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NAD^+ and axon degeneration revisited: Nmnat1 cannot substitute for Wld^S to delay Wallerian degeneration

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The slow Wallerian degeneration protein (Wld^S), a fusion protein incorporating full-length nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1), delays axon degeneration caused by injury, toxins and genetic mutation. Nmnat1 overexpression is reported to protect axons *in vitro*, but its effect *in vivo* and its potency remain unclear. We generated Nmnat1-overexpressing transgenic mice whose Nmnat activities closely match that of *Wld*^{S} mice. Nmnat1 overexpression in five lines of transgenic mice failed to delay Wallerian degeneration in transected sciatic nerves in contrast to *Wld*^{S} mice where nearly all axons were protected. Transected neurites in Nmnat1 transgenic dorsal root ganglion explant cultures also degenerated rapidly. The delay in vincristine-induced neurite degeneration following lentiviral overexpression of Nmnat1 was significantly less potent than for Wld^S, and lentiviral overexpressed enzyme-dead Wld^S still displayed residual neurite protection. Thus, Nmnat1 is significantly weaker than Wld^S at protecting axons against traumatic or toxic injury *in vitro*, and has no detectable effect *in vivo*. The full protective effect of Wld^S requires more N-terminal sequences of the protein.
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Axon degeneration is an early, often contributory, event in many neurodegenerative disorders including amyotrophic lateral sclerosis, multiple sclerosis and Alzheimer's disease.[1–5] Wallerian degeneration distal to an axonal injury is an important and relevant experimental model of axon degeneration in disease. A dominant mutation that delays Wallerian degeneration 10-fold in slow Wallerian degeneration (*Wld*^{S}) mice and rats,[6,7] also delays axon degeneration in genetic and toxic 'dying-back' disorders,[2,8–11] indicating similarities in the underlying mechanisms. Thus, understanding the Wallerian degeneration mechanism should help to understand how axons degenerate in disease. Moreover, the influence of genetics on axon degeneration suggests a regulated, proactive pathway that could be manipulated both as an experimental tool and as a novel protective treatment for axonal degeneration. A clear understanding of the mechanism of axonal protection by Wld^S is thus essential.

The mechanism for axonal protection by Wld^S has been in question since the discovery of the *Wld*^{S} mouse.[6] Our groups showed that the gene identified by positional cloning[12–14] delays Wallerian degeneration in transgenic mice and virally transduced dorsal root ganglion (DRG) explant cultures.[15,16] Wld^S encodes a fusion protein containing 70 N-terminal amino acids of ubiquitination factor Ube4b, full-length nicotinamide adenine dinucleotide (NAD^+)-synthesising enzyme nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1), and a unique 18-amino-acid joining sequence.[15] Surprisingly, Wld^S is abundant in neuronal nuclei[8,11,15,18] and so far undetectable in axons *in vivo*. However, it does enter neurites in virally transduced primary cultures.[16,19] Thus, while a direct protective effect in axons remains possible,[19] Wld^S appears likely to act within nuclei. Thus, it is important to identify downstream factors mediating Wld^S action, to understand and perhaps mimic the protective mechanism.

An important first step is to determine the parts of the Wld^S fusion protein required to delay axon degeneration. Two recent reports[19,20] suggest a primary role for Nmnat1 and its synthetic product NAD^+, but provide conflicting data on how NAD^+ mediates axonal protection. One report concludes that raised NAD^+ levels increase activity of the Sir2 deacetylase Sirt1.[20] Sirtinol, which reduces Sir2 activity, was reported to block NAD^+-dependent axonal protection, whereas resveratrol, a Sir2 enhancer, had the opposite effect. Sirt1 siRNA negated the protective effects of NAD^+ in transected DRG neurites. The other study could not reproduce these results in Sirt1-null mice, and instead concluded that NAD^+ works

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Abbreviations: DRG, dorsal root ganglion; NAD, nicotinamide adenine dinucleotide; Nmnat, nicotinamide mononucleotide adenylyltransferase; Sir2, Sirt1, silent information regulator 2, and its mammalian homologue; *Wld*^{S}, Wld^S, slow Wallerian degeneration gene and protein
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directly within axons independently of Sir2.[19] Both papers also reported that exogenous NAD^+ mimics the protection of neurites by Wld^S but disagreed on the amount of NAD^+ required (0.01–1 and 5–20 mM, respectively).

The implications of these studies are profound. Not only is it important to understand how Wallerian degeneration is controlled, but protection by exogenous agents such as NAD^+ and resveratrol suggests possible leads into pharmacological intervention in axonal disease. Moreover, a role for Sirt1 would link Wallerian degeneration with other forms of ageing-related cell death.[21] However, both studies were carried out exclusively *in vitro* and the potencies of Nmnat1 and Wld^S were not compared. Thus, we studied Wallerian degeneration using lentiviral vectors and transgenic mice to overexpress Nmnat1. Despite matching the Nmnat enzyme activity of *Wld*^{S} heterozygotes, which have a robust slow Wallerian degeneration phenotype, Nmnat1 transgenic mice consistently showed a wild-type rate of Wallerian degeneration. Furthermore, we find that Nmnat1 has significantly lower potency to protect neurites from traumatic or toxic injury *in vitro*. Although we cannot rule out the possibility that Nmnat1 may delay Wallerian degeneration when expressed at very high levels, our data show conclusively that the ability of Wld^S to protect axons is far greater than that of Nmnat1 alone.

Results

Generation of transgenic mice overexpressing Nmnat1. Twenty-three lines of transgenic mice were generated and six high-copy number lines (Supplementary Figure 1) were selected to test for overexpression of enzymatically active Nmnat1. All lines were healthy, fertile and overtly normal.

We tested for transgenic lines expressing Nmnat1 at levels similar to or greater than *Wld*^{S} heterozygotes to use for phenotypic analysis. C57BL/*Wld*^{S} heterozygotes express increased Nmnat1 as part of the Wld^S protein and show a strong axon protection phenotype.[13,15,22] Western blots of sagittally divided half-brain homogenates, probed with anti-Nmnat1 antibody 183, showed that lines 881, 891, 2460, 7103 and 7104 overexpressed Nmnat1 (Figure 1a). Nonexpressing line 884 (data not shown) was not studied further. Nmnat1 levels in lines 881 and 7104 matched or exceeded that of Wld^S protein in *Wld*^{S} heterozygotes, suggesting that these lines were particularly useful to test whether Nmnat1 and Wld^S are functionally interchangeable in their ability to preserve transected axons *in vivo*.

We then tested whether transgenically expressed Nmnat1 possessed NAD^+ synthesis activity. Total Nmnat activity in sagittally divided half-brains of Nmnat1 transgenic line 881 and 7104 hemizygotes was very highly significantly greater than in wild-type mice ($P = 0.0073$ and $P < 0.0001$, respectively; $n = 8$–9). Mean values were increased 3.2–3.5 times, slightly more (although not significantly) than *Wld*^{S} heterozygotes (Figure 1b) and only a little less than the four-fold increase reported in *Wld*^{S} homozygotes.[15]

Wallerian degeneration was tested in sciatic nerve, so we first confirmed transgene expression in neurons projecting axons to this nerve. RT-PCR confirmed the presence of a transgene-specific Nmnat1 mRNA in lumbar DRG (Figure 1c). The same mRNA was detected in DRG explant cultures almost devoid of non-neuronal cells, strongly suggesting the transgene is expressed in sensory neurons (Figure 6n). In motor neurons, we found more definitive evidence that Nmnat1 was overexpressed and targeted to neuronal nuclei like Wld^S. Immunocytochemistry of Nmnat1 transgenic, *Wld*^{S} and wild-type lumbar spinal cords showed clearly stronger signal intensity in nuclei of all transgenic motor neurons compared to wild-type motor neurons (Figure 2), data which were reproducible also with a different anti-Nmnat1 antibody (Supplementary Figure 2). Wld^S mice showed an intermediate level of nuclear staining (Figure 2b). We cannot rule out targeting of some additional overexpressed Nmnat1 to the

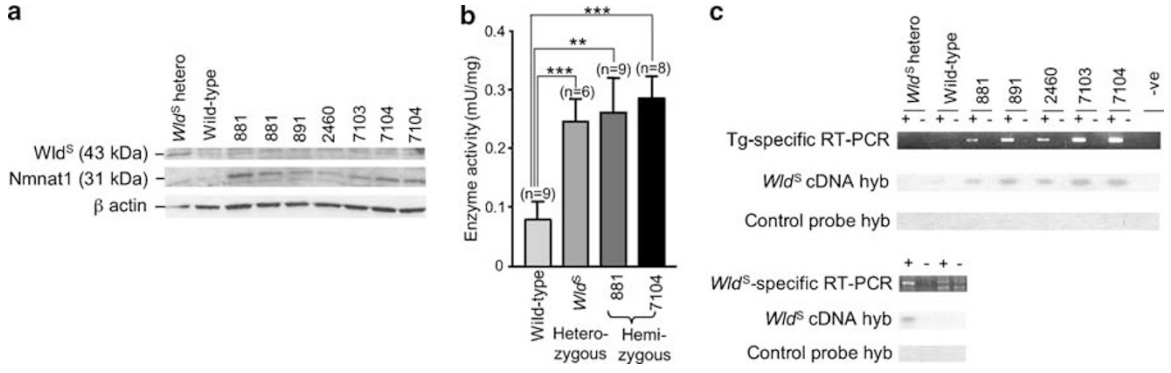


Figure 1 Overexpression of Nmnat1 in transgenic mice. (**a**) Western blot of mouse brain homogenate probed with antibody 183 against Nmnat1. The strong band at 43 kDa in heterozygous *Wld*^{S} brain is Wld^S protein, indicating that this antipeptide antiserum detects the intact protein (see also Conforti *et al*.[14]). Transgenic lines 881, 891, 2460, 7103 and 7104 consistently overexpress a 31 kDa protein compared to wild-type mice, consistent with the M_r of Nmnat1.[17] Loading control: mouse anti-β-actin (**b**) Confirmation that Nmnat1 overexpressed in the transgenic brains is enzymatically active. Total Nmnat activity was very highly significantly increased compared to wild-type brains in hemizygotes of both transgenic lines 881 and 7104, as well as in *Wld*^{S} heterozygotes. The mean values for the transgenics were similar to *Wld*^{S} heterozygotes. Mean $\pm$ SD. $^{**}P < 0.01$; $^{***}P < 0.001$. (**c**) RT-PCR ($+$ and $-$reverse transcriptase, as indicated) confirms that the same five transgenic lines express the transgene in lumbar DRG, some of whose axons project to sciatic nerve. Top panel: Transgene-specific RT-PCR, ethidium-stained gel. Panel 2: Transgene-specific RT-PCR hybridised with *Wld*^{S} cDNA demonstrates specificity of PCR product. Panel 3: RT-PCR hybridised with control probe (Uch-l1 cDNA). Panels 4–6: Same methods to visualise *Wld*^{S}-specific RT-PCR product, expressed specifically in *Wld*^{S} heterozygotes

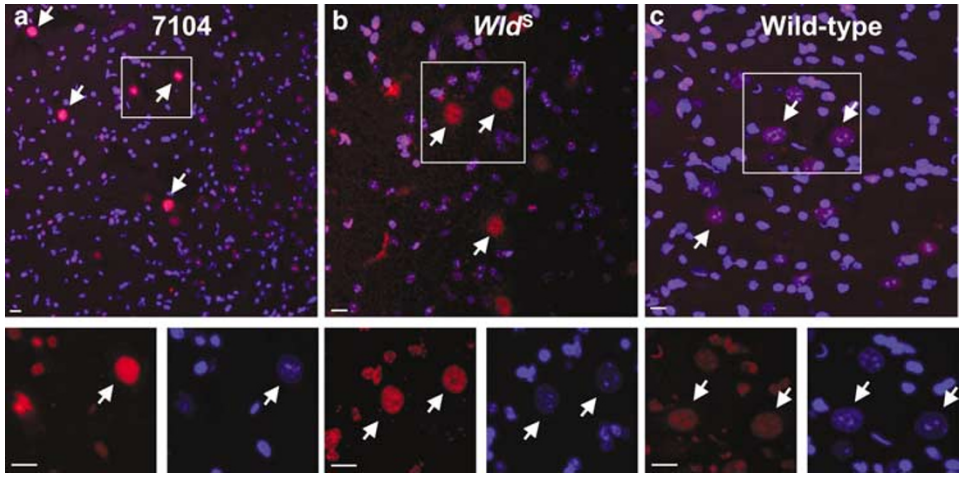


Figure 2 Immunofluorescence of lumbar spinal cord sections stained with affinity-purified anti-Nmnat1 polyclonal antibody[34] (red) and DAPI. Insets: Higher magnification view of boxed areas. Motor neuron nuclei (some indicated by arrows) are at least as strongly stained in transgenic line 7104 (**a**) as in *Wld*^{S} mice (**b**), in agreement with Western blotting and enzyme activity data. Nuclei in both strains are more strongly stained than in wild-type mice (**c**). Identical laser intensities and camera settings were used for each image. Scale bar: 10 μm

cytoplasm or axons, but these data confirm that overexpressed Nmnat1 is targeted to neuronal nuclei at levels exceeding that of Wld^S in *Wld*^{S} mice.

Thus, line 881 and 7104 hemizygotes express enzymatically active Nmnat1 at levels similar to, if not greater than *Wld*^{S} heterozygotes, mice where most axons survive 5 days after nerve lesion.[9,15,22] We therefore chose these lines for most of our subsequent investigations, but used also the other expressing lines, 891, 2460 and 7103, in some assays.

Wallerian degeneration is not delayed in Nmnat1 transgenic mice. We transected sciatic nerves in transgenic, wild-type and *Wld*^{S} heterozygote mice and tested axon survival in the distal stump, studying ultrastructure, axon continuity and protein integrity. To detect even a small delay in Wallerian degeneration, axon ultrastructure and continuity were analysed up to 72 h after nerve lesion, when over 80% of axons remain intact in *Wld*^{S} heterozygotes, but only a negligible proportion survive in wild-type mice.[15] Intact neurofilament protein was assessed at 5 days. To put this low stringency in perspective, most axons in *Wld*^{S} homozygotes survive structurally even 14 days after lesioning.[6,15,23,24]

First, we studied 3-day lesioned distal nerve stumps using electron microscopy (Figure 3 and Supplementary Figure 3). In *Wld*^{S} heterozygotes, most axons displayed normal cytoskeleton, unswollen mitochondria and a regular myelin sheath of normal thickness. In contrast, not one preserved axon was found in an extensive search of nerves from homozygotes or hemizygotes of Nmnat1 transgenic lines 881, 7104 and 891 ($n=5$), despite the low stringency of the assay (Figure 3c–f and Supplementary Figure 3). A very low rate of preservation ($<1\%$) was observed in line 7103, a rate similar to that previously reported in wild-type nerves.[15] Extensive analysis of semithin sections by light microscopy also revealed no evidence of axon preservation in Nmnat1 transgenics (data not shown). Thus, Nmnat1 expressed at levels similar to, or higher than Wld^S protein, does not preserve axon ultrastructure *in vivo*.

We then studied the longitudinal continuity of lesioned, fluorescently labelled distal axons in Nmnat1 transgenic mice crossed with the YFP-H strain.[25] We previously showed that in the absence of Wld^S all labelled axons fragment within 52 h of nerve transection,[26] whereas *Wld*^{S}/YFP-H axons remain continuous after 20 days, showing only localised atrophy.[23] Homozygotes of highly expressing Nmnat1 transgenic line 7104 (Figure 4f) were indistinguishable from wild type (Figure 4b), with every labelled axon fragmented 72 h after nerve transection. Similar results were seen in each line where we obtained Nmnat1/YFP-H double transgenics: lines 7103 (Figure 4e) and 2460 (data not shown). *Wld*^{S} heterozygous nerves showed no loss of continuity at 72 h (Figure 4c), consistent with our previous report that heterozygous *Wld*^{S} is sufficient to maintain axon continuity for 5 days.[9] Thus, Nmnat1 expressed at levels similar to, or higher than Wld^S protein, does not preserve axon continuity *in vivo*.

We then looked for biochemical preservation of heavy neurofilament protein (NF-H) in distal stumps of lesioned Nmnat1 transgenic nerves. As reported previously,[9] heterozygous *Wld*^{S} partially preserved NF-H 5 days after nerve lesion. However, transected nerves from transgenic lines 881 and 7104 were indistinguishable from wild-type nerves with no intact NF-H remaining (Figure 5). Thus, Nmnat1 expression at these levels does not preserve the integrity of cytoskeletal proteins in lesioned axons *in vivo*. Together with its inability to preserve axon ultrastructure and continuity, this leads us to conclude that Nmnat1 is not functionally equivalent to Wld^S. The ability of Wld^S to preserve lesioned axons *in vivo* requires more N-terminal sequences.

Lesioned DRG neurites in Nmnat1 transgenic primary cultures degenerate rapidly. The above data contrast sharply with reports that viral overexpression of Nmnat1 constructs in primary DRG cultures delays degeneration of

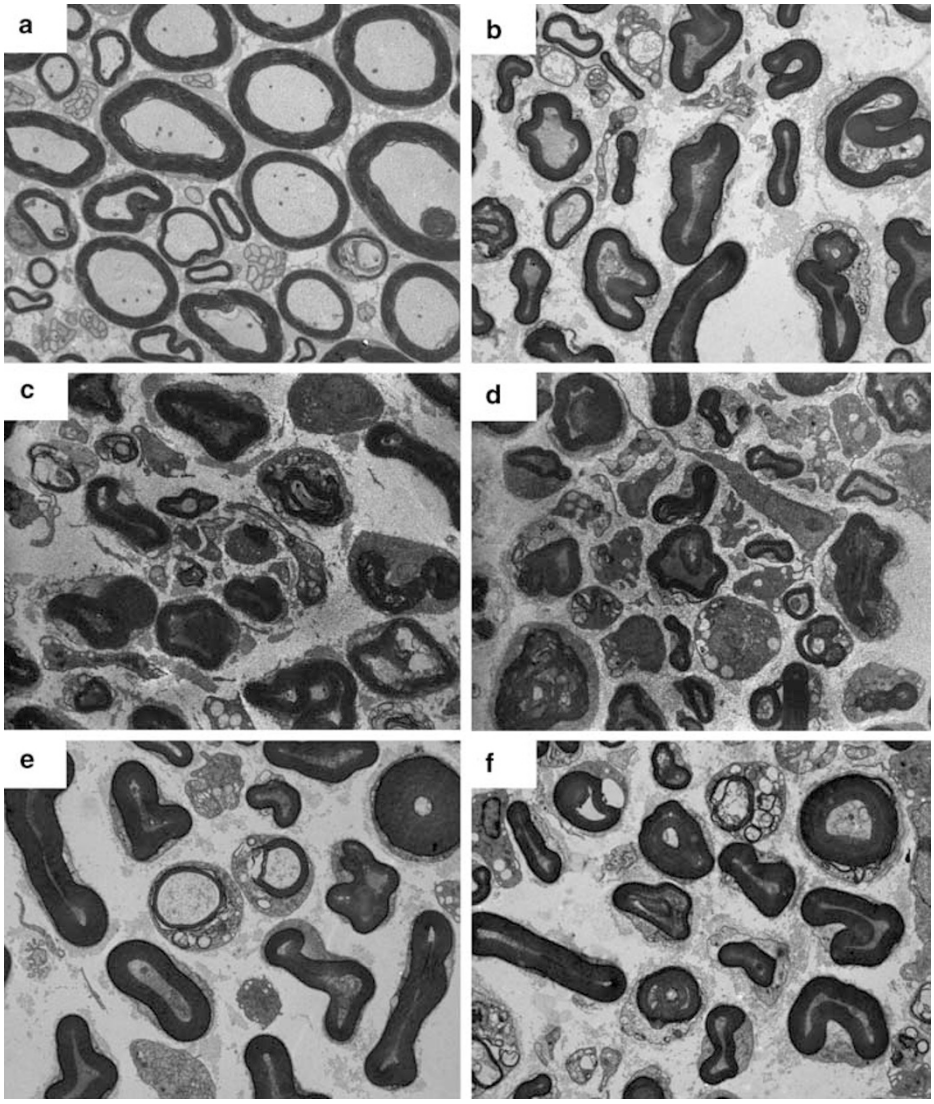


Figure 3 Rapid Wallerian degeneration in Nmnat1 transgenic mice at the ultrastructural level. Transmission electron microscopy ($\times$ 3000 magnification) of distal sciatic nerve stumps removed 72 h after proximal sciatic lesion. (**a**) Both myelinated and unmyelinated axons are well preserved in heterozygous *Wld*^{S} mice, with intact myelin sheaths, uniform, regularly spaced cytoskeleton and unswollen mitochondria. (**b**) In wild-type nerves, myelin collapsed to form ovoids, cytoskeleton was floccular or absent, and mitochondria swollen or absent. (**c** and **d**) Nerves from line 881 and 7104 hemizygotes, respectively, were indistinguishable from wild-type nerves (for further examples, and higher magnification, see Supplementary Figure 3). (**e** and **f**) Axons also continued to degenerate rapidly in lesioned nerves from homozygotes of lines 881 and 7104, respectively

transected neurites for at least 3 days.[19,20] Thus, we tested the effect of Nmnat1 *in vitro* by transecting neurites of DRG explant cultures from highly expressing lines 881 and 7104. Homozygous, hemizygous and wild-type embryos were produced by intercrossing hemizygous transgenic parents, and genotypes were determined only after degeneration had been scored independently by two experienced investigators.

In all 30 heterozygous *Wld*^{S} explants studied, transected neurites survived for more than 3 days (Figure 6a–d). In contrast, cut neurites of all 15 wild-type explants beaded and fragmented within 12 h, and detached or lost all semblance of neurite morphology by 24 h (Figure 6e–g). In every explant from Nmnat1 transgenic embryos (24 homozygous, 60 hemizygous; 39 line 881; 45 line 7104), neurite degeneration was indistinguishable from wild-type cultures. Interestingly, neither Wld^S nor Nmnat1 could be detected at the protein level in *Wld*^{S} and transgenic cultures, respectively (although for *Wld*^{S} there is clearly enough protein to confer the phenotype). However, transgene expression was confirmed at the mRNA level using RT-PCR (Figure 6n). We could find only occasional non-neuronal cells migrating out from aphidicolin-treated explants (Supplementary Figure 4). Thus, it is unlikely that non-neuronal cells made more than a negligible contribution to these strong RT-PCR products, although we cannot completely rule out this possibility. This suggests that Nmnat1 does not prolong the survival of injured axons *in vitro* or *in vivo* when expressed at a similar level to that in *Wld*^{S} heterozygotes.

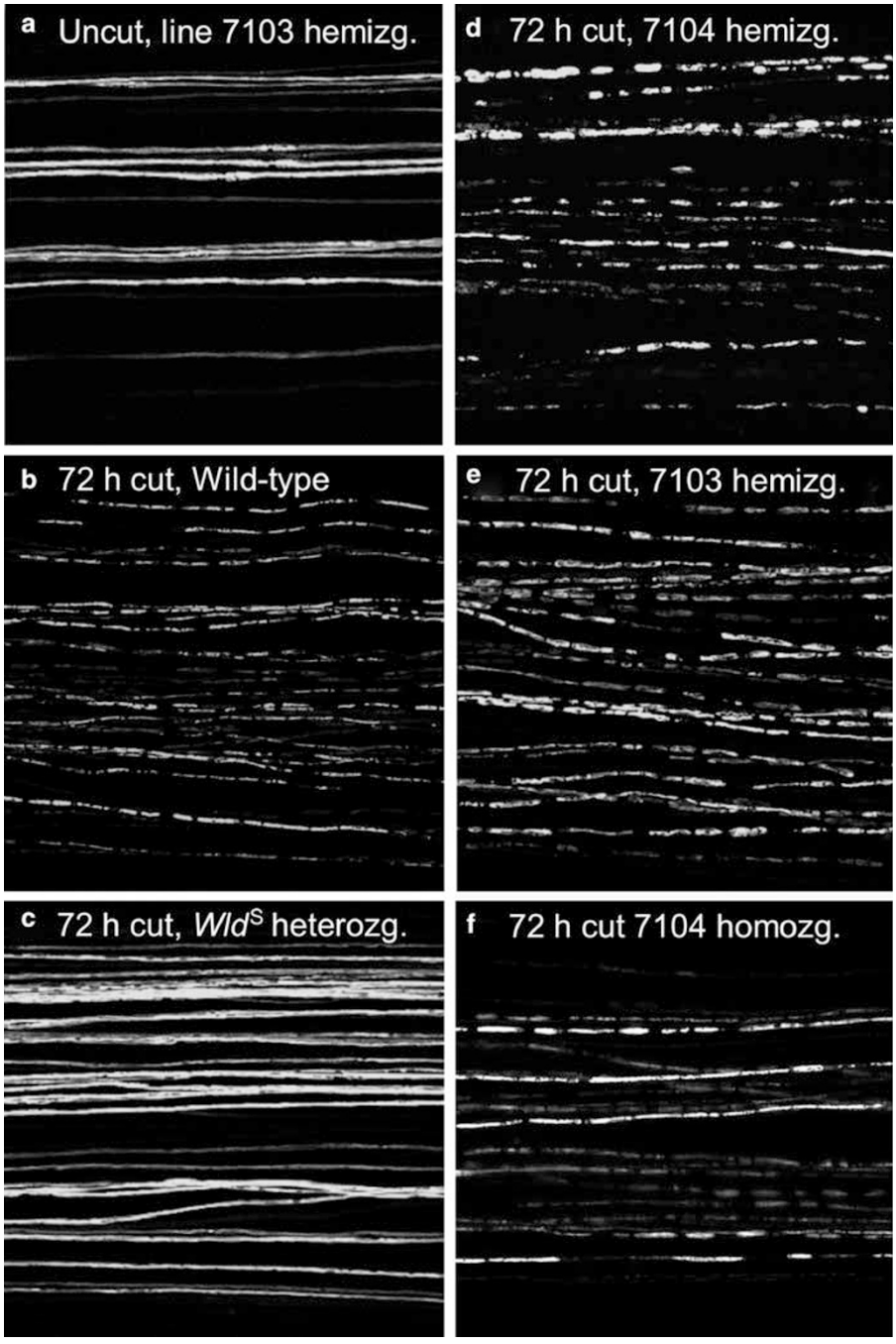


Figure 4 Rapid loss of axon continuity in lesioned transgenic Nmnat1/YFP-H nerves. Representative confocal images of tibial nerves with and without prior sciatic nerve lesion from mice carrying a YFP-H transgene. (**a**) Uncut nerve showing continuous axons. (**b**) All wild-type axons lose continuity within 72 h. (**c**) In contrast, changes in a *Wld*^{S} heterozygous nerve are limited to localised atrophy and continuity is maintained. (**d-f**) As in wild-type nerves, all axons in lesioned Nmnat1 transgenic nerves lost continuity within 72 h, even in strongly expressing line 7104 hemizygotes (**d**) or homozygotes (**f**)

Lentiviral expression of Wld^S shows significantly more axonal protection than Nmnat1. The lack of axonal protection in Nmnat1 transgenic mice led us to re-examine the reported protection provided by *in vitro* expression of Nmnat1 in cultured DRG.[19,20] Lentiviral expression of Wld^S and Nmnat1 was confirmed by Western blotting and endogenous fluorescence, respectively, and the signal in neurites *in vitro* confirmed neuronal expression (Figure 7). Expression of GFP alone showed no protection against vincristine toxicity when compared with uninfected DRG cultures (Figure 8). Expression of Wld^S showed a remarkable level of protection at both 7 and 13 days, similar to that reported previously using adenoviral delivery.[16] Expression of Nmnat1 provided an intermediate level of protection that

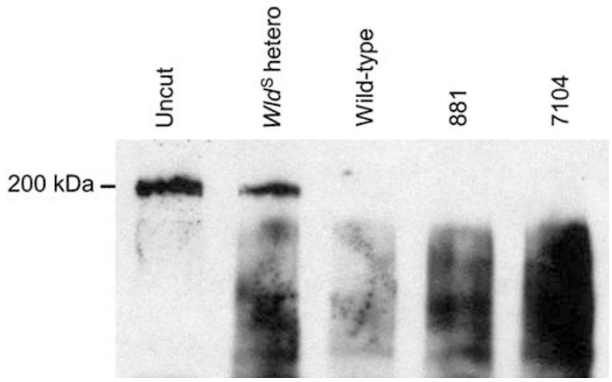


Figure 5 Rapid degradation of neurofilament protein in lesioned Nmnat1 transgenic nerves. Western blot showing NF-H reactivity in distal sciatic nerve 5 days after nerve lesion. A heterozygous dose of *Wld*^{S} is sufficient to preserve some NF-H that comigrates with that in uncut nerve. In contrast, hemizygotes of transgenic lines 881 and 7104, and wild-type mice, show only a smear of degradation products

was significantly less than Wld^S at 7 days ($P<0.01$). Nmnat1 overexpression showed no protection compared to controls after 13 days of vincristine exposure ($P>0.05$).

We also constructed a lentivirus that expressed only the Ube4b portion of Wld^S and tested it in the same experimental paradigm. As with the expression of Nmnat1, there was intermediate protection at day 7 but no protection at day 13 of vincristine exposure. Infection with both the Ube4b and the Nmnat1 showed no additive protective effect (Figure 8b).

The W258A mutation reduces but does not abolish protection by Wld^S. In order to test whether Wld^S confers neuroprotection through its Nmnat1 enzymatic activity, we introduced a specific point mutation in Wld^S that abolishes Nmnat1 activity and thus the additional NAD$^+$ production.[20] Lentiviral expression of W258A mutant in DRG was comparable to the expression of wild-type Wld^S (Figure 7). However, W258A provided a reduced level of protection at

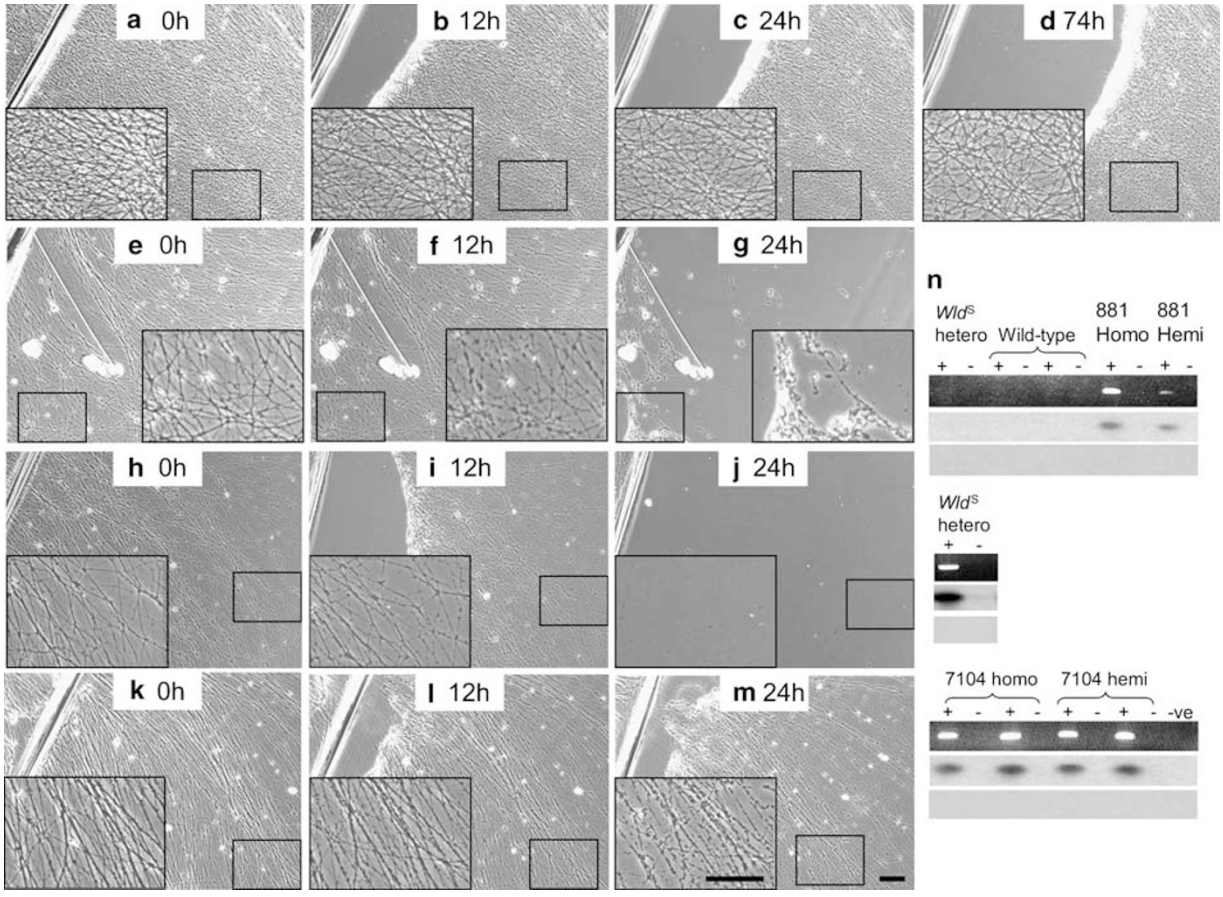


Figure 6 Rapid degeneration of Nmnat1 transgenic neurites in DRG primary cultures. Distal neurites of DRG explant primary cultures after transection with a scalpel. In each case, part of the scalpel impression on the plastic dish is visible in the top left-hand corner, neurites extend towards the bottom right,and cell bodies of the explant are located out of the frame at the top left. Some additional scratch marks made before neurite outgrowth aid orientation of photographs (e.g., **e**–**g**). Photographs marked 0 h (**a**, **e**, **h** and **k**) were taken immediately after cutting, and others at the times indicated. Framed regions are shown enlarged a further 5 $\times$ in boxes. (**a–d**) Culture from *Wld*^{S} heterozygote embryos shows complete preservation of distal neurites for more than 3 days, although neurites do retract just distal to the cut. Neurites in all other cultures degenerated fully within 24 h. (**e**–**g**) Wild-type; (**h**–**j**) transgenic line 881 homozygote and (**k–m**) transgenic line 7104 homozygote. The four cultures are representative of 30, 15, 9 and 15 explants of the same genotypes, respectively. Scale bar 100 μm. (**n**) RT-PCR ($+$ and $-$reverse transcriptase, as indicated) confirms the expression of *Wld*^{S} gene in (**a–d**) and the Nmnat1 transgene in (**h–m**) and in hemizygous cultures (not shown). Each set of three panels depicts ethidium-stained gel (top), *Wld*^{S} cDNA hybridisation of RT-PCR products to indicate product specificity (middle) and control Uch-l1 cDNA probe hybridisation (bottom). Panels 1–3 and 7–9 show Nmnat1 transgene-specific RT-PCR, and panels 4–6 show *Wld*^{S}-specific RT-PCR

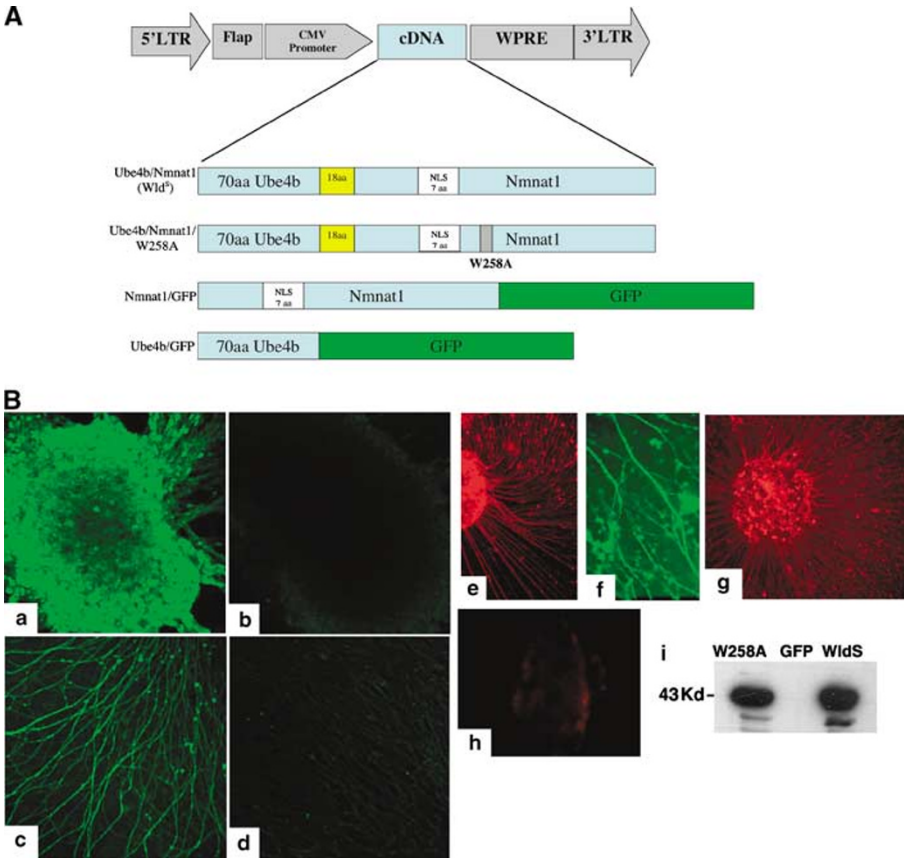
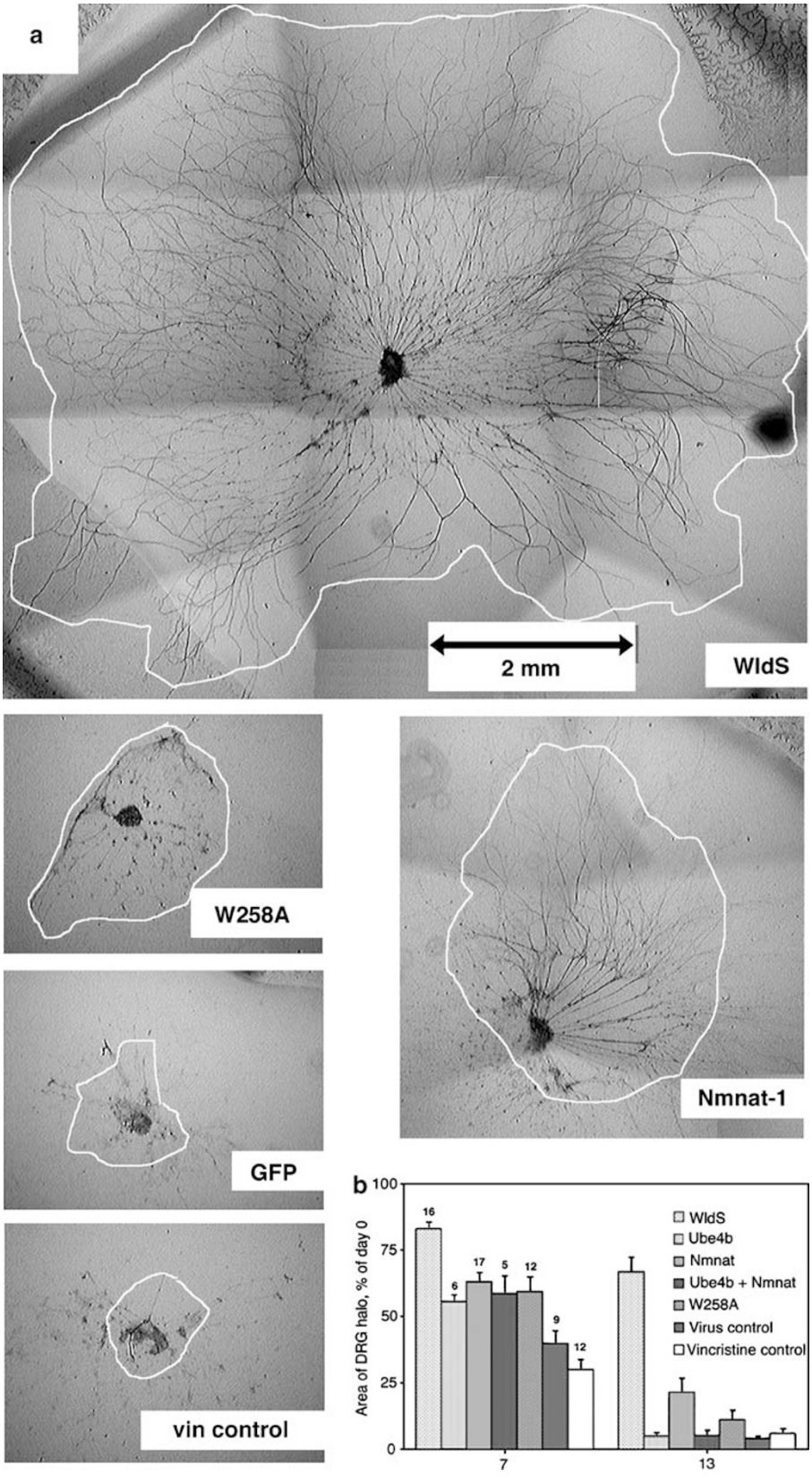


Figure 7 Lentiviral expression of exogenous proteins in DRG cultures. (**a**) Construction maps for the lentiviral expression vectors. These constructs are based on the FUGW backbone. The FLAP sequence promotes nuclear import and increases the amount of integrated vector DNA. WPRE is a post-transcriptional regulatory element of the woodchuck hepatitis virus that stabilises mRNA and improves transgene expression. The Wld^S protein contains the N-terminal 70 amino acids of Ube4b linked by 18 amino acids to Nmnat1. The W258A point mutation is downstream from the 7-amino-acid nuclear localisation signal (NLS). (**b**) Identification of lentiviral-expressed proteins in DRGs. Lentiviruses were added to 6-day-old DRG cultures at a final concentration of 1×10^7 particles/cm^3. Photomicrographs were taken 7 days later, just before the addition of vincristine. (**a**) and (**c**) are the cell bodies and distal axons, respectively, of a DRG explant culture infected with the Nmnat1 lentivirus and stained with anti-Nmnat1 antibody. (**b**) and (**d**) are cell bodies and neurites from a DRG explant culture infected with a control lentivirus and stained with the same antibody. Photomicrographs were taken at the same sitting using the same fluorescent intensity settings and are presented unaltered. Note the relative overexpression of Nmnat1 protein in (**a**) and (**c**) compared to (**b**) and (**d**). The expression of Ube4b/Nmnat1 in DRGs is shown by staining with Ab183 (**e**) and Wld-18 (**f**). Expression of the W258A mutant protein is also shown with Ab183 (**g**). DRGs infected with the control lentivirus show no staining with Ab183 (**h**) or Wld-18 (not shown). The equal expression of Wld^S and W258A proteins by lentiviruses in 293 cells is shown by Western blot (**i**). Cells were homogenised 36 h after lentiviral infection and supernatants were processed for Western blotting with Ab183. The 43 kDa Wld^S protein is identified for each. No signal is seen with the GFP-expressing lentiviral vector

both 7 and 13 days of vincristine exposure (Figure 8). Direct comparison of expression of Wld^S Nmnat1 and W258A showed that the protection provided by W258A was indistinguishable from that provided by Nmnat1. These results suggest that at least a portion of the protective activity of Wld^S relates to the enzymatic activity of Nmnat1. However, the protection provided by W258A was not reduced compared to wild-type Nmnat1. These seemingly conflicting data could reflect a balance between the weakening effect of W258A and the strengthening effect of the N-terminal region of Wld^S, which is not included in the Nmnat1 construct.

Figure 8 Comparative axonal protection by lentiviral-expressed proteins. Lentiviruses were added to DRGs at 6 days after harvest and allowed to express proteins for 7 days before the addition of vincristine (day 0). The photomicrographs are of DRG cultures 13 days after vincristine exposure, and are representative of the findings in total ($n = 10$). At 13 days, the cultures were fixed and stained with an axonal marker (MAP-5 antibody; Sigma) for final size quantitation. Note the impressive protection provided by lentiviral expression of Wld^S, and the relatively little protection provided by Nmnat1 and Wld^S-W258A. The white outlines represent how the size of halos are quantified. Note that this is the best example of protection seen with Nmnat1. (**b**) DRG halos were measured 7 and 13 days after the addition of vincristine and are reported as the percent of size at day 0. Expression of Wld^S was protective at both time points ($P < 0.001$) and was significantly more protective than the expression of either Nmnat1 or Wld^S-W258A. The protective effects of Nmnat1 and W258A, although significant at day 7 ($P < 0.01$), were not significant at day 13. Expression of GFP alone had no effect as compared to noninfected controls


a
2 mm
WldS
W258A
Nmnat-1
GFP
vin control
b
Area of DRG halo, % of day 0
100
75
50
25
0
16
6
17
5
12
9
12
7
13
WldS
Ube4b
Nmnat
Ube4b + Nmnat
W258A
Virus control
Vincristine control

Exogenous NAD$^+$ and resveratrol do not protect against axonal degeneration. To further address the question of whether increased levels NAD$^+$ is the mediator of axonal protection by Wld^S,[20] we added NAD$^+$ (up to 1 mM) in DRG cultures 24 h prior to the addition of vincristine. NAD$^+$ provided no significant protection against vincristine-induced axonal degeneration ($P>0.05$, Figure 9). Treatment of DRG cultures with resveratrol, a compound that increases Sir2 activity, also showed no protective effect ($P>0.05$). Furthermore, neither NAD$^+$ nor resveratrol were protective against transection injury (Figure 9). These results do not support the hypothesis that the direct pathway for axonal protection by Wld^S is through the production of NAD$^+$ and interaction with Sir2.

Discussion

The data we report led our groups to conclude independently that full-length Wld^S protein delays axon degeneration more effectively than Nmnat1. Transgenically overexpressed Nmnat1 was unable to delay Wallerian degeneration *in vivo* or *in vitro*. Nmnat1 overexpressed using a lentivirus vector *in vitro*, protected far more weakly than Wld^S and, at later time points, made no significant difference from wild-type neurons. Moreover, expression of the Ube4b component of Wld^S provided similar levels of protection to Nmnat1, demonstrating that this limited protection is not restricted to the Nmnat1 component. Neither of these constructs showed any significant protection at the 13 days. The fact that lentiviral expression of both the Ube4b sequence and Nmnat1, either separately or together, did not match protection by full-length Wld^S argues that the functional mechanism of Wld^S is more than the sum of its parts. Thus, the proposal that the direct action of Wld^S is mediated solely by Nmnat1[19,20] must be revised to incorporate a role for more N-terminal Wld^S sequence.

Hemizygotes of Nmnat1 transgenic lines 881 and 7104 matched or exceeded the increase in Nmnat1 protein and enzyme activity in *Wld^S* heterozygotes. The 3.2- to 3.5-fold increase in total Nmnat enzyme activity probably underestimates the increase in Nmnat1, as the mitochondrial and cytosolic isoforms of Nmnat also contribute to the baseline figure.[27–29] However, by ultrastructural, topological and biochemical criteria, Wallerian degeneration was not delayed in either hemi- or homozygous Nmnat1 transgenic mice *in vivo*. We previously generated transgenic mice expressing full-length Wld^S protein using the same promoter as here, enabling us to identify the *Wld^S* gene.[15] Full-length Wld^S was targeted to motor neuron nuclei, the same location that we now observe for overexpressed Nmnat1 (Figure 2 and Supplementary Figure 2). Full-length Wld^S significantly delayed Wallerian degeneration for 5 days, even at lower expression levels than in *Wld^S* heterozygotes (e.g., hemizygotes of *Wld^S* transgenic line 4830). Many axons in transgenic lines expressing higher levels of Wld^S survived for 2 weeks. In contrast, Nmnat1 targeted to the same subcellular compartment fails to protect any axons for 3 days, even if the level exceeds that of Wld^S in *Wld^S* heterozygotes.

Also *in vitro*, overexpressed Nmnat1 could not robustly delay axon degeneration like full-length Wld^S protein. DRG cultures established from homozygous embryos of our

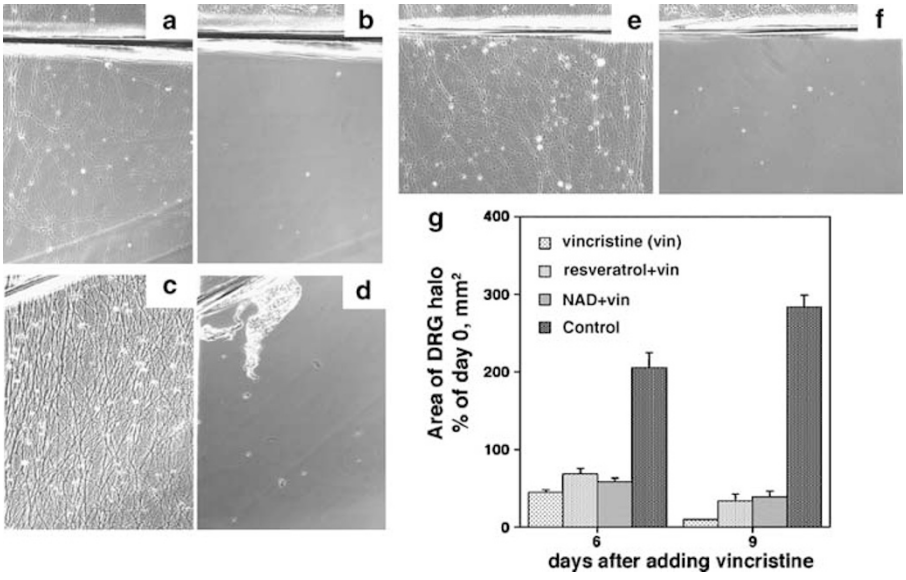


Figure 9 Resveratrol and exogenous NAD$^+$ do not delay axon degeneration (**a–f**) degeneration of transected neurites. We found no protection of transected wild-type DRG neurites with either 100 μM resveratrol or 1 mM exogenous NAD$^+$. Neurites of DRG cultures were (**a** and **b**) untreated, or (**c** and **d**) pretreated 100 μM resveratrol for 24 h, or (**e** and **f**) pretreated with 1 mM NAD$^+$ for 24 h, and were then cut with a scalpel. Fresh medium, with resveratrol or NAD$^+$ as appropriate, was added immediately before cutting. In each case, the scalpel imprint is visible at the top, neurites extend downwards and cell bodies of the explant are out of the frame at the top. Neurites degenerated in 12 h regardless of the presence of resveratrol or NAD$^+$. Each photograph is representative of at least nine explants. Scale bar 100 μm. (**g**) Degeneration in response to vincristine. NAD$^+$ (1 mM) or resveratrol (100 μM) were added to the DRG cultures 24 h before the addition of vincristine. DRG sizes were quantified at 6 and 9 days. In comparison to vincristine alone, there was no significant protection provided by the addition of either compound. Note that control cultures continue to extend neurites over time

strongest lines were indistinguishable from wild-type following neurite transection. In contrast, heterozygous Wld^S routinely delayed degeneration of transected neurites for 3 days. Although both Wld^S and Nmnat1 proteins were not detected in these cultures, the lentiviral experiments did provide clear evidence of Nmnat1 expression in nuclei (Figure 7b). Lentiviral expression of Wld^S protected DRG neurites from vincristine-induced axonal degeneration, consistent with our previous report of protection by Wld^S expression using adenoviral vectors.[16] However, expression of Nmnat1 at similar levels to Wld^S did not protect effectively. If Nmnat1 were the sole functional component of Wld^S, it should substitute fully in this system. While the possibility remains that very strong expression of Nmnat1 might protect axons, our data show conclusively that the potency of Nmnat1 does not match that of Wld^S. Thus, another part of the Wld^S protein is necessary to delay Wallerian degeneration.

Araki *et al*. (2004) also reported a protective effect of 0.01–1.0 mM exogenous NAD^+ on transected neurites.[20] However, like Wang *et al*.,[19] neither of our groups found significant neurite protection at the upper end of this concentration range in either transection or toxic models (Figure 9). Exogenous NAD^+ has also been ineffective at neuroprotection in other laboratories (Pierluigi Nicotera, personal communication). The lack of a robust and fully reproducible effect with exogenous NAD^+ raises questions about the role of Sirt1 in delaying Wallerian degeneration, as Sirt1 was implicated in RNAi experiments using the exogenous NAD^+ method.[20] Resveratrol was also reported to protect axons from degeneration by increasing the activity of Sir2 proteins, analogous to the proposed mechanism of NAD^+,[20] but resveratrol also could not reproduce the *Wld*^{S} phenotype in our laboratories. Our data, together with the recent report that Wld^S delays degeneration of transected neurites even in $Sirt1^{-/-}$ mice,[19] support a different functional mechanism for Wld^S.

Exactly how the N-terminal Ube4b-derived sequence contributes to the Wld^S phenotype remains unclear, but recent data suggest two possibilities. First, valosin-containing protein (VCP/p97) binds to the N-terminal 16 amino acids of Wld^S and Ube4b.[30] Consequently, Wld^S partially redistributes VCP into punctate intranuclear foci in some neuronal subtypes. Removing the N-terminal 16 amino acids of Wld^S abolishes the targeting of VCP to intranuclear foci, and we confirm here that overexpressed Nmnat1 also fails to target VCP to intranuclear foci (Supplementary Figure 5). Second, expression of full-length Wld^S protein alters the expression of a small subset of genes, but these changes are not fully reproduced when N-terminal sequences are deleted.[31] It is not yet known whether either of these effects is required for axon protection, or whether they are linked with one another. Importantly, however, both involve nuclear events, and the nucleus remains the only intracellular location *in vivo* where Wld^S has been detected. Thus, one can hypothesise that binding of Wld^S to VCP alters ubiquitin-proteasome-mediated turnover of a transcription factor, which in turn alters the expression of an axonal regulator of Wallerian degeneration. Our demonstration here that the N-terminal region (including the VCP binding site) is required for strong axon protection supports this model.

The existence of Wld^S is an extraordinary experiment of nature that will no doubt provide valuable clues to the causes of axonal degeneration in human diseases. Wld^S is truly neuroprotective and has been shown to modify disease course in animal models of toxic neuropathy,[10] Charcot–Marie–Tooth neuropathy,[8] motor neuronopathy,[2] amyotrophic lateral sclerosis[32] and Parkinson's disease.[11] Defining the precise mechanism of axonal protection in *Wld*^{S} mice is thus an important step for developing novel therapeutics for peripheral neuropathies and other disorders of axonal degeneration. Our data are inconsistent with the premise that overexpression of Nmnat1 is the sole mechanism underlying the *Wld*^{S} phenotype, and we believe that the question needs further examination. We have no conclusive explanation for why our findings differ from those reported previously,[19,20] but suggest differences in expression level as one possibility. However, the data we report, repeated many times in both our laboratories, conclusively show that Nmnat1 cannot substitute for a similar level of Wld^S.

In summary, we conclude that Nmnat1 and Wld^S protein are not equivalent in their ability to delay Wallerian degeneration in injured axons. Specifically, robust protection of axons by Wld^S is not reproduced when Nmnat1 is expressed at a similar level, as demonstrated by ultrastructural, morphological and biochemical assays *in vivo*, and using both traumatic and toxic lesions *in vitro*. We do not rule out possible involvement of Nmnat1 in the *Wld*^{S} phenotype, but the significantly reduced potency compared to Wld^S and the lack of effectiveness in providing axonal protection *in vivo* suggest that more N-terminal sequences of the Wld^S protein should now be investigated.

Materials and Methods

Generation of transgenic mice. A transgene construct designed to express Nmnat1 from a β-actin promoter was generated as previously described in our successful generation of *Wld*^{S} transgenic mice,[15] except for the use of a different forward PCR primer as follows (underlined bases are 5' *Hin*dIII cloning tag and start codon of Nmnat1): 5′-TAGATCCCAAGCTTAACTTC TCCCCATGGACTCAT-3′.

Pronuclear injection of the ca. 5.7 kbp *Eco*RI/*Nde*I into an F1 C57BL/CBA strain was performed by the in-house Gene Targeting Facility of the Babraham Institute. All animal work in the UK was carried out in accordance with the Animals (Scientific Procedures) Act, 1986, under Project Licence PPL 80/1778.

Genotyping. Founder mice, and their transgene-positive offspring, were identified by Southern blotting of *Bam*HI plus *Hind*III double-digested genomic tail DNA hybridised with a ^{32}P-labelled *Wld*^{S} cDNA probe (Supplementary Figure 1). Twenty-three positive founders with varying transgene copy number were identified from a total of 152 mice. Founders with high-copy number integrations were selected for further study. YFP-H mice[25] were obtained from the Jackson Laboratories and genotyped as described previously.[26]

Western blotting. Sagittally-divided half brains were homogenised in five volumes of RIPA buffer (phosphate-buffered saline (PBS) containing 1% NP40, 0.5% deoxycholate, 0.1% sodium dodecylsulphate). High-speed supernatant was diluted to approximately 0.5 mg/ml total protein according to Bradford Assay (BioRad) and fractionated by standard SDS-PAGE. After semidry blotting (BioRad), nitrocellulose membranes (BioRad) were blocked in PBS plus 0.02% Tween-20 and 5% low-fat milk powder, before incubation with primary antibody and then horseradish peroxidase (HRP)-conjugated secondary antibody (1 : 3000; Amersham Biosciences). Proteins were visualised using the ECL detection kit (Amersham Biosciences) according the manufacturer's instructions. Nmnat1 and Wld^S protein were detected on Western blots using rabbit polyclonal antiserum 183

as described previously,[14] and mouse monoclonal anti-β-actin (Abcam) was used as a loading control.

The distal stumps of transected sciatic nerves (ca. 0.5 cm) were homogenised in 25 μL RIPA buffer. High-speed supernatant was fractionated by SDS-PAGE and gels blotted onto nitrocellulose membranes and blocked as above. Blots were incubated in mouse anti-neurofilament 200 antibody (N52; 1 : 2000; Sigma) and then HRP-conjugated secondary antibody (1:3000; Amersham Biosciences). Proteins were visualised using the ECL detection kit (Amersham Biosciences).

RT-PCR. Lumbar DRG were removed from freshly killed mice, or approximately 20 DRG explants from a primary culture dish and extracted in Trizol (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis and 30 cycles of PCR was then performed as described previously[14] using the following primers:

to detect transgene-specific Nmnat1 mRNA:
5′-GAGCCTCGCCTTTGCCGAT-3′ and
5′-TTCCCACGTATCCACTTCCA-3′
to detect *Wld*^{S} mRNA:
5′-CTTGCTGGTGGACAGACCT-3′ and
5′-TTCCCACGTATCCACTTCCA-3′

Assay of Nmnat enzyme activity. Sagittally divided half mouse brains were flash-frozen immediately post mortem and stored at $^{-}80^{\circ}$C until use. Tissue was suspended in six volumes of 50 mM Hepes, pH 7.4, 0.5 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride and 0.02 mg/ml each of leupeptin, antipain, chymostatin and pepstatin, and homogenised on ice (3×4 s with 10 s intervals at medium speed). Nmnat activity assay was performed at 37°C in a 0.1 ml reaction mixture containing 30 mM Tris-HCl, pH 7.5, 2 mM nicotinamide mononucleotide (NMN), 2 mM ATP, 20 mM $MgCl_2$, 10 mM NaF and an appropriate aliquot of brain homogenate. The reaction was started by adding 4 μl of 50 mM NMN and stopped by the addition of a half-volume of ice-cold 1.2 M $HClO_4$. After 10 min at 0°C, the mixture was centrifuged and 135 μl of supernatant was neutralised by the addition of 36 μl of 0.8 M K_2CO_3. Nmnat activity was calculated after HPLC identification and quantification of the product (NAD^+).[33] One unit of enzyme was defined as the amount capable of producing 1 μmol of NAD^+ per minute at 37°C. Pairwise statistical analyses (unpaired Student's *t*-test) were performed using Prism.

Immunocytochemistry. Cryostat sections (20 μm) were prepared from lumbar spinal cord of TgNmnat1 line 7104 homozygous, *Wld*^{S} homozygous or C57BL/6 control mice that had been perfusion-fixed with 4% paraformaldehyde. Sections were pretreated by incubating overnight at 45°C in 1 mM sodium citrate, pH 6.0, followed by 10 min in 0.1% Trypsin in PBS. They were then immunostained for Nmnat1 using two different affinity-purified rabbit polyclonal antibodies (each diluted 1 : 50) that detect only Nmnat1 on Western blots: one kindly supplied by Professor Mathias Ziegler (Figure 2),[34] and the other (D-20) obtained from Santa Cruz Biotechnology Inc. (Supplementary Figure 2). For each, we used Alexa 568-conjugated anti-rabbit secondary antibody (1 : 200; Molecular Probes). Dispersed primary cultures of DRG neurons were fixed with 4% paraformaldehyde and immunostained for NF-H using monoclonal antibody N52 (1 : 200; Sigma) and Alexa 568 anti-mouse secondary (1 : 200 Molecular Probes).

Nerve lesion. Mice were anaesthetised with a mixture of ketamine (100 mg/kg; Fort Dodge Animal Health, Southampton) and xylazine (5 mg/kg; Parke Davis/Pfizer, Karlsruhe, Germany). Right sciatic nerves were transected at the upper thigh and wounds closed with sutures. After 60 to 72 h, mice were killed by cervical dislocation and the swollen first 2 mm of distal nerve discarded. The remaining distal sciatic nerve stump was used for light and electron microscopy (see below) or Western blotting with antibody to NF-H (see above). For mice carrying a YFP-H transgene, tibial nerve was also removed for confocal microscopy. Except where otherwise indicated, the mutant mice used were hemizygous for Nmnat1 or *Wld*^{S} heterozygotes, produced by crossing C57BL/*Wld*^{S} homozygous males (Harlan UK) to C57BL/6 females.

Light and electron microscopy. Nerves were fixed for at least 24 h in 0.1 M phosphate buffer containing 4% paraformaldehyde and 2.5% glutardialdehyde, embedded in Durcupan resin (Fluka) and processed for light and electron microscopy as described previously.[26]

Analysis of YFP-labelled nerves. Tibial nerves were quickly removed from humanely killed mice and processed as described previously[26] for analysis on a Zeiss LSM 510 Meta Confocal system (LSM software release 3.2) coupled to a Zeiss Axiovert 200 microscope. Only lines 2460, 7103 and 7104 were studied in this assay, as initial crosses of other lines produced no double mutants.

Primary culture for transgenic experiments (Coleman group). DRGs were dissected from E15.5 mouse embryos using sterile technique, initially into L15 medium (Invitrogen) for removal of other tissue, and then plated ca. 20 per dish in 3.5 cm dishes precoated with poly-L-lysine (20 μg/ml for 1 h; Sigma) and laminin (20 μg/ml for 1 h; Invitrogen). Explants were then cultured in Neurobasal medium (Invitrogen) containing 4 μM aphidicolin (Sigma) (which we find reduces non-neuronal cells ca. 250-fold), 2% B27-supplement, 2 mM glutamine, 1% penicillin, 1% streptomycin and 100 ng/ml NGF (all from Invitrogen). Scratches were made on dishes prior to plating the explants to aid subsequent orientation of photographs (e.g., Figure 6e–g). After 5–7 days of neurite outgrowth in standard culture conditions, neurites were transected using a scalpel, immediately photographed with phase contrast using an Olympus IX81 inverted fluorescent microscope, coupled to a PC running SIS imaging software. Further photographs were taken at 3, 6, 9, 12 and 24 h, and after approximately each subsequent 24 h period if neurites had survived (on *Wld*^{S} neurites survived this long). Homozygous and hemizygous transgenic and wild-type embryos were produced by intercrossing hemizygous parents of lines 881 and 7104. Heterozygous *Wld*^{S} embryos were produced by crossing *Wld*^{S} homozygotes with C57BL/6 mice. The liver and tail of each embryo were retained for DNA extraction using the Nucleon II kit (Amersham) and genotyping.

Primary culture for lentiviral vector experiments (Glass group). DRG explant cultures were generated from E15 rats as described previously.[16,35] After allowing for neurite extension for 6 days in culture, lentiviruses titered at 1×10^9 were added to the culture media at a dose of 10 μl in 1 ml (final viral titer 1×10^7). The cultures were maintained under standard conditions for an additional 7 days to allow for the expression of exogenous proteins. For vectors expressing GFP, transduction was monitored in real time by fluorescence microscopy. After 7 days (total 13 days in culture), vincristine sulphate was added to a final concentration 0.02 μM and the cultures monitored by phase-contrast microscopy for an additional 13 days. Quantitative analysis of vincristine neurotoxicity was performed as described previously.[35] Data were compared using a one-way analysis of variance with post-test correction for multiple comparisons (Tukey–Kramer). The expression of exogenous proteins was assessed by immunocytochemistry on cultures prepared at the same sitting as those exposed to vincristine, but fixed and stained after 7 days in culture. Two antibodies raised against the Wld^S protein, 183 and Wld-18, were used to demonstrate expression of Ube4b/Nmnat1 and W258A, as described previously.[8,14] Expression of Nmnat1 was demonstrated with a polyclonal antibody to Nmnat1 (D20 from Santa Cruz Biotech, 1 : 100 concentration).

Addition of resveratrol and NAD^+ to primary cultures. Resveratrol (100 μM) and NAD^+ (1 mM, both from Sigma) were tested for their potential neuroprotective effects against vincristine-induced axonal degeneration in the Glass culture system (above) and against transection-induced degeneration in the Coleman culture system (above). These agents were added 24 h before vincristine (0.02 μM) to 6-day-old cultures and the degree of axonal degeneration was assessed after 6 and 9 days of exposure. For transection experiments, these agents were added for 24 h before neurite transection to 5-day-old cultures, and replaced with new medium immediately before transection containing NAD^+ or resveratrol as appropriate.

Generation of lentiviral vectors. Lentivirus vectors were created on the FUGW backbone using a CMV promotor[36] (Figure 7). The following cDNAs were cloned into the vector: Ube4b/Nmnat1 (*Wld*^{S}), mutant Ube4b/Nmnat1 (W258A), Nmnat1 fused to GFP (Nmnat1) and the 70-amino-acid N-terminal portion of the Wld^S protein fused to GFP (Ube4b). The *Wld*^{S} cDNA was isolated by RT-PCR from the brain of a *Wld*^{S} mouse.[16] The W258A point mutation was generated by PCR-based site-directed mutagenesis.

Vector particles were produced in HEK293 T cells by transient cotransfection with the transfer vector, the HIV-1 packaging vector R8.9 and the VSVG envelope glycoprotein. Approximately 72 h after transfection, virus-containing supernatant was removed, filtered through a 0.45 μM filter unit and concentrated by

centrifugation, aliquoted and frozen at −80°C. Viral titres were calculated using a serial dilution method. Prior to use in DRG cultures, lentiviral expression of the inserted protein was confirmed by Western blot analysis of infected HEK293 cells (Figure 7).

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