EMBO reports

Lysosomal K^+ Channel TMEM175 Promotes Apoptosis and Aggravates Symptoms of Parkinson's Disease

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Prof. Cang

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, from the referee comments it is also clear that a major revision would be required to substantiate your conclusions. I list some of the key concerns that will need to be resolved in a revised version:

- Your findings are in disagreement with a recent study by Wei et al, who reported that TMEM175 deletion promotes rather than inhibits PD-like symptoms. This discrepancy remains unexplained and requires at least a more thorough behavioural analysis and description of the KO mice used here.
- Bath application of Bcl-2 inhibits TMEM175, indicating that it interacts with TMEM175 at the plasma membrane. This observation needs to be resolved.
- HA14-1 has targets beyond Bcl-2 and it indeed shows stronger effects than other inhibitors. The effect of HA14-1 could also be explained by indirect effects on mitochondrial function and ROS production.
- The effect of HA.14 needs to be tested in BAX/BAK dko cells to support the conclusion that the effect is independent of apoptosis.
- The co-IP and interaction data needs further validation.
- Supporting mechanistic data should be provided in a more relevant cell-type.

From the referee comments it is clear that a significant amount of work will be required. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

We invite you to submit your manuscript within three months of a request for revision. This would be October 2nd in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further, also in case you need more time as such for the experiments needed.

***IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.***

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

9) Regarding data quantification
The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
- Please also include scale bars in all microscopy images.

10) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision:
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Yours sincerely

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In this MS by Qu et al., the authors reported that TMEM175, a lysosomal K+ channel, is modulated by the apoptosis regulator Bcl-2. They found that whereas increased expression and activity of TMEM175 caused mitophagy defects, impaired mitochondrial homeostasis, and elevated ROS production, genetic inactivation of TMEM175 is neuroprotective in a pharmacologically-induced mouse model of Parkinson's Disease (PD). The current amplitudes of TMEM175 were shown to be potentiated by several Bcl-2 inhibitors when heterologously-expressed at the plasma membrane of mammalian cell lines. Overall, this is an interesting and comprehensive study on a lysosomal K+ channel, which is a high-risk factor for PD. The paper can benefit from several additional control experiments. My specific comments are as follows.
1. Prolonged drug treatment might cause undesirable effects to the cells and patches. In Fig. 1C, there appeared to be some current increases after 10 min in the non-transfected cells. Are those currents mediated by endogenous TMEM175, or another background K+ channel? Negative control experiments using Bcl-2 KO cells can make the results from the Bcl-2 inhibitor experiments much more convincing.
2. Whole-currents of TMEM175 in the study are generally in high quality, but the lysosomal currents, e.g., in Fig. 7D, are less convincing and require at least the K+ substitution/removal control experiments. In addition, the effects of Bcl-2 peptide and inhibitors on endogenous lysosomal TMEM175 currents are missing.
3. There was a clear difference in the ROS levels between WT and KO in Fig. 4M. The elevated ROS level may induce more cell death in KO cells as shown in Fig 6A. Meanwhile, overexpressing TMEM175 also caused much more cell death in the absence of any stimulation (Fig. 6C). How can do you reconcile both sets of data?
4. Both overexpressing Bcl-2 and bath application of Bcl-2 inhibited whole-cell TMEM175 currents (Fig. 2E-H), suggesting that Bcl-2 can act either extracellularly or intracellularly. In other words, Bcl-2 may bind to either the cytosolic side or extracellular side of the TMEM175 protein. Bcl-2 binding simulation indicated that both Vla145, which is located near the extracellular IS3-4 loop, and Arg377, which is located in the cytosolic side, were critical for Bcl-2 binding. Are there multiple Bcl-2 binding sites in TMEM175?
5. The authors demonstrated that ROS may directly activate TMEM175. Hence, the effect of HA14-1 might be mediated by ROS. Consistent with this prediction, GSH was sufficient to abolish the potentiation effect of HA14-1 (Fig. 4D). Didn't this rule out a direct effect of Bcl-2 binding on TMEM175 activation?
6. The basal currents vary significantly in different experiments. Does that reflect different levels of ROS in the cells?

Referee #2:

The authors provide an interesting manuscript that describes the modulation of TMEM175 activity by Bcl-2 suggesting a potential role of the lysosomal K+ channel in apoptosis. Further the study claims a role of TMEM175 in neurodegeneration in an age dependent manner in the context of Parkinson's disease. The manuscript is well written however some of the claims may need to be further substantiated (see comments below). Additionally, whilst briefly discussed in the text, the discordance between this study and a recent report (Wei et al Nature 2021) that TMEM175 deletion promotes rather than inhibits PD-like symptoms in mice is difficult to understand and warranted further consideration. Ideally this would be addressed experimentally although I appreciate that this could take considerable work to resolve.

General comments
It should be noted that HA14-1 has been shown to kill cells in a Bax/Bak independent fashion indicating that it has cellular target(s) beyond Bcl-2. This has implications for the conclusions drawn on the Bcl-2 inhibitor screening in Figure 1. In particular the statement, "All these agents activated TMEM175 currents similarly to HA14-1, indicating the involvement of the Bcl-2 family in the regulation of TMEM175 (Figure 1D)." is not supported by the data presented in 1D, where the effects of the specific inhibitors on TMEM175 activity appears significantly different. HA14-1 seems to have a much more profound effect, whilst WEHI-539 appears to have minimal effect. This might reflect a non-Bcl-2 target for HA14-1 in influencing TMEM175 activity.

The authors explore the hypothesis that there is an interaction between Bcl-2 and TMEM175 based on the effect of the HA14-1 inhibitor. Whilst plausible that Bcl-2, which is predominantly mitochondrial, contacts TMEM175 on lysosomes, that the effect of HA14-1 was revealed on the channel properties of TMEM175 by whole cell patch-clamp, suggests that Bcl-2 interacts with TMEM175 at the plasma membrane which is harder to reconcile.

To support the functional readout of TMEM175 activity the authors perform coIP analysis to investigate the interactions with BCl-2 proteins. However, there is inconsistency in the functional studies and the coIP. The interaction with Bcl-xl was barely

detectable by IP, yet WEHI-539, a Bcl-xL specific inhibitor, was as effective as the Bc-2 inhibitor ABT199?
Furthermore, the Bax and Bak IPs are difficult to interpret. The input fractions have multiple bands immunoreacting with GFP- which bands are GFP-Bak and GFP-Bax? Also, in the GFP IPs, there appears to be no pull down of GFP-Bak? Why are there two pronounced bands in the middle lane (GFP-Bax)? Also, why are the levels of TMEM175 so variable in these engineered cells? Cocneptually, it is important to recognise that these coIPs do not confirm the the interaction is direct, and neither does the in silico modelling. Definitive evidence could be from interaction of purified protein, cross-linking from cell lysates or rescue mutagenesis of Bcl-2. I would also caution IPs involving Triton X-100, as nonionic detergents have been shown to induce and disrupt interactions of Bcl-2 family members.

Although the binding site on Bcl-2 for HA14-1 is not known, other compounds argued here to block the TMEM175 interaction such as ABT-199 bind to the Bcl-2 hydrophobic groove that also binds BH3-only proteins. Neither E135 or N172 that are implicated from the docking studies map to the Bcl-2 groove, so the basis for the inhibitors disrupting the interaction is not clear. Can the interaction also be displaced by BH3-only proteins eg. Bim?
Additionally, the authors should comment that the proposed interaction site according to Fig 5 models seems to be buried in the membrane and so at odds with binding the soluble portion of Bcl-2?

How can the authors reconcile the proposed interaction of Bcl-2 with extracellular residues of TMEM175 at the plasma membrane (based on the experiments with recombinant Bcl-2 in Figs 2G and 2H), with Bcl-2's ability when expressed in cells to regulate TMEM175's channel properties at the plasma membrane which would involve interaction with cytosol facing residues?

The authors conclude that the effect of inhibiting Bcl-2 was independent of apoptosis and caspase activity based on the lack of effect of zVAD.fmk (Page 5 &Figure 2). The authors need to demonstrate that caspase activation was blocked by Z-VAD-FMK at the dose used. This may be included in a supplementary figure. Moreover, although potentially independent of caspases, the authors need to test if the link to TMEM175 is an indirect consequence of mitochondrial damage (Ca2+, mitochondrial protein release, ROS) by testing HA14-1 in cells that lack both Bax and Bak.

The molecular biology work suggesting the interaction of Bcl-2 with TMEM175 is performed in HEK293 cells. As the study later claims a potential role in pathogenesis of Parkinsons disease a neuronal cell culture model would be more appropriate.

Considering previous reports that the authors have referred to (Jinn et al 2017) have the authors measured mitochondrial function, lysosomal pH/enzyme activity in the TMEM175 KO HEK293 cells used in the present study. This may be useful as cell-based studies (e.g Jinn et al 2017) and a hypomorphic PD patient mutation of TMEM175 (Jinn et al 2019) suggest that loss of TMEM175 function impairs these two critical organelles and is associated with PD.

In the behavioural studies the baseline difference in activity of the WT and KO mice complicates interpretation. The authors should also indicate the age of the mice at which behavioural test was performed and whether these were littermate controls. The authors should also include a prior reference for the behavioural test used. That this motor phenotype conflicts with that reported for TMEM175 ko in Wei et al Nature 2021 strengthens the need to use multiple motor function tests including ideally the rotarod and latency to decline tests used in this previous study.

Specific comments

1. Page 3- details of the compound screening that led to the identification of HA14-1 should be described.

1. Page 3: "Bcl-2 family-specific agents activate TMEM175"
"Agents" may be replaced by inhibitors

2. Figure S3 purports to show interaction of endogenous TMEM1785 and Bcl-2. This should be included in the main figures as it is an important observation. However, it also requires an anti-TMEM175 immunoblot. See also comment above regarding a more relevant neuronal cell model.

3. Studies with MPP+ (Figure 6) need to be repeated at a higher dose of MMP+ to reveal a biological significance given that treatment with MPP+ in these cells only induced 8-9% cell death compared to 3% cell death in untreated controls. This is particularly relevant given that there are more apoptotic cells in non-transfected sample (6E) than WT+vehicle (6A). Figure 6E the non-transfected cells should be replaced by vector control. Furthermore, the authors should specify the error bars in the Figure legends. Ideally they should show the individual data points from the independent experiments, For example in Figure 6 death assays, the error bars (SEM) are much smaller than might be expected for such assays over independent experiments.

4. Figure 6: Expression level of WT and mutant TMEM175 should be shown by western blot to determine if the level of expression was equal.

5. The authors claim (in Figure 7A) that TMEM175 expression increases with age, but the Western appears to show a significant increase between 2W and 2M and no significant increase after that point (2M- 18M). This suggests a role of TMEM175 more

during a developmental stage of maturity than in advanced age. The authors may resolve this to some extent by performing densitometry on samples from more mice and highlight the stages in which there is a significant increase of TMEM175 levels.

6. Figure 7I the GAPDH band is less intense in the MPTP treated mouse samples making interpretation difficult. At the moment it appears that a) TH levels are increasing in both WT and KO mice on MPTP treatment. B) TH levels of WT and KO are similar in the untreated condition. The authors may include normalized densitometry for the blot to address this. Further, mRNA levels may not be very informative of the levels and activity of an enzyme.

7. It is unclear whether 7J and 7K refer to MPTP treated mice samples or untreated. This should be made clearer in the text and figure.

8. Figure 5F- Represents data from one cell. The authors should use ratiometric FACS analysis to analyse mtKeima in multiple cells across multiple experiments. Also, if mitophagy is impaired by TMEM175 expression the 445 emission spectra should remain high, but this appears low in TMEM175 expressing cells. This suggests a defect in mtKeima expression/mitochondrial import, not a defect in mitophagy.

9. Please show Western blots of brain tissue in ko mice to confirm TMEM175 deletion.

Referee #3:

The paper from the Cang's group provides interesting new insights into the regulation of the lysosomal potassium channels TMEM175 and how these channels are implicated in apoptosis and the development of Parkinson's Disease. The authors show that TEMEM175 is inhibited by binding of Bcl-2 and that pharmacological inhibitors of Bcl-2 release this inhibition and increase the activity of the channel. Importantly this mechanism appears to be independent of caspases. Increased activity of TEMEM175 induces ROS production, which further increases TEMEM175 activity via a positive feedback mechanism. Together this leads to increased apoptosis. Finally, the authors demonstrate that KO of TMEM175 leads to milder impairment of motor function and decreased loss of dopaminergic neurons in a mouse model of Parkinson's Disease.

In summary the paper provides important new insight into the physiological and pathophysiological significance of TMEM175. The paper is well written, the data sound and the conclusions drawn are convincing. The paper would be of high interest to the readership of EMBO Reports and I recommend that the paper be published. However, before publication the following minor points need to be addressed:

#1: for each experiment, please indicate in the text which cell type was used.

#2: page 4, last paragraph: is it possible to confirm the conclusion that channel activity is increased by HA14-1 using single channel recordings or estimate the effect using noise analysis?

#3: Page 5, second paragraph: is the binding of bcl-xl functionally relevant? How was this tested?

#4: page: 7, second paragraph: is there further experimental evidence (detailed confocal microscopy) confirming "intracellular complexity" in addition to simple side scatter FACS signals?

#5: page 8, second paragraph: how have the off-target effects been tested by genetic analysis in KO cell lines and also in the KO mice?

#6: Is it possible to include patch clamp recordings of TMREM currents from native dopaminergic neurons?

Point-by-point responses

Introduction:

We thank the referees and editor for your encouragement and very insightful comments. We have followed your suggestions and have completed experiments to address the comments. The manuscript is now much improved. New data are now added to the revised manuscript and are discussed in more detail in the Point-by-Point Responses.

Editor

- Your findings are in disagreement with a recent study by Wei et al, who reported that TMEM175 deletion promotes rather than inhibits PD-like symptoms. This discrepancy remains unexplained and requires at least a more thorough behavioural analysis and description of the KO mice used here.

Response:

Different animals and treatment were used in these two studies, and we believe that this is the main cause of the difference in the behavioral data. We used homozygous TMEM175 knockout mice while they used heterozygous mice. We treated the mice with MPTP to generate the model of Parkinson's disease, while they used untreated mice. Therefore, our model may be more representative of induced (or sporadic) PD, while Wie's model may be more representative of inherited (or familial) PD. The variance between the two studies indicates that TMEM175 may play distinct roles in different forms of PD.

In addition, different behavioral tests were performed in the two studies. We performed cylinder test, and they conducted rotarod, wire hang, grip strength and pole tests. In the revised paper, we added new data of wire hang, gait and rotarod tests (new Fig 7I-K). There is significant difference between WT and KO in wire hang test, but not in gait or rotarod test. The disagreement in our and Wie's behavioral results is understandable, because it is common for different animal models of Parkinson's disease to exhibit different behavioral phenotypes.

- Bath application of Bcl-2 inhibits TMEM175, indicating that it interacts with TMEM175 at the plasma membrane. This observation needs to be resolved.

Response:

The data showing bath application of Bcl-2 protein (Figure 2G and H) were acquired from lysosomal recordings, not from whole-cell recordings. We have labeled this on the revised Fig 2L and M to make it clear. We also tried bath application of Bcl-2 in whole-cell recording, but did not detect inhibitory effect (Fig R1 below), indicating that Bcl-2 interacts with TMEM175 on the cytosolic side.

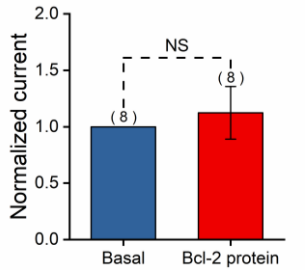

Fig R1: Bath application of 1 μg Bcl-2 protein failed to inhibit TMEM175 current in whole-cell recordings.

- HA14-1 has targets beyond Bcl-2 and it indeed shows stronger effects than other inhibitors. The effect of HA14-1 could also be explained by indirect effects on mitochondrial function and ROS production.

Response:

We agree with the referee and editor that HA14-1 may activate TMEM175 through a Bcl-2-independent mechanism, possibly by indirectly affecting mitochondrial function and ROS production. Our data showed that ROS depletion by GSH greatly abolished the effect of HA14-1 on TMEM175 activation (Fig 4C and D). The effects of several other Bcl-2 inhibitors had proved the involvement of Bcl-2 inhibition in the activation of TMEM175, therefore, we believe that HA14-1 activates TMEM175 through both Bcl-2-dependent and -independent pathways.

- The effect of HA.14 needs to be tested in BAX/BAK dko cells to support the conclusion that the effect is independent of apoptosis.

Response:

We thank the editor and referee for the insightful suggestion. We generated Bax/BAK1 double knockout HEK293T cells using CRISPR/Cas9 technique and found that 10 μM HA14-1 could still significantly increase whole-cell TMEM175 currents, which further supported the conclusion that a non-caspase mechanism was involved (new Fig 2E and F).

- The co-IP and interaction data needs further validation.

Response:

As recommended by referee #2, new co-IP experiments have been performed to test the interaction between TMEM175 and Bcl-2 proteins using CHAPS IP and lysis buffer which is a zwitterionic detergent that is especially well suited for protecting the native state of proteins (new Fig 2G and H).

In addition, the interaction of endogenous TMEM175 and Bcl-2 were also detected using mouse midbrain tissue and untransfected SH-SY5Y, HEK293T, and HeLa cells (new Fig 2I). The binding between the two proteins could be detected in all above

experiments.

- Supporting mechanistic data should be provided in a more relevant cell-type.
Response:
As mentioned above, we added the verification of the endogenous binding of TMEM175 and Bcl-2 in SH-SY5Y and mouse midbrain (new Fig 2I). We have also recorded the effect of HA14-1 on TMEM175 currents in lysosomes of SH-SY5Y cells (new Fig EV2I and J).

Referee #1:
In this MS by Qu et al., the authors reported that TMEM175, a lysosomal K+ channel, is modulated by the apoptosis regulator Bcl-2. They found that whereas increased expression and activity of TMEM175 caused mitophagy defects, impaired mitochondrial homeostasis, and elevated ROS production, genetic inactivation of TMEM175 is neuroprotective in a pharmacologically-induced mouse model of Parkinson's Disease (PD). The current amplitudes of TMEM175 were shown to be potentiated by several Bcl-2 inhibitors when heterologously-expressed at the plasma membrane of mammalian cell lines. Overall, this is an interesting and comprehensive study on a lysosomal K+ channel, which is a high-risk factor for PD. The paper can benefit from several additional control experiments. My specific comments are as follows.
1. Prolonged drug treatment might cause undesirable effects to the cells and patches. In Fig. 1C, there appeared to be some current increases after 10 min in the non-transfected cells. Are those currents mediated by endogenous TMEM175, or another background K+ channel? Negative control experiments using Bcl-2 KO cells can make the results from the Bcl-2 inhibitor experiments much more convincing.
Response:
We agree with the referee that prolonged drug treatment might cause undesirable effects, especially to patch-clamp recording. There should be no endogenous TMEM175 expressed on the cell membrane. And there was TEA in our recording solutions which blocks endogenous voltage-gated potassium channels. The slight increase of current after 10 min in Fig 1C was probably due to the instability of the seal. The current amplitude at -100 mV is about -100 pA, so the input resistance was still at GΩ range. Therefore, these recordings were retained.

The involvement of Bcl-2 in regulating TMEM175 was showed by multiple lines of evidence. First, multiple Bcl-2 inhibitors with different structures all activated TMEM175 current (Fig 1B-D). Second, our co-IP data revealed a binding between Bcl-2 and TMEM175. Third, we predicted the binding interface of the two proteins and found potential binding sites. Mutation in these sites weakened the co-IP signals and reduced the activation effect of Bcl-2 inhibitor on TMEM175 currents (Fig 3). Last, Bcl-2 knockout increased the basal TMEM175 currents (Fig EV2F). All these data indicated that Bcl-2

interacts with TMEM175 and inhibits TMEM175 activity.

2. Whole-currents of TMEM175 in the study are generally in high quality, but the lysosomal currents, e.g., in Fig. 7D, are less convincing and require at least the K+ substitution/removal control experiments. In addition, the effects of Bcl-2 peptide and inhibitors on endogenous lysosomal TMEM175 currents are missing.

Response:

The endogenous lysosomal TMEM175 currents are usually small, and it is easily interfered by noise and currents from other endogenous channels. So, the lysosomal currents did not look as good as the whole-cell currents. In fact, we recorded larger lysosomal currents in cells overexpressing TMEM175, and such currents looked much better (new Fig 2L). All our recordings were verified by substitution of K^+ with $NMDG^+$. The current traces of NMDG solution were added to the lysosomal recordings in the revised new Fig 7F. It should be noted that the lysosomal current is reversed because the lysosomal membrane potential (Ψ) is defined as Vcytosol – Vlumen. Therefore 0 K^+ bath bath will eliminate the outward current, which is the opposite of whole-cell recording.

We have added the data of Bcl-2 protein and inhibitors on the endogenous lysosomal TMEM175 currents. 1ug Bcl-2 suppressed lysosomal TMEM175 currents in HEK293T cells (new Fig EV2G and H) and 10 μM HA14-1 enhanced the endogenous currents in SH-SY5Y lysosomes (new Fig EV2I and J) as predicted.

3. There was a clear difference in the ROS levels between WT and KO in Fig. 4M. The elevated ROS level may induce more cell death in KO cells as shown in Fig 6A. Meanwhile, overexpressing TMEM175 also caused much more cell death in the absence of any stimulation (Fig. 6C). How can do you reconcile both sets of data?

Response:

ROS has long been considered as a double-edged sword in cellular processes including apoptosis. Low to modest level of ROS plays important roles in cellular signaling and promotes cell survival, while high level of ROS induces apoptosis. ROS is a byproduct of the mitochondrial respiratory chain. Therefore, enhanced mitochondrial function can moderately increase the generation of ROS, which may be the reason why the ROS level in TMEM175 KO cells was slightly increased. More importantly, the KO of TMEM175 prevented the dramatic increase in ROS caused by external stimuli, thereby preventing the induction of apoptosis.

4. Both overexpressing Bcl-2 and bath application of Bcl-2 inhibited whole-cell TMEM175 currents (Fig. 2E-H), suggesting that Bcl-2 can act either extracellularly or intracellularly. In other words, Bcl-2 may bind to either the cytosolic side or extracellular side of the TMEM175 protein. Bcl-2 binding simulation indicated that both Vla145, which is located near the extracellular IS3-4 loop, and Arg377, which is located in the cytosolic side, were critical for Bcl-2 binding. Are there multiple Bcl-2 binding sites in TMEM175?

Response:
As mentioned in the above response to the editor, Fig 2G and H in the initial submission (new Fig 2L and M) are actually lysosomal recordings. We have labeled this on the revised Fig 2 to make it clear. In addition, we have tried bath application of Bcl-2 in whole-cell recording, but did not detect inhibitory effect (Fig R1 above), indicating that Bcl-2 interacts with TMEM175 on the cytosolic side. The data from binding site mutation (new Fig 3B-F) indicated that there are multiple Bcl-2 binding sites in TMEM175, and the Arg377 residue is more important than Vla145.

5. The authors demonstrated that ROS may directly activate TMEM175. Hence, the effect of HA14-1 might be mediated by ROS. Consistent with this prediction, GSH was sufficient to abolish the potentiation effect of HA14-1 (Fig. 4D). Didn't this rule out a direct effect of Bcl-2 binding on TMEM175 activation?
Response:
Our data of new Fig 3D-F indicated that Bcl-2 is also important for the effect of HA14-1. In addition, GSH did not completely abolish the effect of HA14-1 (Fig 4C and D). It is possible that the full activation of TMEM175 requires both the release of Bcl-2 inhibition and the action of ROS. Another possibility is that the elevation of ROS level is a consequence of the inhibition of Bcl-2, thus ROS act as a downstream effector of Bcl-2 inhibition to activate TMEM175.

6. The basal currents vary significantly in different experiments. Does that reflect different levels of ROS in the cells?
Response:
The largest difference in the basal currents appears in new Fig 2J-M. In fact, Fig 2J-K represented whole-cell recordings and Fig 2L-M were whole-lysosomal recordings performed on HEK293T cells overexpressing TMEM175. As an organellar membrane protein, the expression density of TMEM175 on the lysosome is much higher than that on the plasma membrane. That’s the reason why the basal current densities vary significantly in Fig 2. Furthermore, the amount of TMEM175 protein expressed in different cells or lysosomes is quite different, which may be a more important factor affecting the basal current of TMEM175 compared to the level of ROS.

Referee #2:
The authors provide an interesting manuscript that describes the modulation of TMEM175 activity by Bcl-2 suggesting a potential role of the lysosomal K+ channel in apoptosis. Further the study claims a role of TMEM175 in neurodegeneration in an age dependent manner in the context of Parkinson's disease. The manuscript is well written however some of the claims may need to be further substantiated (see comments below). Additionally, whilst briefly discussed in the text, the discordance between this study and a recent report (Wei et al Nature 2021) that TMEM175 deletion promotes rather than

inhibits PD-like symptoms in mice is difficult to understand and warranted further consideration. Ideally this would be addressed experimentally although I appreciate that this could take considerable work to resolve.

General comments

It should be noted that HA14-1 has been shown to kill cells in a Bax/Bak independent fashion indicating that it has cellular target(s) beyond Bcl-2. This has implications for the conclusions drawn on the Bcl-2 inhibitor screening in Figure 1. In particular the statement, "All these agents activated TMEM175 currents similarly to HA14-1, indicating the involvement of the Bcl-2 family in the regulation of TMEM175 (Figure 1D)." is not supported by the data presented in 1D, where the effects of the specific inhibitors on TMEM175 activity appears significantly different. HA14-1 seems to have a much more profound effect, whilst WEHI-539 appears to have minimal effect. This might reflect a non-Bcl-2 target for HA14-1 in influencing TMEM175 activity.

Response:

We agree that the statement is inaccurate. It was edited in the revised manuscript to read as “All these inhibitors activated TMEM175 currents, indicating the involvement of the Bcl-2 family in the regulation of TMEM175 (Fig 1D)”.

These Bcl-2 family inhibitors with different structures can all activate TMEM175, suggesting that Bcl-2 plays an important role in regulating the activity of TMEM175. However, these results does not rule out the possibility of other non-Bcl-2 mechanisms acting at the same time. As mentioned in the above response to the editor and referee #1, HA14-1 may activate TMEM175 through both Bcl-2 dependent and independent pathways, and the latter may be achieved by indirectly affecting mitochondrial function and ROS generation. This may be the reason why HA14-1 is more effective than other inhibitors.

The authors explore the hypothesis that there is an interaction between Bcl-2 and TMEM175 based on the effect of the HA14-1 inhibitor. Whilst plausible that Bcl-2, which is predominantly mitochondrial, contacts TMEM175 on lysosomes, that the effect of HA14-1 was revealed on the channel properties of TMEM175 by whole cell patch-clamp, suggests that Bcl-2 interacts with TMEM175 at the plasma membrane which is harder to reconcile.

Response:

Whole-cell recording is used for compound screening mainly considering that lysosomal recording is performed on lysosomes extracted from cells, so some cytoplasmic signal molecules may be missing. In addition, we also performed lysosomal patch-clamp recording and found that application of purified Bcl-2 protein inhibited lysosomal TMEM175 currents (Figs 2L and M, and EV2G and H).

Bcl-2 is mainly located in mitochondria, but studies have also found its expression on the cell membrane (Bruce-Keller, Begley et al., 1998, Chen-Levy, Nourse et al., 1989). In addition, there are mitochondria-plasma membrane contact sites, where mitochondrial

proteins can interact with plasma membrane proteins (Szymanski, Janikiewicz et al., 2017, Westermann, 2015). These studies show that it is possible for Bcl-2 to regulate TMEM175 on the cell membrane.

References:

Bruce-Keller AJ, Begley JG, Fu W, Butterfield DA, Bredesen DE, Hutchins JB, Hensley K, Mattson MP (1998) Bcl-2 protects isolated plasma and mitochondrial membranes against lipid peroxidation induced by hydrogen peroxide and amyloid beta-peptide. J Neurochem 70: 31-9

Chen-Levy Z, Nourse J, Cleary ML (1989) The bcl-2 candidate proto-oncogene product is a 24-kilodalton integral-membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14;18) translocation. Mol Cell Biol 9: 701-10

Szymanski J, Janikiewicz J, Michalska B, Patalas-Krawczyk P, Perrone M, Ziolkowski W, Duszynski J, Pinton P, Dobrzyn A, Wieckowski MR (2017) Interaction of Mitochondria with the Endoplasmic Reticulum and Plasma Membrane in Calcium Homeostasis, Lipid Trafficking and Mitochondrial Structure. Int J Mol Sci 18

Westermann B (2015) The mitochondria-plasma membrane contact site. Curr Opin Cell Biol 35: 1-6

To support the functional readout of TMEM175 activity the authors perform coIP analysis to investigate the interactions with BCl-2 proteins. However, there is inconsistency in the functional studies and the coIP. The interaction with Bcl-xl was barely detectable by IP, yet WEHI-539, a Bcl-xL specific inhibitor, was as effective as the Bc-2 inhibitor ABT199?

Response:

The IC50 of WEHI-539 to Bcl-xL is 1.1nM. Its selectivity for Bcl-xL is 500-fold over Bcl-2 (Lessene, Czabotar et al., 2013), which means that the IC50 for Bcl-2 is also at the sub-micromolar level. The WEHI-539 used in our study is 10 μM, a concentration that can effectively inhibit Bcl-2. We tried 10 nM WEHI-539 and found that it could not activate the TMEM175 current (Fig R2 below), which indicated that the 10 μM WEHI-539 mainly works by inhibiting Bcl-2.

Reference:

Lessene G, Czabotar PE, Sleebs BE, Zobel K, Lowes KN, Adams JM, Baell JB, Colman PM, Deshayes K, Fairbrother WJ, Flygare JA, Gibbons P, Kersten WJ, Kulasegaram S, Moss RM, Parisot JP, Smith BJ, Street IP, Yang H, Huang DC et al. (2013) Structure-guided design of a selective BCL-X(L) inhibitor. Nat Chem Biol 9: 390-7

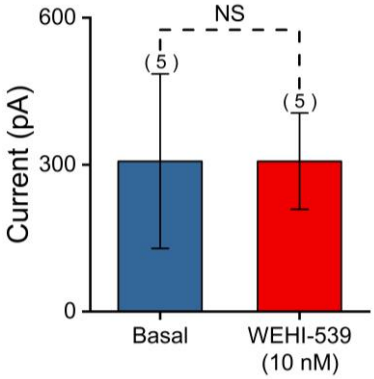

Fig R2：10 nM WEHI-539 does not activate TMEM175.

Furthermore, the Bax and Bak IPs are difficult to interpret. The input fractions have multiple bands immunoreacting with GFP- which bands are GFP-Bak and GFP-Bax? Also, in the GFP IPs, there appears to be no pull down of GFP-Bak? Why are there two pronounced bands in the middle lane (GFP-Bax)? Also, why are the levels of TMEM175 so variable in these engineered cells? Cocneptually, it is important to recognise that these coIPs do not confirm the the interaction is direct, and neither does the in silico modelling. Definitive evidence could be from interaction of purified protein, cross-linking from cell lysates or rescue mutagenesis of Bcl-2. I would also caution IPs involving Triton X-100, as nonionic detergents have been shown to induce and disrupt interactions of Bcl-2 family members.

Response:

New co-IP experiments have been performed to test the interaction between TMEM175 and Bcl-2 proteins using CHAPS IP and lysis buffer which is a zwitterionic detergent that is especially well suited for protecting the native state of proteins (new Fig 2G and H). The calculated protein molecular weights of GFP-BAK and GFP-Bax are approximately 53 kDa and 51 kDa respectively as indicated in new Fig 2H. The pull down of GFP-BAK turned more obvious when we increased the amount of protein loaded. It was very close to IgG heavy chain band at 55 kDa which probably masked the GFP-BAK band in previous Fig 2D. As the two pronounced bands in the middle lane (GFP-Bax) mentioned, there are still two adjacent protein bands in new GFP-tag IP image which is consistent with the product specification of the GFP Tag Polyclonal Antibody (50430-2-AP, Proteintech) for the IP results of anti-GFP tag.

We understand the referee's concerns. However, our conclusion that TMEM175 interacts with Bcl-2 is not solely based on the results of a single co-IP. Our data showed that multiple Bcl-2 inhibitors with different structure all activated TMEM175 currents (Fig 1). We also predicted the binding interface of the two proteins and found potential binding sites. Mutation in these sites weakened the co-IP signals and reduced the activation effect of Bcl-2 inhibitor on TMEM175 currents (Fig 3). Furthermore, Bcl-2 knockout

increased the basal TMEM175 currents (new Fig EV2F). All these data indicated that Bcl-2 interacts with TMEM175 and inhibits TMEM175 activity.

Although the binding site on Bcl-2 for HA14-1 is not known, other compounds argued here to block the TMEM175 interaction such as ABT-199 bind to the Bcl-2 hydrophobic groove that also binds BH3-only proteins. Neither E135 or N172 that are implicated from the docking studies map to the Bcl-2 groove, so the basis for the inhibitors disrupting the interaction is not clear. Can the interaction also be displaced by BH3-only proteins eg. Bim?

Response:

According to the referee's suggestion, we tested the effect of the BH3-only protein Bim on TMEM175. As shown in the Fig R3 below, overexpression of Bim does not affect the basal TMEM175 current, indicating that it does not affect the inhibitory effect of Bcl-2 on TMEM175.

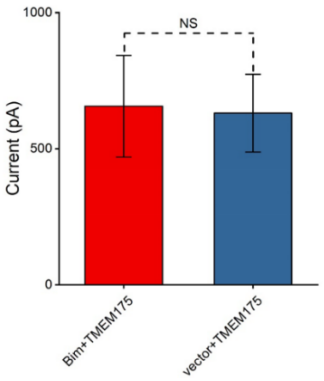

Fig R3：Expression of Bim does not affect TMEM175 current.

Additionally, the authors should comment that the proposed interaction site according to Fig 5 models seems to be buried in the membrane and so at odds with binding the soluble portion of Bcl-2?

Response:

Since Bcl-2 is mainly located in mitochondria while TMEM175 is mainly located in lysosomes, we speculate that their interaction mainly occurs at the mitochondria-lysosome contact sites. The membrane-spanning C terminus of Bcl-2 is embedded in the mitochondrial membrane, so it can only interact with TMEM175 with its soluble portion containing the predicted binding sites. We discussed this in the revised manuscript.

How can the authors reconcile the proposed interaction of Bcl-2 with extracellular residues of TMEM175 at the plasma membrane (based on the experiments with recombinant Bcl-2 in Figs 2G and 2H), with Bcl-2's ability when expressed in cells to

regulate TMEM175's channel properties at the plasma membrane which would involve interaction with cytosol facing residues?

Response:

As mention in the above response to the editor and referee #1, the Fig 2G and H (new Fig 2L and M) refer to lysosomal recordings, not whole-cell recordings. We have labeled this on the revised new Fig 2L to make it clear. We also tried bath application of Bcl-2 in whole-cell recording, but did not detect inhibitory effect (Fig R1 above), indicating that Bcl-2 interacts with TMEM175 on the cytosolic side.

The authors conclude that the effect of inhibiting Bcl-2 was independent of apoptosis and caspase activity based on the lack of effect of zVAD.fmk (Page 5 &Figure 2). The authors need to demonstrate that caspase activation was blocked by Z-VAD-FMK at the dose used. This may be included in a supplementary figure. Moreover, although potentially independent of caspases, the authors need to test if the link to TMEM175 is an indirect consequence of mitochondrial damage (Ca2+, mitochondrial protein release, ROS) by testing HA14-1 in cells that lack both Bax and Bak.

Response:

The concentration of z-VAD-FMK used in this study (10 μM) is widely used in the inhibition of caspase. Studies have shown that high concentrations of z-VAD-FMK sometimes promote apoptosis, so we did not choose higher concentrations (Cowburn, White et al., 2005). To verify the effectiveness of 10 μM z-VAD-FMK, we treated cells with staurosporine (STS) and used caspase 3 Activity Assay Kit to monitor caspase activation. As shown in Fig R4 below, 10 μM z-VAD-FMK greatly inhibited STS-induced activation of caspase 3. Since EMBO reports limits the number of supplementary figures to no more than 5, we cannot include this data in the revised manuscript.

According to the referee's suggestion, we generated a Bax/BAK1 double knockout cell line and used it to test the effect of HA14-1. As shown in the revised Fig 2E and F, HA14-1 could still activate TMEM175 currents in Bax/BAK1 double knockout cells, indicating that Bax/BAK1 is not involved.

Reference:

Cowburn AS, White JF, Deighton J, Walmsley SR, Chilvers ER (2005) z-VAD-fmk augmentation of TNF alpha-stimulated neutrophil apoptosis is compound specific and does not involve the generation of reactive oxygen species. Blood 105: 2970-2

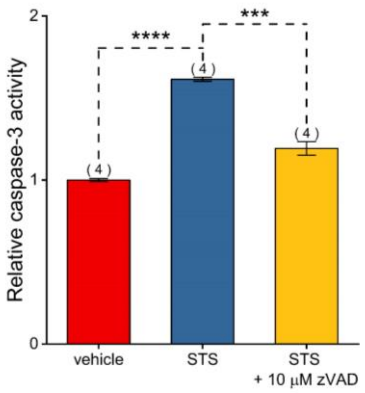

Fig R4：10 μM z-VAD-FMK effectively inhibited caspase activation. HEK293T cells were pre-incubated with 10 μM z-VAD-FMK for 2 hours before application of 2 μM staurosporine. 5 hours treatment of staurosporine induced activation of caspase and Z-VAD-FMK inhibited the effect.

The molecular biology work suggesting the interaction of Bcl-2 with TMEM175 is performed in HEK293 cells. As the study later claims a potential role in pathogenesis of Parkinsons disease a neuronal cell culture model would be more appropriate.

Response:

We have performed co-IP experiments using mouse brain and SH-SY5Y cells. The human neuroblastoma SH-SY5Y cell line has been widely used as a dopaminergic cell model for Parkinson's disease research. Co-IP signals could be detected in both preparations (new Fig 2I).

Considering previous reports that the authors have referred to (Jinn et al 2017) have the authors measured mitochondrial function, lysosomal pH/enzyme activity in the TMEM175 KO HEK293 cells used in the present study. This may be useful as cell-based studies (e.g Jinn et al 2017) and a hypomorphic PD patient mutation of TMEM175 (Jinn et al 2019) suggest that loss of TMEM175 function impairs these two critical organelles and is associated with PD.

In the behavioural studies the baseline difference in activity of the WT and KO mice complicates interpretation. The authors should also indicate the age of the mice at which behavioural test was performed and whether these were littermate controls. The authors should also include a prior reference for the behavioural test used. That this motor phenotype conflicts with that reported for TMEM175 ko in Wei et al Nature 2021 strengthens the need to use multiple motor function tests including ideally the rotarod and latency to decline tests used in this previous study.

Response:

The age of the mice used in behavioural tests were 3-4 months. We have indicated this in the Materials and Methods, Animals section of the revised manuscript. The mice were not

littermate controls because the KO mice were obtained by mating homozygous KO mice. The KO mice used for mating were obtained from heterozygous mice that have been backcrossed to WT for more than 5 times. The references for the behavioural tests were included in the revised manuscript.

As mentioned in the above response to the editor, different animals and treatments were used in these two studies, and we believe that this is the main cause of the difference in the behavioral data. We used TMEM175 knockout mice, while they used heterozygous mice. We treated the mice with MPTP to generate the model of Parkinson's disease, while they used untreated mice. Therefore, our model may be more similar to the induced (or sporadic) PD, while Wie's model may be more similar to inherited (or familial) PD. The difference between the two studies indicates that TMEM175 may play various roles in different types of PD.

In the revised paper, we added new data of wire hang, gait and rotarod test (new Fig 7I-K). There is significant difference between WT and KO in wire hang test, but not in gait or rotarod test. The difference in the behavioral result between our and Wie's paper is understandable, because it is common for different animal models of Parkinson's disease to exhibit distinct behavioural phenotypes.

Specific comments

1. Page 3- details of the compound screening that led to the identification of HA14-1 should be described.

Response:

The text in our initial submission was misleading. Those compounds were not screened out by us, but were known activators/inhibitors of intracellular signaling pathways. What we did was to use whole-cell patch clamp to test the effect of these compounds on the current of TMEM175. The text has been edited to read as "We next used whole-cell patch-clamp to test the effect of a numerous compounds known to target intracellular signaling molecules on the current of TMEM175, and found that the Bcl-2 inhibitor HA14-1 strongly activated TMEM175."

1. Page 3: "Bcl-2 family-specific agents activate TMEM175" "Agents" may be replaced by inhibitors

Response:

We apologize for the imprecise word. The "agents" was changed to "inhibitors" in the revised manuscript.

2. Figure S3 purports to show interaction of endogenous TMEM1785 and Bcl-2. This should be included in the main figures as it is an important observation. However, it also requires an anti-TMEM175 immunoblot. See also comment above regarding a more relevant neuronal cell model.

Response:

We have performed the experiments of Figure S3 again and added the anti-TMEM175 immunoblots. In addition, we have also tested the binding of TMEM175 and Bcl-2 in mouse brain and SH-SY5Y cells. These new data were included in the revised Fig 2I.

3. Studies with MPP+ (Figure 6) need to be repeated at a higher dose of MMP+ to reveal a biological significance given that treatment with MPP+ in these cells only induced 8-9% cell death compared to 3% cell death in untreated controls. This is particularly relevant given that there are more apoptotic cells in non-transfected sample (6E) than WT+vehicle (6A). Figure 6E the non-transfected cells should be replaced by vector control. Furthermore, the authors should specify the error bars in the Figure legends. Ideally they should show the individual data points from the independent experiments, For example in Figure 6 death assays, the error bars (SEM) are much smaller than might be expected for such assays over independent experiments.

Response:

Compared with other studies, the concentration of MPP^+ we used is already high. More importantly, this set of experiments is to show the promoting effect of TMEM175 on apoptosis. If we use a higher concentration of MPP^+ to induce robust cell apoptosis, it may mask the effect of TMEM175. Therefore, we chose a concentration of MPP^+ that can induce slight apoptosis.

The control group of cells in Fig 6E was indeed cells transfected with empty vector. We previously labeled it as "non-transfected" by mistake. Transfection is sometimes harmful to cells, especially when the cell density is low. This might be the reason why the apoptosis of these cells is slightly higher than the control of Fig 6A-D. We have added the statement of “Data were presented as mean ± SEM.” to the legends to make it clear. The individual data points were also showed in the revised Fig 6.

4. Figure 6: Expression level of WT and mutant TMEM175 should be shown by western blot to determine if the level of expression was equal.

Response:

The expression level of WT and TMEM175 mutants were determined by western blot with 3 repeats (new Fig 3C).

5. The authors claim (in Figure 7A) that TMEM175 expression increases with age, but the Western appears to show a significant increase between 2W and 2M and no significant increase after that point (2M- 18M). This suggests a role of TMEM175 more during a developmental stage of maturity than in advanced age. The authors may resolve this to some extent by performing densitometry on samples from more mice and highlight the stages in which there is a significant increase of TMEM175 levels.

Response:

New western blotting experiment was performed with more samples. With the increase of age, the expression of TMEM175 in mice midbrain trend to increase gradually. There is a

significant difference between 2 and 18-26 months of age (new Fig 7B).

6. Figure 7I the GAPDH band is less intense in the MPTP treated mouse samples making interpretation difficult. At the moment it appears that a) TH levels are increasing in both WT and KO mice on MPTP treatment. B) TH levels of WT and KO are similar in the untreated condition. The authors may include normalized densitometry for the blot to address this. Further, mRNA levels may not be very informative of the levels and activity of an enzyme.

Response:

We have done more western-blot experiments to obtain the statistics of the TH/GAPDH ratio (new Fig 7M). Because we recently have only a limited amount of KO mice of the appropriate age, all of which were treated with MPTP for behavioral testing, we can only use these MPTP-treated animals to detect the protein level of TH. We agree with the referee that the mRNA level may not be informative, thus these data was removed.

7. It is unclear whether 7J and 7K refer to MPTP treated mice samples or untreated. This should be made clearer in the text and figure.

Response:

Fig 7J and 7K refer to MPTP-treated mice. This was indicated in the legend. We have also labeled it in the revised Fig 7N.

8. Figure 5F- Represents data from one cell. The authors should use ratiometric FACS analysis to analyse mtKeima in multiple cells across multiple experiments. Also, if mitophagy is impaired by TMEM175 expression the 445 emission spectra should remain high, but this appears low in TMEM175 expressing cells. This suggests a defect in mtKeima expression/mitochondrial import, not a defect in mitophagy.

Response:

The expression level of mt-mKeima was low in TMEM175 overexpressing cells. Although this can reflect the abnormality of mitochondria to a certain extent, it will bring trouble to the analysis of mitophagy. To avoid this issue, we adopted another method. Tom20-mCherry and LysoTracker green were used to label mitochondria and lysosomes, respectively. The co-localization of Tom20 and lysosome means that the autophagic mitochondria are delivered to lysosomes. Our results showed that mitophagy was enhanced in TMEM175-KO cells (new Fig 5H and I), indicating that TMEM175 inhibits mitophagy.

9. Please show Western blots of brain tissue in ko mice to confirm TMEM175 deletion.

Response:

The western blots of brain tissue from WT and KO mice have been included in the revised manuscript (new Fig 7E).

Referee #3:

The paper from the Cang's group provides interesting new insights into the regulation of the lysosomal potassium channels TMEM175 and how these channels are implicated in apoptosis and the development of Parkinson's Disease. The authors show that TEMEM175 is inhibited by binding of Bcl-2 and that pharmacological inhibitors of Bcl-2 release this inhibition and increase the activity of the channel. Importantly this mechanism appears to be independent of caspases. Increased activity of TEMEM175 induces ROS production, which further increases TEMEM175 activity via a positive feedback mechanism. Together this leads to increased apoptosis. Finally, the authors demonstrate that KO of TMEM175 leads to milder impairment of motor function and decreased loss of dopaminergic neurons in a mouse model of Parkinson's Disease.

In summary the paper provides important new insight into the physiological and pathophysiological significance of TMEM175. The paper is well written, the data sound and the conclusions drawn are convincing. The paper would be of high interest to the readership of EMBO Reports and I recommend that the paper be published.

Response:

We thank the referee for the positive comments and kind encouragement.

However, before publication the following minor points need to be addressed:

#1: For each experiment, please indicate in the text which cell type was used.

Response:

The cell types used are indicated in the revised manuscript.

#2: Page 4, last paragraph: is it possible to confirm the conclusion that channel activity is increased by HA14-1 using single channel recordings or estimate the effect using noise analysis.

Response:

We have performed single-channel recording based on the referee's comments, but failed to record the single-channel event of TMEM175. In fact, as early as 2015 when we first identified TMEM175 as a lysosomal potassium channel, we had already tried to record its single-channel current, but also failed. Considering that we can record large TMEM175 currents (more than 1 nA) on a tiny lysosome, we speculate that its single channel conductance should be relatively large. The reason why the single-channel event could not be recorded is probably because this leak-type channel stays in the open state all the time. In this case, we cannot conduct single-channel recording and noise analysis. We speculate that HA14-1 activates TMEM175 by increasing the conductance of TMEM175, rather than increasing its open probability.

#3: Page 5, second paragraph: is the binding of bcl-xl functionally relevant? How was this tested?

Response:
The binding of Bcl-xL is much weaker than that of Bcl-2. Our new data showed that overexpression of Bcl-2, but not Bcl-xL, significantly inhibits the basal TMEM175 current (new Fig 2J and K), indicating that Bcl-xL does not play an important role in the regulation of TMEM175.

#4: Page: 7, second paragraph: is there further experimental evidence (detailed confocal microscopy) confirming "intracellular complexity" in addition to simple side scatter FACS signals?
Response:
Intracellular granular structure is an important contributor to the “intracellular complexity”. Since TMEM175 promotes apoptosis and the generation of ROS, it may promote the generation of stress granules (SGs). G3BP1 (Ras GTPase-activating protein-binding protein 1) is a common SG constituent. We stained HEK293T cells with anti-G3BP1 antibody and observed more SGs in TMEM175-overexpressing cells (new Fig 5F and G). It further supported that TMEM175 overexpressing generated a higher intracellular complexity.

#5: Page 8, second paragraph: how have the off-target effects been tested by genetic analysis in KO cell lines and also in the KO mice?
Response:
For each sgRNA used, we analyzed the genomic sites with the highest probability of off-target. By comparing with WT, we found that none of these sites were mutated in KO cells and animals (Figs R5 and R6 below). For the KO mouse line, heterozygous mice were backcrossed to WT for more than five generations before being used to generate homozygous knockouts. This also reduced the risk of off-target.

#6: Is it possible to include patch clamp recordings of TMEM currents from native dopaminergic neurons?
Response:
The culture of native dopaminergic neurons is very difficult. Usually, only a small portion of the neurons obtained from the primary culture of embryonic mouse midbrain are dopaminergic neurons. The identification of these dopaminergic neurons often requires immunocytochemical staining, and the stained cells cannot be used for patch clamp experiments. The SH-SY5Y neuroblastoma cells have been widely used as a cell model of dopaminergic neurons for PD research. In the revised manuscript, we added new data obtained from SH-SY5Y cells, including co-IP (new Fig 2I) and lysosomal patch clamp data (new Fig EV2I and J).

Fig R5: Off-target detection of the TMEM175 KO cells.

(A) Electrophoresis of the PCR products of 3 potential off-target sites with the highest probability. (B) Comparison of the DNA sequences of WT and KO mice at the suspected off-target sites.

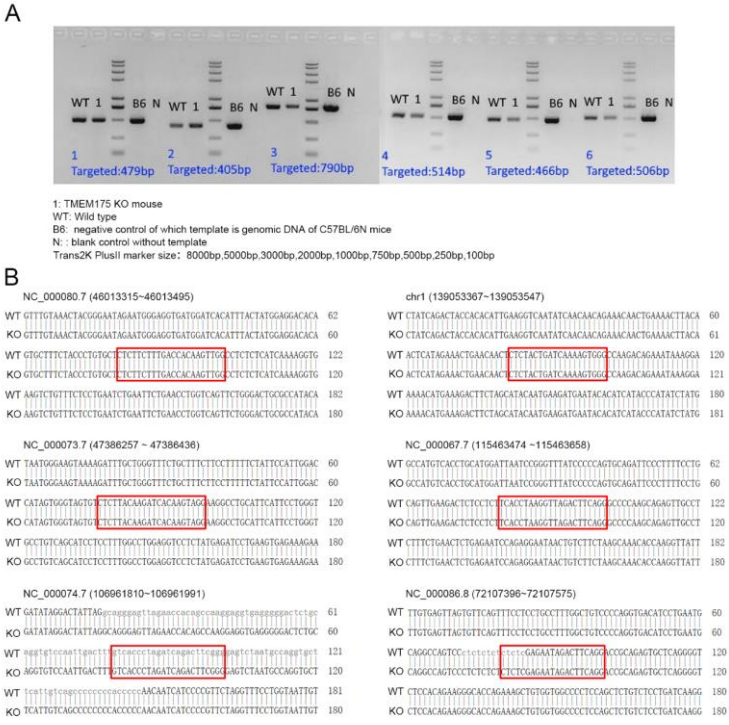

Fig R6: Off-target detection of the TMEM175 KO mice.

(A) Electrophoresis of the PCR products of 6 potential off-target sites with the highest probability. (B) Comparison of the DNA sequences of WT and KO mice at the suspected off-target sites.

Dear Prof. Cang

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the revision has strengthened your study, but they also indicate that further experiments and clarification will be required before the study can be published. I would like to give you the chance to address the remaining reviewer concerns in a second round of revision. Please also provide a point-by-point response.

From the editorial side, there are also a number of points that need your attention.

1) Data availability section: This section should be used to provide access to data deposited in external databases. In case you have not generated such data, please simply state this, e.g. 'No data have been deposited in a public database.' or alike.

2) References: please list only the first 10 authors, followed by et al.

3) Please provide the Expanded View figures as individual figure files, i.e., one file per figure.

4) Please also note that the supplemental material is not limited to 5 EV figures. Additional supplementary figures or tables can be provided in the form of an Appendix. The Appendix is a single PDF file called *Appendix*, which should start with a short Table of Content incl. page numbers. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. The size of the Appendix is not limited.

5) Please provide callouts to the following figure panels:
Fig EV1 panels and Fig EV5E.

6) During our routine image analysis we noticed that the blots in Figure 7E appear to have high contrast settings. Please provide the blots with as little processing as possible in the figure and please also provide the unmodified source data for these blots.

7) I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In the revised MS by Qu et al., the authors made significant efforts and added substantial amount of new data to address the reviewers' comments. It remains to be an interesting and comprehensive story. However, it is worrisome that some of the discrepancies in the results from different experiments were not fully explained. For example, based on the results in Fig. 4D, one would expect that the stimulatory effects of Bcl-2 inhibitors should be seen in the intact cells, e.g., whole-cell recordings, but not in the isolated lysosomes, as the proposed ROS-dependent mechanisms are operative only under the former, but not the latter conditions. On the other hand, the inhibitory effects of the Bcl-2 proteins are expected to be seen in both whole-cell and lysosomal recordings. Note that GSH treatment almost completely (> 90%) abolished the activation effect of HA 14-1, suggesting that slow ROS increases might underlie the activation effects of Bcl-2 inhibitors and apoptosis/ROS-inducers. Therefore, it is puzzling that robust activation by HA 14-1 was seen in the lysosomal recordings (Fig. EV2I, J). No time course (of activation) was shown. Overall, the recordings on the endogenous lysosomal currents were not convincing in the current study. Were those blind experiments? If there were seal instability issues even for non-transfected cells, how can you be confident about your study dealing with much smaller endogenous currents over the time course of 20 min or more? In my view, the proposed mechanisms regarding Bcl-2 regulation of TMEM175 may not require lysosomal recordings in the current study, especially if those data are not convincing, and the results are inconsistent with the proposed model. The results from the Bcl-2 KO cells are very interesting. Have you tested whether there are any remaining activation effects of HA 14-1, as expected for the Bcl-2-dependent and Bcl-2-independent interpretation? Finally, as Dr. Cang was also a co-author in the Wie et al paper, and

conclusions in these two studies, neurodegeneration vs. neuroprotection, are completely opposite (!), more extensive discussions about this major discrepancy are required.

Referee #2:

The authors have in the main adequately addressed my comments, with considered manuscript changes and additional experiments. A couple of comments on the revised manuscript below that I think should be addressed.

I think it would be useful to the reader to discuss the rationale for the discrepancy with Wie et al. The explanation that the Wie et al study involved heterozygous loss compared with homozygous loss used here supports a dual role for TMEM175 in PD has implications for targeting TMEM175 in PD as argued in the Abstract. I think these implications should be discussed.

The authors show new data supporting interaction between endogenous TMEM175 and Bcl-2. What is the "a-Ctrl" lane in this data? The authors should define this in the legend. As requested in the original review, they also need to show a TMEM175 blot of the IP (now Fig 2I). Recommend that the authors align the Bcl-2 blots rather than crop the blots differently.

The explanation regarding the effect of the WEHI-539 and the respective binding affinities of Bcl-2 and Bcl-xl is a reasonable one. Suggest they also incorporate this discussion in their text.

In Figure 6 death assays I made a comment regarding clearly defining error bars and showing individual data points, which the authors now show for the n=4 experiments. However, I am puzzled why the SEM, n=4 error bars are now different than in the original submission despite being SEM of the same data? Can the authors explain this?

Point-by-Point Responses

Introduction:

We thank the reviewers and editors for your effort in processing our manuscript. We have revised the manuscript to address the points raised by the reviewers. The comments are discussed in detail in the point-by-point responses.

Editor

From the editorial side, there are also a number of points that need your attention.

1) Data availability section: This section should be used to provide access to data deposited in external databases. In case you have not generated such data, please simply state this, e.g. 'No data have been deposited in a public database.' or alike.

Response:

We have not generated data that needs to be deposited in external databases in this study. The statement “No data have been deposited in a public database” has been added to the Data availability section.

2) References: please list only the first 10 authors, followed by et al.

Response:

The references have been revised as requested.

3) Please provide the Expanded View figures as individual figure files, i.e., one file per figure.

Response:

Individual figure files for Expanded View figures are provided.

*4) Please also note that the supplemental material is not limited to 5 EV figures. Additional supplementary figures or tables can be provided in the form of an Appendix. The Appendix is a single PDF file called *Appendix*, which should start with a short Table of Content incl. page numbers. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. The size of the Appendix is not limited.*

Response:

We have moved Fig EV2C to Appendix as Appendix Figure S1. A new figure showing the effect of WEHI-539 at low concentration (10 nM) is added as Appendix Figure S2.

5) Please provide callouts to the following figure panels:

Fig EV1 panels and Fig EV5E.

Response:

The callouts for the panels have been provided in the text.

6) During our routine image analysis we noticed that the blots in Figure 7E appear to have high contrast settings. Please provide the blots with as little processing as possible in the figure and please also provide the unmodified source data for these blots.

Response:

Figure 7E has been revised as requested. Unmodified images for these blots are

provided.

7) I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

Response:

A revised manuscript file with tracked changes is also submitted along with a final version of manuscript.

Referee #1:

In the revised MS by Qu et al., the authors made significant efforts and added substantial amount of new data to address the reviewers' comments. It remains to be an interesting and comprehensive story. However, it is worrisome that some of the discrepancies in the results from different experiments were not fully explained.

For example, based on the results in Fig. 4D, one would expect that the stimulatory effects of Bcl-2 inhibitors should be seen in the intact cells, e.g., whole-cell recordings, but not in the isolated lysosomes, as the proposed ROS-dependent mechanisms are operative only under the former, but not the latter conditions. On the other hand, the inhibitory effects of the Bcl-2 proteins are expected to be seen in both whole-cell and lysosomal recordings. Note that GSH treatment almost completely (> 90%) abolished the activation effect of HA 14-1, suggesting that slow ROS increases might underlie the activation effects of Bcl-2 inhibitors and apoptosis/ROS-inducers. Therefore, it is puzzling that robust activation by HA 14-1 was seen in the lysosomal recordings (Fig. EV2I, J). No time course (of activation) was shown. Overall, the recordings on the endogenous lysosomal currents were not convincing in the current study. Were those blind experiments? If there were seal instability issues even for non-transfected cells, how can you be confident about your study dealing with much smaller endogenous currents over the time course of 20 min or more? In my view, the proposed mechanisms regarding Bcl-2 regulation of TMEM175 may not require lysosomal recordings in the current study, especially if those data are not convincing, and the results are inconsistent with the proposed model.

Response:

In the first round of revision, we added the data on the effects of Bcl-2 peptide and HA14-1 on endogenous lysosomal TMEM175 currents (Fig EV2G–J in last revision) as requested by the reviewer. HA14-1 did activate endogenous lysosomal currents, but the activation was much weaker than that in whole-cell recordings (Fig EV2J compared to Fig 1C). Although the activation effect of HA14-1 was largely abolished by GSH, a small fraction of activation remained. Therefore, we believe that HA14-1 activates TMEM175 through both ROS-dependent and ROS-independent mechanisms. The HA14-1-induced activation we found in the lysosomal recordings should only involve the ROS-independent mechanism and thus do not represent the full view of the regulation of TMEM175 by Bcl-2. We agree with the reviewer that these data are not required in the current study and are even somewhat misleading. Therefore, we have removed the Fig EV2I, J in this revision.

Lysosomal recording is generally more stable than whole-cell recording and can therefore

handle smaller endogenous currents. There are several reasons for this. First, some issues that may disturb the stability of whole-cell recording are not present in lysosomal recording configuration, such as decreased cell viability or activation of intracellular signaling pathways that affect plasma membrane stability. Second, lysosomal membrane has fewer types and number of proteins than plasma membrane. Third, the recording pipette sometimes drift slightly due to mechanical reasons during long-term recordings. In whole-cell recordings, this drift will cause relative displacement at the contact interface of the electrode tip and the cell membrane, which may affect the recording stability. In lysosome recordings, the tiny and free lysosomes can be attached to the pipette tip as a whole and move with it, avoiding the instability caused by drifting of pipette.

The results from the Bcl-2 KO cells are very interesting. Have you tested whether there are any remaining activation effects of HA 14-1, as expected for the Bcl-2-dependent and Bcl-2-independent interpretation?

Response:

We have performed new experiments to test the effect of HA14-1 in Bcl-2 KO cells. Although the activation was largely abolished, HA14-1 still induced a two-fold increase in TMEM175 currents (included in revised manuscript as new Fig EV2E–G). This result further indicates that there are both Bcl-2-dependent and Bcl-2-independent mechanisms underlying the activation of TMEM175 by HA14-1. This may also be the reason why HA14-1 works better than other Bcl-2 inhibitors in activating TMEM175.

Finally, as Dr. Cang was also a co-author in the Wie et al paper, and conclusions in these two studies, neurodegeneration vs. neuroprotection, are completely opposite (!), more extensive discussions about this major discrepancy are required.

Response:

In the Wie et al paper, Dr. Cang only contributed some early exploratory recordings demonstrating that the AKT activator SC79 activates TMEM175 and that TMEM175-KO lysosomes lacks TMEM175 currents. The conclusions of the two papers on neurodegeneration and neuroprotection were based on data from different PD models and different animals, suggesting that TMEM175 may play different roles in different types of Parkinson's disease. In addition, the extent to which TMEM175 function is altered may have completely different effects on PD.

We have added more extensive discussion about this discrepancy in the revised manuscript.

Referee #2:

The authors have in the main adequately addressed my comments, with considered manuscript changes and additional experiments. A couple of comments on the revised manuscript below that I think should be addressed.

I think it would be useful to the reader to discuss the rationale for the discrepancy with Wie et al. The explanation that the Wie et al study involved heterozygous loss compared with

homozygous loss used here supports a dual role for TMEM175 in PD has implications for targeting TMEM175 in PD as argued in the Abstract. I think these implications should be discussed.

Response:

We have discussed the implication in the current version of manuscript.

The authors show new data supporting interaction between endogenous TMEM175 and Bcl-2. What is the "a-Ctrl" lane in this data? The authors should define this in the legend. As requested in the original review, they also need to show a TMEM175 blot of the IP (now Fig 2I). Recommend that the authors align the Bcl-2 blots rather than crop the blots differently.

Response:

The α-Ctrl in Fig 2I represents that the magnetic beads used in IP were not pre-incubated with any antibody. It was described in the “Materials and Methods” part and we have now described it in the legend as suggested. We have also added the TMEM175 blots of the IP and aligned the Bcl-2 blots in Fig 2I.

The explanation regarding the effect of the WEHI-539 and the respective binding affinities of Bcl-2 and Bcl-xl is a reasonable one. Suggest they also incorporate this discussion in their text.

Response:

We thank the reviewer for the suggestion. This discussion has been incorporated in the “Results” section.

In Figure 6 death assays I made a comment regarding clearly defining error bars and showing individual data points, which the authors now show for the n=4 experiments. However, I am puzzled why the SEM, n=4 error bars are now different than in the original submission despite being SEM of the same data? Can the authors explain this?

Response:

We thank the reviewer for the meticulous review. In the original submission, one of the authors carelessly used the wrong formula when calculating the SEM for this set of data, and we amended it accordingly when submitting the revision. We apologize for the mistake. We have now thoroughly checked all the data and ensured their accuracy in the current version.

Dear Prof. Cang

Thank you for the submission of your revised manuscript to EMBO reports. It was assessed again by referee 1 who now supports publication.

Thank you also for supplying the source data for Fig. 7. You outlined that the band you observe for TMEM175 in the KO brains is likely unspecific. I would like to point out that it is not good scientific practice to selectively remove data by increasing the contrast and brightness settings. While the residual band could indeed be unspecific, as you suggest, it could also constitute residual protein that is stable. With the current data is difficult to unambiguously discriminate between these options. Since the TMEM175 KO phenotype you observe is distinct from the phenotype reported before, I feel that it is important to further validate the KO. I have discussed this issue also with our chief editor, Bernd Pulverer, and we both agree that this issue needs to be resolved before the study can be published in EMBO reports.

Does the antibody work in immunostainings and could you verify the absence of TMEM175 protein in the KO mice using this technique? Alternatively, you might run the gel for a prolonged period or on a lower percentage gel to resolve the band. An IP of TMEM175 prior to running the sample on the Western blot might also help to remove unspecific bands. In either case, additional data will be required to validate the TMEM175 KO. Please also provide the source data for the mRNA quantification shown in Fig. 7D.

There are also some editorial issues that need your attention:

- Legend for Figure 4: You indicate for panel (M) that n refers to the number of cells counted within one experiment but in 'Data information' you indicate that 'n' means the number of biological replicates. Please clarify whether the measurements in the other panels were indeed from independent replicates, i.e., cells from different preparations/experiments or whether the same applies as for (M), i.e., cells from one experiment were counted.
- The same applies to Fig. 5, Fig. EV2, EV4, and EV5, Data information. Please note that different cells in one dish are not true biological replicates but rather technical replicates. Please be clear whether you quantified different cells within one experiment or whether you quantified 'n' cells from 'N' independent experiments.

- Please remove the Appendix legends from the main manuscript file.

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee 1:
[recommends publication without further revision]

Dear Dr. Rembold,

Thank you for giving us the chance to revise our manuscript "Lysosomal K^+ Channel TMEM175 Promotes Apoptosis and Aggravates Symptoms of Parkinson's Disease" (Ms. No. EMBOR-2021-53234). We have completed the revision and are pleased to submit it for your consideration.

We have made the following changes to address your concerns.

1. We agree that the source data for Fig. 7E were not convincing enough. So, we improved experimental conditions and verified the knockout of TMEM175 in mice again using Western blotting. Firstly, we significantly shortened the time to dissect the brain tissue on ice. Secondly, we lysed the protein right away instead of storing the brains at -80°C. Thirdly, freshly-made RIPA lysis buffer (Biosharp) containing a working concentration of protease inhibitor (Thermo Fisher, A32953), 10 mM sodium fluoride and 1 mM sodium orthovanadate were used. Fourthly, we homogenized the tissue using a precooled glass Dounce homogenizer on ice this time. And fifthly, the protein samples were centrifuged at 18506 ×g at 4°C for 10 min to obtain the supernatants. Fortunately, we got a much better result this time. As shown in the revised Figure 7E and the source data, there was no visible band in the KO lane at the position of TMEM175. The source data of western blotting were included in the Figure7_Source_Data.zip file. Besides the unprocessed raw images, we also provided images with labels for your convenience. We have also made corresponding changes in the method section of western blotting.

2. To further validate the KO of TMEM175, we repeated the RT-qPCR experiment with midbrains of three WT and three KO mice and got the same results as before. These new data were included in the revised Figure 7D. The source data of RT-qPCR were also included in the Figure7_Source_Data.zip file, including the original files generated by the Roche LightCycler® 96 System (.lc96p files), the exported data file for each trial (.txt files), the summary of the data (RT-qPCR data summary.xlsx) and the summary of calculation (RT-qPCR calculation.xlsx).

3. The n values in Figs. 4M, 5G, 5I, EV2B, EV4D refer to the numbers of cells counted within one experiment. We have now clearly stated in the data information that the n values in these panels mean the number of technical replicates. We have also checked other data and confirmed that n values in other data mean the number of biological replicates (data collected from different dishes of cells or different animals). For example, in patch clamp recordings, we performed only one recording in the same dish of cells.

4. We have removed the Appendix legends from the main manuscript file.

We very much hope the revised manuscript is acceptable for publication in EMBO reports and look forward to hearing from you soon.

With best wishes,

Chunlei Cang
On behalf of the authors

3rd Revision - Editorial Decision **2nd Jun 2022**

Dear Chunlei,

Thank you once more for submitting your further revised manuscript and for providing new Western blot data for Figure 7E and the source data for Fig. 7E and the RT-qPCR in Fig. 7D. I have meanwhile uploaded the updated source data to your manuscript. Please check the uploaded files.

As you know, I have discussed the new data once more with our chief editor, Bernd Pulverer. We both agreed that the new blot looks cleaner and more specific, now that you modified the procedure to prepare brain lysate for blotting. However, given that the antibody seems to cross-react with an unspecific band, which you noticed in your own experiments and likely also in another publication (https://doi.org/10.1038/s41586-021-03185-z), and given that the phenotype of your TMEM175 KO mice differs from that previously published, we felt that it is in our and your best interest to verify the KO and the specificity of the antibody as rigorously as possible.

In response to this you have meanwhile supplied data on antibody verification on HEK293 cell and on brain lysate. Having this validation data at hand, together with the RT-PCR and Western blot data of the TMEM175 KO, we have decided to move forward and proceed with the publication of your manuscript. Please include the data on antibody validation in your manuscript, e.g., as an Appendix figure.

Moreover, we need you to update the 'Conflict of interest' paragraph to our new 'Disclosure and competing interests statement'. For more information see
https://www.embopress.org/page/journal/14693178/authorguide#conflictsofinterest

I am looking forward to receiving the final version of your manuscript.

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

June 4, 2022

Dear Dr. Rembold,

Thank you for giving us the chance to revise our manuscript “Lysosomal K^+ Channel TMEM175 Promotes Apoptosis and Aggravates Symptoms of Parkinson's Disease” (Ms. No. EMBOR-2021-53234).

As per your request, we have included the data on antibody validation in the revised manuscript as Appendix Figure S3, and have updated the 'Conflict of interest' to 'Disclosure and competing interests statement'.

We hope the revised manuscript is acceptable for publication in EMBO reports and look forward to hearing from you soon.

With best wishes,

Chunlei Cang
On behalf of the authors

Prof. Chunlei Cang
University of Science and Technology of China
School of Basic Medical Sciences
443 Huangshan Road
Hefei, Anhui 230027
China

Dear Prof. Cang,

Thank you for sending your further revised manuscript. I am now very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: Chunlei Cang
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2021-53234

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	At least 3 independent triplicates were performed in all experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size is shown in each figure or described in respective figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All samples and animals were analyzed.
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For animal studies, include a statement about randomization even if no randomization was used.	The statement has been included in "Animals"section of "Materials and Methods".
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For cylinder and wire hang tests, the videos were scored by an experimenter blinded to the genotype and treatment condition within the experiment.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Animal behavior studies data were analyzed by the experimenter unaware of the study design.
5. For every figure, are statistical tests justified as appropriate?	Statistical tests used to derive the significance are described in the respective figure legends section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistically significant differences were determined using Student t-test or post hoc tests with ANOVA.
Is there an estimate of variation within each group of data?	Standard error of the mean were calculated as indicated.

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Is the variance similar between the groups that are being statistically compared?	The variation was similar.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	They have been provided in the materials and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T, Hela and SH-SY5Y cells were obtained from the American Type Culture Collection. All cell lines were regularly tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Can be found in the manuscript materials and methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Can be found in the manuscript materials and methods section.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Can be found in the manuscript materials and methods section.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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