nature portfolio

Peer Review File

Naturally Occurring Hyperactive Mutants of Human Parkin

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The study by Huq et al. is an interesting and well-written report on naturally occurring potentially hyperactive PRKN variants that might be relevant for drug development in the future.

The study is relevant to the field because it is essential in times of whole exome and whole genome sequencing to establish assays deciphering the functional impact of specific variants in disease-associated genes. Although the data presented is not entirely straightforward, the approach and results are interesting and relevant.

I have some comments that might be helpful to ameliorate the manuscript further:

The authors discuss findings that are not statistically significant but do not mention which tests were conducted, and there is no information on statistics in the methods section.

The variant K161N is mentioned to be a „PD mutation“ (line 86). First, the term “mutation“, which is used throughout the manuscript, should be avoided due to impreciseness; second, this variant is not claimed to be definitely pathogenic and rated as only probably pathogenic in the MDSgene database (www.mdsgene.org) as well as applying the ACMG criteria.

Why did the authors subdivide the presentation of results into three graphs in Figure 1d, 2b, 4b and d, and 7c, although, if I understood it correctly, the same analyses were performed? In the current way of presentation, the positive and negative controls (e.g., WT and W403A) are only shown in one of the graphs, although the variants in the other graphs are also compared to these controls. This is somehow misleading, although I understand that presenting all variants and controls in one graph is difficult to present. However, the authors might explain this issue in the figure legend.

It might be worth mentioning that the R234Q variant has been described in a female with compound-heterozygous PRKN mutations and typical early-onset PD (PMID: 16643317).

Based on the partly conflicting data presented here, without convincing evidence for increased Parkin activity compared to e.g., the artificial W403 variant, „Targeting these interfaces is the most promising route to increasing parkin activity in cells.“ (Line 317) does not represent a

suitable conclusion based on the presented data. I suggest toning down this sentence.

Reviewer #2 (Remarks to the Author):

The authors have performed a series of biochemical experiments to characterize the activity of 5 parkin variants that have previously been reported to lead to increased parkin activity.

Overall, the experiments are of high quality and appear to have been performed carefully. The manuscript is well-written. The results are of interest to the parkin field (but probably less so to people without specific interest in parkin).

I have only some minor comments:
- Instead of using the term hyperactive 'mutants', I would suggest saying hyperactive 'variants' throughout the manuscript. The term 'mutation' implies pathogenicity. The 5 variants studied in this manuscript are not considered definitely pathogenic (cfr. also MDSGene website)
- P.6: 'The reaction was verified on a Phos-tag gel'. It would be worth showing this in a supplementary figure.
- P. 11, last sentence: 'Curiously, the 5 naturally occurring hyperactive mutants showed slightly less phosphorylation than wild-type parkin but the difference was not statistically reproducible (Figure 4a,b).' Please delete this sentence because the difference is not significant but also because the gels in Fig. 4a do not really show a trend in that direction.
- P. 13: R104A, L102K etc. Please explain why R104 was replaced by A (and not by another amino acid), L102 by K etc.
- Fig. 1a: the drawing indicates that pUb binds to RING0 while the text in the Introduction states that pUb binds to RING1. Please clarify.
- Fig. 1D, right graph: the error bars are not visible. Either they are not shown, or the variability was extremely low (rather unlikely for this kind of experiment).
- Fig. 1D: please perform a statistical test to test for statistical significance of the differences.
- Fig. 4: please add in the legend that these are Phos-tag gels
- Fig. 7c: these data reflect an n of only 2 experiments. N = 3 should be a minimum.

Reviewer #3 (Remarks to the Author):

In this manuscript, the authors validated the hyperactivities of identified Parkin mutants in vitro and compared their activity levels to those of the wild-type and the well-characterized

hyperactive mutant, W403A. Additionally, they also investigated the impact of mutating the parkin ACT (activating element) on parkin activity in vitro. This study enhances our comprehension of the pathogenicity of parkin variants and provides new insights in developing molecules to enhance parkin activity. This manuscript is well-written and providing comprehensive information for researchers who are interested in this field. I have some suggestions as follows to strengthen the manuscript and enhance its persuasiveness.

1.In the manuscript , it is mentioned that enhanced phosphorylation of Parkin could account for parkin hyperactivity. If possible, the authors should consider including a classic Parkin substrate as a positive control to validate whether the enhanced phosphorylation of different Parkin mutants indeed enhances Parkin's catalytic activity towards the substrate, rather than solely focusing on Parkin's autoubiquitination and phosphorylation.
2.The authors should create a table detailing specific information about the different Parkin mutants selected in the manuscript, such as the age of onset in PD patients, mutation frequency in all PD patients, and whether these mutations are pathogenic or represent SNP sites in the general population.
3.Please provide a Western blot showing the total levels of Parkin protein expression for the different Parkin mutant forms used in the study to determine if different Parkin mutation forms affect the stability and expression of total Parkin protein.
4.Please provide more detailed figure legends, especially for Figure 5, Figure 6, and Supplementary Figure 1. Please describe in the figure legends the number of independent repetitions for each experiment.
5.In Figure 7b, the homology comparison of ACT amino acids across different species can include monkeys as well.
6.Numerous in vitro studies have suggested that PINK1 can phosphorylate Parkin, and together they regulate mitochondrial function through mitophagy. However, compelling in vivo evidence under physiological conditions to support this theory is still lacking. It has been reported that PINK1 protein is abundantly expressed in the primate brain under physiological conditions (PMID: 34800266, PMID: 38287905, PMID: 30770867). The authors are encouraged to discuss in the discussion section how the differential expression of PINK1 protein in different species under physiological conditions may affect the phosphorylation, activity, and function of Parkin. This discussion could help elucidate the inconsistent results observed by different research groups using the same Parkin mutant, as the limitations of in vitro experiments make it challenging to replicate gene function accurately under genuine in vivo physiological conditions.

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We thank the reviewers for their time and effort in reviewing our manuscript. As detailed in the following point-by-point rebuttal, we responded to their suggestions to best of our abilities. In addition to changes to the text, we have added a figure panel and table to the main text and three figures to the supplemental material.

R1.1. The authors discuss findings that are not statistically significant but do not mention which tests were conducted, and there is no information on statistics in the methods section.

We thank the reviewer for pointing this out. We have modified the manuscript to include the statistical analysis in the methods section and included an additional panel in Figure 1.

> Statistical Analysis. Two-tailed, paired t-tests were performed on the data from the autoubiquitination assays, UbVS assays and kinase assays of the full-length proteins. P-values less than 0.05 were considered to indicate statistically significant differences, while p-values above 0.05 were considered to indicate statistically insignificant differences. *(Materials and Methods, page 9, lines 154 to 157)*

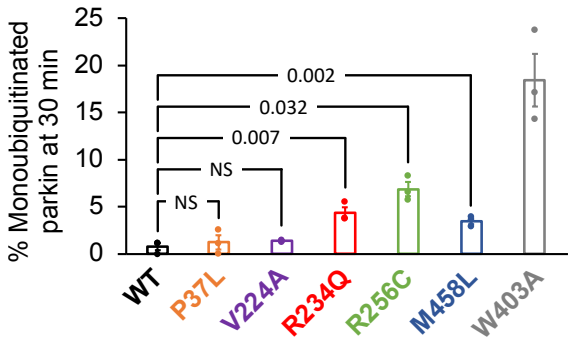

Fig 1e Statistical comparison of p-values for the increase in monoubiquitinated GST-parkin. NS, not significant.

R1.2. The variant K161N is mentioned to be a „PD mutation“ (line 86). First, the term “mutation“, which is used throughout the manuscript, should be avoided due to impreciseness; second, this variant is not claimed to be definitely pathogenic and rated as only probably pathogenic in the MDSgene database (www.mdsgene.org) as well as applying the **ACMG criteria**.

We thank the reviewer for the suggestion of replacing the word “mutation”. We have edited the manuscript to use the word “variant” instead, including in the title of our manuscript. Additionally, we would like to point out that according to MDSGene, the K161N variant of parkin is classified as “definitely pathogenic” (see

https://www.mdsgene.org/sequence_variations/36585). Furthermore, in the paper by Yi. et al cited in our manuscript, it is also reported as pathogenic according to Sherloc and listed as “Pathogenic” under PDmutDB.

R1.3. Why did the authors subdivide the presentation of results into three graphs in Figure 1d, 2b, 4b and d, and 7c, although, if I understood it correctly, the same analyses were performed? In the current way of presentation, the positive and negative controls (e.g., WT and W403A) are only shown in one of the graphs, although the variants in the other graphs are also compared to these controls. This is somehow misleading, although I understand that presenting all variants and controls in one graph is difficult to present. However, the authors might explain this issue in the figure legend.

We apologize for the lack of clarity and thank the reviewer for pointing this out. We subdivided the graphs and only showed the controls in one graph to avoid cluttering and kept the graphs to scale with each other to allow for comparison. We also found it easier to distinguish the line from each mutant if they were in separate graphs. To avoid confusion by repeating the same information multiple times in the same figure, we decided to show the control graphs only once. As recommended, we have clarified this in the figure legends and have added the following lines:

> The activity levels of the controls (WT and W403A) are shown only on the first graph for clarity. (*Figure 1d, page 25, line 474-475)*
>
> The activity levels of the controls (pWT and pW403A) are shown only on the last graph for clarity. (*Figure 2b, page 26, line 482-483)*
>
> The phosphorylation levels of the controls (WT and W403A) are shown only on the first graph for clarity. *(Figure 4b and d, page 28, line 501-502)*
>
> The activity levels of the controls (WT, pWT and pK161N) are shown only on the first graph for clarity. *(Figure 7c, page 31, line 522-523)*

R1.4. It might be worth mentioning that the R234Q variant has been described in a female with compound-heterozygous PRKN mutations and typical early-onset PD (PMID: 16643317).

We agree that this is a very interesting report and thank the reviewer for the suggestion. We have now included a table (Table 1, page 24), in accordance with a recommendation by reviewer 3 (see R3.2 below. Table 1 includes this information as being the first report of the R234Q variant listed in MDSGene.

> The first reported carrier of the R234Q variant was a woman with PD who was compound heterozygous with another PD mutation (R33Q) [41]. *(Discussion,*

page 16, lines 293-295)

R1.5. Based on the partly conflicting data presented here, without convincing evidence for increased Parkin activity compared to e.g., the artificial W403 variant, „Targeting these interfaces is the most promising route to increasing parkin activity in cells.“ (Line 317) does not represent a suitable conclusion based on the presented data. I suggest toning down this sentence.

We agree that the data is not sufficient to back up this claim and thank the reviewer for pointing this out. We have rephrased this paragraph and toned down this conclusion as recommended. The reworded paragraph is:

> Targeting these interfaces could be a possible route to increasing parkin activity in cells. With the… the fight against PD. *(Discussion, page 18, lines 341 to 345)*

R2.1. Instead of using the term hyperactive ‘mutants’, I would suggest saying hyperactive ‘variants’ throughout the manuscript. The term ‘mutation’ implies pathogenicity. The 5 variants studied in this manuscript are not considered definitely pathogenic (cfr. also MDSGene website)

We thank the reviewer for this valuable feedback and acknowledge the need to use clearer terminology in our study. As mentioned in our response to reviewer 1’s comment as well (R1.2), we have replaced the term “mutants” when referring to the with “variants” as recommended. This includes the word “mutant” in the title of our manuscript.

R2.2. P.6: ‘The reaction was verified on a Phos-tag gel’. It would be worth showing this in a supplementary figure.

We appreciate this recommendation and have included a gel verifying the phosphorylation of ubiquitin in our supplementary data. This is supplementary figure S2.

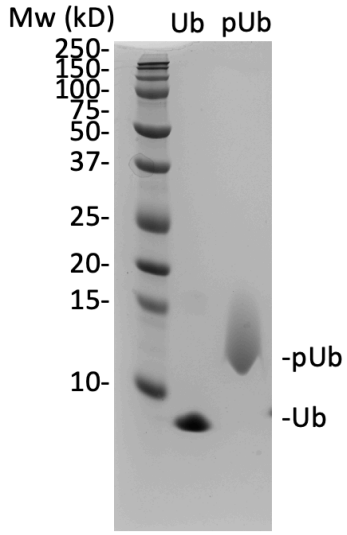

Figure S2: Phos-tag gel showing the phosphorylation of ubiquitin, used to verify the generation of phosphorylated ubiquitin (pUb) from before the assays.

R2.3. P. 11, last sentence: 'Curiously, the 5 naturally occurring hyperactive mutants showed slightly less phosphorylation than wild-type parkin but the difference was not statistically reproducible (Figure 4a,b).' Please delete this sentence because the difference is not significant but also because the gels in Fig. 4a do not really show a trend in that direction.

We agree that the data does not support this conclusion and as per the reviewer's recommendation, we have deleted it from the manuscript.

R2.3. P. 13: R104A, L102K etc. Please explain why R104 was replaced by A (and not by another amino acid), L102 by K etc.

We appreciate the need to justify each mutation. An expanded explanation is provided below:

One of the goals of the experiments on the ACT mutants was to replicate and verify the results reported in Gladkova et al. (2018). The authors of that paper used the mutants R104A and del102-109, based on which, we generated the same mutants. As for the other residues, the authors reported the importance and nature of their interactions, but did not show any experiments with associated point mutants. Based on their crystal structure, the Gladkova study reported that the three hydrophobic residues (L102, V105, and L107) make crucial contacts with a hydrophobic region in RING0 which become exposed when parkin is actived. We wanted to test the hypothesis that disrupting these contacts would decrease parkin activity. In addition, Gladkova reported that polar residues of ACT make contacts with pUbl. To test the importance of the various contacts described by Gladkova et al., we mutated charged/polar residues to alanine and hydrophobic residues to lysine.

L102K → Hydrophobic L into charged K

T103A → Polar T into hydrophobic A
V105K → Hydrophobic V into charged K
D106A → Charged D into hydrophobic A
L107K → Hydrophobic L into charged K

We have provided a brief explanation of this in our updated manuscript as recommended:

> The variants R104A and del101-109 were generated to replicate the experiments performed by Gladkova et al. [26] with additional variants designed to test the importance of specific ACT residues. *(Results, page 13, lines 236 to 238)*

R2.4. Fig. 1a: the drawing indicates that pUb binds to RING0 while the text in the Introduction states that pUb binds to RING1. Please clarify.

We apologize for this error and thank the reviewer for bringing the error to our attention. The label in Figure 1a has been corrected to show that pUbl (not pUb) binds to RING0. *(page 25)*

R2.5. Fig. 1D, right graph: the error bars are not visible. Either they are not shown, or the variability was extremely low (rather unlikely for this kind of experiment).

For the M458L variant, the error bars are indeed very small, reflecting the chance occurrence of three similar values.

R2.6. Fig. 1D: please perform a statistical test to test for statistical significance of the differences.

We thank the reviewer for this comment and would like to apologize for not specifying the statistical significance of the differences. We have added a panel to Figure 1 to indicate the p-values when comparing autoubiquitination of the mutants and WT parkin at 30 min. *(shown above in reply to R1.1)*

In addition, we have included a description in methods section now. *(also mentioned in response to R1.1).*

R2.7. Fig. 4: please add in the legend that these are Phos-tag gels

We apologize for this leaving this out of the figure legend and thank the reviewer for the suggestion. The legend has been updated accordingly:

> Representative phos-tag gels are shown in panels a and c along with the

percentage of phosphorylated parkin measured by densitometry. *(Figure 4, page 28, line 492-494).*

R2.8. Fig. 7c: these data reflect an n of only 2 experiments. N = 3 should be a minimum.

We thank the reviewer for this valuable recommendation. While we agree that 3 replicates are ideal, we were limited in the number of experiments we could perform for this particular test as the contributing author who had performed these experiments has graduated and it was not practical to perform a third repetition. We apologize for this inconsistency. As an alternative, we have now included the results from a preliminary experiment which replicates the autoubiquitination assays for the two variants - the R104A and ACT deletion - tested in Gladkova et al. (2018). This preliminary experiment, while performed by a different hand under slightly different assay conditions, is consistent with our findings from the two trials of the assay presented in the manuscript. It is referenced as Supplemental Figure S4 in the updated manuscript on page 14, line 248. We hope this will be sufficient to support our data in lieu of a third replicate.

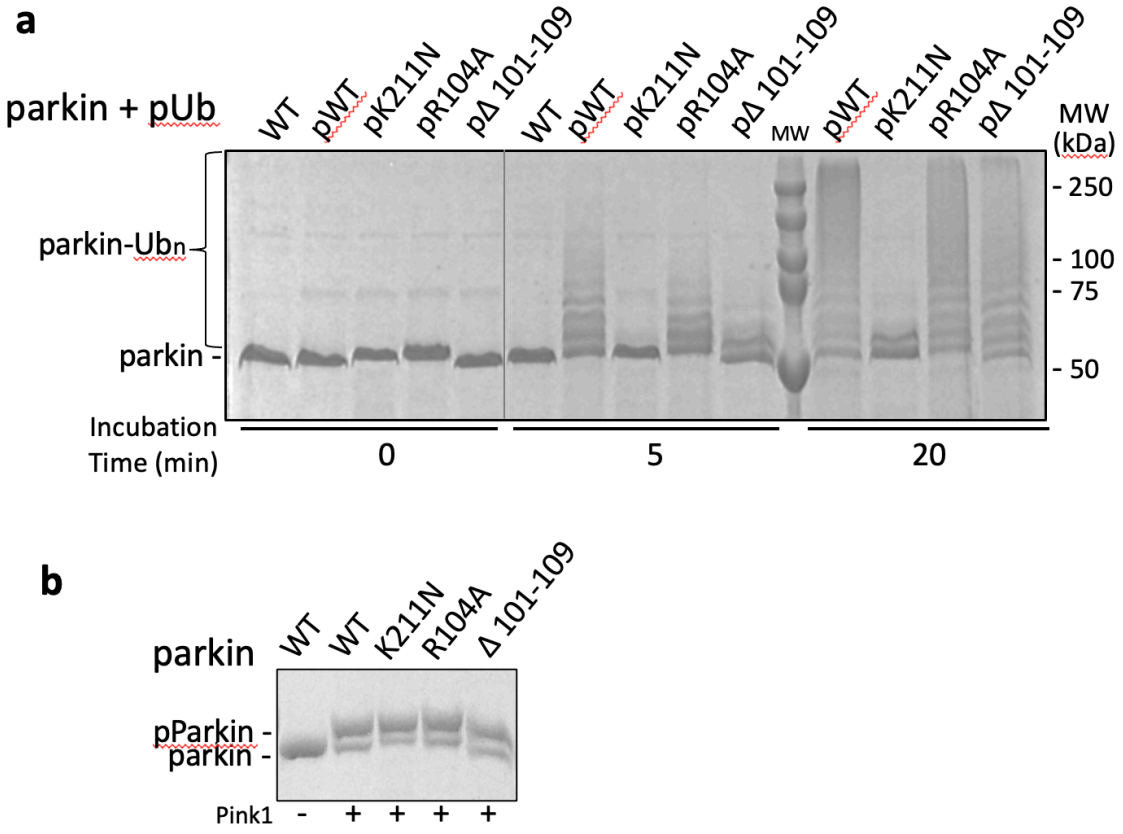

Figure S4: Preliminary experiment showing autoubiquination activity of ACT variants. **a** Time course of the autoubiquitination assay of the two ACT mutants, compared to unphosphorylated wild-type (WT), phosphorylated wild-type (pWT), and phosphorylated K211N parkin (pK211N). The phosphorylated R104A mutant had

activity similar to phosphorylated wild-type while the phosphorylated truncation Δ101-109 had decreased activity. **b** Phos-tag gel confirming that the proteins used in the experiment were equally phosphorylated. The experiment was performed once (n=1).

R3.1 In the manuscript, it is mentioned that enhanced phosphorylation of Parkin could account for parkin hyperactivity. **If possible**, the authors should consider including a classic Parkin substrate as a positive control to validate whether the enhanced phosphorylation of different Parkin mutants indeed enhances Parkin's catalytic activity towards the substrate, rather than solely focusing on Parkin's autoubiquitination and phosphorylation.

We thank the reviewer for this recommendation. However, as the authentic substrates for parkin are mitochondrial membrane proteins, it would not be feasible to perform the suggested experiment *in vitro* with soluble parkin. While this would be a valuable addition to the investigation, we do not feel this is within the scope of our study.

R3.2. The authors should create a table detailing specific information about the different Parkin mutants selected in the manuscript, such as the age of onset in PD patients, mutation frequency in all PD patients, and whether these mutations are pathogenic or represent SNP sites in the general population.

We appreciate this recommendation and agree that such a table would provide valuable insight to readers. The added Table 1 is cited in the discussion section *(Discussion, page 16, line 312).* We have included a discussion of the fact that frequency of these variants is too rare to definitively classify them as pathogenic or benign. The information provided are from the first publications which reports the mutations, and from a variety of databases (dbSNP, MDSGene, ClinVar, gnomAD). We could not directly check PDmutDB (which was also mentioned in Yi et al.) as the website is no longer available.

TABLE 1: Details of the natural variants included in this study

Variant	MDSGene classification	Age at onset[1]	Type of mutati	Allele frequency (gnomAD)	Population database (dpSNP)	Disease database (ClinVar)	Findings in this study
P37L	Possibly pathogenic	30/36	Homozygous	4.09e-4	Yes	Yes (Conflicting reports of pathogenicity)	Activity similar to WT
V224A	--			4.15e-5	Yes	No	Activity similar to WT
R234Q	Possibly pathogenic	35	Compound heterozygous	1.26e-4	Yes	Yes (Uncertain pathogenicity)	Increased activity when unphosphorylated

R256C	Probably pathogenic	37/56	Heterozygous	6.03e-4	Yes	Yes (Uncertain pathogenicity)	Increased activity when unphosphorylated
M458L	--			1.76e-4	Yes	Yes (Uncertain pathogenicity)	Increased activity when unphosphorylated

[1]Age of onset and type of mutation are from the first reported case of the variant (as per MDSGene and ClinVar). For variants with two ages of onset, multiple patients were tested, and the mutation appeared in two different individuals.

R3.3. Please provide a Western blot showing the total levels of Parkin protein expression for the different Parkin mutant forms used in the study to determine if different Parkin mutation forms affect the stability and expression of total Parkin protein.

The parkin variants studied were all prepared as `proteins purified from bacteria. We have added a figure to the supplemental material (Figure S1) to show the purity and final concentration of each variant.

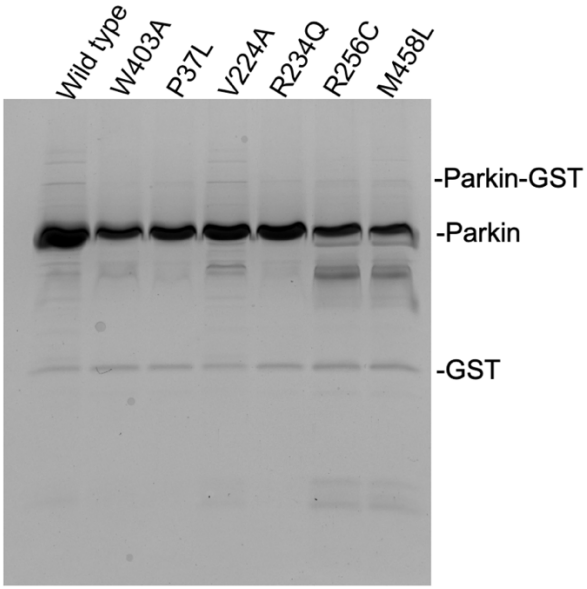

Figure S1: The purified proteins were verified by SDS-PAGE analysis.

As discussed on page 16, line 302, a study of synthetic, rationally designed variants observed a correlation between destabilizing mutations at the RING0:RING2 and REP:RING1 interfaces and increased basal ubiquitination activity (Stevens et al, ref 42). A similar trend might be inferred from the lower concentration and presence of lower weight bands in the preparations of R356C and M458L mutant proteins. However, the

trend does not hold for the P37L and V224A mutants that showed normal levels of basal activity. We did test if the P37L mutation affected stability of the Ubl domain but did not observe an effect (Figure 6).

While our manuscript was under review, a deep mutational scanning study was published, which examined the expression levels of several thousand parkin missense mutations in HEK293T (Clausen et al, 2024). The authors attributed most low abundance variants to proteasomal degradation unrelated to their activity as ubiquitin ligases. Of the putative hyperative variants studied here, only R356C showed lower expression.

variant	abundance_score*
P37L	0.804933092281774
V224A	1.02618620113165
R234Q	0.949426140014516
R256C	0.535553834325303
M458L	1.13910994656872

*Parkin variant abundance relative to WT as reported by Clausen et al, 2024.

R3.4. Please provide more detailed figure legends, especially for Figure 5, Figure 6, and Supplementary Figure 1. Please describe in the figure legends the number of independent repetitions for each experiment.

We appreciate this suggestion and have now incorporated the number of repetitions for each experiment in the figure legends.

R3.5. In Figure 7b, the homology comparison of ACT amino acids across different species can include monkeys as well.

We thank the reviewer for this suggestion to and have included the sequence from monkey in Figure 7b. *(page 31)*

R3.6. Numerous in vitro studies have suggested that PINK1 can phosphorylate Parkin, and together they regulate mitochondrial function through mitophagy. However, compelling in vivo evidence under physiological conditions to support this theory is still lacking. It has been reported that PINK1 protein is abundantly expressed in the primate brain under physiological conditions (PMID: 34800266, PMID: 38287905, PMID: 30770867). The authors are encouraged to discuss in the discussion section how the differential expression of PINK1 protein in different species under physiological conditions may affect the phosphorylation, activity, and function of Parkin. This discussion could help elucidate the inconsistent results observed by different research groups using the same Parkin mutant, as the limitations of in vitro experiments make it challenging to replicate gene function accurately under genuine in vivo physiological conditions.

We thank the reviewer for this valuable input, and for suggesting the papers which substantiate this. We have expanded our discussion of parkin signalling and cited the papers recommended. *(pages 16, 17, lines 312-324)*

> Animal studies of parkin variants are an additional source of variability due to the differences in the expression of PINK1 protein in different species [44-46]. A recent study by Chen et al. [45] reported that while PINK1 plays an important neuroprotective role in primates, it does not appear to do so in pigs**.** Unlike cultured monkey cells, cultured pig cells lacking PINK1 were still found to maintain normal levels of phosphorylated parkin suggesting the existence of alternative phosphorylation and activation pathways. In flies, parkin overexpression has long been known to genetically rescue PINK1 mutants, pointing to PINK1-independent functions of parkin [47]. In patient-derived dopaminergic neurons, parkin was shown to be phosphorylated by calmodulin-dependent protein kinase 2 (CaMK2) to trigger its recruitment to synaptic vesicles and facilitate endocytosis [48]. Mutations in parkin led to defective recycling of vesicles and accumulation of toxic oxidized dopamine. Other studies have also implicated parkin in synaptic vesicle recycling and ubiquitination of synaptic membrane proteins [49, 50].

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The manuscript has improved upon revision by the authors, and most of my comments were appropriately addressed.

I have only some minor remarks.

R1.1. The authors now report the statistical tests in the methods section. However, paired t-tests may not be the most suitable statistical test for the data presented, considering the comparison of more than two groups. I recommend using statistical tests appropriate for the type and distribution of the data, and, if significant, following up with appropriate post-hoc tests.

R1.2. I agree that there is sufficient evidence to assume that the K161N variant is likely pathogenic. However, this variant does not meet the criteria of a class 5 variant (definitely pathogenic) in a clinical setting, as reflected by applying the ACMG criteria (https://franklin.genoox.com/clinical-db/variant/snp/chr6-162622214-T-A?app=acmg-classification). The authors might consider mentioning this detail when describing this variant.

Reviewer #2 (Remarks to the Author):

The authors have adequately addressed my comments.

Reviewer #3 (Remarks to the Author):

In the revised manuscript, the authors almost adequately addressed the points raised by the reviewers. The manuscript is reasonably sound, and the evidence can support the conclusions the authors present.