the interaction with Sec16 rather than the many other functional interactions of LRRK2 that have been described. As such, one is left with the sense that this is a little under-developed for publication in EMBO Journal.
Response: We appreciate the reviewer's generally positive response to this study. As requested by he reviewer, we have carried out substantial additional experiments and added more discussion to further establish the role of LRRK2 at ERES and its interaction with Sec16A in the revised manuscript.

Q1: Given that LRRK2 regulates both the actin and microtubule networks, the authors should show that the outcomes shown here are not down to such indirect effects.
Response: The reviewer raised an excellent point here. LRRK2 regulates microtubule and actin dynamics especially in developing neurons (Parisiadou & Cai, 2010). Since depolymerization of actin has no obvious effect on directed transport from ER to Golgi (Scales et al, 1997), we focused the role of LRRK2 in microtubules. Microtubules are essential in ER-to-Golgi transport (Presley et al, 1997). There is substantial evidence demonstrating that microtubules associate with ER–Golgi intermediate compartment (ERGIC) and mediate the vesicle transport via dynein and kinesin motor proteins from ERGIC to Golgi (Appenzeller-Herzog & Hauri, 2006). However, whether microtubules directly contribute to the vesicle transport from ERES to ERGIC is less clear (Zanetti et al, 2012). On one hand, depolymerization of microtubules seems not affect the transport of COPII cargos from ERES to ERGIC, suggesting a microtubule-independent mechanism of ERES export to ERGIC (Hammond & Glick, 2000; Presley et al, 1997; Scales et al, 1997). On the other hand, Watson and colleagues show that ERES co-localizes with microtubules and can rapidly associate with the newly polymerized microtubules (Watson et al, 2005). Dynactin $p150^{glued}$, a microtubule plus end binding protein, mediates the association of ERES with the dynamic end of microtubules

through interaction with COPII protein Sec23A at (Watson et al, 2005). Interestingly, a recent study also demonstrates that LRRK2 preferentially binds to the dynamic microtubules that undergo rapid polymerization and depolymerization (Law et al, 2014). LRRK2 may play a similar role as $p150^{glued}$ in association with both ERES protein Sec16A and microtubule network. Like $p150^{glued}$, the binding of LRRK2 with the dynamic microtubules may direct the clustering of Sec16A and other ERES proteins at the vicinity of nucleus. To test this hypothesis, we treated *Lrrk2*$^{+/+}$ and *Lrrk2*$^{-/-}$ fibroblasts with microtubule-depolymerizing agent nocodazole and microtubule-polymerizing agent taxol. Nocodazole treatment caused dispersion of Sec16A staining in *Lrrk2*$^{+/+}$ cells, a phenomenon similar to non-treated *Lrrk2*$^{-/-}$ fibroblasts (Fig. S6A). In parallel, nocodazole treatment led to further dispersion of Sec16A staining in *Lrrk2*$^{-/-}$ fibroblasts, which showed a rather homogenous staining pattern in *Lrrk2*$^{-/-}$ cells (Fig. S6B). By contrast, taxol treatment did not significantly alter the subcellular distribution of Sec16A in *Lrrk2*$^{+/+}$ fibroblasts, but appeared to increase the clustering of Sec16A staining near the nucleus of *Lrrk2*$^{-/-}$ cells (Fig. S6). However, the treatment of taxol did not fully restore the juxtanuclear clustering of Sec16A in *Lrrk2*$^{-/-}$ cells compared to the non-treated *Lrrk2*$^{+/+}$ fibroblasts (Fig. S6). It worth to point out that a lack of LRRK2 did not cause overt disruption of microtubule network compared cells treated with nocodazole (Fig. S6B). Nonetheless, taxol may still help to stabilize and align microtubules in the absence of LRRK2. It may allow the stable microtubules concentrate near the nucleus (Fig. S6B). ERES has been shown to attach to the stable microtubules (Mizuno & Singer, 1994). We suspect that one function of LRRK2 is to direct the attachment of ERES protein complex to the dynamic ends of microtubules, which may contribute to the clustering of ERES near the nucleus. In addition, LRRK2 is also important in anchoring Sec16A at ERES. A loss of LRRK2 led to more Sec16A into cytosol and less attached to the microsomes (Fig. 3F). Our findings raise an interesting perspective that LRRK2 may play two roles in organizing ERES. The first one is to bind with Sec16A and facilitate the attachment of Sec16A with ERES. The second one is by binding with the dynamic end of microtubules LRRK2 not only stabilizes the microtubules but also establishes the polarity of ERES clustering in the vicinity of nucleus. Together, our study suggests that LRRK2 play an important role in clustering ERES proteins near the nucleus through association with both Sec16A and the dynamic ends of microtubules. We added these new data and discussions in the Discussion Section of the revised manuscript.

Q2: Similar arguments could be made for its interaction and regulation of and by ArfGAP1 - highly relevant in the context of the current work.

Response: The reviewer raised another interesting question. LRRK2 has been shown to interact with ArfGAP1 and reciprocally regulate the activity of each other (Stafa et al, 2012; Xiong et al, 2012). ArfGAP1 is primarily associated with Golgi and plays an important role in the formation of COPI vesicles that transport between ER and cis-Golgi (Shiba & Randazzo, 2012). To directly test whether ArfGAP1 regulates the clustering of Sec16A at ERES, we transfected wild-type fibroblasts with *Arfgap1* or control siRNA. We found an inhibition of ArfGAP1 did not affect the localization of Sec16A near nucleus (Fig. L1), suggesting that in contrast to LRRK2 ArfGAP1 may not actively regulate the subcellular localization of Sec16A.

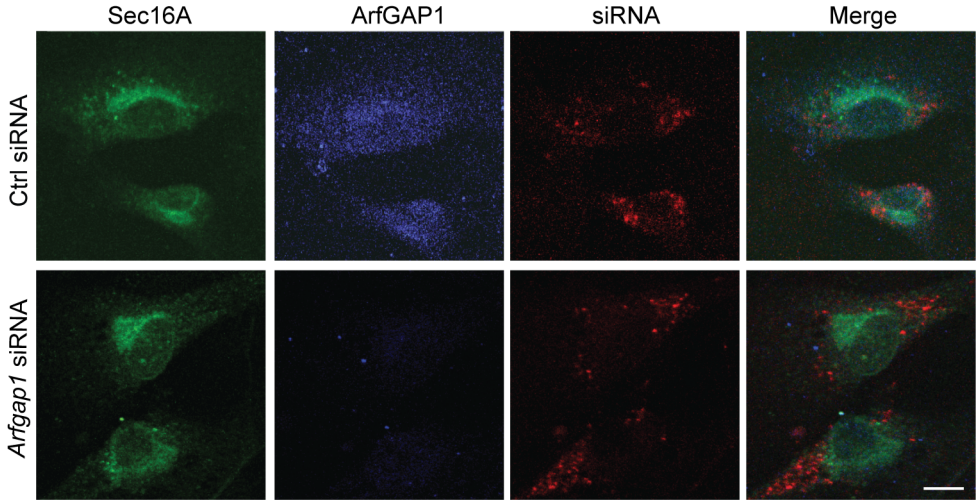

Fig. L1 Knock-down of ArfGAP1 does not affect the clustering of Sec16A near nucleus. Wilde-type mouse fibroblasts were transfected with control (Ctrl) or ArfGAP1 siRNA (red) for 48 hrs and stained with antibodies against Sec16A (green) and ArfGAP1 (blue). Scale bar: 10 mm.

Q3: Specifically it seems to me that one could explain the findings not as a loss of ERES function but as a change in their organization.
Response: We agree with the reviewer that a loss of LRRK2 did not completely disrupt the ERES function. The *Lrrk2*-deficiency altered both the association of Sec16A with ERES and the clustering of ERES near the nucleus (Figs. 2 and 3). The change in ERES organization may contribute to the impairment of cargo transport from ER to Golgi observed in $Lrrk2^{-/-}$ cells (Fig. 4). Nonetheless, in the revised manuscript we used "impaired ERES organization and function" to replace the statement of "loss of ERES function".

Q4: If LRRK2 is so critical for Sec16 function then I would be very surprised since the knockout cells are seemingly viable and have minimal perturbation of secretory pathway function.
Response: Our data showed that LRRK2 is important in clustering ERES in the vicinity of nucleus though interaction with Sec16A. However, the loss of LRRK2 did not lead to a complete detachment of Sec16A with ERES (Figs. 2 and 3). The remaining Sec16A at ERES may be still functional in $Lrrk2^{-/-}$ cells. In line with this notion, *Lrrk2*-deficiency only caused a delay in the VSVG-GFP experiment (Fig. 4A, B). In the revised manuscript, we also checked the ER-Golgi transport of endogenous protein nicastrin. It has been shown previously that the presence of Parkinson's disease-related mutant a-synuclein impairs the transport of nicastrin from ER to Golgi (Chung et al, 2013). In the Endo H assay, we treated $Lrrk2^{+/+}$ and $Lrrk2^{-/-}$ cell extracts with or without Endo H and then examined the expression of various forms of nacastrin by Western blot. We found more ER-form of nicastrin in the $Lrrk2^{-/-}$ cell extracts (Fig. 4C, D). Our data suggest that LRRK2 may facilitate the ER-Golgi transport via maintaining the normal organization of ERES through association with ERES residential protein Sec16A.

Q5: Therefore I find I cannot agree with the conclusion that "These results suggest a critical role of LRRK2 in anchoring Sec16 at ERES".
Response: We changed the sentence to "These results suggest that LRRK2 is important in clustering ERES in the vicinity of nucleus though interaction with Sec16A".

Q6: An alternative explanation is that the number, size and organization of ERES is perturbed. Higher resolution data are required to reconcile these alternatives.
Response: We appreciate the reviewer for raising this important point. Our data actually do not exclude either scenario. Our results suggest that LRRK2 may play both roles in anchoring Sec16A at ERES and organizing ERES in the vicinity of nucleus. LRRK2 plays the first one role through association with Sec16A that facilitates the attachment of Sec16A to the ERES. LRRK2 plays the second one is by binding with the dynamic end of microtubules LRRK2 that stabilizes the microtubules and establishes the polarity of ERES clustering in the vicinity of nucleus. Together, our study suggests that LRRK2 play an important role in clustering ERES proteins near the nucleus through association with both Sec16A and the dynamic ends of microtubules. As requested by the reviewer, we also added new high-magnification images to Figs. 2A and 3A. We also performed immnuno-EM of Sec16A on $Lrrk2^{+/+}$ and $Lrrk2^{-/-}$ fibroblasts, counted the number of Sec16A-positive gold particle clusters (two or more particles within 100nm radius), and measured the shortest distance between two particles. We found less numbers of Sec16A clusters and longer distance between the particles in $Lrrk2^{-/-}$ cells (Fig. 2C-E). These new EM data are consistent with the early observations with light microscopy that Sec16A staining was more spreading in $Lrrk2^{-/-}$. We added these new data and discussions in the Discussion Section of the revised manuscript.

Q7: Some assay for COPII assembly is essential for one to properly interpret this work. Wither this should take the form of an in vitro budding assay in the presence and absence of LRRK2 or by exploiting FRAP of COPII subunits, including Sec16, inner and outer coat components, to define the role of LRRK2 in the assembly of COPII. The knockout and knock-in cells provide an exceptional opportunity to do this in a very clean background.
Response: Our FPLC data showed the shift of molecular weights of Sec16A and Sec31A-containing protein complex (Figs. 2E-F and 3G), suggesting that a loss of *Lrrk2* may affect the efficiency of COPII assembly. As suggested by the reviewer, we performed additional in vitro budding assay using microsomes purified from $Lrrk2^{+/+}$ and $Lrrk2^{-/-}$ mouse fibroblasts. The reaction was assembled

using rat liver cytosol. Using ERGIC marker protein, ERGIC-53/58, we were able to measure the efficiency of COPII-dependent vesicle budding. When we quantitated the ERGIC-53/58 from budding performed from *Lrrk2*$^{+/+}$ and *Lrrk2*$^{-/-}$ fibroblasts, the budding was attenuated in *Lrrk2*$^{-/-}$ cells as compared to the controls (Fig. 4E). By contrast, the total levels of ERGIC-53/58 were comparable between *Lrrk2*$^{+/+}$ and *Lrrk2*$^{-/-}$ cell lysates (Fig. 4E). These results support a role for LRRK2 in vesicle budding. We added these new data in the revised manuscript.

Q8: In terms of conclusions, I find that the BFA assay does not provide an accurate reflection of increased cargo load. That the ERES do not enlarge further in the presence of BFA suggests a size limit (as has been proposed by Glick previously) such that no further increase is seen. This is not the same as there being no effect. Farhan 2008 is also not the first time that the effect of BFA on ERES was reported.
Response: We agree with the reviewer on the interpretation of BFA data. Since we had carried out only limited experiments on this subject that is not essential to the main theme of this manuscript, we removed the BFA data from the revised manuscript.

Q9: The VSV-G assay, although widely used, requires further support from the use of an endogenous cargo e.g. BFA washout and repopulation of the Golgi with glycosyltransferases. An EndoH assay for VSV-G traffic would be a possible alternative.
Response: We agree with the reviewer that it is important to examine an endogenous cargo. We decided to check the ER-Golgi transport of nicastrin in *Lrrk2*$^{+/+}$ and *Lrrk2*$^{-/-}$ mouse fibroblasts. It has been shown previously that the presence of Parkinson's disease-related mutant a-synuclein impairs the transport of nicastrin from ER to Golgi (Chung et al, 2013). In the Endo H assay, we treated *Lrrk2*$^{+/+}$ and *Lrrk2*$^{-/-}$ cell extracts with or without Endo H and then examined the expression of various forms of nacastrin by Western blot. We found more ER-form of nicastrin in the *Lrrk2*$^{-/-}$ cell extracts (Fig. 4C, D). We added these new data in the revised manuscript.

Q10: I also do not understand the term cytoplasmic membrane in the context of these assays (the absence of page and line numbers is frustrating in terms of providing a reference point).
Response: We changed "cytoplasmic membrane" to "cell surface" in the revised manuscript. We also added page numbers.

Q11: A similar point arises from the neuronal trafficking work, which while very nicely executed is similarly open to other interpretations. The labeling for Sec16A for example is suggestive of a cytoplasmic pool, not a bulk ER pool (Figure 7D). On what basis do the authors conclude that this is ER associated?
Response: In Figs. 7B and 7D, we found an abnormal redistribution of Sec16A into the dendritic spines of *Lrrk2*$^{-/-}$ neurons. To investigate whether Sec16A remains associated with ER, we co-transfected GFP-Sec16A and ER marker Sec61b into cultured hippocampal neurons. We found Sec61b signals were restricted within the dendritic shaft of *Lrrk2*$^{-/-}$ neurons, while Sec16A staining was presented in both the dendritic shaft and spines. These observations demonstrate that a pool of Sec16A within the dendritic spines was detached from the ER and stay in the cytosol. However, our present data cannot determine whether Sec16A is in the cytosol or associated with ER in the dendritic shaft. Nonetheless, these results from neurons further support the notion that LRRK2 is important in anchoring Sec16A at ERES. Interestingly, the dynamic ends of microtubules also enrich at the base and stem of dendritic spines (Jaworski et al, 2009). LRRK2 may anchor the dendritic ERES (dERES) near the dendritic spines through interaction with the dynamic ends of microtubules, and facilitates the cargo transport from dERES to the dendritic spines in response to strong neuron activation (Fig. 8F).

Q12: Similarly, I find that the data showing localization of Sec16 are unconvincing in many panels. The Sec16 labeling in Figure 2 looks dramatically different to that of Sec31 in Figure 3 and especially Figure 4. Some ultrastructural characterization would be of enormous benefit here.
Response: In the original Figs. 2A and 3A, we examined the distribution of Sec16A and Sec31A in HeLa cells, whereas in Figs. 2B, 3B, and 4, we checked the subcellular localization of Sec 16A and Sec31A in mouse fibroblasts. In HeLa cells, Sec16A and Sec31A staining was more compact near one side of nucleus. In mouse fibroblasts, Sec16A and Sec31A signals, however, seem to form a gradient, stronger near the nucleus as demonstrated in the signal intensity plots (Figs. 2B and 3B). We suspect that cell types may contribute to the difference of Sec16A and Sec31A distribution in

these cells. Nonetheless, to avoid potential confusion, we removed the HeLa cell data from the revised manuscript.

Q13: In terms of the biochemistry, if the important interaction is with the CCD of Sec16, a critical question is whether this affects the association of Sec13 which binds tightly to this region. This could have profound mechanistic implication if Sec13 is displaced by LRRK2.
Response: We appreciate the reviewer raised this very interesting point. As suggested by the reviewer, we performed in vitro recombinant protein binding assays that showed LRRK2 competed with Sec13 in binding with the CCD of Sec16A. We purified FLAG-tagged Sec16A CCD from HEK293 cells, and then ran the in vitro binding assay with recombinant GST-tagged LRRK2 and His-tagged Sec13 proteins. We found the presence of LRRK2 but not the control GST protein replaced the binding of Sec13A with Sec16A (Fig. 1E, F). Sec16A may serve a scaffold protein in the formation of COPII vesicles. A structural study shows that Sec16A binds to Sec13A to form edge elements similar to those formed by Sec13-31, suggesting the interaction of Sec13-16 with Sec23-24 at the ERES may set up the platform for the eventual COPII cage polymerization of Sec13-31 and Sec23-24 proteins (Whittle & Schwartz, 2010). A competition of LRRK2 with Sec13A in binding with the CCD of Sec16A raises an interesting perspective that LRRK2 may involve in COPII vesicle formation at the ERES, although the detailed mechanism remains to determine. We included these new data and discussions in the revised manuscript.

Q14: Specific examples within the discussion where I do not find that the data support the conclusion are the headings: "LRRK2 regulates COPII vesicle trafficking" - LRRK2 regulates the distribution of Sec16A is supported but not that this is reflected in a change in COPII vesicle trafficking.
Response: We agree with the reviewer that our data do not support a direct involvement of LRRK2 in COPII vesicle trafficking. We changed the heading to “COPII vesicle trafficking is impaired in *Lrrk2*$^{-/-}$ cells” in the revised manuscript.

Q15: Similarly "LRRK2 regulates ERES in response to load" is similarly not supported by the limited data shown here. In conclusion, I find this an interesting story that might be published in EMBO Journal but the conclusions require substantial support from other experiments.
Response: We agree with the reviewer that we had carried out only limited experiments on this topic. Since it is not essential to the main theme of this manuscript, we removed the BFA data and discussion from the revised manuscript.

Referee #2:

In this manuscript Cho et reported a new molecular interaction between LRRK2 and components of the ER exit sites (ERES). Their finding suggest that one of the normal functions of LRRK2 is to regulate ER-Golgi transport, and that defects in this process may underlie the pathogenic effects of at least the R1441C mutation. Overall, the biochemical and cell biological experiments were carried out adequately to support the LRRK2-Sec16A interaction. However, it is not clear at this stage the contribution of this newly identified molecular interaction to the normal function of LRRK2 or to LRRK2 pathogenesis, or its general relevance to Parkinson's disease.

Main comments:

Pathogenic forms of LRRK2, including mutations at the R1441 site, have previously been shown to cause neurotoxicity in cultured neurons or Tg animals, reducing neurite length and branching. It will be important to test whether impairment of ERES alone is sufficient to cause such toxic effects, and whether genetic manipulation of ERES-related genes would modify LRRK2 toxicity in neurons. It would also be important to test whether the altered glutamate receptor trafficking documented in the cell biological studies in cultured neurons has electrophysical consequence on neurotransmission.
Response: The reviewer raised some very interesting questions. It has been shown previously that inhibition of Sar1, a key mediator in the assembly of COPII vesicles at ERES, impairs the axon outgrowth (Aridor & Fish, 2009). To investigate whether inhibition of Sec16A affects neuron morphogenesis, we transfected cultured hippocampal neurons with Sec16A siRNA, and observed a

significant reduction of neurite outgrowth (Fig. L2). These observations further support the notion that ERES is important in the elongation of neurites during neuron development.

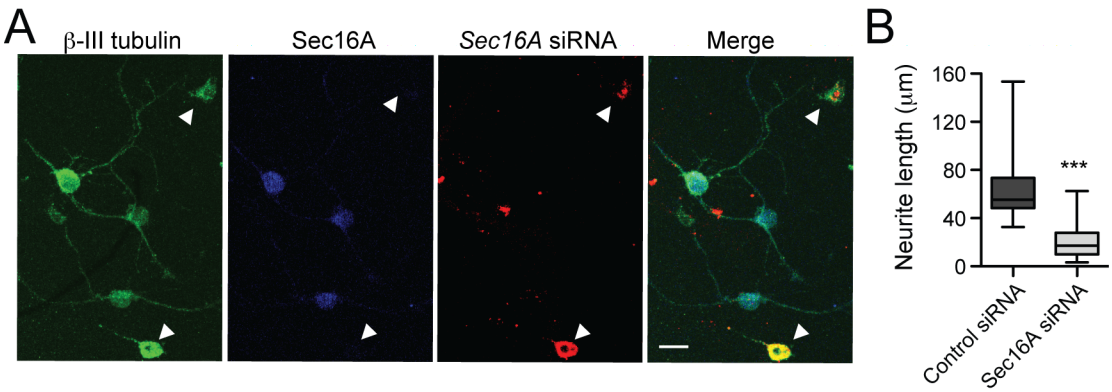

Fig. L1 Knock-down of Sec16A impairs the extension of neurites. (A) Wilde-type mouse hippocampal neurons were transfected with control (Ctrl) or Sec16A siRNA (red) for 48 hrs and stained with antibodies against bIII-tubulin to mark the neurons (green) and Sec16A (blue). Scale bar: 20 mm. (B) Whisker box graph shows a significant reduction of neurite length in *Sec16A* siRNA-transfected neurons. 55 (ctrl siRNA) and 54 (*Sec16A* siRNA) neurons were analyzed. P<0.0001.

Our present study identified Sec16A as a downstream target for LRRK2. A loss of LRRK2 or the PD-related R1441C mutation compromised the organization of ERES and impaired ER-Golgi transport. To restore or improve the functions of ERES and COPII vesicle transport may be beneficial to ameliorate LRRK2-mediated toxicity in neurons. A number of studies have shown that regulating of ER-related protein degradation and COPI transport can prevent a-synuclein-induced ER export deficiency (Chung et al, 2013). We will test if such manipulations also apply for LRRK2-medaited ER dysfunction. However, we are afraid this line of study is beyond the scope of the present manuscript.

We did observe impairments of postsynaptic transmission in *Lrrk2*$^{-/-}$ striatal medium spiny neurons (Parisiadou et al, 2014). While we emphasized an aberrant of PKA pathway underlying this electrophysiological abnormalities in this published study, we suspect the deficiency of dERES may also contribute to the overall reduction of synaptic transmission in *Lrrk2*$^{-/-}$ neurons.

Minor comments:
1. Although other pathogenic mutation do not seem to show altered binding to Sec16A, it would be important to test whether ERES structure or function is affected by these mutations. This will test whether ERES dysfunction is more generally involved in LRRK2 pathogenesis.
Response: As requested by the reviewer, we examined the effects of different Parkinson's disease-related LRRK2 mutations on the organization of ERES. To test whether the mutations compromise the role of LRRK2 in clustering Sec16A at the ERES, we introduced these mutations into the *Lrrk2*$^{-/-}$ mouse fibroblasts and determined which mutation was able to restore the normal juxtanuclear localization of Sec16A. We found the G2019S, Y1699C, and G2385 mutations rescued the Sec16A distribution defects in *Lrrk2*$^{-/-}$ fibroblasts, while the R1441C mutation failed (Fig. S3). These new cell biology data are consistent with the biochemical analyses that only the R1441C mutation compromised the interaction with Sec16A (Fig. 5A). We included these new data in the revised manuscript.

2. In the VSVG-GFP trafficking experiments, it is not clear whether the different time points were derived from the same cell. Based on the outlines, they are different cells. It would be important to follow the reporter trafficking in the same cell over time.
Response: We agree with the reviewer that it is ideal to follow individual cells through the tracing process. Unfortunately, we don't have the setup to carry out such live imaging experiments. Instead, we studied a population of cells at different stages and compared the difference based on thorough statistical analyses, a method also used by many other labs.

3. In Figure 4, it was shown that ERES and Golgi structures are abnormal in LRRK(-/-) cells. To demonstrate specificity of LRRK2 effect on ER and Golgi, other membrane structures such as endosomes, lysosomes, etc. should be shown.
Response: There is increasing evidence that LRRK2 participates retrograde vesicle transport in endosomes and lysosomes through interacting with Rab5, Rab7, Rab7L, and other proteins (Beilina et al, 2014; Dodson et al, 2012; Heo et al, 2010; MacLeod et al, 2013). Our study is the first to demonstrate the involvement of LRRK2 in the anterograde vesicle transport from ER to Golgi. We do not think LRRK2 only specifically affects ER and Golgi. It is more likely that LRRK2 play important roles in both anterograde and retrograde vesicle transport.

4. In Figure 5A, myc-LRRK2 WB panel, why the control lane has a drastically different background?
Response: We think a slight over-exposure in the image may exaggerate the background difference. We tuned down the brightness of the whole image to show the consistency of gray background in the "control" and "WT" lanes in the figure.

5. In Figure 6, the specificity of the antibodies should be shown.
Response: The specificity of Sec16A and LRRK2 was shown in supplementary Figs. S1B, C, E, and G.

6. In Figure 7, the GFP-Sec16A signals are weaker in LRRK(+/+) than that in LRRK2(-/-). This may contribute to the weaker spine signal in the LRRK(+/+) cells. To make a convincing argument, images with equal GFP-Sec16A signals should be shown.
Response: The redistribution of Sec16A into the dendritic spines of *Lrrk2*$^{-/-}$ neurons are not resulted from over-exposure of GFP-Sec16A signals. In the revised Figs. 7A and 7B, we added histogram analyses of GFP-Sec16A and mCherry signal intensities across the dendritic spines and shaft in both *Lrrk2*$^{+/+}$ and *Lrrk2*$^{-/-}$ neurons. They clearly show that GFP-Sec16A signals stayed within the dendritic shaft of *Lrrk2*$^{+/+}$ neurons, while they distributed comparably in both dendritic shaft and spine of *Lrrk2*$^{-/-}$ neurons. In addition, GFP-Sec16A presented as punctate staining pattern, consistent with the distribution pattern of dERES along the dendritic shaft of *Lrrk2*$^{+/+}$ neurons. In contrast, GFP-Sec16A appeared an even distribution inside the dendritic shaft of *Lrrk2*$^{-/-}$ neurons. The distinct distribution pattern of GFP-Sec16A along the dendritic shaft of *Lrrk2*$^{+/+}$ and *Lrrk2*$^{-/-}$ neurons is impossible resulted from different exposures of the images.

7) In Figures 6 & 7, Some kind of data quantification would be helpful.
Response: We performed histogram analyses of GFP-Sec16A and mCherry signal intensities across the dendritic spines and shaft in both *Lrrk2*$^{+/+}$ and *Lrrk2*$^{-/-}$ neurons. In the line graphs, we showed that GFP-Sec16A signals stayed within the dendritic shaft of *Lrrk2*$^{+/+}$ neurons, while they distributed comparably in both dendritic shaft and spine of *Lrrk2*$^{-/-}$ neurons (Bottom panels, Figs. 7A and B).

Referee #3:

LRRK2 is a most important gene linked to Parkinson's disease (PD). It encodes a large gene product that contains GTPase and kinase enzymatic domains in a characteristic configuration. The physiological role(s) of LRRK2 are largely unknown. It has been noted previously that a portion of LRRK2 is associated with intracellular membrane structures. Here Cho et al. discover that LRRK2 binds to Sec16A, a protein regulating ER to Golgi traffic at endoplasmatic reticulum exit sites (ERES). The functional interaction is perfectly validated by co-immunoprecipitation and co-fractionation experiments, and co-localization is documented. Using RNAi technology as well as their battery of genetically engineered LRRK2 mice and cells derived from these animals, that authors prove that LRRK2 positively influences the localization of Sec16A at ERES. Excitingly, among a number of PD mutations, R1441C within the GTPase domain stands out as having lost the functional interactions with Sec16A.
Indeed, the GTPase domain, but not the phosphotransferase domain of LRRK2 mediates Sec16A interactions. Therefore, this fantastic paper not only provides novel information about the regulation of ER-Golgi trafficking through ERES, but provides also important novel information about the specific molecular genetics of PARK8/LRRK2. Thus, this work provides a very large advance in knowledge. The experiments are well done and convincing, just a few details:

Response: We appreciate the very positive responses from the reviewer.

1) Reference to Fig. S3I should be made already in the results section, not only late in the discussion.
Response: As suggested by the reviewer, we described the data in Fig. 3I in the result section.

2) Fig. S4 is comparatively less convincing. Total NR2B tends to increase after TTX. Clear effects as for NR1 and NR2A as shown in Fig. 8A should be shown to prove this particular point.
Response: We agree with the reviewer that NR2B tends to increase after TTX treatment. To control for this kind of variations, we normalized the levels of receptor proteins at cell surface by total protein levels after each treatment in Figs. 8B-E.

3) It would be interesting to discuss the present work with reference to LRRK2 interactions with Rab7L1 recently published by MacLeod et al. (2013) Neuron 77:425. This also was reported to influence ER-Golgi sorting. Could this point to a more comprehensive GTPase network?
Response: There is increasing evidence that LRRK2 participates retrograde vesicle transport in endosomes and lysosomes through interacting with Rab5, Rab7, Rab7L1, and other proteins (Beilina et al, 2014; Dodson et al, 2012; Heo et al, 2010; MacLeod et al, 2013). Our study is the first to demonstrate the involvement of LRRK2 in the anterograde vesicle transport from ER to Golgi. We do not think LRRK2 only specifically affects ER and Golgi. It is more likely that LRRK2 play important roles in both anterograde and retrograde vesicle transport. As indicated by the reviewer, the GTPase activity of LRRK2 may contribute to these processes. We added these discussions in the revised manuscript.

4) Although I find this manuscript should be published as soon as possible, the authors might find the time to check if LRRK2 regulates ERES trafficking also in immune cells that express LRRK2 in abundance.
Response: It would be interesting to investigate whether LRRK2 regulates ERES organization in immune cells. However, the immune cells tend to have very compact cytosol, and they would be very difficult for cell biology studies of ERES or other organelles compared to HeLa cells, fibroblasts and neurons.

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2nd Editorial Decision 01 August 2014

Thank you for submitting a revised version of your manuscript to us. It has now been seen by two of the original referees, and I attach their comments below.

I am happy to accept your manuscript in principle for publication here. However, a few amendments are still required and I would like to ask you to send a final version of your manuscript by response email to me.

Please address the following points:

1. The outlined parts of the dendrites in figure 7 do not all correspond to the zoomed images depicted, please correct this.

2. Please add a conflict of interest statement as well as author contributions to your manuscript.

3. Please check your manuscript once more carefully for grammar or spelling mistakes.

4. I would be grateful at this stage if you were to provide original source data, particularly uncropped/-processed electrophoretic blots for figures 1-5 and 8 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.

Thank you very much for contributing to our journal, I am looking forward to receiving the final version of your manuscript!

Referee #1:
The new data within the manuscript do add somewhat to the story but I am still left wondering what LRRK2 actually does in relation to the function of Sec16, ERES, or COPII-dependent budding. As such I maintain my initial view that while this might be published in EMBO J, the story remains somewhat under-developed. It is really now an editorial decision as to whether this is "exciting" enough for EMBO J.

Referee #2:
The authors have adequately addressed my concerns.

