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Increased Nuclear NAD Biosynthesis and SIRT1 Activation Prevent Axonal Degeneration

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Axonal degeneration is an active program of self-destruction that is observed in many physiological and pathological settings. In Wallerian degeneration slow (*wld*^{s}) mice, Wallerian degeneration in response to axonal injury is delayed because of a mutation that results in overexpression of a chimeric protein (Wld^s) composed of the ubiquitin assembly protein Ufd2a and the nicotinamide adenine dinucleotide (NAD) biosynthetic enzyme Nmnat1. We demonstrate that increased Nmnat activity is responsible for the axon-sparing activity of the Wld^s protein. Furthermore, we demonstrate that SIRT1, a mammalian ortholog of Sir2, is the downstream effector of increased Nmnat activity that leads to axonal protection. These findings suggest that novel therapeutic strategies directed at increasing the supply of NAD and/or Sir2 activation may be effective for treatment of diseases characterized by axonopathy and neurodegeneration.

Axonopathy is a critical feature of many peripheral neuropathies, and axonal degeneration often precedes the death of neuronal cell bodies in neurodegenerative diseases such as Parkinson's and Alzheimer's disease (*1*). These axonal deficits are an important component of the patient's disability and potentially represent a therapeutic target for combating these diseases (*2*).

The discovery of a spontaneous dominant mutation in mice that results in delayed axonal degeneration, the Wallerian degeneration slow (*wld*^{s}) mice, suggests that axonal degeneration is an active process of self-destruction (*3*). Genetic analysis has shown that the *wld*^{s} mutation comprises an 85-kb tandem triplication, which results in overexpression of a chimeric nuclear molecule (Wld^s protein). This protein is composed of the N-terminal 70 amino acids of Ufd2a (ubiquitin fusion degradation protein 2a), a ubiquitin-chain assembly factor, fused to the complete sequence of nicotinamide mononucleotide adenylyltransferase1 (Nmnat1), an enzyme in the NAD biosynthetic pathway that generates NAD within the nucleus (*4*, *5*). The Wld^s protein has Nmnat activity but lacks ubiquitin ligase function, suggesting that axonal protection is derived from either increased Nmnat1 activity or a "dominant negative" inhibition of Ufd2a function.

To determine the mechanism of delayed axonal degeneration mediated by the Wld^s protein, we used an in vitro Wallerian degeneration model. Primary dorsal root ganglion (DRG) explant neurons were infected with lentivirus expressing recombinant proteins, and axons were injured by either removal of the neuronal cell body (transection) or growth in vincristine (toxic). We first demonstrated that transected axons from neurons expressing the Wld^s protein degenerated with the delayed kinetics characteristic of neurons derived from *wld*^{s} mice (Fig. 1A) (*6*). Next, we compared axonal degeneration after transection in wild-type neurons that express the chimeric Wld^s protein with those that express only the Ufd2a or Nmnat1 portions of the Wld^s protein, linked to enhanced green fluorescent protein (EGFP) (Fig. 1B). We found that expression of EGFP-Nmnat1 delayed axonal degeneration comparable to Wld^s protein itself, whereas the N-terminal 70 amino acids of Ufd2a (fused to EGFP), targeted to either the nucleus or cytoplasm, did not affect axonal degeneration. Quantification of these effects was performed by counting the percentage of remaining neurites at various times after removal of neuronal cell bodies. This analysis showed that EGFP-Nmnat1, like Wld^s protein itself, resulted in a >10-fold increase in intact neurites 72 hours after injury. To further exclude direct involvement of the ubiquitin-proteasome system in Wld^s protein-mediated axonal protection, we examined the effect of Ufd2a inhibition using either a dominant-negative Ufd2a mutant or a Ufd2a small interfering RNA (siRNA) construct. However, neither method of Ufd2a inhibition resulted in delayed axonal degradation in response to axotomy. Together, these experiments demonstrated that the Nmnat1 portion of the Wld^s protein is responsible for the delayed axonal degeneration observed in *wld*^{s} mice.

Nmnat1 is an enzyme in the nuclear NAD biosynthetic pathway that catalyzes the conversion of nicotinamide mononucleotide (NMN) and nicotinate mononucleotide (NaMN) to NAD and nicotinate adenine dinucleotide (NaAD), respectively (*7*). The axonal protection observed in Nmnat1 overexpressing neurons could be mediated by its ability to synthesize NAD (i.e., its enzymatic activity), or perhaps by other unknown functions of this protein. To address this question, we used the Nmnat1 crystal structure to identify several residues predicted to participate in substrate binding (*8*). A mutation in one of these residues was engineered into full length Nmnat1 (W170A) and Wld^s (W258A) protein. In vitro enzymatic assays confirmed that both of these mutant proteins were severely limited in their ability to synthesize NAD (fig. S2). Each of these mutants and their respective wild-type counterparts was singly introduced into neurons to assess their ability to protect axons from degradation. We found that neurons expressing these enzymatically inactive mutants had no axonal protective effects (Fig. 1C), which indicates that NAD/NaAD production is responsible for the ability of Nmnat1 to prevent axonal degradation.

In addition to mechanical transection, axonal protection in *wld*^{s} mice is also observed against other damaging agents such as ischemia and toxins (*2*, *9*). We sought to determine whether increased Nmnat activity would also delay axonal degradation in response to other types of axonal injury, such as vincristine, a cancer chemotherapeutic reagent with well-characterized axonal toxicity. Neurons expressing either Nmnat1 or EGFP (control) were grown in 0.5 μM vincristine for up to 9 days. We found that axons of neurons expressing Nmnat1 maintained their original length and refractility, whereas axons emanating from uninfected neurons or those expressing EGFP gradually retracted and had mostly degenerated by day 9 (Fig. 2). These results indicate that increased Nmnat activity itself can protect axons from both mechanical and toxic insults.

Previous experiments have shown that neuronal cells express membrane proteins that can bind and transport extracellular NAD into the cell (*10*). This encouraged us to investigate whether exogenously administered NAD could prevent axonal degeneration. We added various concentrations of NAD to neuronal cultures before axonal transection and examined the extent of axonal degradation. We found that 0.1 to 1 mM NAD added 24 hours before axotomy significantly delayed axonal degeneration, although exogenously applied NAD (1 mM) was slightly less effective in protecting axons than lentivirus-mediated Nmnat1 expression (Fig. 3A). These results provide direct support for the idea

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that increased NAD supply can prevent axonal degradation.

NAD plays a variety of roles in the cell. In the mitochondria, it is involved in electron-transport processes important in energy metabolism, whereas in the nucleus NAD regulates aspects of DNA repair and transcription. In yeast, the Nmnat homologs are nuclear proteins that participate in the nuclear NAD salvage pathway (*11*, *12*), which suggests that NAD could be mediating its axonal protective effects by a nuclear mechanism. Indeed, both Wld^s and Nmnat1 were found in the nucleus with immunohistochemistry and EGFP fluorescence (fig. S3). Interestingly, the activation of the NAD salvage pathway in yeast does not alter total cellular NAD levels (*11*). Similarly, tissue NAD levels in wild-type and *wld^s* brain are similar, despite the increased NAD synthetic activity in *wld^s* tissues (*5*). We measured NAD levels in wild-type and Nmnat1-expressing cells using sensitive microscale enzymatic assays (*13*) and found that increased Nmnat activity did not result in changes in overall cellular NAD levels (*14*). Together, these data suggest that an NAD-dependent enzymatic activity in the nucleus, as opposed to cytoplasmic NAD-dependent processes, is likely to mediate the axonal protection observed in response to increased Nmnat activity.

To gain further insight into the mechanism of NAD-dependent axonal protection (NDAP), we examined whether NAD was required prior to the removal of the neuronal cell bodies or whether direct exposure of the severed axons to high levels of NAD was sufficient to provide protection (Fig. 3B). Neuronal cultures were prepared, and 1 mM NAD was added to the culture medium at the time of axonal transection or at various times (4 to 48 hours) before injury. We found that administering NAD at the time of axonal transection or for up to 8 hours before injury had no protective effects on axons. However, significant axon sparing was observed when neurons were incubated with NAD for longer periods of time before injury, with the greatest effects occurring after 24 hours of NAD pretreatment. These results indicate that NDAP is not mediated by a rapid posttranslational modification within the axons themselves. Instead, they suggest that the protective process requires de novo transcriptional and/or translational events. The active nature of axonal self-destruction was further emphasized by our observations that treatment of neurons for 24 hours before axotomy with inhibitors of either RNA (actinomycin D) or protein (cycloheximide) synthesis resulted in axonal protection (*15*).

The Sir2 family of protein deacetylases and poly(ADP-ribose) polymerase (PARP) are involved in major NAD-dependent nuclear enzymatic activities. Sir2 is an NAD-

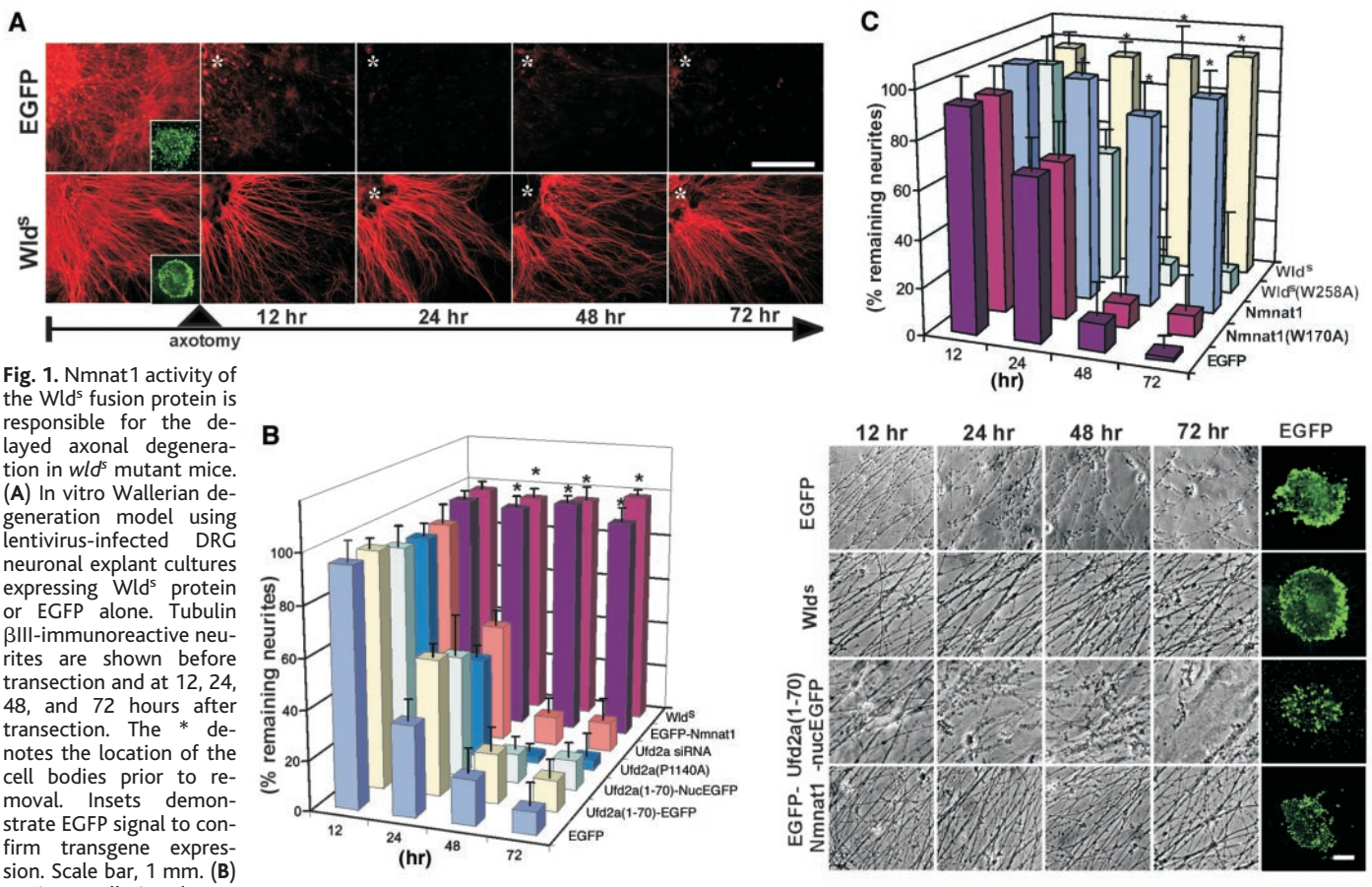

Fig. 1. Nmnat1 activity of the Wld^s fusion protein is responsible for the delayed axonal degeneration in *wld^s* mutant mice. (**A**) In vitro Wallerian degeneration model using lentivirus-infected DRG neuronal explant cultures expressing Wld^s protein or EGFP alone. Tubulin βIII-immunoreactive neurites are shown before transection and at 12, 24, 48, and 72 hours after transection. The * denotes the location of the cell bodies prior to removal. Insets demonstrate EGFP signal to confirm transgene expression. Scale bar, 1 mm. (**B**) In vitro Wallerian degeneration model with lentivirus-infected DRG neurons expressing EGFP only, Wld^s protein, Ufd2a portion (70 residues) of Wld^s protein fused to EGFP [Ufd2a(1-70)-EGFP], Ufd2a(1-70)-EGFP with C-terminal nuclear localization signal, Nmnat1 portion of Wld^s protein fused to EGFP, dominant-negative Ufd2a [Ufd2a(P1140A)], or Ufd2a siRNA construct. Representative images of neurites and quantitative analysis of remaining neurite numbers (percentage of remaining neurites relative to pretransection ± SD) at the indicated time point with each construct are shown. The * indicates significant difference ($P < 0.0001$) with EGFP-infected neurons. The EGFP signal before transection confirms transgene expression (bottom row). Scale bar, 50 μm. (**C**) In vitro Wallerian degeneration model of lentivirus-infected DRG neurons expressing Nmnat1 or Wld^s protein, mutants of these proteins that lack NAD-synthesis activity Nmnat1(W170A) and Wld^s(W258A), or EGFP (see color code). Quantitative analysis of the number of remaining neurites at the indicated time points for each construct (percentage of remaining neurites relative to pretransection ± SD). The * indicates significant difference ($P < 0.0001$) with EGFP-infected neurons.

dependent deacetylase of histones (*15*) and other proteins, and its activation is central to promoting increased longevity in yeast and *Caenorhabditis elegans* (*17*, *18*). PARP is activated by DNA damage and is involved in DNA repair (*19*). The importance of these NAD-dependent enzymes in regulating gene activity prompted us to investigate their potential role in the self-destructive process of axonal degradation. We tested whether inhibitors of Sir2 (Sirtinol) (*20*) and PARP [3-aminobenzamide (3AB)] (*21*) could affect NDAP (Fig. 4A). Neurons were cultured in the presence of 1 mM NAD and either Sirtinol (100 μM) or 3AB (20 mM). Axonal transection was performed by removal of the neuronal cell bodies, and the extent of axonal degradation was assessed 12 to 72 hours later. We found that, although Sirtinol had no axonal toxicity on uninjured axons (fig. S5), it effectively blocked NDAP after transection, indicating that Sir2 proteins are likely effectors of this process. In contrast, 3AB had no effect on NDAP, indicating that PARP does not play a role in axonal protection. Interestingly, 3AB alone did stimulate limited axonal protection (Fig. 4A), presumably as a consequence of PARP inhibition, which decreases NAD consumption and raises nuclear NAD levels. To confirm the involvement of Sir2 proteins in NDAP, we tested the effects of resveratrol (10 to 100 μM), a polyphenol compound found in grapes that enhances Sir2 activity (*22*). We found that neurons treated with resveratrol prior to axotomy showed a decrease in axonal degradation that was comparable to that obtained with NAD (Fig. 4B), providing further support for the idea that Sir2 proteins are effectors of the axonal protection mediated by increased Nmnat activity.

In humans and rodents, seven molecules that share the Sir2 conserved domain [sirtuin (SIRT) 1 to 7] have been identified (*23*). SIRT1 is located in the nucleus and is involved in chromatin remodeling and the regulation of transcription factors such as p53 (*24*), whereas other SIRT proteins are located within the cytoplasm and mitochondria (*25*, *26*). To determine which SIRT protein(s) is involved in NDAP, we performed knockdown experiments using siRNA constructs to specifically target each member of the SIRT family. Neurons were infected with lentiviruses expressing specific SIRT siRNA constructs that effectively suppressed expression of their intended target (table S1). The infected neurons were cultured in 1 mM NAD, and axonal transection was performed by removing the cell bodies. Inhibiting the expression of most SIRT proteins did not significantly affect NDAP; however, the knockdown of SIRT1 blocked NDAP as effectively as Sirtinol (Fig. 4C). Like Sirtinol treatment, SIRT1 inhibition by siRNA did not affect the rate of degeneration in untreated neurons or the axonal integrity in uninjured neurons (fig. S5). These results indicate that SIRT1 is the major effector of the increased NAD supply that effectively prevents axonal self-destruction. Although, SIRT1 may deacetylate proteins directly involved in axonal stability, its predomi-

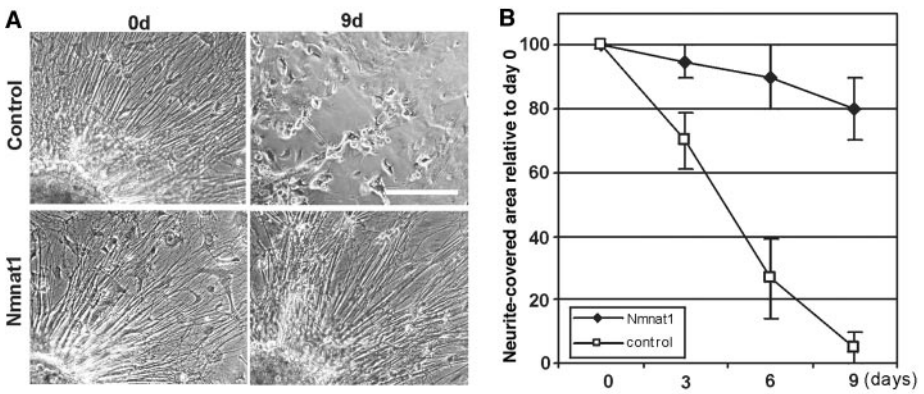

Fig. 2. Increased Nmnat1 activity protects axons from degeneration caused by vincristine toxicity. (**A**) DRG neuronal explants expressing either Nmnat1 or EGFP (control) were cultured with 0.5 μM vincristine. Representative images of neurites (phase-contrast) at the indicated times after vincristine addition are shown. Scale bar, 1 mm. (**B**) Quantification of the protective effect at the indicated time points is plotted as the area covered by neurites relative to that covered by neurites before treatment.

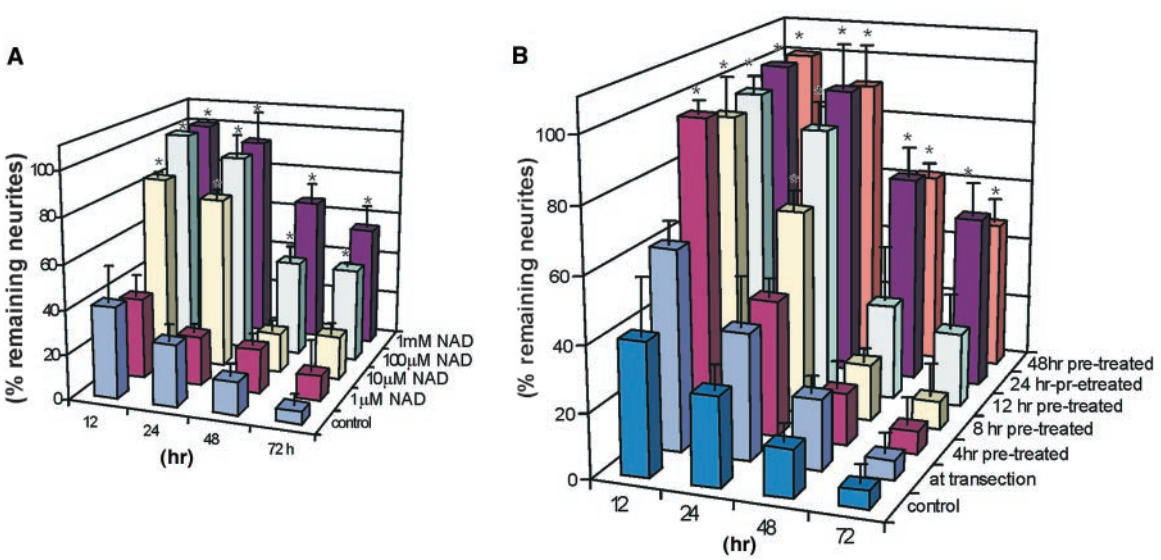

Fig. 3. Axonal protection requires pretreatment of neurons with NAD before injury. (**A**) In vitro Wallerian degeneration using DRG explants cultured in the presence of various concentrations of NAD added 24 hours before axonal transection. (**B**) DRG explants preincubated with 1mM NAD for 4, 8, 12, 24, or 48 hours prior to transection. In each experiment, the number of remaining neurites (percentage of remaining neurites relative to pretransection ± SD) is shown at each of the indicated time points. The * indicates significant axonal protection compared with control ($P < 0.0001$).

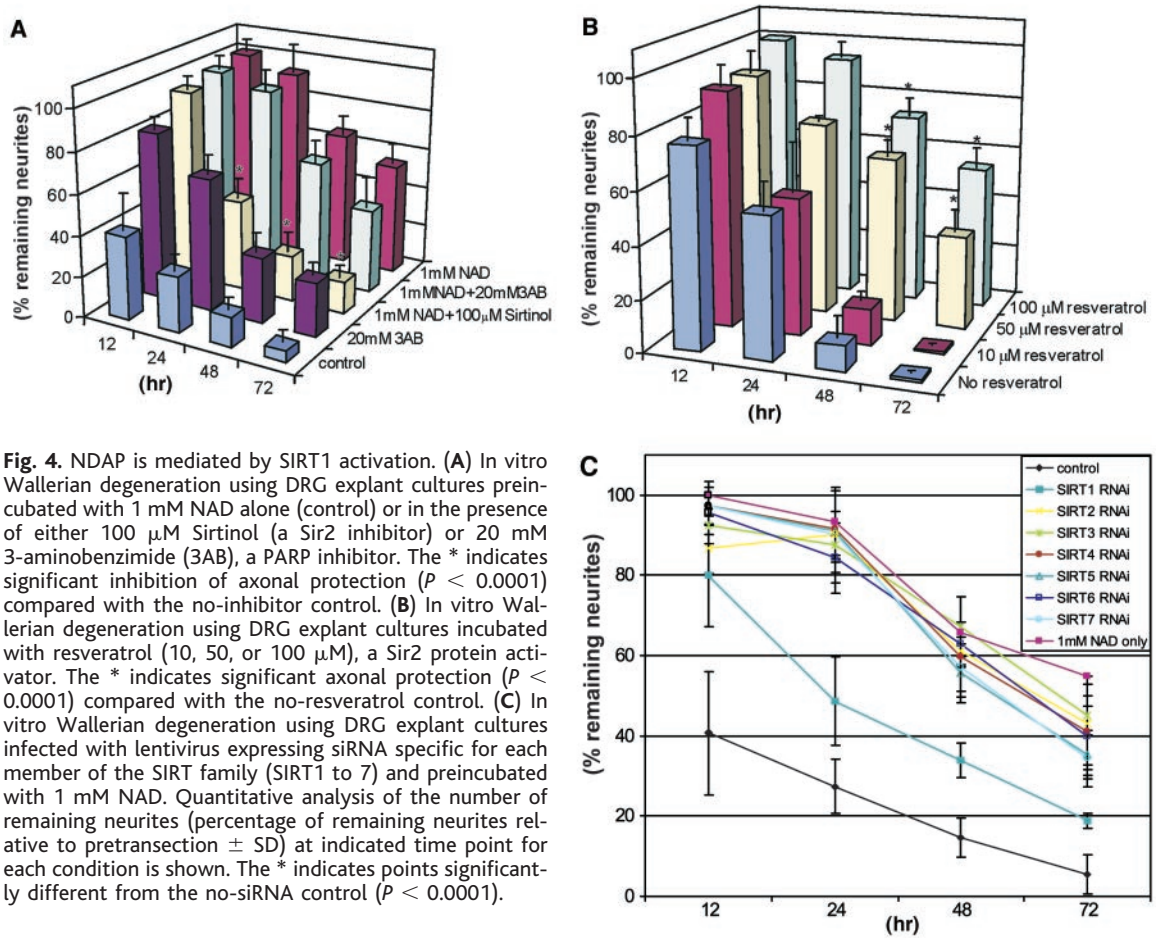

Fig. 4. NDAP is mediated by SIRT1 activation. (**A**) In vitro Wallerian degeneration using DRG explant cultures preincubated with 1 mM NAD alone (control) or in the presence of either 100 μM Sirtinol (a Sir2 inhibitor) or 20 mM 3-aminobenzimide (3AB), a PARP inhibitor. The * indicates significant inhibition of axonal protection ($P < 0.0001$) compared with the no-inhibitor control. (**B**) In vitro Wallerian degeneration using DRG explant cultures incubated with resveratrol (10, 50, or 100 μM), a Sir2 protein activator. The * indicates significant axonal protection ($P < 0.0001$) compared with the no-resveratrol control. (**C**) In vitro Wallerian degeneration using DRG explant cultures infected with lentivirus expressing siRNA specific for each member of the SIRT family (SIRT1 to 7) and preincubated with 1 mM NAD. Quantitative analysis of the number of remaining neurites (percentage of remaining neurites relative to pretransection ± SD) at indicated time point for each condition is shown. The * indicates points significantly different from the no-siRNA control ($P < 0.0001$).

nantly nuclear location, along with the requirement for NAD ~24 hours prior to injury for effective protection, suggest that SIRT1 regulates a genetic program that leads to axonal protection.

In *wld*^{s} mice, axonal protection through Wld^s protein overexpression has been demonstrated in models of motor neuron and Parkinson's disease and in peripheral sensory neurons affected by chemotherapeutic agents (*2*, *27*). Our results indicate that the molecular mechanism of axonal protection in the *wld*^{s} mice is due to the increased nuclear NAD biosynthesis that results from increased Nmnat1 activity and consequent activation of the protein deacetylase SIRT1. Other intracellular events that affect NAD levels or NAD/NADH ratios, such as energy production through respiration, may also affect physiological and pathological processes in the nervous system through SIRT1-dependent pathways (*28*). It is possible that the alteration of NAD levels by manipulation of the NAD biosynthetic pathway, Sir2 protein activity, or other downstream effectors will provide new therapeutic opportunities for the treatment of diseases involving axonopathy and neurodegeneration.

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Supporting Online Material

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