SUPPLEMENTAL MATERIALS

EXTENDED EXPERIMENTAL PROCEDURES

Limb bud isolation, culture, and degeneration

The assay for measuring axon degeneration in limb buds is based on an existing assay for studying axon growth in slice cultures (Brachmann et al., 2007). Mouse E10.5 embryos were isolated from the uterus, cleared of the amnion and chorion, and kept in ice-cold Leibovitz's L-15 medium. Embryos were then moved to a plastic dish on ice, and excess media was removed using Whatmann paper. Embryos were then embedded in 4% agarose (low melting point) in PBS, which had been kept at approximately 40°C. Once hardened, excess agarose was trimmed away, and the embedded embryo was affixed to the microtome chuck using super glue. We attempted to position the embryo on the chuck such that the limb buds were parallel to the chuck. Embryos were sectioned on a Leica VT1000S vibrating blade microtome using the following settings: thickness, 400 µm; frequency, 50; amplitude, 1.2; velocity, 8; continuous stroke mode. Sectioning was performed in buffer containing 1x HBSS, 10 mM HEPES buffer pH 7.3, and 500 U/mL penicillin/streptomycin. Slices were removed from the chamber using a Pasteur pipette from which the tip was removed to increase the diameter. We collected 2-3 sections per embryo. Any remaining agarose was removed from the tissue under a dissection microscope, and the sections were transferred to 30-mm Millicell inserts (Millipore, PICMORG50) that had been rinsed with culture medium. Inserts were placed into a 6 well plate with 1 mL of culture medium (DMEM, 25% HBSS, 25% fetal bovine serum, 0.5% glucose, 1 mM L-glutamine, 2.5 mM HEPES pH 7.3, and 500 U/mL penicillin/streptomycin) and incubated at 37°C and 5% CO_2.

Limb bud slices were fixed in 4% paraformaldehyde in PBS at 4°C overnight. Slices were carefully removed from the culture inserts using forceps and transferred into PBS. Washes and incubations were performed in a 12-well plate. Slices were washed in PBS + 0.3% triton X-100 (PBST) and blocked using 5% BSA in PBST. Primary (anti-NFH; DSHB 2H3; 1:1000) and secondary (AlexaFluor 568; Life Technologies; 1:1000) antibodies were diluted in 5% BSA in PBST and incubated overnight at 4°C on a moving platform. Finally, slices were mounted on glass coverslips using Prolong Gold with DAPI (Life Technologies).

To induce axon degeneration, slices were incubated in culture media containing vinblastine (VB; 100 nM) for 8 hr. Where indicated, slices were incubated in culture media containing NAD^+ (5 mM) for 24 hr prior to the addition of VB.

Quantification of axon degeneration in limb buds

Axons were visualized in tissue sections using anti-neurofilament H immunostaining, and images were recorded using a 4x objective. In most cases, slices were large and required at least 2 images to visualize the entire section. In these cases, images were stitched together using Fiji (Schindelin et al., 2012). Due to the nature of degenerating axons in tissue, it was not possible to use the automated, degeneration index quantification commonly used to quantify axon degeneration (Sasaki et al., 2009). Instead, we developed a classification system to quantify degeneration: 0, no degeneration; 1, partial degeneration; 2, complete degeneration. We considered degeneration to be partial when blebbing occurred along the length of an axon and complete when only particles remained, with no clearly connected segments. We avoided using multiple classes for partially degenerated axons to reduce subjectiveness. Axons were only counted when single axon shafts were clearly distinguishable, typically at the ends of the axons. We included an additional score for major bundled regions of axons. This score was weighted as 10 axons, although it likely contained greater than 50, to limit subjectivity during measurement.

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Someya, S., Yu, W., Hallows, W.C., Xu, J., Vann, J.M., Leeuwenburgh, C., Tanokura, M., Denu, J.M., and Prolla, T.A. (2010). Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. Cell *143*, 802-812.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Wld^S expression and NR treatment prevent decreases in cochlear NAD^+ levels following noise exposure.

To determine the effect of NR treatment on NAD^+ levels in the cochlea, C57BL/6 mice, Wld^S C57BL/6 mice or NR-treated C57BL/6 mice were evaluated for cochlear NAD levels before and after noise exposure. NR treatments consisted of 5 days of BID IP injections of 1000 mg/kg NR prior to noise exposure followed by BID injections for a further 48 hours after noise exposure. All

groups were exposed to a 90 dB octave band for 2 hours. **(a)** Cochlear levels of NAD^+ prior to noise exposure. NAD^+ measurements are normalized to the NAD^+ levels in the control C57BL/6 sample. (NS = non-significant difference) **(b)** Cochlear NAD^+ levels after noise exposure. A total of 48 hours after noise exposure cochlea were isolated and their NAD^+ content was quantified. Ratios of NAD concentrations comparing [NAD^+] after noise to untreated mice was then determined. Noise exposure resulted in a significant drop in cochlear NAD^+ levels. This effect was not seen in Wld^S mice or in mice subjected to NR treatment. (unpaired, one-tailed Student's t-test, *P < 0.10, **P < 0.05).

Supplemental Figure 2. Prevention of NIHL by NR is independent of mouse background.

C57BL/6 mice, CBA mice and BalbC mice were tested for hearing loss at 24 hr and 14 d after exposure to 2 hr of 90 dB noise. NR/vehicle was administered at 1,000 mg/kg by intraperitoneal injection twice daily either for 5 d prior to noise exposure and 14 d after (NR All), NR treatment in all mouse backgrounds showed protection against transient hearing loss at 24 hr and permanent hearing loss at 14 d in comparison to vehicle controls, most notably at higher frequencies affected by noise exposure (16,000 and 32,000 Hz). Each point is the average threshold shift measured in 5 mice per group. Statistical analysis was performed using a two-way ANOVA followed by a Bonferroni post-test (**P < 0.01, ***P < 0.001).

Supplemental Figure 3. SIRT3 expression levels in wild type, *SIRT3* $Tg^{+/-}$ and *SIRT3* $^{-/-}$ mice.

(**a**) SIRT3 protein levels were measured in hepatic lysates from wild type (WT), *SIRT3* $Tg^{+/-}$ and *SIRT3* $^{-/-}$ mice. Fluorescence intensity was measured using a Biorad ChemiDoc imaging system and were normalized to beta actin levels. As shown in Supplementary Figure 2b, the SIRT3-overexpressing mice show an ~3.5-fold increase in SIRT3 levels. The endogenous SIRT3 protein is not clearly seen because the gel was not run out far enough to separate SIRT3 from FLAG-tagged SIRT3. The knockout mouse liver shows no detectable SIRT3 protein.

(**b**) ß-actin western blot. ß-actin staining was used to confirm equal loading of protein in the experiment shown in (a).

Supplemental Figure 4. SIRT3 is required for NAD to delay vinblastine-induced sensory axon degeneration.

(**a-b**) **Schematic of a limb bud.** To develop an assay for axon degeneration that combines the experimental amenability of standard in vitro assays with an *in vivo* environment for axon growth, we modified an assay developed by Brachmann et al. (2007) used to study peripheral neuron growth. In this assay, we isolate transverse sections through the developing limb buds of

E10.5 mice. Shown is a schematic of the transverse section (**a**). In these tissue sections, we can observe the growth of sensory and motor axons from the spinal column into the limb. This is schematized in (**b**).

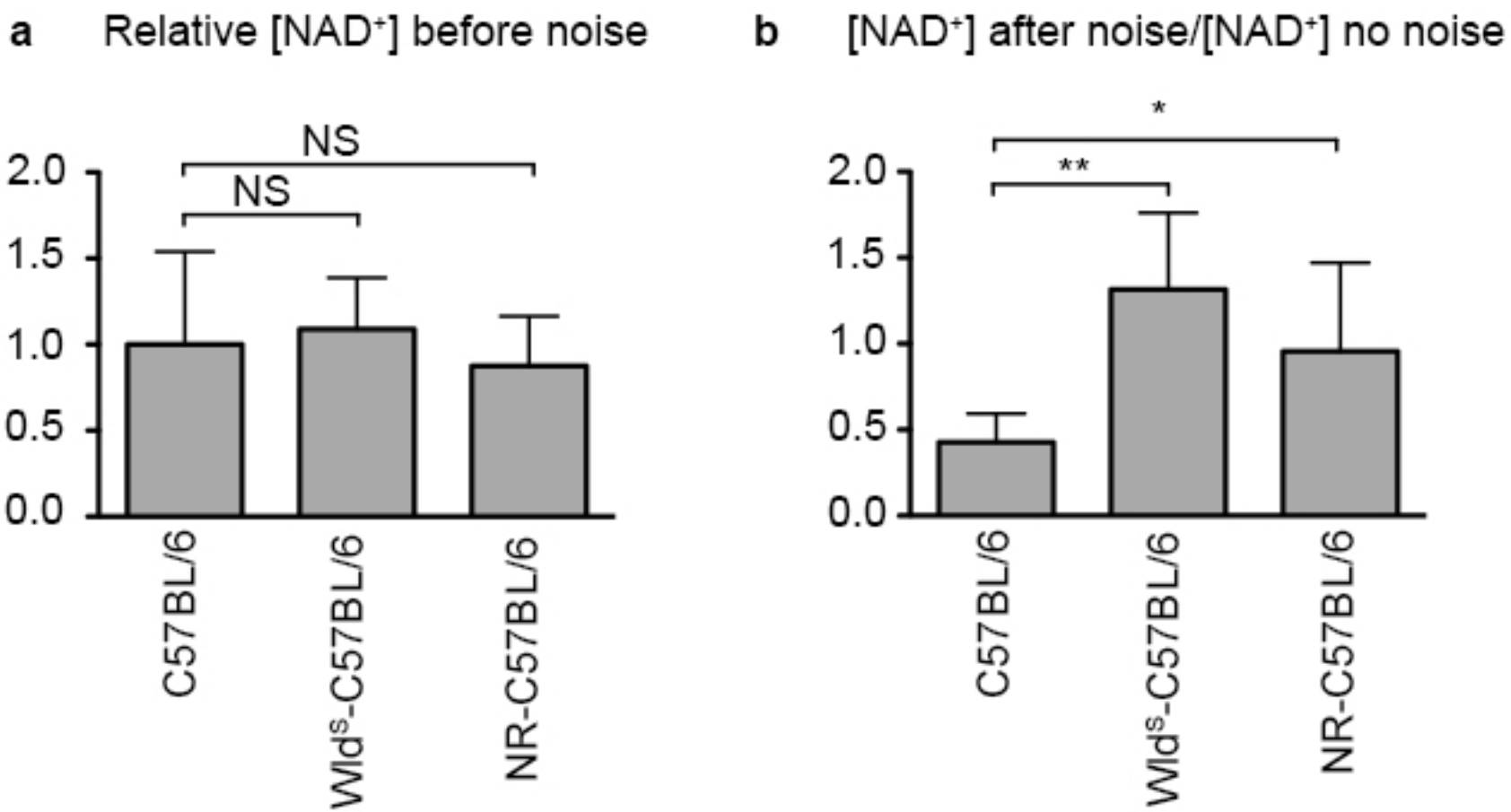
(**c**) **Vinblastine as model of peripheral neuropathy.** To induce axon degeneration in our model system, we utilized vinblastine (VB), which serves as a model of peripheral neuropathy. Sections were treated with VB (100 nM) for 8 hr which induced moderate axon degeneration. When tissue sections were treated with NAD^+ (5 mM) prior to VB treatment, however, axons were significantly preserved, consistent with studies using cultured neurons. Axons were visualized in tissue sections using anti-neurofilament H immunostaining, and images were recorded using a 4x objective. n=4 sections containing ~100 axons each per condition. Scale bar, 50 μm.

(**d**) **Quantification of results in C.** We quantified axon degeneration in tissue sections by calculating the degeneration rank, a classification score described in the methods. These results validate our limb bud assay as a model of NAD-sensitive axon degeneration. Bar graph represents the mean ± s.e.m. ***p<0.001 (one-way ANOVA with Tukey's post-hoc test).

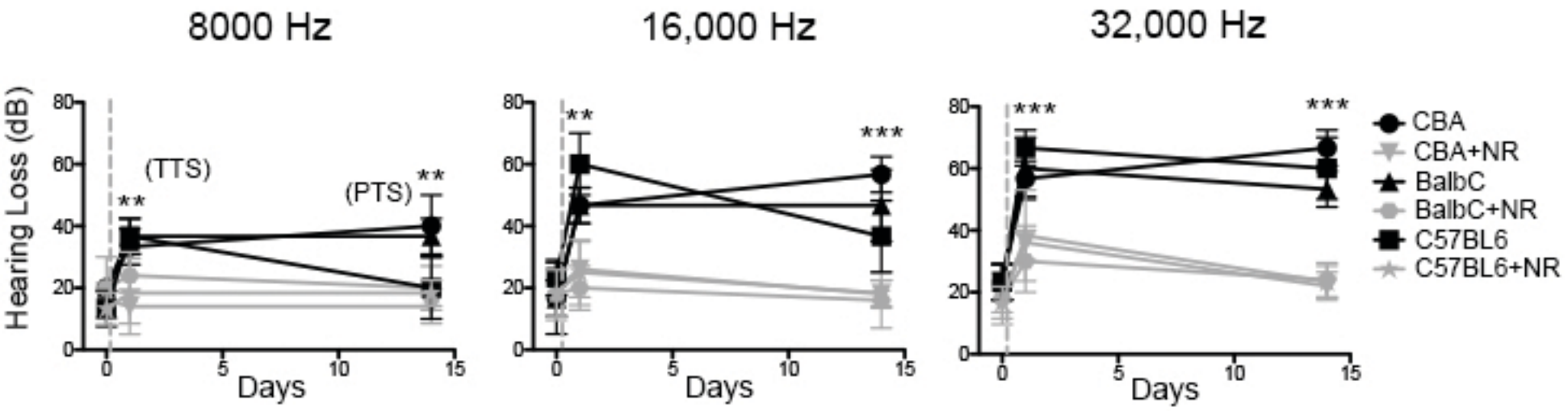
(**e**) **SIRT3 is required for NAD to delay axon degeneration.** To determine if SIRT3 is necessary for NAD-mediated axon protection, we bred *SIRT3* homozygous knockout mice with *SIRT3* heterozygous knockout mice, and we isolated embryos on E10.5. Tissue from each embryo was saved for genotyping after sectioning. Tissue sections from both heterozygous (+/-) and homozygous (-/-) *SIRT3* knockout embryos were treated with vinblastine (VB; 100 nM) in the presence or absence of NAD^+ (5 mM). In *SIRT3* heterozygous knockout tissue, NAD^+ delayed axon degeneration similar to what we observed in wild-type tissue (**C**). In *SIRT3* homozygous knockout tissue, however, NAD^+ no longer delays axon degeneration.

(**f**) **Quantification of results in e.** Results were quantified by calculating the degeneration rank, as described in the methods. These results suggest that SIRT3 mediates the effects of NAD^+ in delaying axon degeneration. n=4 sections containing ~100 axons each per condition. Scale bar, 50 μm. ***p<0.001 (one-way ANOVA with Tukey's post-hoc test).

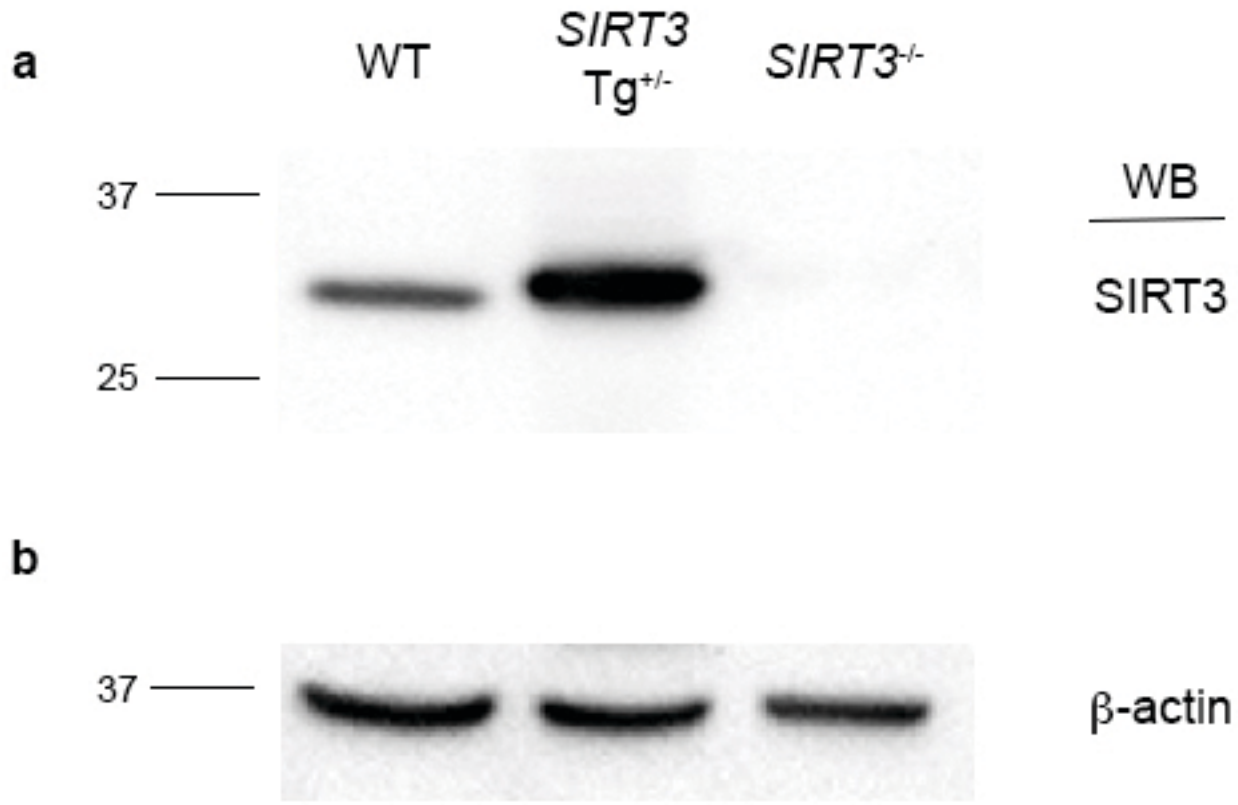
Supplemental Figure 1 Brown et al, Related to Figures 1 and 2



Supplemental Figure 2 Brown et al, Related to Figure 2



Supplementary Figure 3, Brown et al, Related to Figures 3, 4 and 5



Supplemental Figure 4, Brown et al, Related to Figure 6