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NDST3 Deacetylates α-tubulin and Suppresses V-ATPase Assembly and Lysosomal Acidification

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1st Editorial Decision 10th Dec 2020

Thank you for submitting your manuscript entitled "NDST3 catalyzes deacetylation of α-tubulin and suppresses V-ATPase assembly and lysosomal acidification" [EMBOJ-2020-107204] to the EMBO Journal. Your study has been sent to three referees for evaluation and we have now received their reports, which are enclosed below for your information. In light of these reports, I am afraid that the manuscript is not a sufficiently strong candidate for publication here.

As you will see, while the referees find the study potentially interesting, they also indicate that the data do not support the main conclusion that NDST3-mediated deacetylation of tubulin controls V-ATPase assembly and lysosomal acidification in mammalian cells.

Given these opinions from trusted experts in the field and the extensive additional data that would be needed to substantiate your model, I am afraid that we cannot offer to invite a revised version of your manuscript at this stage.

However, considering the potential interest of your findings, we would be willing to reconsider your study as a new submission at a later time, if the referees' concerns could be fully addressed and their suggestions implemented. Please note that the novelty of your study will be taken into consideration at the time of resubmission.

I thank you for the opportunity to consider this manuscript. While I am sorry that we cannot communicate more positive news, I hope that you will find our referees' comments helpful to improve your manuscript.

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Referee #1:

The work of Tang et al. highlights an interesting novel function of the histone deacetylase NDST3 in the regulation microtubule deacetylation and assembly of the lysosomal V-ATPase. A loss of NDST3 results in increased microtubule acetylation, which in turn promotes the assembly of V-ATPase enzyme and results in over acidification of lysosomes which reduces their degradative capacity. It is a beautiful concept and well-done experiments which create convincing case of MT acetylation/deacetylation and lysosomal pH interdependencies but there are some major gaps on how could this work mechanistically. Hopefully, the authors have a possibility to conduct a few more experiments/ analysis in this direction.

Major comments:

1. Figure 2: It is not directly convinced that NDST3 KO results only to changes in the lysosomal pH. The observed effects might also be a result of changes in the number of lysosomes (acidification of late-endosomes) and/or differences in the size of the lysosomes in the cells. For example, in panel A and B the authors claim that the enhanced fluorescence is an indication of stronger acidified lysosomes. This should be supported with more control experiments/ analysis and not only by measuring a total cell fluorescence. From given representative examples it seems that in KO cells lysosomes are bigger. It would be important to quantification of the lysosomes per cell (or per area) as the authors suggest but do not directly quantify ('... an evaluated lysosomal acidification but not an increased number of lysosomes'). The authors may also check if the fluorescent intensity of LysoTracker/ Acridine Orange scales with the pH as performed in panel Fig.2 F for DND-160. In the panel C LAMP1 shows smaller molecular weight in KO cells. Same in Fig. 4A. Do the authors have an explanation for this?

2. In the manuscript the authors claim that the maturation of Cat. D and B is lysosomal pH dependent (Fig. EV4). Can the authors show some evidence that the treatment performed in Fig. EV4 changes the pH of lysosomes?

3. The authors show very interesting link between the deacetylation capabilities of NDST3 and the assembly of the V-ATPase complex. A reduction of NDST3 leads to stronger acetylated microtubules. How this can work at the mechanistic level? The acetylated Lys40 is inside of the microtubule lumen and is not likely to have any direct impact on the transmembrane V-ATPase complex. There must be some downstream changes in the lattice like other post-translational modifications of MT surface, association of MAPs, ect. Are there any pH-dependent changes in lysosomal motility? Perhaps one can check if lysosomes in NDST3 KO cells are stronger or weaker associated with acetylated MTs? This manuscript will strongly benefit from some live imaging experiments and higher quality colocalization studies (for example lysosomes + ac-MT and lysosomes + tyr-MT, which are usually deacetylated).

4. The part when it comes to neurological phenotypes and ALS is a bit loose from the rest of the manuscript.

5. The overall interdependencies mechanism seems to work well in several types of non-polarized cells. I'm really curious if this is true in neurons. In adult neurons, most of the axonal MTs are acetylated however there are very few functional lysosomes in distal compartments, although LAMP1-positive endo-lysosomes and cathepsin-containing vesicles are present along the entire length. The protein removal is mostly mediated by an autophagy pathway. Autophagosomes are transported towards the proximal axonal compartment and there they fuse with functional lysosomes. Addressing the neuronal interplay of MT acetylation and lysosomal acidification is probably beyond scope of this manuscript but it should be at least discussed.

Minor points:

1. It is highly appreciated that the authors display each of their measurements as dots in the bar graphs. Hereby the standard deviation bars sometimes become hardly visible, especially in graphs with many measurements. For clarity, I would like to ask if the authors can plot the standard deviation bars on the foreground.

2. In the model in Fig. 7H, MT acetylation is shown on the surface of the lattice, which is incorrect.

Referee #2:

In this manuscript, the authors have identified a loss of function mutation in the NDST3 gene in retinal pigment epithelia (RPE) cells as a suppressor of sensitivity to the potent V-ATPase inhibitor bafilomycin. They provide evidence that the NDST3 protein is able to deacetylate a-tubulin and destabilize microtubules. They present a model in which NDST3-mediated deacetylation of tubulin controls V-ATPase assembly and lysosomal acidification, and loss of this activity leads to increased V-ATPase assembly and hyperacidification of the lysosome. Previous data do implicate microtubules in control of V-ATPase assembly, so it is possible that levels of microtubule deacetylation could affect V-ATPase assembly and activity. Compromised lysosomal acidification is associated with several neurological diseases, including ALS, but hyperacidification has not been observed in these diseases and in fact, is quite rare. Thus, if correct, this model could be very significant. However, as it stands the data do not fully support the model, some results are not fully explained, and alternative explanations need to be considered.

Major issues to be considered:
1). The bafilomycin-resistant phenotype of the NDST3 mutant is not easily explained by increased assembly of the V-ATPase, and this is central to the model. Bafilomycin inhibits by binding to the membrane domain of the enzyme and preventing rotation, and simply assembling more of the V1 subcomplex on the membrane would not overcome this inhibition. The mechanism of bafilomycin resistance in the NDST3 mutant, which is the starting point for the study, could be addressed more thoroughly by showing that lysosomes are actually acidified in the NDST3 mutant, but not the wild-type cells, in the presence of high bafilomycin concentrations.
2). Throughout the paper the authors assume that it is the lysosomal V-ATPase that is affected by the NDST3 mutant. However, some of the results could be explained by altered trafficking en route to the lysosome, which could also arise from altered microtubule function and could result in mislocalization of V-ATPases, increased assembly of V-ATPases resident in other organelles and even hyperacidification of compartments other than the vacuole. In Figure 2, there is more staining with fluorescent weak bases like Lysotracker, but it would also be good to demonstrate that Lysotracker colocalizes well with a lysosomal protein like LAMP1 (Figure 2C does not address this) in order to rule out increased acidity in multiple compartments. Increased acidification of earlier compartments in the secretory pathway due to trafficking defects could explain the reduced co-localization of Bodipy-pepstatin with LAMP1 in the mutant (Figure 3L and M) as well as altered maturation of lysosomal proteases, so this possibility must be addressed.

Minor points:
3). In other places, the authors do not fully address the results they show. For example, why is the mobility of LAMP1 on SDS-PAGE different between wt and the NDST3 KO in Figures 2 and 3? (This could suggest altered modification as a result of perturbed trafficking to the lysosome.). Why would the NDST3 KO incorporate 50% more 14C valine in Figure 3G? This result undermines the claim that protein degradation is reduced from Figure 3H. In Figure 5, why does the ratio of V0D to LAMP1 increase in TSA, to about the same extent as the increase in the V1A or V1C to V0D ratios? Using a "correction coefficient" does not explain the unexpected effect on V0D. While these are not individually major points, they undermine the overall story.
4). In Figure EV4, the authors argue that because less mature cathepsin is seen in cells clamped to either lower pH or higher pH, then low lysosomal pH must compromise protease maturation. This is not a legitimate argument, since the agents used to clamp pH will also perturb trafficking to the lysosome and this could cause accumulation of immature forms of the protease.

Referee #3:

The manuscript by Tang and colleagues proposes that the protein NDST3 regulates lysosomal acidification by promoting acetylation of alpha-tubulin. The authors identified NDST3 and ATF2 as necessary factors for bafilomycin-induced cell death through a CRISPR screen. The follow-up lead the authors to focus only on NDST3.
The conclusions on the role of NDST3 on the lysosomes are not supported by the data. There are key controls lacking. Some of the methodology is innapropriate (in particular, lysosomal pH assessment needs to be completely redone).
One key aspect that remains completely ignored is the reason why cells die when treated with bafilomycin. This question has been explored (PMID 28296633, 31793879, 31983508), and it has been shown unequivocally that bafilomycin-induced death is due to iron deficiency. Nevertheless, this theme was not even brough up by the authors. Given that restoring cytoplasmic iron homeostasis is sufficient to rescue bafilomycin-induced death, it is pivotal to test if the hits that the authors got on the CRISPR screen interfere with the iron homeostasis pathway. Secondly, there are numerous factors that regulate the acetylation of alpha-tubulin, but none of them appeared in the CRISPR screen. Third, the rescue experiments are only able to recover a small part of the phenotypes.

- 1G - expression of FLAG-NDST3 in NDST3-KO cells results in 40-50% viability, which is ~5-fold higher than the WT cells. This is a big difference for what one would expect from a "rescue" experiment. What is the reason for this?

2A-2C - Lisotracker staining is not just dependent on the acidic pH, it is also dependent on the size of the lysosomal particles, which appear to be bigger in the NDST3-KO cells. The size of the lysosomes should be quantified (for example, by immunocytochemistry against LAMP1. Furthermore, per the images shown, there is too much lisotracker staining outside the expected lysosomal particles, which raises further doubts that this experiment can be informative at all. In the acridine orange experiment, the one result that seems evident is, again, the increase in the size of the lysosomal particles. The fact that there is no change in the amount of LAMP1 protein does not mean that there is no change in the amount of LAMP1 per lysosomal particle.

2D-2G - Lysosensor is a set of dies that are known to buffer the lysosomal pH, and therefore to introduce perturbations in the very system they are supposed to measure. For this reason, the assessment of lysosomal pH needs to be done by a different method, for example using dextrans or a genetically-encoded probe. It is also fundamental to measure the lysosomal pH in cells lacking NDST3 that are treated with bafilomycin. The authors are implying that NDST3 can increase lysosomal acidity, and for that reason counteract the effect of bafilomycin, therefore that very idea has to be demonstrated. Otherwise, the possibility remains that NDST3 is affecting other components besides the vATPase.

3A-3D - no effect in autophagy is observed between the WT and NDST3-KO cells in basal conditions. This does not seem compatible with an effect of NDST3 specifically in the lysosomal component. Again, it would be important to show what happens to the autophagic flux in WT and KO cells treated with bafilomycin for a long time. Importantly, the chloroquine experiment in 3D argues that there is less autophagosomal cargo being delivered to the lysosomes in NDST3-KO cells, because less of it accumulates when the lysosomes are shut down by the cloroquine (and the

effect of chloroquine is to serve as a short-circuit of the lysosomal pH gradient, so even if NDST3 would be working to acidify the lysosomes, it would not be able to restore acidity to cloroquine-treated lysosomes - the experiment would have been a lot more informative with bafilomycin, for the reasons already stated above.

3G-3H - the measurement of protein stability cannot be used as a readout for lysosomal function, as there are too many other interfering components (for example, the protein synthesis rate was different between WT and NDST3-KO cells, which alone makes the interpretation of this result borderline impossible; secondly, the authors used whole cells, which is not able to distinguish between lysosomal proteolysis, excretion towards the extracellular space, proteasomal degradation, etc. If the experiment would have been done with isolated lysosomes, it might be a closer readout, provided the protein synthesis and incorporation of the tracer was similar. In these conditions, I don't see how this experiment can be used.
3I-K - these results do not imply that the lysosomal pH is more acidic. They show that there is less mature cathepsins, but it doesn't show if this is an issue of maturation (i.e., lysosomal or expression (i.e., lower transcriptional levels?. The outcome is that there is less cathepsin activity, but this is not an evidence that the pH is more acidic.

4A - there seems to be also more V1C1 in cytoplasm in the KO cells. The total levels would be important here.

4E-4F - the effect of the KO on a-tub acetylation is much higher than the observed when the protein is reexpressed. As mentioned above, this raises questions regarding the specificity of the findings.

Dear Editor and Reviewers:

Thank you very much for the detailed and insightful suggestions on improving our manuscript. We appreciate that the editor and reviewers considered our study interesting and novel. We have conducted several lines of new experiments and addressed all reviewers' main concerns. Accordingly, we have made multiple revisions in the figures and throughout the text. Altogether, these revisions have significantly strengthened our conclusions and substantially improved the manuscript, which we hope is now suitable for publication.

Our itemized response to the comments of each reviewer is presented below.

Referee #1:

The work of Tang et al. highlights an interesting novel function of the histone deacetylase NDST3 in the regulation microtubule deacetylation and assembly of the lysosomal V-ATPase. A loss of NDST3 results in increased microtubule acetylation, which in turn promotes the assembly of V-ATPase enzyme and results in over acidification of lysosomes which reduces their degradative capacity. It is a beautiful concept and well-done experiments which create convincing case of MT acetylation/deacetylation and lysosomal pH interdependencies but there are some major gaps on how could this work mechanistically. Hopefully, the authors have a possibility to conduct a few more experiments/ analysis in this direction.

Reply: We have performed new experiments and provided further findings on the mechanism of the pathways. The detailed results are described in the replies to the comments below.

Major comments:

1. Figure 2: It is not directly convinced that NDST3 KO results only to changes in the lysosomal pH. The observed effects might also be a result of changes in the number of lysosomes (acidification of late-endosomes) and/or differences in the size of the lysosomes in the cells. For example, in panel A and B the authors claim that the enhanced fluorescence is an indication of stronger acidified lysosomes. This should be supported with more control experiments/ analysis and not only by measuring a total cell fluorescence. From given representative examples it seems that in KO cells lysosomes are bigger. It would be important to quantification of the lysosomes per cell (or per area) as the authors suggest but do not directly quantify ('... an evaluated lysosomal acidification but not an increased number of lysosomes'). The authors may also check if the fluorescent intensity of LysoTracker/ Acridine Orange scales with the pH as performed in panel Fig.2 F for DND-160. In the panel C LAMP1 shows smaller molecular weight in KO cells. Same in Fig. 4A. Do the authors have an explanation for this?

Reply: We appreciate the reviewer's comments and suggestions on Figure 2. We agree with the reviewer that the experiments using only LysoTracker or Acridine orange staining may not be sufficient to represent the changes in the lysosomal pH, since these dyes are not very sensitive to small changes in pH. In the revised manuscript, we have added several new lines of evidence supporting conclusions. First, we have now added new results using a well-established ratiometric dye for measuring lysosomal pH (i.e. LysoSensor dextran). This dye enters the lysosome via endocytosis and provides a ratiometric measurement of the lysosomal pH independent of lysosomal size, number, or other potential variables. Together with our initial results using LysoSensor DND-160 (a free cell-permeant ratiometric dye preferably accumulating in acidic organelles such as lysosome), these measurements consistently demonstrate a decrease in the lysosomal pH upon loss of NDST3. These results are now shown in Fig 2A-D and Fig EV2 in the revised manuscript. Second, we have performed a few control experiments to support the LysoTracker-based assays. There was no change in the number or size of the lysosomes as a result of loss of NDST3 (Fig EV2A), and nearly all of the acidic vesicles stained with the LysoTracker probe were LAMP1-GFP-positive lysosomes (Fig EV2F), indicating that the observed increase in the staining of LysoTracker reflects the increased acidification of the lysosomes (Fig EV2B). Lastly, the decrease of lysosomal pH observed in NDST3 KO cells in our study is consistent with a previous report showing that the

treatment with trichostatin A (TSA, an inhibitor of HDAC6, thus capable of increasing the microtubule acetylation level) also reduced lysosomal pH (Eriksson *et al*, 2013).

We also thank the reviewer for pointing out the smaller molecular weight of LAMP1 in NDS3 KO cells in the western blotting results. We have found that it was due to a change in the glycosylation of LAMP1. Treatment with swainsonine (SWN), an inhibitor of protein glycosylation, resulted in the same shift in the migration of LAMP1 from WT cells while not affecting LAMP1 from NDST3 KO cells (Appendix Figure S4A), suggesting the migration shift was a consequence of deglycosylation of LAMP1. The SWN treatment alone did not cause lysosomal hyperacidification or dysfunction (Appendix Figure S4B and C), as observed in NDST3 KO cells, indicating that the deglycosylation of LAMP1 was not responsible for the changes in lysosomal pH and functions in the NDST3 KO cells. We have added the explanation in the manuscript (Page 9, paragraph 2).

2. In the manuscript the authors claim that the maturation of Cat. D and B is lysosomal pH dependent (Fig. EV4). Can the authors show some evidence that the treatment performed in Fig. EV4 changes the pH of lysosomes?

Reply: To address the reviewer's comments, we have performed new experiments to confirm that the cathepsin enzyme activity is indeed dependent on the pH in the environment. To remove the complication from treating the whole cells with buffers of different pH, we have now performed an *in vitro* cathepsin B maturation activity assay to confirm that the activation of cathepsin B indeed depends on the pH of the environment. Using recombinant Cat B protein to measure the ration of mature Cat B to its precursor form, as incubated in the activation buffer with a series of pH values, we showed that both over- and under-acidification would impair the maturation of Cat B (Cat D was not examined due to lack of similar assays available to us). These observations are consistent with a previous report that over-acidification of the lysosome suppresses the proteolytic activity of cathepsins (Mach *et al*, 1994). The newly added data and descriptions are shown in Fig EV3A and Page 7, paragraph 2.

3. The authors show very interesting link between the deacetylation capabilities of NDST3 and the assembly of the V-ATPase complex. A reduction of NDST3 leads to stronger acetylated microtubules. How this can work at the mechanistic level? The acetylated Lys40 is inside of the microtubule lumen and is not likely to have any direct impact on the transmembrane V-ATPase complex. There must be some downstream changes in the lattice like other post-translational modifications of MT surface, association of MAPs, ect. Are there any pH-dependent changes in lysosomal motility? Perhaps one can check if lysosomes in NDST3 KO cells are stronger or weaker associated with acetylated MTs? This manuscript will strongly benefit from some live imaging experiments and higher quality colocalization studies (for example lysosomes + ac-MT and lysosomes + tyr-MT, which are usually deacetylated).

Reply: We thank the reviewer for raising these questions on the mechanism through which NDST3 regulates the assembly of the V-ATPase complex. We have provided the following new results indicating a mechanism in which loss of NDST3 increases microtubule acetylation and stability, which in turn facilitate the trafficking and assembly of the V-ATPase subunits on the lysosomes.

i) First, to test whether the microtubule is required for the recruitment of V-ATPase V1 subunits to the lysosome, we treated RPE1 cells with a microtubule depolymerization agent nocodazole (Noco) or a stabilizing agent Taxol, and observed significantly decreased or increased colocalization of ATP6V1C1 with the lysosomes marked by LAMP1-GFP, respectively (Fig EV4 A and B), suggesting that the microtubule stability is critical for the recruitment of V1 subunits to the lysosome.

ii) Next, to understand the role of microtubule acetylation in the recruitment of V1 subunits to the lysosome, we analyzed the localization pattern of lysosomes marked by LAMP1-GFP in relationship to ATP6V1C1 and α-tubulin acetylated at Lys40 via immunostaining in RPE1 cells. LAMP1-GFP and ATP6V1C1 colocalized in a perinuclear area where acetylated microtubules are relatively enriched (Fig 4A), suggesting that the acetylated microtubules help spatially

constrain the lysosomes and V-ATPase V1 subunits in the perinuclear area.

iii) Furthermore, to test whether microtubule acetylation plays a crucial role in the assembly of the V-ATPase holoenzyme, we modulated the level/activity of HDAC6, a α-tubulin deacetylase, or treated the cells with Noco, which substantially reduced the level of microtubule acetylation, and confirmed that microtubule acetylation plays a critical role in mediating the V-ATPase holoenzyme assembly (Fig 4C-L).

iv) Lastly, we observed higher levels of microtubule acetylation (Fig 4M and N) and increased colocalization of the lysosomes and V-ATPase V1 subunits (Fig 4M and O) in NDST3 KO cells, consistent with our data in the last submission that HDAC6 decreased the V-ATPase assembly (Fig 4P-T).

Taken together, our data indicate that the increase in the assembly of the V-ATPase V1-V0 holoenzyme induced by loss of NDST3 was dependent on the microtubule acetylation, which increases microtubule stability and enhances the colocalization of the lysosomes and V-ATPase subunits (Page 10-12). We thank the reviewer for the suggestions on the colocalization studies, which have significantly strengthened the evidence in support of our conclusions.

4. The part when it comes to neurological phenotypes and ALS is a bit loose from the rest of the manuscript.

Reply: We have now strengthened the data that support an important implication of NDST3 in the disease mechanism underlying C9orf72-linked ALS. In the revised manuscript, we have added new results showing a regulation of NDST3 by C9orf72 and described the connection of NDST3 to C9orf72-linked ALS in three levels.

i) First, we found that NDST3 is down-regulated in patients' tissues and cells carrying the C9orf72 hexanucleotide repeat expansions (Fig 6A-D).

ii) One of the consequences of the hexanucleotide repeat expansions is reduced expression of C9orf72 in patient cells and tissues. We found that the reduction of NDST3 in the patient tissues is a result of the C9orf72 haploinsufficiency, as demonstrated by a positive regulation of C9orf72 on NDST3 protein levels in cell models (Fig 6G-L).

iii) Functionally, we demonstrated that loss of NDST3 exacerbates the proteotoxicity of poly-dipeptide repeats generated by the C9orf72 repeat expansions, an effect that is dependent on microtubule integrity (Fig 6M-P).

Taken together, our results (Fig 6 and Page 13-14) demonstrate that NDST3 provides an important link between the loss-of-function and gained toxicity mechanisms of C9orf72-linked ALS, a finding that should be of interest to the readers in the field of ALS and neurodegeneration.

5. The overall interdependencies mechanism seems to work well in several types of non-polarized cells. I'm really curious if this is true in neurons. In adult neurons, most of the axonal MTs are acetylated however there are very few functional lysosomes in distal compartments, although LAMP1-positive endo-lysosomes and cathepsin-containing vesicles are present along the entire length. The protein removal is mostly mediated by an autophagy pathway. Autophagosomes are transported towards the proximal axonal compartment and there they fuse with functional lysosomes. Addressing the neuronal interplay of MT acetylation and lysosomal acidification is probably beyond scope of this manuscript but it should be at least discussed.

Reply: We appreciate very much the reviewer sharing the insights into the transport and localization of lysosomes/autophagosomes in neurons.

i) In the revised manuscript, we have included new results on the functions of NDST3 in neurons (Fig EV1D-F and Fig EV5D-F), confirming that the resistance to Baf A1 rendered by the loss of NDST3 and the regulatory role of NDST3 on microtubule acetylation can also be observed in neurons.

ii) Following the reviewer's suggestion, we have further discussed the need for future studies to

fully understand the role of NDST3 in the complex interplay of the autophagy-lysosome pathway and the microtubule-meditated transport in neurons and other cells relevant to the diseases in the *Discussion* section (Page 16, Paragraph 1).

Minor points:

1. It is highly appreciated that the authors display each of their measurements as dots in the bar graphs. Hereby the standard deviation bars sometimes become hardly visible, especially in graphs with many measurements. For clarity, I would like to ask if the authors can plot the standard deviation bars on the foreground.

Reply: In the revised manuscript, we have plotted the standard deviation bars on the foreground in all figures.

2. In the model in Fig. 7H, MT acetylation is shown on the surface of the lattice, which is incorrect.

Reply: We thank the reviewer for pointing this out and have redrawn the model, in which the acetylated tubulin is shown facing the microtubule lumen (Fig 7) in the revised manuscript.

Referee #2:

In this manuscript, the authors have identified a loss of function mutation in the NDST3 gene in retinal pigment epithelia (RPE) cells as a suppressor of sensitivity to the potent V-ATPase inhibitor bafilomycin. They provide evidence that the NDST3 protein is able to deacetylate a-tubulin and destabilize microtubules. They present a model in which NDST3-mediated deacetylation of tubulin controls V-ATPase assembly and lysosomal acidification, and loss of this activity leads to increased V-ATPase assembly and hyperacidification of the lysosome. Previous data do implicate microtubules in control of V-ATPase assembly, so it is possible that levels of microtubule deacetylation could affect V-ATPase assembly and activity. Compromised lysosomal acidification is associated with several neurological diseases, including ALS, but hyperacidification has not been observed in these diseases and in fact, is quite rare. Thus, if correct, this model could be very significant. However, as it stands the data do not fully support the model, some results are not fully explained, and alternative explanations need to be considered.

Reply: We appreciate the reviewer's recognition of the significance of our work and have added more experimental data in the revised manuscript to support our conclusions.

Major issues to be considered:
1). The bafilomycin-resistant phenotype of the NDST3 mutant is not easily explained by increased assembly of the V-ATPase, and this is central to the model. Bafilomycin inhibits by binding to the membrane domain of the enzyme and preventing rotation, and simply assembling more of the V1 subcomplex on the membrane would not overcome this inhibition. The mechanism of bafilomycin resistance in the NDST3 mutant, which is the starting point for the study, could be addressed more thoroughly by showing that lysosomes are actually acidified in the NDST3 mutant, but not the wild-type cells, in the presence of high bafilomycin concentrations.

Reply: We appreciate the reviewer's comment and have now characterized the lysosomal pH in the mutant cells more thoroughly, including the condition of a high concentration of bafilomycin A1 (Baf A1). These results demonstrated that loss of NDST3 promotes acidification of lysosomes and thereby neutralizes the Baf A1-induced basification of the lysosomes.

i) We have used multiple approaches to firmly establish that loss of NDST3 promotes acidification of lysosomes. These approaches include the use of ratiometric LysoSensor dyes (Fig 2A and Fig EV2C) and the measurement of absolute lysosomal pH values based on a stand curve (Fig 2B-C and Fig EV2D-E).

ii) Following the reviewer's suggestion, we have measured the lysosomal pH values in WT and NDST3 KO cells under the treatment of a high concentration of Baf A1 (100 nM, 1 h). The Baf A1 treatment increased the lysosomal pH in WT and NDST3 KO cells from 4.8 and 4.0 to 5.4 and 4.8, respectively (Fig 2D). Notably, the lysosomal pH remains lower in the NDST3 KO cells than in the WT cells, but the lysosomal pH in the NDST3 KO cells treated with Baf A1 was comparable to that in the WT cells without the Baf A1 treatment, confirming that the acidification effect of loss of NDST3 neutralized the Baf A1-induced basification of the lysosomes.

iii) The functional data also support the conclusion that loss of NDST3 neutralizes the Baf A1-induced basification of the lysosomes. For example, we have found that the Cathepsin B activity and the lysosomal degradative activity are higher in NDST3 KO cells than in WT cells under Baf A1 treatment (Fig 2F and G), consistent with the abovementioned observation that the lysosomal pH in the NDST3 KO cells treated with Baf A1 was comparable to that in the WT cells without the Baf A1 treatment. Furthermore, under the condition in which Baf A1 completely blocked the autophagic flux in WT cells, NDST3 KO was able to maintain a higher autophagic flux (Fig 2J), consistent with the compensatory effect of lysosomal acidification upon loss of NDST3 that renders cells resistant to Baf A1-induced toxicity.

With regard to the mechanism of Baf A1's action, we think it is conceivable that enhanced V-ATPase assembly and activities could alter the dynamic effects of Baf A1, since Baf A1 is a reversible inhibitor of V-ATPase and may not block 100% of the V-ATPase activity but maintains an equilibrium with its targets in live cells. Indeed, in our experiments, Baf A1-treated cells can live several days before they die, suggesting that the V-ATPase functions critical for cell survival are not completely blocked. Moreover, we have observed more cell survival conferred by the loss of NDST3 in the presence of a low concentration of Baf A1 than in the presence of a relatively higher concentration of the drug (Appendix Figure S1B). Relevant modifications for the texts have been made under the section "Loss of NDST3 leads to lysosomal hyperacidification and dysfunction" in *Results*.

2). Throughout the paper the authors assume that it is the lysosomal V-ATPase that is affected by the NDST3 mutant. However, some of the results could be explained by altered trafficking en route to the lysosome, which could also arise from altered microtubule function and could result in mislocalization of V-ATPases, increased assembly of V-ATPases resident in other organelles and even hyperacidification of compartments other than the vacuole. In Figure 2, there is more staining with fluorescent weak bases like Lysotracker, but it would also be good to demonstrate that Lysotracker colocalizes well with a lysosomal protein like LAMP1 (Figure 2C does not address this) in order to rule out increased acidity in multiple compartments. Increased acidification of earlier compartments in the secretory pathway due to trafficking defects could explain the reduced co-localization of Bodipy-pepstatin with LAMP1 in the mutant (Figure 3L and M) as well as altered maturation of lysosomal proteases, so this possibility must be addressed.

Reply: We appreciate the reviewer's suggestions to check potential hyperacidification of compartments other than the vacuole and additional trafficking defects. We have performed several experiments and now added the new data to exclude the possibilities that were raised to potentially change the interpretation of our results.

i) As suggested, we have performed the colocalization experiments using the LysoTracker Red DND-99 probe to track acidic vesicles and the stably expressed LAMP1-GFP to label lysosomes in live cells. We observed that nearly all of the acidic vesicles (>95%) stained with the Red DND-99 probe were LAMP1-GFP-positive lysosomes in the cells and that loss of NDST3 did not alter this ratio (Fig EV2F), indicating that the loss of NDST3 did not cause detectable acidification of compartments other than the lysosomes.

ii) Additionally, to exclude the possibility that the observed reduction of cathepsin activity in NDST3 KO cells was a result of defective trafficking of the enzyme to the lysosome, we have performed two new experiments to analyze the localization of Cathepsin B in the lysosome. First, using double immunofluorescence staining, we found no change in the colocalization of Cathepsin B with the lysosomal marker LAMP1 (Fig EV3B). Second, we isolated lysosomes

from cells and found that it was the maturation of Cathepsin B but not the amount of its precursors in the lysosome that was significantly reduced upon loss of NDST3 (Fig 2E), indicating that there was no major defect in the trafficking of Cathepsin B precursors to the lysosome.

Together with other results on the changes related to the lysosomal V-ATPase, our results clearly demonstrate that loss of NDST3 leads to increased assembly of V-ATPases, which causes hyperacidification of the lysosomes and impairs the maturation of Cathepsin B and lysosomal degradation activity, without increasing the acidification of non-lysosomal compartments or decreasing the trafficking Cathepsin B to the lysosomes.

Minor points:

3). In other places, the authors do not fully address the results they show. For example, why is the mobility of LAMP1 on SDS-PAGE different between wt and the NDST3 KO in Figures 2 and 3? (This could suggest altered modification as a result of perturbed trafficking to the lysosome.). Why would the NDST3 KO incorporate 50% more 14C valine in Figure 3G? This result undermines the claim that protein degradation is reduced from Figure 3H. In Figure 5, why does the ratio of V0D to LAMP1 increase in TSA, to about the same extent as the increase in the V1A or V1C to V0D ratios? Using a "correction coefficient" does not explain the unexpected effect on V0D. While these are not individually major points, they undermine the overall story.

Reply: We thank the reviewer for the detailed questions and have performed multiple experiments to address them.

i) For the differential migration of LAMP1 on SDS-PAGE, we found that it was due to a change in the glycosylation. Treatment with swainsonine (SWN), an inhibitor of protein glycosylation, resulted in the same shift in the migration of LAMP1 from WT cells while not affecting LAMP1 from NDST3 KO cells (Appendix Figure S4A), suggesting the migration shift was a consequence of deglycosylation of LAMP1. The SWN treatment alone did not cause lysosomal hyperacidification or dysfunction (Appendix Figure S4B and C), as observed in NDST3 KO cells, indicating that the deglycosylation of LAMP1 was not responsible for the changes in lysosomal pH and functions in the NDST3 KO cells. The mechanism of the deglycosylation of LAMP1 is not known and could be studied beyond the scope of this work in the future.

ii) Regarding the measurements of lysosomal degradative functions, in our original assay based on ^{14}C-valine-labelled proteins, there was a higher level of initial steady-state ^{14}C-valine-labelled protein signals in NDST3 KO cells, which could be explained by the slower degradation of these proteins upon loss of NDST3. To avoid the complication caused by other unknown factors that might affect ^{14}C-valine incorporation, we have now replaced this assay with a chase assay that measures the degradation of a lysosomal substrate, Alexa Fluor 488-dextran, independently of its uptake. The results from this chase experiment clearly demonstrated that the degradation of the lysosomal probe was impaired in NDST3 KO cells, a defect that could be rescued by bafilomycin treatment (Fig 2G and Page 8, Paragraph 2).

iii) We agree with the reviewer that the data from the TSA treatment in the original Figure 5 was complicated, which could be a result of the fact that TSA targets all members of the HDAC family. In the revised manuscript, we have replaced this drug with tubacin, which is a specific HDAC6 inhibitor. The tubacin treatment did not change the ratio of V0D to LAMP1 but still increased the ratio of ATP6V1A and ATP6V1C1 to V0D (Fig 4C-G and Page 11, Paragraph 2).

4). In Figure EV4, the authors argue that because less mature cathepsin is seen in cells clamped to either lower pH or higher pH, then low lysosomal pH must compromise protease maturation. This is not a legitimate argument, since the agents used to clamp pH will also perturb trafficking to the lysosome and this could cause accumulation of immature forms of the protease.

Reply: We appreciate this comment from the reviewer. As explained in our reply to the comment #2 above, we have demonstrated loss of NDST3 does not affect the trafficking of Cathepsin B (Cat B) to the lysosome. Furthermore, to demonstrate that the maturation of Cat B can be directly affected

by either under- or over-acidification of its environment, we performed an *in vitro* maturation assay, in which the activity of purified Cat B was examined in different buffers with a range of pH values. We observed that the maturation of Cat B from its inactive precursor proCat B to the lower-molecular-weight mature form was impaired in under- or over-acidificated conditions, such as that with a pH below 4.5 (Fig EV3A). This observation is consistent with a previous report that over-acidification of the lysosome suppresses the proteolytic activity of cathepsins (Mach *et al*, 1994).

Referee #3:

The manuscript by Tang and colleagues proposes that the protein NDST3 regulates lysosomal acidification by promoting acetylation of alpha-tubulin. The authors identified NDST3 and ATF2 as necessary factors for bafilomycin-induced cell death through a CRISPR screen. The follow-up lead the authors to focus only on NDST3.

1. The conclusions on the role of NDST3 on the lysosomes are not supported by the data. There are key controls lacking. Some of the methodology is innapropriate (in particular, lysosomal pH assessment needs to be completely redone).

Reply: We appreciate the reviewer's comments and have performed multiple experiments resulting in new data that substantially improved the conclusions in the revised manuscript. We have added controls whenever appropriate and necessary. In particular, we have reanalyzed the lysosomal pH as suggested, using widely used ratiometric dyes (i.e. LysoSensor Yellow/Blue dextran and LysoSensor Yellow/Blue DND-160) (Fig 2A-D and Fig EV2C-E). Additionally, we have added results from other improved methods, including a cathepsin maturation assay (Fig 2E), a lysosomal degradation assay (Fig 2G), and a pH-dependent cathepsin B maturation assay (Fig EV3A). Together with other improvements made during the revision, our data have strengthened the conclusions in this study.

2. One key aspect that remains completely ignored is the reason why cells die when treated with bafilomycin. This question has been explored (PMID 28296633, 31793879, 31983508), and it has been shown unequivocally that bafilomycin-induced death is due to iron deficiency. Nevertheless, this theme was not even brough up by the authors. Given that restoring cytoplasmic iron homeostasis is sufficient to rescue bafilomycin-induced death, it is pivotal to test if the hits that the authors got on the CRISPR screen interfere with the iron homeostasis pathway.

Reply: We appreciate that the reviewer brought to our attention the link between bafilomycin and iron homeostasis. In fact, the main conclusions from the abovementioned references (PMID 28296633, 31793879, 31983508) are that iron homeostasis is regulated by lysosomal acidity. These previous reports are consistent with our findings in the present study that NDST3 is a critical regulator of lysosomal acidity, which is upstream of the iron homeostasis pathway. We recognize that lysosomal dysfunction triggers a variety of downstream deleterious events, including but likely not limited to apoptosis (Boya *et al*, 2005), accumulation of damaged organelles and misfolded proteins (Platt *et al*, 2012), and iron deficiency (Yambire *et al*, 2019; Weber *et al*, 2020; Miles *et al*, 2017). Indeed, it was shown that, although iron supplement alleviated the bafilomycin-induced toxicity, it did not completely rescue the bafilomycin-induced cell death (Yambire *et al*, 2019; Weber *et al*, 2020), suggesting the presence of parallel pathways mediating the bafilomycin-induced cell death. The focus of our present study is on the mechanism through which NDST3 regulates microtubule acetylation and lysosomal acidity. Given the large amount of data already existing in the manuscript and its limited scope, the studies on the bafilomycin-induced toxicity would belong to a future study. Accordingly, we have de-emphasized the bafilomycin resistance screen in our revised manuscript but only used it to introduce the identification of NDST3 as a critical regulator of lysosomal acidity. Nevertheless, we have now included a discussion of all three references as mentioned by the reviewer above in relation to our newly identified mechanism in the revised manuscript (Page 15, Paragraph 2).

3. Secondly, there are numerous factors that regulate the acetylation of alpha-tubulin, but none of them appeared in the CRISPR screen.

Reply: There are two possible reasons that we haven't identified other factors regulating tubulin acetylation in our CRISPR screen: 1) Our screen is far from being saturated, which is quite likely; 2) most acetylases/deacetylases have many different targets that could complicate the phenotype related to the function of any single target. For the revision, we have de-emphasized the bafilomycin resistance screen in our revised manuscript by only describing it to introduce the identification of NDST3 as a critical regulator of lysosomal acidity. Since the mechanism of bafilomycin resistance is not the focus of the present study, a saturated screen for a complete list of underlying factors would belong to a future study.

4. Third, the rescue experiments are only able to recover a small part of the phenotypes.
- 1G - expression of FLAG-NDST3 in NDST3-KO cells results in 40-50% viability, which is ~5-fold higher than the WT cells. This is a big difference for what one would expect from a "rescue" experiment. What is the reason for this?

Reply: We have performed new experiments to improve this rescue experiment. In the originally submitted manuscript, we conducted the rescue experiment by transiently transfecting the NDST3-expressing plasmid into NDST3 KO cells, an approach that can only mediate the expression of NDST3 in a subset of cells for a short period and result in a partial rescue of the phenotype. In the revised manuscript, we have generated lentiviruses expressing NDST3 and used the viruses to make stable cell lines expressing NDST3 in the parent KO background, enabling uniform and stable expression of NDST3. The improved approach has resulted in a full rescue of the phenotype. The new results are shown in Fig 1E and 1G in the revised manuscript.

5. 2A-2C - Lisotracker staining is not just dependent on the acidic pH, it is also dependent on the size of the lysosomal particles, which appear to be bigger in the NDST3-KO cells. The size of the lysosomes should be quantified (for example, by immunocytochemistry against LAMP1. Furthermore, per the images shown, there is too much lisotracker staining outside the expected lysosomal particles, which raises further doubts that this experiment can be informative at all. In the acridine orange experiment, the one result that seems evident is, again, the increase in the size of the lysosomal particles. The fact that there is no change in the amount of LAMP1 protein does not mean that there is no change in the amount of LAMP1 per lysosomal particle.

Reply: We thank the reviewer for the suggestions and have performed additional control experiments as suggested. Using immunofluorescent staining against LAMP1, we found no change in the number or size of the lysosomes as a result of loss of NDST3 (Fig EV2A). However, we confirmed that the staining with LysoTracker Red DND-99 that tracks acidic vesicles showed an increase in both the number and sizes of positive puncta in NDST3 KO cells compared to WT cells (Fig EV2B), consistent with the increase in the lysosomal acidification in the absence of NDST3. As a control, a co-staining of the LysoTracker probe and the lysosomal marker LAMP1-GFP stably expressed in live cells showed that nearly all of the acidic vesicles stained with the LysoTracker were LAMP1-GFP-positive lysosomes in both WT and NDST3 KO cells (Fig EV2F). The revised text is shown on Page 7. The measurements of the changes in the lysosomal pH are supported by other experiments including those described in the answer to the comment below.

6. 2D-2G - Lysosensor is a set of dies that are known to buffer the lysosomal pH, and therefore to introduce perturbations in the very system they are supposed to measure. For this reason, the assessment of lysosomal pH needs to be done by a different method, for example using dextrans or a genetically-encoded probe. It is also fundamental to measure the lysosomal pH in cells lacking NDST3 that are treated with bafilomycin. The authors are implying that NDST3 can increase lysosomal acidity, and for that reason counteract the effect of bafilomycin, therefore that very idea

has to be demonstrated. Otherwise, the possibility remains that NDST3 is affecting other components besides the vATPase.

Reply: We thank the reviewer for the comment and help suggestions. We have addressed it by clarifying our methods and performing new experiments as suggested.

i) The LysoSensor Yellow/Blue DND-160 dye can exhibit an alkalizing effect on the lysosomes, and a long-time incubation with this dye can induce an increase in lysosomal pH. Therefore, the manufacturer of this dye suggests that this dye is a useful pH indicator only when it is incubated with cells for 1-5 minutes. Similar to many previous reports (Yagi *et al*, 2021; Deriy *et al*, 2009; Chung *et al*, 2019), we have used this dye very carefully by setting the incubation time set at 3 minutes and using a pH calibration curve to measure the lysosomal pH (Fig EV2C-E).

ii) Following the reviewer's suggestion, we have now measured the lysosomal pH using the LysoSensor Yellow/Blue dextran that would not interfere with the lysosome pH. We have used the dextran probe to measure the lysosomal pH in NDST3 KO and WT cells, including the conditions with bafilomycin treatment. The results are consistent with those obtained by other methods: loss of NDST3 promotes lysosomal acidity, causing over-acidification of lysosomes in WT cells but offsetting bafilomycin-induced increase in lysosomal pH (Fig 2A-D, and Page 6-7).

7. 3A-3D - no effect in autophagy is observed between the WT and NDST3-KO cells in basal conditions. This does not seem compatible with an effect of NDST3 specifically in the lysosomal component. Again, it would be important to show what happens to the autophagic flux in WT and KO cells treated with bafilomycin for a long time. Importantly, the chloroquine experiment in 3D argues that there is less autophagosomal cargo being delivered to the lysosomes in NDST3-KO cells, because less of it accumulates when the lysosomes are shut down by the cloroquine (and the effect of chloroquine is to serve as a short-circuit of the lysosomal pH gradient, so even if NDST3 would be working to acidify the lysosomes, it would not be able to restore acidity to cloroquine-treated lysosomes - the experiment would have been a lot more informative with bafilomycin, for the reasons already stated above).

Reply: We appreciate the reviewer's comment and have provided clarification of our original results and added new experimental results as suggested to address the comment.

i) There might have been a misunderstanding of our original results on the difference in autophagic activities between WT and NDST3 KO cells in Figure 3A-3D (now Fig 2H and 2I). In fact, we had shown a statistically significant difference in autophagic activities, as demonstrated by the changes in the ratio of LC3B-II/LC3B-I and the level of p62 in basal conditions, in the original Figure 3A-3C (now Fig 2H, Rapa -).

ii) In the original Figure 3D (now Fig 2I), we performed a standard autophagy flux assay by measuring the turnover of LC3B-II before and after treatment with chloroquine (CQ) as a lysosomal inhibitor. In general, both CQ and Baf A1 can be used as the lysosomal inhibitor in the autophagy flux assay. The reason we chose CQ instead of Baf A1 in our flux assay is that NDST3 KO cells are resistant to Baf A1-induced lysosomal inhibition, as demonstrated in our original Baf A1 resistance screen, but NDST3 KO and WT cells had no differential effects in response to CQ-induced toxicity (not shown), likely due to the different mechanism through which CQ affects lysosomal functions. The results from our flux assay indicated that NDST3 KO cells have a slower autophagic flux than WT cells (Fig 2I), consistent with the impaired lysosomal functions in the NDST3 KO cells.

iii) We agree with the reviewer that it would be important to examine the autophagy flux under the Baf A1 treatment conditions to verify our conclusions. As suggested by the reviewer, we treated the WT and NDST3 KO cells with Baf A1 for a relatively long time (25 nM, 48 h) and then conducted the flux assay to measure the turnover of LC3B-II upon blockade of lysosomal activity by CQ. The results showed that, although the prolonged Baf A1 treatment completely blocked the turnover of LC3B-II in WT cells, the NDST3 KO cells was able to maintain a partial autophagic flux (Fig 2J), consistent with the compensatory effect of lysosomal acidification

upon loss of NDST3 that renders cells resistant to Baf A1-induced toxicity.

Together, these results about the levels of autophagic markers and the status of the autophagic flux are consistent with our conclusions that loss of NDST3 causes lysosomal over-acidification and functional impairment but in the meantime compensates for the effects of Baf A1 by keeping the lysosomal pH relatively low and thus rendering cells resistant to Baf A1 treatment with partial lysosomal activities (Page 7-9).

8. 3G-3H - the measurement of protein stability cannot be used as a readout for lysosomal function, as there are too many other interfering components (for example, the protein synthesis rate was different between WT and NDST3-KO cells, which alone makes the interpretation of this result borderline impossible); secondly, the authors used whole cells, which is not able to distinguish between lysosomal proteolysis, excretion towards the extracellular space, proteasomal degradation, etc. If the experiment would have been done with isolated lysosomes, it might be a closer readout, provided the protein synthesis and incorporation of the tracer was similar. In these conditions, I don't see how this experiment can be used.

Reply: We agree with the reviewer that the measurement of protein stability could be influenced by factors other than the lysosomal function. In our original manuscript, the measurement of the turnover rate of ^{14}C-valine-labelled long-lived proteins, as used by previous studies (Lee *et al*, 2010; Dupont *et al*, 2017), was employed to provide supportive evidence to reflect the status of lysosomal proteolytic functions.

In the revised manuscript, to avoid the complication caused by other unknown factors in the ^{14}C-valine labeling assay, we have now replaced this assay with a chase assay that measures the degradation of a direct lysosomal substrate, Alexa Fluor 488-dextran, using a previously established method (Liu *et al*, 2018). The results from this dextran chase experiment clearly demonstrated that the degradation of the lysosomal probe was impaired in NDST3 KO cells, a defect that could be rescued by bafilomycin treatment (Fig 2G and Page 8, Paragraph 2).

9. 3I-K - these results do not imply that the lysosomal pH is more acidic. They show that there is less mature cathepsins, but it doesn't show if this is an issue of maturation (i.e., lysosomal) or expression (i.e., lower transcriptional levels?). The outcome is that there is less cathepsin activity, but this is not an evidence that the pH is more acidic.

Reply: We agree with the reviewer that the cathepsin maturation assay using the whole cells could be complicated by other factors such as expression or trafficking. We have now redone this experiment using isolated lysosomes to clearly show that the cathepsin maturation inside the lysosomes is impaired, consistent with the hyperacidification of the lumen as shown by other assays in our studies. In brief, we isolated the lysosome fraction and calculated the ratio of mature cathepsin B to its immature form in the lysosomes, which indicated a clear defect in the maturation of cathepsin B (now Fig 2E). Together with other supporting evidence, including an *in vitro* maturation assay using purified cathepsin proteins showing the dependence of their maturation on pH (Fig EV3A) and the Magic Red Cat B assays measuring the cathepsin proteolytic activities with or without bafilomycin (Fig 2F), our results are consistent with a previous report that both over- and under-acidification of the lysosome suppresses the proteolytic activity of cathepsins (Mach *et al*, 1994) (Page 7-8).

10. 4A - there seems to be also more V1C1 in cytoplasm in the KO cells. The total levels would be important here.

Reply: We thank the reviewer for pointing this out. To address the potential relevance of the observed change in the level of ATP6V1C1 on the assembly of V-ATPase holoenzymes, we have performed a control experiment with over-expression of ATP6V1C1. We observed that over-expression of ATP6V1C1 in WT cells did not change the level of membrane-bound ATP6V1C1 or the level of other subunits such as ATP6V1A (Appendix Figure S6A-E and Page 10, Paragraph 2). Therefore, the changes in the level of ATP6V1C1 does not explain the regulation of V-ATPase

holoenzymes. We have then focused on studying how microtubules affect V-ATPase assembly.

11. 4E-4F - the effect of the KO on a-tub acetylation is much higher than the observed when the protein is reexpressed. As mentioned above, this raises questions regarding the specificity of the findings.

Reply: There might be a misunderstanding of our experiments, so we have revised the text to clarify the description of our experiments shown in the original Figure 4E-4F (now Fig 5A-B). In fact, the experiment in the original Figure 4F (now Fig 5B) is an over-expression experiment rather than a rescuing/re-expression experiment. We conducted this experiment to confirm that NDST3 is a deacetylase of α-tubulin. As expected, the over-expression of NDST3 produced an opposite effect on the acetylation of tubulin compared to that in NDST3 KO cells, which is consistent with our findings that NDST3 acts as a tubulin deacetylase.

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1st Revision - Editorial Decision 22nd Jun 2021

Thank you for submitting your revised study. The manuscript has been sent back to the original referees, whose comments are appended below.

As you will see, while referee #1 and #3 find that their criticisms have been adequately addressed and recommend the study for publication, referee #2 requests you to address some remaining points.

In addition, there are few editorial issues concerning the text and the figures that I need you to address before we can officially accept your manuscript.

Referee #1:

The revised manuscript is significantly improved and authors addresses most of my previous comments. Although they did not manage to do experiments in real neurons and used N2a cells instead, their findings are consistent with results in non-neuronal cels. I have minor suggestions related to the organisation of figures. They are very heterogeneous in terms of the size and panel alignment. I would recommend to improve it. Otherwise I am happy with the new version and would recommend it for publishing.

Referee #2:

This manuscript addresses a novel link between microtubule acetylation, V-ATPase assembly, and lysosomal acidification. This major revision is greatly improved over the previous submission and provides significant new insights that will be valuable to the community. The use of ratiometric pH indicators to measure lysosomal pH is a major improvement, particularly because bafilomycin actually raises the lysosomal pH in the NDST3 mutant to the wild-type level, accounting for the effects on downstream processes. The new connection between NDST3 reduction and c9orf72 is intriguing, if not fully developed. However, there are still some points that are confusing that need to be clarified before publication, so that the authors' data and conclusions are clear.

1) The data clearly indicate relatively constant levels of V-ATPase membrane subunits and higher levels of association with peripheral subunits when tubulin deacetylation is inhibited. This is consistent with regulation of V-ATPase activity at the level of assembly and with increased acidification and the downstream effects on proteolysis that are described. However, there are places where the authors seem to confuse this mechanism with altered trafficking or localization of the V-ATPase, which is not supported by the data and confuses the model. For example, on page 11, the statement that "the acetylated microtubules help constrain the lysosomes and V-ATPase V1 subunits in the perinuclear area" is supported only by a correlation of localization with no real causation. Similarly, on p. 10, the reference to Duan et al, 2010 describes the role of microtubules in V-ATPase trafficking, not assembly. On p. 15, it would also be more accurate to say that NDST3 is connected to "assembly" of the V-ATPase rather than "formation", which would suggest an effect on biosynthesis that is not tested.

2) On page 8, a reference should be provided for use of Alexa488-dextran degradation as a measure of lysosome degradation, as this assay is not commonly used.
3) Lamp1 fluorescence indicates that there is no change in lysosome size or number in cells lacking NDST3 (p. 7), but there is an increase with Lysotracker Red DND-99. This is not "consistent with the increase in lysosomal acidification", but rather suggestive that the Lysotracker is having effects beyond pH sensing. I would recommend omitting the Lysotracker Red data now that the Lysosensor data is available.
4) Minor point: on p. 2, mammalian V-ATPases have no c' subunit.

Referee #3:

The manuscript by Tang and colleagues was significantly improved by the addition of multiple controls and optimization of experimental procedures. In particular, the effect of short-term bafilomycin on the pH of lysosomes in WT and NDST3-KO cells is a very strong result that supports the model proposed by the authors. In my view, the manuscript is ready for publication. I congratulate the authors on their findings.

Dear Editor and Reviewers:

Thank you for the further suggestions on our manuscript. We have made additional revisions according to the remaining points of the reviewers.

Referee #1:

The revised manuscript is significantly improved and authors addresses most of my previous comments. Although they did not manage to do experiments in real neurons and used N2a cells instead, their findings are consistent with results in non-neuronal cels. I have minor suggestions related to the organisation of figures. They are very heterogeneous in terms of the size and panel alignment. I would recommend to improve it. Otherwise I am happy with the new version and would recommend it for publishing.

Reply: Thank you and we have adjusted the sizes and the panel alignment of the figures, particularly for Figure 1 and Figure 4, to make them more uniform and easier to follow.

Referee #2:

This manuscript addresses a novel link between microtubule acetylation, V-ATPase assembly, and lysosomal acidification. This major revision is greatly improved over the previous submission and provides significant new insights that will be valuable to the community. The use of ratiometric pH indicators to measure lysosomal pH is a major improvement, particularly because bafilomycin actually raises the lysosomal pH in the NDST3 mutant to the wild-type level, accounting for the effects on downstream processes. The new connection between NDST3 reduction and c9orf72 is intriguing, if not fully developed. However, there are still some points that are confusing that need to be clarified before publication, so that the authors' data and conclusions are clear.

Reply: We appreciate the reviewer's further points and have addressed them in the revision.

1). The data clearly indicate relatively constant levels of V-ATPase membrane subunits and higher levels of association with peripheral subunits when tubulin deacetylation is inhibited. This is consistent with regulation of V-ATPase activity at the level of assembly and with increased acidification and the downstream effects on proteolysis that are described. However, there are places where the authors seem to confuse this mechanism with altered trafficking or localization of the V-ATPase, which is not supported by the data and confuses the model. For example, on page 11, the statement that "the acetylated microtubules help constrain the lysosomes and V-ATPase V1 subunits in the perinuclear area" is supported only by a correlation of localization with no real causation. Similarly, on p. 10, the reference to Duan et al, 2010 describes the role of microtubules in V-ATPase trafficking, not assembly. On p. 15, it would also be more accurate to say that NDST3 is connected to "assembly" of the V-ATPase rather than "formation", which would suggest an effect on biosynthesis that is not tested.

Reply: We have revised the texts as suggested to clarify the descriptions.

i) We have deleted the original statement "the acetylated microtubules help constrain the lysosomes and V-ATPase V1 subunits in the perinuclear area", but simply describe the colocalization of acetylated-α-tubulin, LAMP1-GFP and ATP6V1C1 in the context of other experiments (Page 11, Paragraph 2).

ii) We have removed the reference to Duan et al, 2010 on page 10 from our manuscript.

iii) We have changed the word "formation" into "assembly" on page 15.

2). On page 8, a reference should be provided for use of Alexa488-dextran degradation as a measure of lysosome degradation, as this assay is not commonly used.

Reply: We have added the reference (Liu B, Palmfeldt J, Lin L, Colaço A, Clemmensen KKB, Huang J, Maeda K, Luo Y & Jäättelä M (2018) STAT3 associates with vacuolar H + -ATPase and regulates cytosolic and lysosomal pH. *Cell Res* 28: 996–1012) for the Alexa488-dextran degradation assay on page 8.

3). Lamp1 fluorescence indicates that there is no change in lysosome size or number in cells lacking NDST3 (p. 7), but there is an increase with Lysotracker Red DND-99. This is not "consistent with the increase in lysosomal acidification", but rather suggestive that the Lysotracker is having effects beyond pH sensing. I would recommend omitting the Lysotracker Red data now that the Lysosensor data is available.

Reply: As suggested, we have removed the LysoTracker Red data from the original Fig EV2 and deleted the relevant texts in the Result section (Page 7, Paragraph 1).

4). Minor point: on p. 2, mammalian V-ATPases have no c' subunit.

Reply: We have made the correction as follows: “The V0 domain comprises membrane-embedded subunits a, c, c", d, e, Ac45, and (pro)renin receptor (PRR), responsible for pumping protons across the membrane against the electrochemical gradient (Abbas *et al*, 2020).” (Page 3, Paragraph 2)

Referee #3:

The manuscript by Tang and colleagues was significantly improved by the addition of multiple controls and optimization of experimental procedures. In particular, the effect of short-term bafilomycin on the pH of lysosomes in WT and NDST3-KO cells is a very strong result that supports the model proposed by the authors. In my view, the manuscript is ready for publication. I congratulate the authors on their findings.

Reply: We appreciate the reviewer’s support for publication.

2nd Revision - Editorial Decision 20th Jul 2021

I am pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jiou Wang
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2020-107204

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;
  - definition of error bars as s.d. or s.e.m.

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?	The sample size for each experiment was chosen based on pilot studies to ensure adequate power for the data analyses.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from the analysis. Therefore, no criteria was set in the manuscript for the inclusion/exclusion of samples.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Samples were allocated randomly.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	Investigators were not blinded to allocation during experimets and outcome assessment. When capturing images under the microscope, we selected the fields randomly to avoid an effect of subjective bias when assessing results.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	Yes. We reported standard deviations and plotted individual data in all analyzed experiments.

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	The p values from the F tests for comparing variances between groups in all figures were greater than 0.05, except for that in Fig 1E, 2C, 2F, 2G, 2N, EV2F, EV4B, and Appendix Figure S1, S3, and S4.

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).	All commercial antibodies used in this study were fully validated for the indicated use. The NDST3 antibody generated in-house was validated by knockout samples. We provided the catalog number for any commercial antibodies used in this study.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of cell lines used in this study is shown in *Materials and Methods* .

* 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.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	No restriction exists on the availability of human data and samples.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	A subsection named "Data Availability" is shown at the end of the Materials and Methods. This study does not include any data required for public deposition.
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).	N/A
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).	N/A
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.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.