

Expanded View Figures

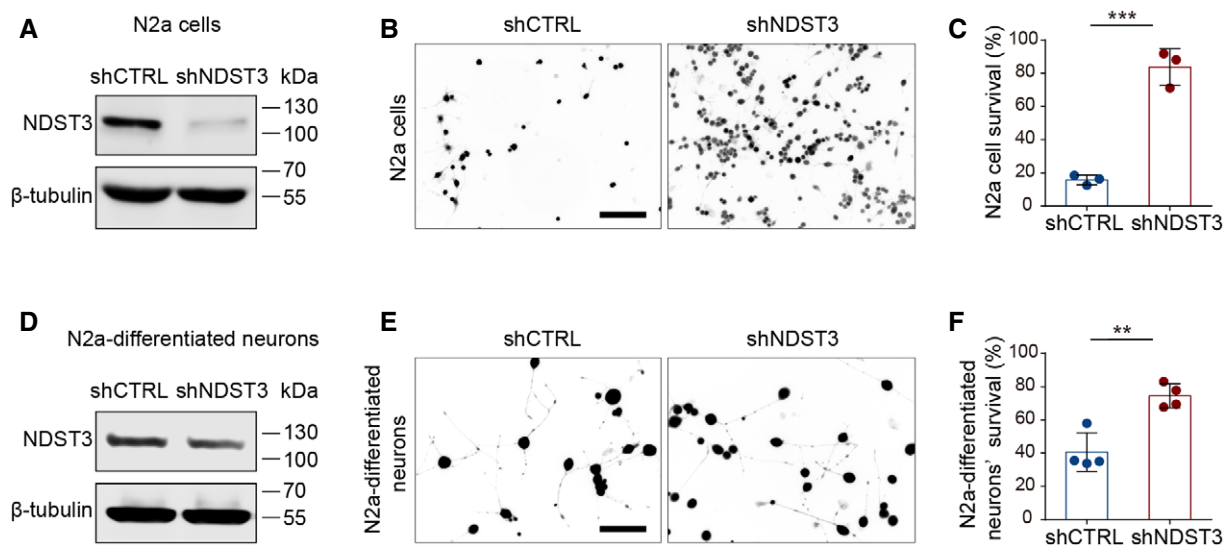


Figure EV1. NDST3 knockdown renders N2a cells and differentiated neurons resistant to Baf A1 toxicity (related to Fig 1).

A–C Baf A1 sensitivity assay in N2a cells. NDST3 is knocked down in N2a cells with transfection of *NDST3* shRNA (shNDST3) for 72 h (A). N2a cells transfected with shNDST3 or control shRNA (shCTRL) were incubated with 25 nM Baf A1 for 48 h, and cell survival was measured using calcein AM staining (B) and quantified (C) ($n = 3$ independent cultures, *** $P = 0.0005$).

D–F Baf A1 sensitivity assay in N2a-differentiated neurons. NDST3 is knocked down in N2a-differentiated neurons infected with shNDST3-containing lentivirus for 72 h (D). The N2a-differentiated neurons with NDST3 knockdown and the shCTRL-treated N2a-differentiated neurons were incubated with 25 nM Baf A1 for 6 h, and cell survival was measured using calcein AM staining (E) and quantified (F) ($n = 4$ independent cultures, ** $P = 0.0026$).

Data information: Error bars represent $\pm$ standard deviation. Scale bar, 200 μ m.

Source data are available online for this figure.

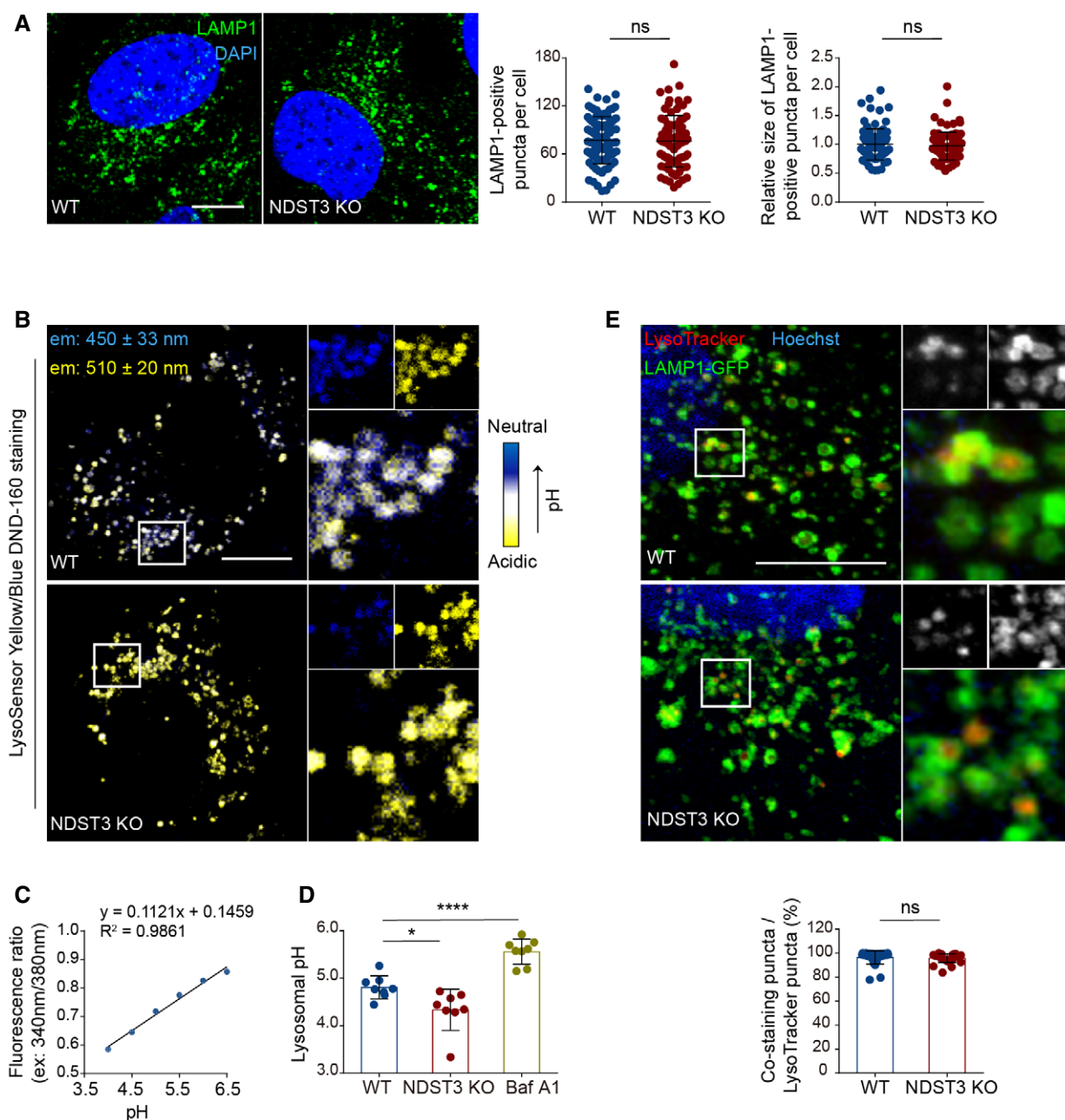


Figure EV2. Analysis of lysosomal acidity with LysoSensor Yellow/Blue DND-160 (related to Fig 2).

- A Immunofluorescent staining for the lysosomal marker LAMP1. WT and NDST3 KO RPE1 cells were fixed and subjected to immunofluorescence staining with antibodies against LAMP1. The number of the LAMP1-positive puncta per cell and their relative sizes were quantified ($n = 91$ cells in the WT group, $n = 83$ cells in the NDST3 KO group, ns represents non-significant).
- B Determination of lysosomal pH with the ratiometric dye LysoSensor Yellow/Blue DND-160. WT and NDST3 KO RPE1 cells were stained with LysoSensor Yellow/Blue DND-160 for a short period of 3 min to minimize its alkalizing effect and then quantified for fluorescence signals. Acidic organelles have predominantly yellow fluorescence and less acidic organelles have more blue fluorescence.
- C The calibration curve for the lysosome pH measured with LysoSensor Yellow/Blue DND-160. RPE1 cells were incubated with a series of pH calibration buffers (pH 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5) in the presence of 10 μ M monensin and 30 μ M nigericin and examined for the fluorescence ratio of LysoSensor Yellow/Blue DND-160 staining by a plate reader.
- D Lysosomal pH values in WT and NDST3 KO RPE1 cells calculated from the fluorescence ratio of LysoSensor Yellow/Blue DND-160 staining against the pH calibration curve in (C) ($n = 8$ independent cultures, $*P = 0.0172$, $****P < 0.0001$). Baf A1 was used as a positive control.
- E The colocalization of LysoTracker with LAMP1-GFP. WT and NDST3 KO cells stably expressing LAMP1-GFP were incubated with LysoTracker Red DND-99 (50 nM, 1 h) and then analyzed ($n = 30$ cells, $P = 0.6242$).

Data information: Error bars represent $\pm$ standard deviation. Scale bar, 10 μ m.

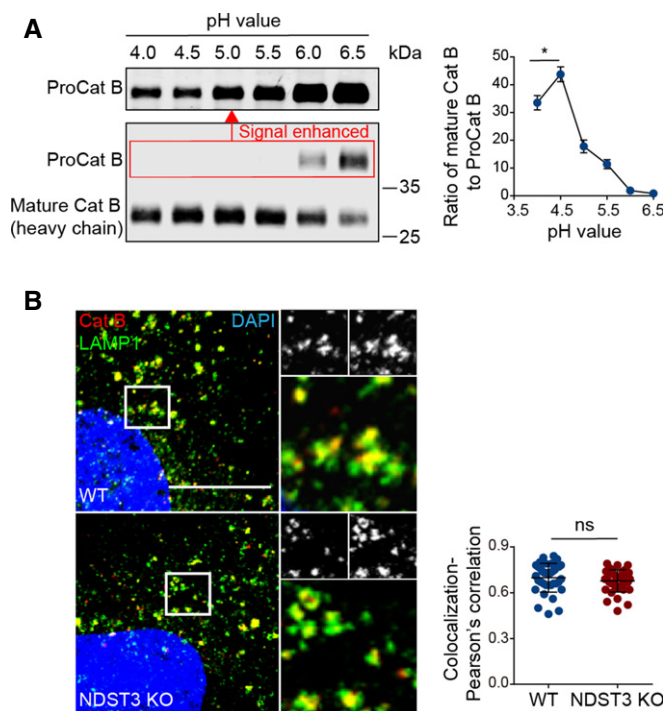


Figure EV3. *In vitro* assay for pH-dependent maturation of Cathepsin B and the colocalization of Cat B with LAMP1 (related to Fig 2).

- A** *In vitro* assay for Cat B maturation under various pH conditions. Recombinant human Cat B was incubated in activation buffers with different pH values (pH4.0–pH6.5) at room temperature for 10 min and then subjected to immunoblotting analysis using an antibody against Cat B. The ratio of mature Cat B to ProCat B was quantified ($n = 3$ independent experiments, $*P = 0.0177$).
- B** The colocalization of Cat B with LAMP1 is not altered by the loss of NDST3. WT and NDST3 KO cells were fixed and subjected to immunofluorescent staining using antibodies against Cat B and LAMP1. The nucleus was stained with DAPI. Representative images of the staining are shown, and Pearson's correlation is used to evaluate the colocalization of Cat B and LAMP1 ($n = 37$ cells in the WT group, $n = 38$ cells in the NDST3 KO group, $P = 0.2959$). Scale bar, 10 μm .

Data information: Error bars represent $\pm$ standard deviation. Source data are available online for this figure.

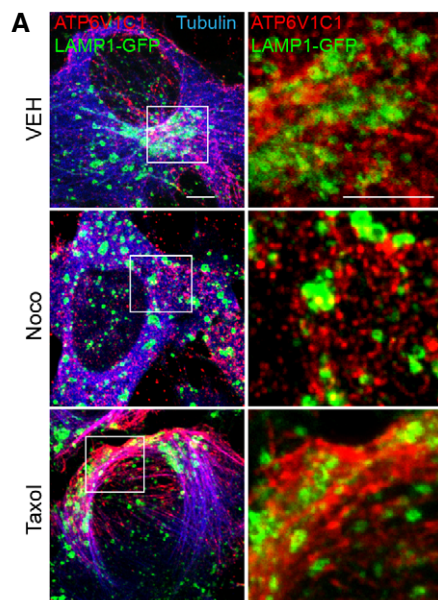
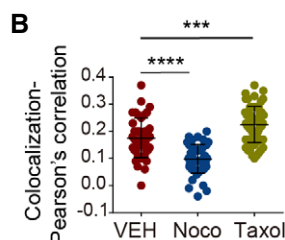


Figure EV4. Stable polymerized microtubules are crucial for the recruitment of V-ATPase V1 subunits to lysosomes (related to Fig 4).

- A** Immunofluorescent staining of ATP6V1C1 and tubulin as well as imaging of LAMP1-GFP upon the treatment of VEH, Noco, or Taxol. Representative images are shown in the left panel, with magnified insets of ATP6V1C1 and LAMP1-GFP imaging shown in the right panel. Scale bar, 5 μm .
- B** Quantification of the colocalization of ATP6V1C1 with LAMP1-GFP from the imaging in (A) ($n = 45$ cells in the VEH-treated group, $n = 50$ cells in the Noco-treated group, $n = 62$ cells in the Taxol-treated group, $***P = 0.0004$, $****P < 0.0001$). The data were analyzed by one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc analysis.

Data information: Error bars represent $\pm$ standard deviation.



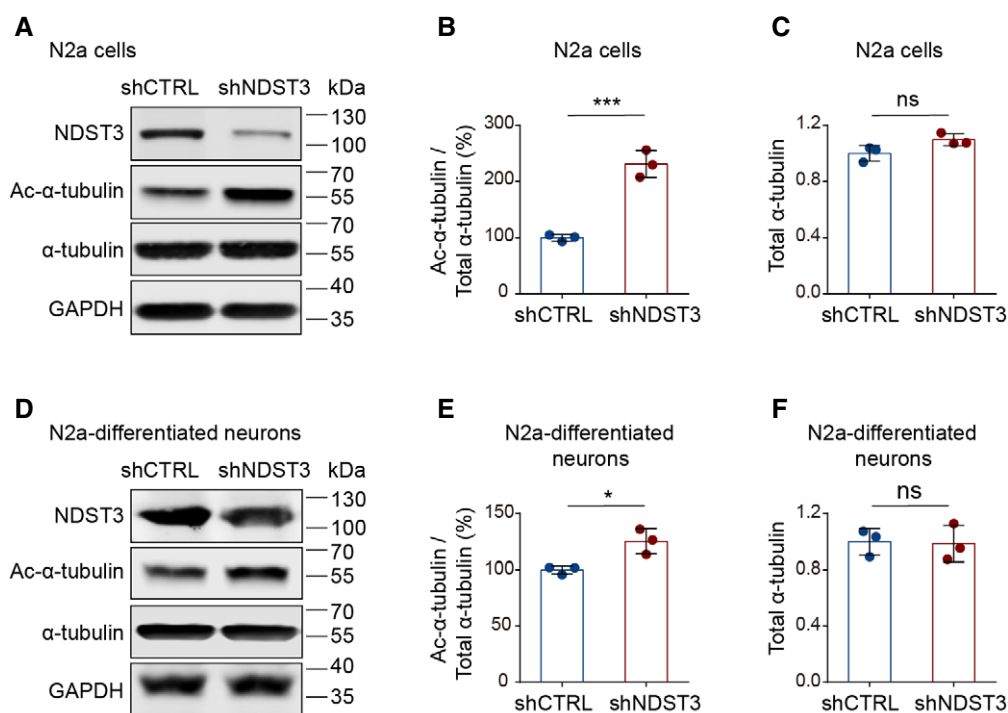


Figure EV5. NDST3 knockdown increases α-tubulin acetylation in N2a cells and differentiated neurons (related to Fig 5).

A–C The levels of acetylated and total α-tubulin in N2a cells under the NDST3 knockdown condition were determined by SDS–PAGE and immunoblotting analysis. The percentage of acetylated-α-tubulin (Ac-α-tubulin) relative to total α-tubulin (B) ($n = 3$ independent experiments, $***P = 0.0008$) and the level of total α-tubulin normalized against GAPDH (C) ($n = 3$ independent experiments, $P = 0.0739$) were quantified.

D–F The levels of acetylated and total α-tubulin in N2a-differentiated neurons were determined by SDS–PAGE and immunoblotting analysis. The percentage of Ac-α-tubulin relative to total α-tubulin (E) ($n = 3$ independent experiments, $*P = 0.0203$) and the level of total α-tubulin normalized against GAPDH (F) ($n = 3$ independent experiments, $P = 0.8881$) were quantified.

Data information: Error bars represent $\pm$ standard deviation. ns means non-significant.

Source data are available online for this figure.