## Title

# Nicotinic acid mononucleotide is an allosteric SARM1 inhibitor promoting axonal protection

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## Abstract

SARM1 is an inducible $NAD^+$ hydrolase that is the central executioner of pathological axon loss. Recently, we elucidated the molecular mechanism of SARM1 activation, demonstrating that SARM1 is a metabolic sensor regulated by the levels of $NAD^+$ and its precursor, nicotinamide mononucleotide (NMN), via their competitive binding to an allosteric site. In healthy neurons with abundant $NAD^+$, binding of $NAD^+$ blocks access of NMN to this allosteric site. However, with injury or disease the levels of the $NAD^+$ biosynthetic enzyme NMNAT2 drop, increasing the NMN/$NAD^+$ ratio and thereby promoting NMN binding to the SARM1 allosteric site, which in turn induces a conformational change activating the SARM1 $NAD^+$ hydrolase. Hence, $NAD^+$ metabolites both regulate the activation of SARM1 and, in turn, are regulated by the SARM1 $NAD^+$ hydrolase. This dual upstream and downstream role for $NAD^+$ metabolites in SARM1 function has hindered mechanistic understanding of axoprotective mechanisms that manipulate the $NAD^+$ metabolome. Here we reevaluate two methods that potently block axon degeneration via modulation of $NAD^+$ related metabolites, 1) the administration of the NMN biosynthesis inhibitor FK866 in conjunction with the $NAD^+$ precursor nicotinic acid riboside (NaR) and 2) the neuronal expression of the bacterial enzyme NMN deamidase. We find that these approaches not only lead to a decrease in the levels of the SARM1 activator NMN, but also an increase in the levels of the $NAD^+$ precursor nicotinic acid mononucleotide (NaMN). We show that NaMN competes with NMN for binding to the SARM1 allosteric site, that NaMN inhibits SARM1 activation, and that this NaMN-mediated inhibition is important for the long-term axon protection induced by these treatments. Together, these results demonstrate that the SARM1 allosteric pocket can bind a diverse set of metabolites including NMN, $NAD^+$, and NaMN to monitor cellular $NAD^+$ homeostasis and regulate SARM1 $NAD^+$ hydrolase activity. The relative promiscuity of the allosteric site may enable the development of potent pharmacological inhibitors of SARM1 activation for the treatment of neurodegenerative disorders.

## Introduction

Axon degeneration is a neuropathological consequence of both acute neuronal injury and neurodegenerative disease. SARM1 is the central executioner of axon degeneration, and a key driver of pathology in models of traumatic nerve injury [1-3], chemotherapy induced peripheral neuropathy [4-6], traumatic brain injury [7,8], and glaucoma [9]. SARM1 can also induce neuronal cell death [10], and promotes photoreceptor loss in models of retinitis pigmentosa [11] and Leber congenital amaurosis [12]. In addition, we identified human genetic variants in patients with amyotrophic lateral sclerosis that render SARM1 constitutively active, suggesting that SARM1 variants may be a risk factor for this devastating neurodegenerative disease [13]. The identification of SARM1 as a therapeutic target for this wide range of neurodegenerative disorders has motivated detailed studies of the SARM1 mechanism of action in hopes of identifying novel therapeutics [14,15].

SARM1 is the founding member of the TIR-domain family of $NAD^+$ hydrolases [16-19]. The SARM1 protein comprises an N-terminal Armadillo repeat motif (ARM) autoinhibitory domain, SAM domains required for oligomerization, and a C-terminal TIR domain that catalyzes $NAD^+$ hydrolysis [18,20]. SARM1 forms an octamer [19,21], and interactions across multiple domain interfaces both within and across subunits are required to keep the SARM1 octamer inactive [22]. Upon injury, the autoinhibitory N-terminus releases the TIR domain to activate the $NAD^+$ hydrolase. Interestingly, SARM1 not only acts upon $NAD^+$, but its activation state is also regulated by $NAD^+$ and the $NAD^+$ precursor nicotinamide mononucleotide (NMN) [20,23-25]. In the off-state, $NAD^+$ binds to an allosteric site in the autoinhibitory ARM domain [20,23]. Upon axon injury, levels of the $NAD^+$ biosynthetic enzyme NMNAT2 fall, thereby leading to a drop in the levels of $NAD^+$ and an increase in the levels of NMN [26,27]. NMN binds the allosteric site much more tightly than does $NAD^+$, and so when the NMN/ $NAD^+$ ratio rises, NMN displaces $NAD^+$ from the allosteric site, initiating a conformational change that activates the $NAD^+$ hydrolase [24]. This understanding of the SARM1 activation mechanism explains prior findings such as 1) overexpression of the $NAD^+$ synthesis enzyme NMNAT1 inhibits SARM1 activation [28] and 2) inhibition of NAMPT, the NMN synthesizing enzyme, reduces cellular NMN levels and protects axons from degeneration [26,29]. However, there are some prior findings that are harder to understand. Why, for example, does reduction of NMN due to treatment with the NAMPT inhibitor FK866 only lead to short-term axonal protection [26,29], while expression of *Escherichia coli (E. coli)* NMN deamidase, which also reduces NMN levels and yet provides dramatically longer-term axonal protection [26,30]? Moreover, how is the short-term protection afforded by FK866 transformed into long-lasting axon preservation by the addition of NaR, a NAMPT-independent $NAD^+$ precursor that bypasses NMN production [31]? Interestingly, both scenarios with profound axon protection boost the levels of deamidated $NAD^+$ precursors.

$NAD^+$ biosynthesis can occur along two parallel tracks. The salvage pathway works on amidated molecules, with NAMPT converting nicotinamide to NMN and NMNAT enzymes then converting NMN to $NAD^+$. The Preiss Handler pathway works on deamidated molecules, with nicotinic acid phosphoribosyl transferase (NaPRT) converting nicotinic acid to nicotinic acid mononucleotide (NaMN) and NMNAT enzymes then converting NaMN to nicotinic acid adenine dinucleotide (NaAD), with $NAD^+$ synthetase finally generating $NAD^+$ from NaAD. The two axoprotective treatments described above both lead to the production of NaMN. The enzyme NMN deamidase directly converts NMN to NaMN, while the precursor NaR is converted to NaMN by the cellular enzymes NRK1 and NRK2 (nicotinamide riboside kinases 1&2) [32,33]. This observation led us to test the hypothesis that NaMN, or another component of the deamidated pathway, may inhibit SARM1 and thereby contribute to the strong axon protection induced by these treatments. We demonstrate that the addition of NaR to FK866 treated axons post-axotomy promotes strong axonal protection without boosting $NAD^+$ biosynthesis. Instead, NaMN derived from NaR binds the SARM1 ARM domain and inhibits the activation of the SARM1 $NAD^+$ hydrolase. Likewise, we show that the strong axonal protection afforded by neuronal expression of *E. coli* NMN deamidase is

mediated not only by an NMN decrease, but also by an NaMN increase. These results demonstrate that the SARM1 allosteric pocket binds diverse components of the $NAD^+$ metabolome to monitor cellular $NAD^+$ homeostasis and regulate SARM1 $NAD^+$ hydrolase activity. The identification of NaMN as a SARM1 inhibitor may facilitate the development of novel approaches for blocking SARM1 activation as treatments for neurodegenerative disorders.

## Results

The application of Nicotinic acid riboside (NaR) and FK866, an inhibitor of NAMPT that synthesizes NMN, strongly delays vincristine-induced axon degeneration [31]. To test the effect of the co-administration of NaR and FK866 on axon degeneration after axotomy, dorsal root ganglion (DRG) neurons were treated with various concentrations of FK866 and NaR pre- and post-axotomy. As previously reported [26,28,29], the pretreatment of FK866 (100 nM) for 24 hours before axotomy provided short-term (6 hours) axon protection (Figure 1A, B). In contrast, in FK866 pretreated axons, NaR (100 μM) addition to the culture medium at 10 minutes post-axotomy conferred prolonged axonal protection (24 hours) (Figure 1A, B). This effect is dose dependent, with an $EC_{50}$ of ~18μM NaR in the culture media (Figure 1E). NaR alone provides no axonal protection, demonstrating that combination treatment is required for the long-term axon preservation. Moreover, this effect is selective for NaR, as FK866 preincubation with nicotinic acid, another deamidated $NAD^+$ precursor, provided no additional protection to FK866 preincubation. It is striking that NaR can be added after injury. We have previously demonstrated that inhibiting SARM1 function as much as three hours after injury is sufficient to block axon degeneration [34,35]. We varied the timing of NaR application in conjunction with FK866 pretreatment and found that NaR addition at 10 minutes, two, or three hours post-axotomy provided indistinguishable and potent axonal protection lasting at least 72 hours (Figure 1C, D, F). It is notable that NaR potently protects axons when added three hours post-axotomy, as by this time NMNAT2, the major NMNAT enzyme in the axon, is largely degraded [27,36]. A prior study demonstrating that a combination treatment with FK866 and NaR could protect axons from vincristine treatment, suggested that the role of NaR was to boost $NAD^+$ levels [31]; however, our finding that NaR can be added after the $NAD^+$ biosynthetic pathway is impaired calls this $NAD^+$ synthesis hypothesis into questions.

To investigate the mechanism of NaR mediated axonal protection, we first explored the hypothesis that NaR functions through enhanced $NAD^+$ synthesis [31]. For NaR to promote axonal $NAD^+$ synthesis, it must be converted to NaMN by either NRK1 or NRK2, then to NaAD by NMNAT2, and finally to $NAD^+$ by $NAD^+$ synthetase (NADSYN) [31] (Figure 2A). However, the short-lived NMNAT2 enzyme is quickly lost after either axotomy or vincristine treatment [5,27,36]. We first asked whether NaR or FK866 influenced the level of axonal NMNAT2 in injured axons. Neurons were incubated with FK866 for 24 hours before axotomy, then NaR was added 10 minutes after injury (Figure 2B). We used Western blotting to monitor NMNAT2 levels and found that NMNAT2 was reduced more than four-fold within three hours under all conditions tested (Figure 2C), indicating that NaR and FK866 inhibition of axon degeneration is not

mediated by increased NMNAT2 stability. We next analyzed the effects of NaR and FK866 on axonal $NAD^+$ levels before and after axotomy. Neurons were incubated with FK866 for 24 hours before axotomy, then NaR was added three hours after injury, a time at which it is potently protective (Figure 2D). Axonal metabolites were collected at 0 or 27 hours after axotomy and measured using LC-MSMS (Figure 2E). Consistent with previous reports [28,29], basal axonal $NAD^+$ levels were dramatically reduced by FK866 treatment (Figure 2E, panel $NAD^+$, 0h control vs 0h FK866) and axotomy (Figure 2E, panel $NAD^+$, 0h control vs 27h control). The addition of NaR significantly increased NaMN and NaAD (Figure 2E panels NaR, NaMN and NaAD); however, it did not rescue axotomy-induced or FK866-mediated $NAD^+$ depletion. Hence, NaR is not conferring protection by increasing $NAD^+$ levels, and so must work through a different mechanism.

Nicotinamide riboside kinases (NRK1 or 2) metabolize NaR as well as nicotinamide riboside (NR, the amidated form of NaR) in mammalian cells [32,33]. NRK1/2 phosphorylates NaR and produces NaMN. We set to determine the role of NRK enzymes in NaR-mediated axonal protection. We generated NRK1/2 double knockout mice and found they are viable and fertile without overt behavioral phenotypes (Supplemental Figure 1A). NRK1 deficient mice were shown to display no overt phenotypes [37] and our results indicated dispensable roles of NRK1 and 2 dependent processes for mouse survival. We did not test the glucose intolerance, insulin resistance, and hepatosteatosis that were reported in NRK1 deficient mice [38]. NRK1/2 double knockout (dKO) DRG neurons were healthy with qualitatively normal axonal growth (Supplemental Figure 1B). To assess the metabolic fate of NaR, NRK1/2 dKO neurons were treated with NaR in the presence or absence of lentiviral-mediated expression of NRK1. Unlike in NaR treated wild type neurons (Figure 2E), the synthesis of NaMN and NaAD was completely blocked in NRK1/2 dKO neurons after NaR addition (100 $\mu$M for 24 hours), confirming the depletion of NRK1/2 in these neurons. This phenotype was rescued by the exogenous expression of NRK1 (Figure 3A, B). Hence, NRK1 or NRK2 are absolutely required for the conversion of NaR and NR (Supplemental Figure1C), but are not required for cellular or animal viability. Next, we measured axonal degeneration in add-back experiments in which NRK1 was re-introduced into NRK1/2 dKO neurons using lentivirus. The addition of NaR 10 minutes post-axotomy to FK866-treated wild type neurons showed strong axonal protection. This treatment regimen was ineffective in NRK1/2 dKO axons, with robust axon degeneration in the presence of NaR and FK866 (Figure 3C, D). When NRK1 was re-expressed in these dKO neurons, NaR and FK866 joint treatment once again provided strong axonal protection. Hence, NRK-mediated NaMN synthesis from NaR is necessary for NaR to promote axon preservation.

SARM1 activation is regulated by the balance between NMN and $NAD^+$, the substrate and product of NMNAT enzymes, respectively [24]. This model can explain the short-lived protection afforded by FK866 alone—at baseline NMN is depleted promoting axon protection, but as NMNAT2 is lost, post-axotomy, $NAD^+$ levels fall, enabling even low levels of NMN to activate SARM1. However, this model cannot explain the enhanced protection provided by addition of NaR to FK866, because NaR does not rescue $NAD^+$ levels (Figure 2E). Instead, NaR promotes the synthesis of NaMN (Figure 3A-D), which

is structurally similar to NMN. Therefore, we hypothesize that NaMN or its derivatives compete with NMN to bind SARM1 and, hence, block NMN-mediated SARM1 activation. As a first test of this hypothesis, we increased NMN production in injured axons preserved by combining NaR and FK866 treatment. Neurons were pre-treated with FK866 for 24 hours before axotomy, and then NaR was added three hours post-axotomy. At 24 hours post-axotomy, these non-degenerating axons were treated with NR (100 μM) to increase NMN levels in a NAMPT-independent manner [24] (Figure 4A). As expected, axonal NMN was reduced by FK866 treatment (Figure 2E, control 0h vs FK866 0h), and these levels were similar to those in neurons treated with NaR or NaR plus FK866. In contrast, the addition of NR to neurons treated with NaR plus FK866 twenty-four hours post-axotomy resulted in large increases in NMN within three hours (Figure 2E, panel NMN). Notably, $NAD^+$ was not increased in these axons likely due to the loss of NMNAT2 (Figure 2E, panel $NAD^+$). We also observed a decrease of NaMN after NR treatment, potentially due to competition for the NRK enzymes that convert both NR and NaR to NMN and NaMN, respectively (Figure 2E, panel NaMN). We next assessed axonal integrity in these injured, but preserved axons, after NR addition (Figure 4A). While axons treated with combination of NaR and FK866 were intact at 48 hours post-axotomy, the addition of NR at 24 hours post-axotomy resulted in very rapid and robust axonal degeneration of these NaR and FK866 treated axons (Figure 4B, C). Hence, high levels of NMN eliminate the axon protection provided by NaR and FK866 treatment, and are consistent with the model that NaMN competes with NMN for binding to the allosteric site of SARM1.

Our metabolite and axon degeneration data support the hypothesis that NaMN or its derivatives directly inhibit SARM1 activation by competing with NMN for binding to the allosteric pocket in the autoinhibitory N-terminal ARM domain. In this model, 1) NaMN or its derivatives will directly inhibit SARM1 $NAD^+$ hydrolysis activity; 2) the SARM1 N-terminal autoinhibitory domain will be required for NaMN-mediated inhibition; and 3) NMN will mitigate this inhibition. To directly test these predictions, we performed an *in-vitro* SARM1 $NAD^+$ hydrolysis assay. Strep-tagged human SARM1 (hSARM1) was expressed in HEK293T cells and affinity purified and mixed with NMN, NaMN, or NaAD. The $NAD^+$ hydrolase reaction was initiated by addition of $NAD^+$ (10 μM) and the rate of ADPR production, monitored by HPLC, was quantified to calculate hSARM1 $NAD^+$ hydrolase activity. NMN activated hSARM1 $NAD^+$ hydrolase activity with a Ka of 8.7 μM (Figure 5A), which is very similar to the binding constant of NMN for the SARM1 N-terminal domain [24]. We next tested the effects of NaMN and NaAD, the major metabolites derived from NaR (Figure 2E), on hSARM1 $NAD^+$ hydrolase activity. We found that NaMN inhibits ADPR production in a dose-dependent manner with an $IC_{50}$ of 93 μM in the absence of NMN, demonstrating that NaMN can directly inhibit SARM1 $NAD^+$ hydrolase activity (Figure 5B). In contrast, NaAD did not inhibit hSARM1 $NAD^+$ hydrolase activity (Figure 5B), suggesting that the active inhibitory component in neurons is NaMN and not NaAD. This NaMN-mediated inhibition could either be due to binding to the allosteric site in the N-terminus, or the orthosteric site in the C-terminal TIR domain NAD hydrolase. To distinguish between these two possibilities, we performed similar activity assays using a truncated version of hSARM1 lacking the N-terminal ARM domain (ΔARM-hSARM1) that houses the allosteric site. ΔARM-hSARM1

maintains enzymatic activity, but was not inhibited by NaMN (Figure 5D). Hence, NaMN does not inhibit SARM1 activity at the orthosteric site. To test whether NaMN inhibition competes with NMN-mediated activation, we assessed NaMN inhibition of full-length SARM1 in the presence of 5 μM NMN. We found that this small amount of NMN shifts the $IC_{50}$ of NaMN by more than 50 μM to 147 μM, consistent with the much higher affinity of NMN for binding to SARM1 (Figure 5C). These results support the hypothesis that NaMN binds to the SARM1 N-terminal allosteric NMN/ $NAD^+$ binding site and competes with NMN to inhibit SARM1 $NAD^+$ hydrolase activity.

Next, we directly assessed NaMN binding to SARM1 using saturation-transfer difference (STD) NMR[24]. STD NMR assays demonstrated that NaMN binds to the hSARM1and the *Drosophila* SARM1 ARM domain (dSARM1 ARM) (Figure 5E, F). Furthermore, NaMN binding to hSARM1 was decreased by 31% and 84% upon addition of NMN, at ratios of NMN:NaMN = 1:100 and 1:10, respectively. Similarly, NaMN binding to dSARM1 ARM was also inhibited by 52% and 94% at ratios of NMN:NaMN = 1:100 and 1:10 respectively. NMN completely abolished NaMN binding to hSARM1 and dSARM1 ARM when the two metabolites are present at equal concentrations, demonstrating that NaMN and NMN are binding to the same allosteric site (Figure 5E, F). The competition between NMN and NaMN, together with the lower affinity of NaMN for binding, explain why NMN levels must be reduced in order to observe NaMN-mediated (i.e. the addition of NaR) axonal protection.

Having discovered the molecular mechanism of axonal protection mediated by NaR plus FK866, we next addressed the mechanism of protection for expression of *E. coli* NMN deamidase, which catalyzes the conversion of NMN to NaMN and potently blocks injury-induced axon degeneration both *in vitro* and *in vivo* [26,28,30]. Prior studies attributed NMN deamidase mediated protection to reduction in NMN levels, however its axoprotective effects are dramatically greater than those observed with FK866 treatment, which also decreases NMN levels but does not increase NaMN levels. Hence, we hypothesized that NaMN also plays a role in long-term axonal protection mediated by NMN deamidase. If this were the case, then elevation of axonal NMN or reduction of NaMN should inhibit the long-term axonal protection by NMN deamidase just as it inhibits NaMN and FK866-mediated axonal protection. To boost NMN without the confound of a concomitant increase in $NAD^+$ or NaMN 1) we applied NR, a cell permeable precursor of NMN, to axons 24 hours post-axotomy when the majority of NMNAT2 is lost and the conversion of NMN to $NAD^+$ is suppressed (Figure 6A); and 2) we limited the amount of NMN deamidase in the axon to minimize the conversion of NMN to NaMN. To limit axonal NMN deamidase, protein transduction using lentivirus particles was used. We previously showed that the cytosolic form of NMNAT1 (cytNMNAT1) is able to block axon degeneration when it is introduced into axons as a protein using virus particles containing cytNMNAT1 [34]. To test if NMN deamidase also provides strong axonal protection via protein transduction, NMN deamidase virus particles were applied to the culture medium 10 minutes after axotomy (Figure 6A). We found that axonal degeneration was strongly inhibited for at least 48 hours (Figure 6B). Axonal metabolite measurements showed a significant elevation of NaMN at 24 hours post-axotomy in NMN deamidase protein-transduced axons

compared with control or FK866 treated axons (Figure 6C, D). In contrast, the level of NMN was comparable to the control axons at 24 hours post axotomy and higher than FK866-treated axons at 0 and 24 hours post-axotomy. In addition, the $NAD^+$ levels were not restored in NMN deamidase transduced axons compared with control axons at 0 or 24 hours post-axotomy. Hence, protein transduction of NMN deamidase provides much longer lasting protection than does FK866 (compare Figure 6B to Figure 1D), yet NMN levels are higher and $NAD^+$ levels are comparable. Therefore, the protection afforded by NMN deamidase is not solely due to changes in the levels of NMN or $NAD^+$.

Next, we tested whether NR treatment to injured axons would reverse NMN deamidase-mediated protection, similar to what we observed in axons preserved by combined NaR/FK866 treatment. We found that NR (100 μM) addition 24 hours post-axotomy potently stimulated axon degeneration in axons transduced with NMN deamidase protein (Figure 6B). We then measured axonal metabolites in NMN deamidase protein transduced axons in the presence of NR. NMN deamidase containing virus particle was added 10 minutes post-axotomy, NR was added 24 hours post-axotomy, and axonal metabolites were measured 6 hours later (30 hours post-axotomy, Figure 6E). Interestingly, axonal NMN was not increased, demonstrating that the NMN deamidase can still suppress NMN levels even following the addition of NR. However, NR addition did lead to a large decrease in NaMN to the levels of control axons lacking NMN deamidase, and also induced a robust increase in the levels of NaR (Figure 6F). While this result was initially surprising, it is likely explained by the generation of NaMN by NMN deamidase, followed by the dephosphorylation of NaMN to NaR by the purine nucleoside phosphorylase family of phosphatases [33]. We speculate that NR supplementation competes with NaR for access to the NRK enzymes that convert NaR to NaMN, leading to unopposed phosphatase activity that keeps NaMN low and NaR high. Since the addition of NR blocks axon protection mediated by protein transduction of NMN deamidase, these findings strongly suggest that NMN deamidase inhibits axon degeneration via two steps: by reducing levels of the SARM1 activator, NMN, and by elevating levels of the SARM1 inhibitor, NaMN. These steps are also carried out by a combination of FK866 (NMN reduction) and NaR (NaMN increase) to inhibit axon degeneration. These findings revise our understanding of NMN deamidase and FK866/NaR mediated axon protection and highlight the potential of NMN analogs as key modulators of SARM1 activation.

## Discussion

The mechanism of SARM1 activation was recently elucidated, identifying SARM1 as a metabolic sensor regulated by the ratio of NMN-to-$NAD^+$ via their competitive binding to an allosteric pocket in the SARM1 autoinhibitory domain [24]. Here we demonstrate that NaMN, the deamidated form of NMN, competes with NMN for binding to the SARM1 allosteric pocket and thereby inhibits SARM1 activation. The identification of NaMN as a SARM1 inhibitor provides a new mechanistic understanding for previously described treatments that can block degeneration of injured axons [26,30,31]. In addition, the identification of a third $NAD^+$ metabolite binding and regulating SARM1 underscores the

central role of $NAD^+$ metabolism in regulating the activation of SARM1 $NAD^+$ hydrolysis and subsequent neurodegeneration. Moreover, these findings highlight the potential of SARM1 allosteric inhibitors as novel therapeutics for the treatment of neurodegeneration.

Here we identify NaMN as a SARM1 inhibitor and reveal the mechanism of strong axonal protection provided by the combination of NaR and FK866 or the neuronal expression of NMN deamidase. A series of elegant experiments from the Coleman, Conforti, and Zhao labs highlighted the role of NMN in promoting SARM1-dependent axon degeneration [25-27,30], and our recent demonstration that NMN binds to an allosteric pocket and activates SARM1 provides the mechanistic explanation for these observations [24]. However, a few inconsistencies persisted in the literature. Most strikingly, two mechanisms that each reduce NMN levels yield dramatically different degrees of axonal protection [26,29]. Inhibition of NMN synthesis by treatment with the NAMPT inhibitor FK866 results in less than one day of protection for severed axons, while the conversion of NMN to NaMN by the neuronal expression of the bacterial enzyme NMN deamidase provides at least 72 hours of protection [26,28]. While the simplest explanation for this discrepancy would be that the NMN deamidase lowers NMN levels more than does FK866, we show here that FK866 can lower NMN levels even more than the NMN deamidase while providing much poorer protection. The second surprising finding was the dramatic enhancement of FK866-mediated protection by the addition of NaR in axons injured by the neurotoxin vincristine [31]. While it was originally suggested that NaR could be bypassing the $NAD^+$ salvage pathway to generate $NAD^+$ in these injured axons, we questioned this explanation because the generation of axonal $NAD^+$ requires the $NAD^+$ biosynthetic enzyme NMNAT2, which is rapidly lost in injured axons following both axotomy and vincristine treatment [27,36]. Here we resolve these discrepancies by demonstrating that: 1) both of these strongly protective treatments lead to the generation of NaMN; 2) NaMN directly binds to SARM1 and inhibits its activation; 3) NaMN competes with NMN for SARM1 binding; and 4) NaMN binds SARM1 more weakly than does NMN, explaining why NMN levels must be lowered to observe protection by NaMN.

We only observe NaMN-mediated protection when NMN levels are artificially lowered, raising the question of if and when this mechanism might be relevant to axonal maintenance in an untreated neuron. NMN is generated by the $NAD^+$ salvage pathway, while NaMN is generated by both the *de novo* and Preiss-Handler $NAD^+$ synthesis pathways. In mammals, the salvage pathway is generally predominant, and we certainly find that to be true in the DRG neurons tested here. However, there may be neuronal cell types or conditions in which there is a relative preference for the *de novo* or Preiss-Handler pathways, which would boost the NaMN/NMN ratio and thereby decrease the likelihood of SARM1 activation. For example, in a neuronal subtype with abundant NaPRT using the common vitamin B3 analog nicotinic acid would lead to high levels of NaMN. We speculate that differences in $NAD^+$ biosynthetic routes are a candidate mechanism for differential vulnerability of neurons due to the antagonistic effects of NMN and NaMN on SARM1 activation.

The SARM1 allosteric regulatory pocket binds NaMN, $NAD^+$, and NMN, with NaMN and $NAD^+$ inhibiting NMN binding and thereby antagonizing SARM1 activation [20,23,24]. Hence, the SARM1 allosteric pocket is relatively promiscuous, binding diverse components of the $NAD^+$ metabolome. This suggests that additional endogenous and exogenous molecules could bind this pocket and regulate SARM1 activation. This relative promiscuity gives hope that small molecules could be developed that would bind the pocket much more tightly than either NaMN or $NAD^+$ to block NMN binding and to inhibit SARM1 activation. The ability to target both SARM1 activation at the allosteric site and SARM1 activity within the TIR enzymatic pocket may enable powerful synergistic approaches to inhibit SARM1 for the prevention and treatment of neurodegenerative diseases [39-41].

## Materials and Methods

Chemicals: FK866 was obtained from National Institute of Mental Health Chemical Synthesis and Drug Supply Program (MH number F-901). Nicotinic acid riboside was purchased from Santa Cruz Biotechnology (sc-478242). Nicotinamide riboside (NR) was a gift from ChromaDex, Inc.. $NAD^+$ (Sigma, N1636), NMN (Sigma, N3501), cADPR (Sigma, C7344) were purchased from Sigma.

Mouse studies: CD1 mice (gestation day 12.5; Charles River Laboratories) were housed (12 hr dark/light cycle and less than 5 mice per cage) and used under the direction of institutional animal study guidelines at Washington University in St. Louis. NMRK1 knockout mice: $Nmrk1^{tm1a(KOMP)Wtsi}$ ($NRK1^{-/-}$) and NMRK2 knockout mice: $Nmrk2^{tm1.1(KOMP)Vlcg/J}$ ($NRK2^{-/-}$) were obtained from Wellcome Trust Sanger Institute and Jackson laboratory respectively and were used to generated $NRK1^{-/-}$:$NRK2^{-/-}$ (NRK1/2 dKO, Supplemental Figure1).

DRG neuronal culture and axon degeneration assay: mouse DRG neuron culture was performed as described before [28]. Mouse dorsal root ganglion (DRG) was dissected from embryonic days 13.5 CD1 mouse embryo (50 ganglia per embryo) and incubated with 0.05% trypsin solution containing 0.02% EDTA (Gibco) at 37°C for 15 min. Then cell suspensions were triturated by gentle pipetting and washed 3 times with the DRG growth medium (Neurobasal medium (Gibco) containing 2% B27 (Invitrogen), 100 ng/ml 2.5S NGF (Harlan Bioproduts), 1 μM uridine (Sigma), 1 μM 5-fluoro-2'-deoxyuridine (Sigma), penicillin, and streptomycin). Cells were suspended in DRG growth medium at a ratio of 50 μl medium/50 DRGs. The cell density of these suspensions was ~$1x10^7$ cells/ml. Cell suspensions (1 μl/96 well, 5 μl/24 well) were placed in the center of the well using either 96- or 24-well tissue culture plates (Corning) coated with poly-D-Lysine (0.1 mg/ml; Sigma) and laminin (3 μg/ml; Invitrogen). Cells were allowed to adhere in humidified tissue culture incubator (5% $CO_2$) for 15 min and then DRG growth medium was gently added (100 μl/96 well, 500 μl/24 well). Lentivirus transfer vector constructs harboring cDNAs including Venus, cytNMNAT1 (mouse), NRK1 (mouse), and NMN deamidase (E. coli) were previously described[28]. Lentiviruses were added (1-$10x10^3$ pfu) at 1–2 days *in vitro* (DIV) and metabolites were extracted or axon degeneration

assays[29] were performed at 6–7 DIV. For protein transduction of NNM deamidase, lentivirus particles (5-50x$10^3$) were added at indicated time post axotomy. When using 24 well DRG cultures, 50% of the medium was exchanged for a fresh medium at DIV4. For axotomy experiments, cell bodies and axons were separated by a microsurgical blade.

Metabolite extraction: At DIV6, tissue culture plates were placed on ice and culture medium replaced with ice-cold saline (0.9% NaCl in water, 500 µl per well). For collection of axonal metabolites, cell bodies were removed using a pipette. For collection of intracellular metabolites, saline was removed and replaced with 160 µL ice cold 50% MeOH in water. The axons were incubated for a minimum of 5 min on ice with the 50% MeOH solution and then the solution was transferred to tubes containing 50 µl chloroform on ice, shaken vigorously, and centrifuged at 20,000 x g for 15 min at 4ºC. The clear aqueous phase (140 µl) was transferred into a microfuge tube and lyophilized under vacuum. Lyophilized samples were stored at -20ºC until measurement.

Metabolite measurements: Lyophilized samples were reconstituted with 5 mM ammonium formate (15 µl), centrifuged (13,000 x g, 10 min, 4ºC), and 10 µl clear supernatant was analyzed. NMN, $NAD^+$, and cADPR were measured using LC-MS/MS [42]. Samples were injected into a C18 reverse phase column (Atlantis T3, 2.1 x 150 mm, 3 µm; Waters) using HPLC (Agilent 1290 LC) at a flow rate of 0.15 ml/min with 5 mM ammonium formate for mobile phase A and 100% methanol for mobile phase B. Metabolites were eluted with gradients of 0–10 min, 0–70% B; 10–15 min, 70% B; 16–20 min, 0% B. The metabolites were detected with a triple quad mass spectrometer (Agilent 6470 MassHunter; Agilent) under positive ESI multiple reaction monitoring (MRM) using parameters specific for each compound ($NAD^+$, 664>428, fragmentation (F) = 160 V, collision (C) = 22 V, and cell acceleration (CA) = 7 V: NMN 335 > 123, F=135 V, C=8 V, CA = 7 V: cADPR, 542>428, F = 100 V, C = 20 V, and CA = 3 V: NaMN 336>124, F=80 V, C=10V, CA = 7 V: NaAD 669>428, F=150 V, C=20 V, CA = 7 V: NaR, 256>124, F=35 V, C=10V, CA = 3 V: NR 255 >123, F=130 V, C=20 V, CA = 1 V). Serial dilutions of standards for each metabolite in 5 mM ammonium formate were used for calibration. Metabolites were quantified by MassHunter quantitative analysis tool (Agilent) with standard curves and normalized by the protein.

Western blot: SDS-PAGE was performed using Bolt™ 4 to 12%, Bis-Tris (Invitrogen) with Bolt MOPS SDS Running Buffer (Invitrogen) and proteins were transferred to nitrocellulose membrane (Amersham, 10600002). Membranes were incubated with blocking solution (5% BSA in PBS containing 0.1% Triton X-100 (PBS-T)) for one hour then with NMNAT2 antibody (Santa Cruz, sc-515206, 1:1000 dilution in blocking solution) overnight at 4ºC. Membranes were washed in PBS-T and incubated with HRP-conjugated secondary antibody (Jackson Immuno Research, 115-007-003, 1:5000 dilution in PBS-T) for one hour and developed with chemiluminescent reaction (VWR, 490005-008).

*In-vitro* SARM1 $NAD^+$ hydrolase assay: human SARM1 without mitochondria targeting sequence and with N-terminal tandem StrepTag II was expressed in HEK293T cells

expressing NRK1 supplemented with NR[18] and purified with MagStrep (Strep-Tactin) type 3 XT beads suspension (IBA Lifesciences). The purified protein attached on beads was incubated with $NAD^+$ (10 μM for Figure 5 B, C and 50μM for Figure 5A) for 0, 5, 15, and 30 minutes at 25ºC in the presence or absence of various concentrations of NMN, NaMN, or NMN plus NaMN that were preincubated with SARM1 before $NAD^+$ addition. Metabolites were extracted with the chloroform/methanol method, lyophilized, and measured by HPLC as previously described[18].

Saturation-transfer difference (STD) NMR: both hSARM1 and dSARM1ARM were produced as per Figley et al [24], and STD NMR was performed in a similar fashion as described previously [24]. Briefly, samples for STD NMR were prepared in a total volume of 200 µL, with each sample consisting of 175 µL HBS buffer, 20 µL $D_2O$, and 5 µL DMSO-d6. STD NMR spectra were acquired with a Bruker Avance 600 MHz NMR spectrometer equipped with $^1H/^{13}C/^{15}N$ triple resonance cryoprobe at 298K. The pulse sequence STDDIFFGP19.3, built-in within the TopSpin™ program (Bruker), was employed to acquire STD NMR spectra[43]. This pulse sequence consists of a 3-9-19 water suppression pulse, the parameters of which were obtained from the water suppression pulse program (P3919GP), to suppress the resonance from $H_2O$. The on-resonance irradiation was set close to protein resonances at 0.8 ppm, whereas the off-resonance irradiation was set far away from any protein or ligand resonances at 300 ppm. A relaxation delay of 4 s was used, out of which a saturation time of 3 s was used to irradiate the protein with a train of 50 ms Gaussian shaped pulses. The number of scans was 512. All spectra were processed by TopSpin (Bruker) and Mnova 11 (Mestrelab Research).

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**Author Contributions**
Conceptualization, Y.Sasaki, A.D., J.M. ;Investigation, Y.Sasaki., J.Z., Y.S., W.G., B.K., T.V., A.D., J.M.; Writing –Original Draft, Y.Sasaki, A.D., J.M. ; Writing – Review &

Editing, All authors; Funding Acquisition, B.K., T.V., A.D., J.M.; Supervision, B.K., T.V., A.D., J.M.

**Declarations of interests**

A.D and J.M. are co-founders, scientific advisory board members, and shareholders of Disarm Therapeutics, a wholly owned subsidiary of Eli Lilly & Company. B.K. is shareholder of Disarm Therapeutics. B.K. is a consultant to Disarm Therapeutics. B.K. and T.V receive research funding from Disarm Therapeutics. Y. Sasaki and J.M. may derive benefits from a licensing agreement with ChromaDex, which did not provide any support for this work.

**Figure 1**

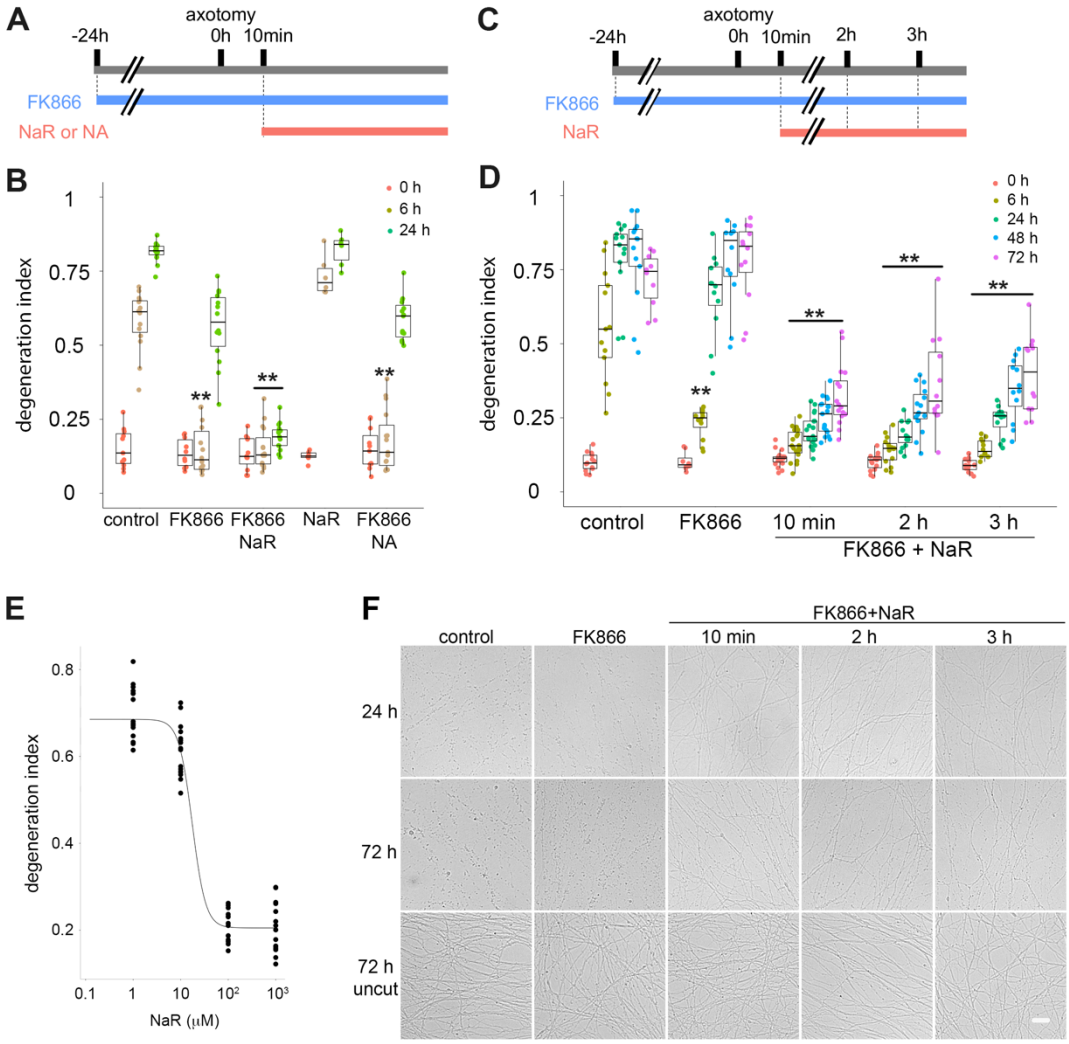



**Figure 1. NaR provides prolonged axonal protection after axotomy in the presence of FK866.** A) Time scheme showing the timing of axotomy, chemical addition, and axon degeneration assay used in (B). B) Axonal degeneration 0 to 24 h post-axotomy with treatments described in (A) was quantified. Statistical analysis was performed by two-way ANOVA with Tukey post-hoc test. $F(4, 164)=178.4$, $p<1.0\times10^{-16}$ among groups including control, FK866, FK866+NaR, NaR, and FK866+NA. **$p<1\times10^{-5}$ denotes the significant difference compared with the degeneration index of control at the same time post-axotomy. C) Time scheme showing the timing of axotomy, chemical addition, and axon degeneration assay used in (D). D) Axonal degeneration 0 to 72 h post-axotomy with treatments described in (C) was quantified. While control or NaR-treated axons degenerate within 6 h after axotomy, FK866 showed axonal protection at 6 h, but not 24 h. NaR addition 10 min to 3 h post axotomy provided strong axonal protection up to 72 h. Statistical analysis was performed by two-way ANOVA with a Tukey post-hoc test. $F(4, 317)=269.1$, $p<1.0\times10^{-16}$ among groups including control, FK866, FK866+NaR (10 min, 2h, 3h). **$p<1 \times 10^{-5}$ denotes the significant difference

compared with control 0h post-axotomy. E) Dose response curve showing NaR-mediated axonal protection. $EC_{50}$ = 18 μM. F) Representative images of DRG axons at 24 h or 72 h after axotomy or 72 h without injury (uncut) with the indicated treatments. Scale bar, 20μm.

**Figure2**

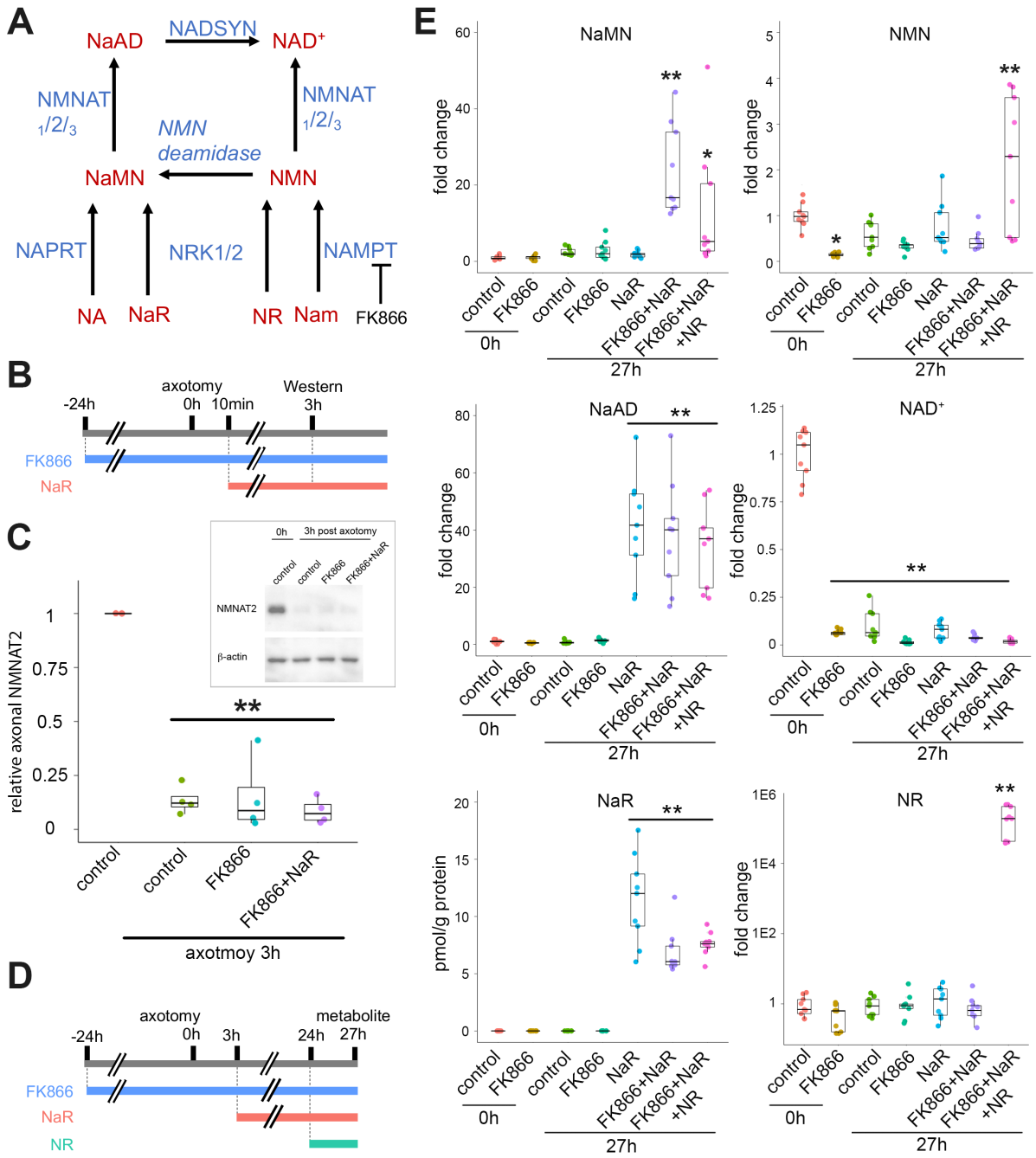



**Figure 2. NaR-mediated NaMN and NaAD increases do not support NAD⁺ synthesis in transected axons.** A) Diagram of mammalian nicotinamide adenine dinucleotide (NAD⁺) biosynthesis pathways. Nam; nicotinamide, NA; nicotinic acid, NMN, nicotinamide riboside, NaR; nicotinic acid riboside, NaMN; nicotinic acid mononucleotide, NAD⁺; nicotinamide adenine dinucleotide, NaAD; nicotinic acid adenine dinucleotide. FK866 is an inhibitor of NAMPT. B) Time scheme showing the

timing of axotomy, chemical addition, and Western blotting used in (C). C) Axonal NMNAT2 normalized by actin at 0 or 3 h post-axotomy are shown. Data were normalized to the control axons 0 h post-axotomy. Statistical analysis was performed by one-way ANOVA with a Tukey post-hoc test. $F(3, 11)=57.6$, $p=1.5\times10^{-6}$ among groups. **$p<5.1\times10^{-7}$ denotes the significant difference compared with the amount of NMNAT2 in control axons at 0 h post-axotomy. Inset shows a representative Western blot, showing axonal NMNAT2 0 or 3 h post-axotomy with indicated conditions. D) Time scheme showing the timing of axotomy, chemical addition, and metabolite measurement used in (E). E) Axonal metabolite analysis 0 and 27 h post-axotomy with treatments described in (D) are shown. Statistical analysis was performed by one-way ANOVA with Benjamini-Hochberg post-hoc test. $F(6, 56)=11.3$, $p=3\times10^{-8}$ for NMN, $F(6, 56)=10.4$, $p=1.1\times10^{-7}$ for NaMN, $F(6, 56)=27.7$, $p=4.4\times10^{-15}$ for NaAD, $F(6, 56)=311.7$, $p<2\times10^{-16}$ for $NAD^+$, $F(6, 56)=74.1$, $p<2\times10^{-16}$ for NaR, $F(6, 56)=14.4$, $p=7.8\times10^{-10}$ for NR. **$p<1\times10^{-5}$ and *$p<0.05$ denote the significant difference compared with the amounts of metabolites in control axons at 0 h post-axotomy.

**Figure 3**

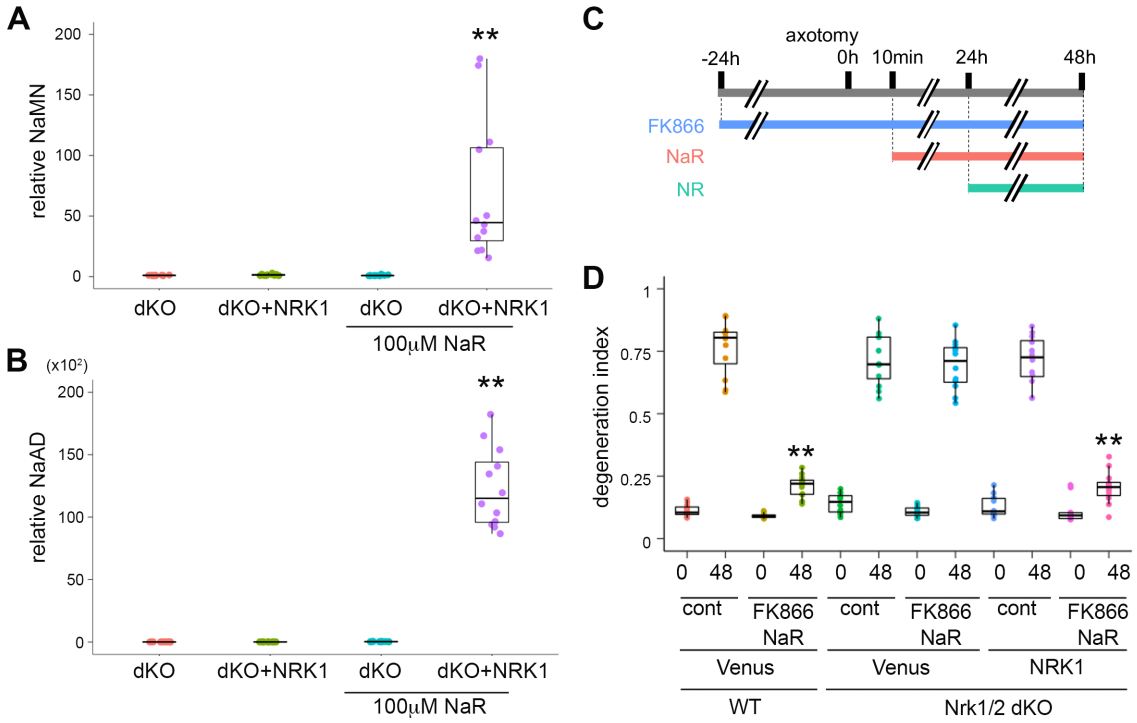



**Figure 3. NaMN synthesis is required for axonal protection by NaR.** A, B) Metabolite analysis from NRK1 and NRK2 double knockout (NRK1/2 dKO) cells expressing Venus (dKO) or mouse NRK1 (dKO+NRK1) via lentivirus. NaMN (A) or NaAD (B) were low in NRK1/2 dKO neurons with or without NaR addition (100 μM, 24 hours). Exogenously expressed NRK1 produces high NaMN and NaAD in NRK1/2 dKO neurons. Statistical analysis was performed by one-way ANOVA with a Tukey post-hoc test. $F(3, 44)=16.5$, $p=2.5\times10^{-7}$ for NaMN and $F(3 ,44)=181.1$ $P<2 \times10^{-16}$ for NaAD. $^{**}p<1\times10^{-5}$ denotes the significant difference compared with metabolites of the NRK1/2 dKO control neurons. C) Time scheme showing the timing of axotomy, chemical addition, and axon degeneration assay used in (D). D) Axonal degeneration 0 or 48 h post-axotomy with treatments described in (C) was quantified. Wild-type axons were protected by NaR with FK866. NRK1/2 dKO axons were not protected by NaR with FK866, however exogenous expression of NRK1 in NRK1/2 dKO neurons rescued the axonal protection by NaR with FK866. Statistical analysis was performed by two-way ANOVA with a Tukey post-hoc test. $F(5, 130)=68.7$, $p<2.0\times10^{-16}$ among groups. $^{**}p<1\times10^{-5}$ denotes the significant difference compared with the degeneration index of control at the same time post-axotomy.

**Figure 4**

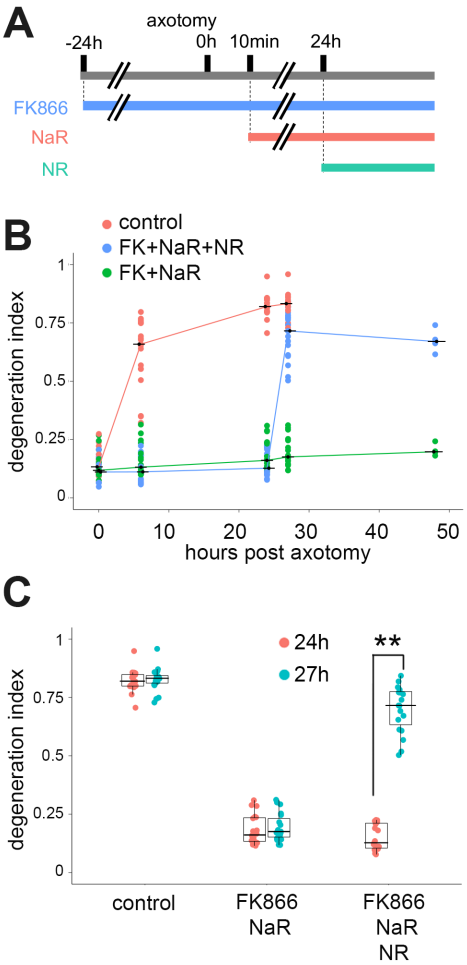



**Figure 4. NMN blocks NaR-mediated axonal protection.** A) Time scheme showing the timing of axotomy, chemical addition, and axon degeneration assay used in (B). B) Axonal degeneration 0 to 48 h post-axotomy with treatments described in (A) was quantified. While axons treated with NaR and FK866 (FK+NaR) showed axonal protection up to 48 h, NR addition at 24 h post-axotomy induced axon degeneration within three hours in NaR+FK866 treated axons (FK+NaR+NR). C) Statistical analysis of axon degeneration at 24 and 27 h post axotomy in (B) was performed by two-way ANOVA with Tukey post-hoc test. $F(2, 108)=924.9$, $p<2.0\times10^{-16}$ among groups. **$p<1\times10^{-5}$ denotes the significant difference compared with the degeneration index of control at the same time post-axotomy.

**Figure 5**

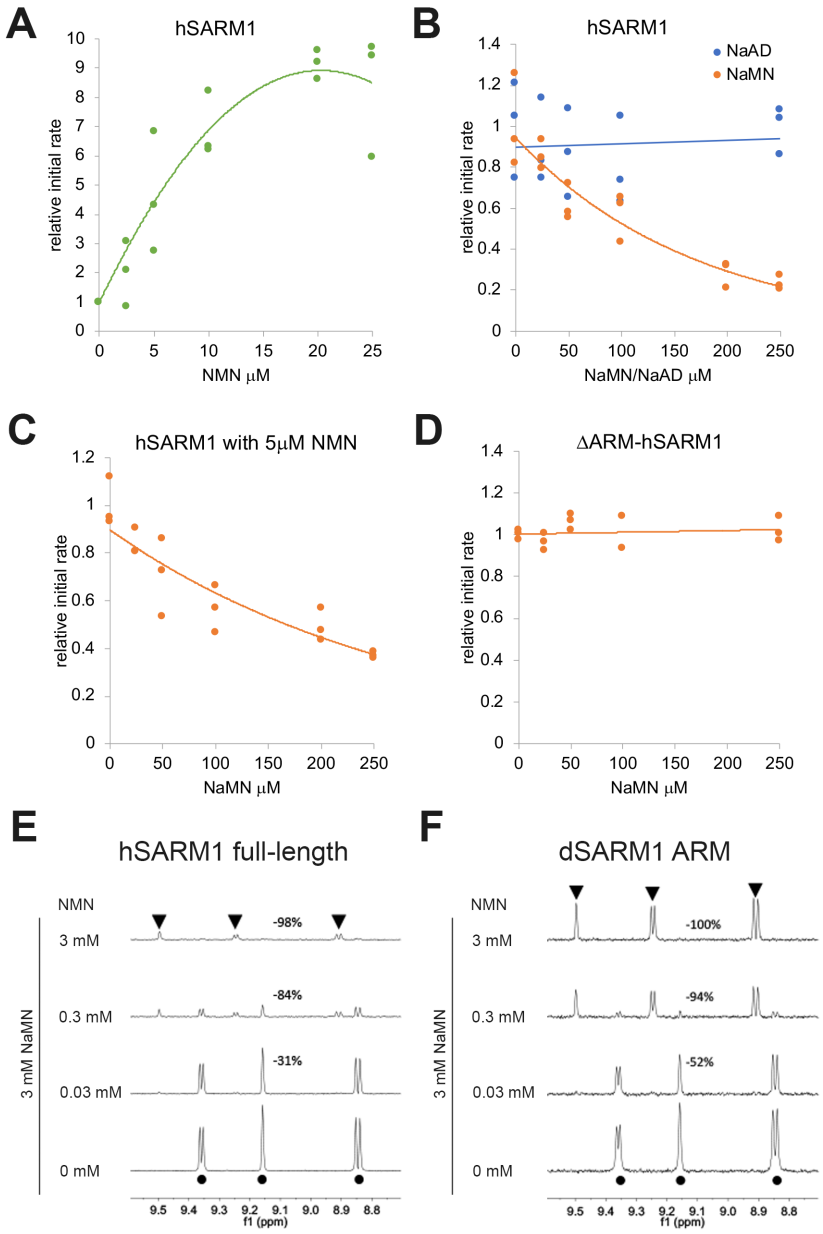



**Figure 5. NaMN inhibits SARM1 NAD⁺ hydrolase activity.** A-C) In-vitro SARM1 $NAD^+$ hydrolase assay using full-length human SARM1 full-length without the mitochondrial localization signal (hSARM1), using different concentrations of NMN or NaMN. Initial rates of the $NAD^+$ hydrolase reaction normalized to control (0 μM NMN or NaMN) are shown. (A) NMN activated hSARM1 with Ka = 8.7 μM, (B) NaMN inhibited SARM1 with $IC_{50}$ of 93.3 μM. NaAD did not show inhibition or activation of hSARM1 $NAD^+$ hydrolase activity. (C) NaMN inhibited SARM1 with $IC_{50}$ of 147.2 μM . (D) NaMN up to 250 μM did not show inhibition or activation of $NAD^+$ hydrolase activity of hSARM1 lacking the N-terminal ARM domain (ΔARM-hSARM1). E-F) Expansions of saturation-transfer

difference (STD) NMR spectra showing E) NaMN binding to 7.35 μM hSARM1 in the absence and presence of NMN and F) NaMN binding to 50 μM *Drosophila* SARM1 ARM domain (dSARM1 ARM) in the absence and presence of NMN. Four samples with 3 mM NaMN and increasing concentrations of NMN (from bottom to top: 0, 0.03 mM, 0.3 mM, and 3 mM) are shown. "●" indicates NaMN signals while "▾" indicates NMN signals. The percentage decrease of NaMN signals relative to no-NMN control is labelled.

**Figure 6**

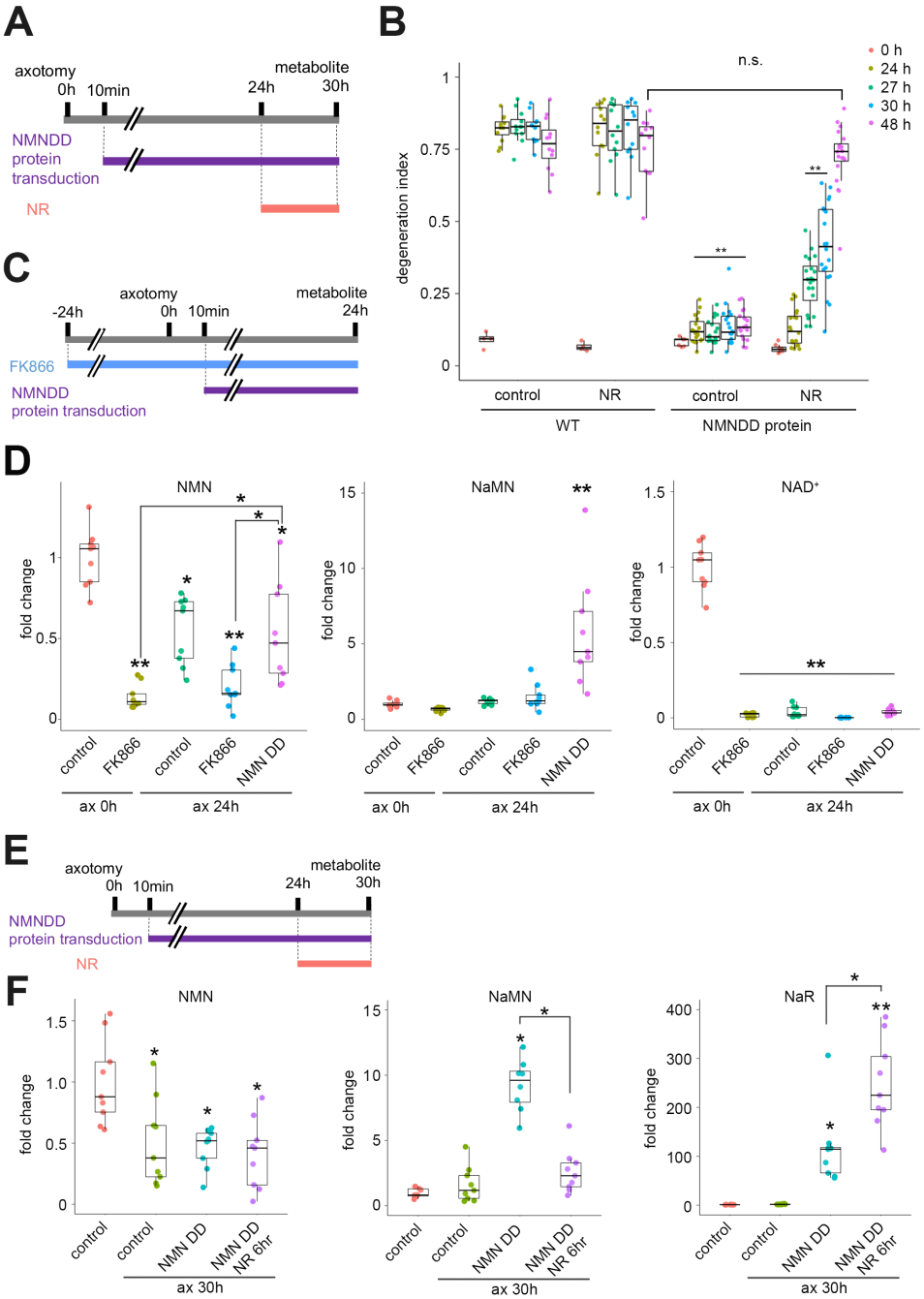
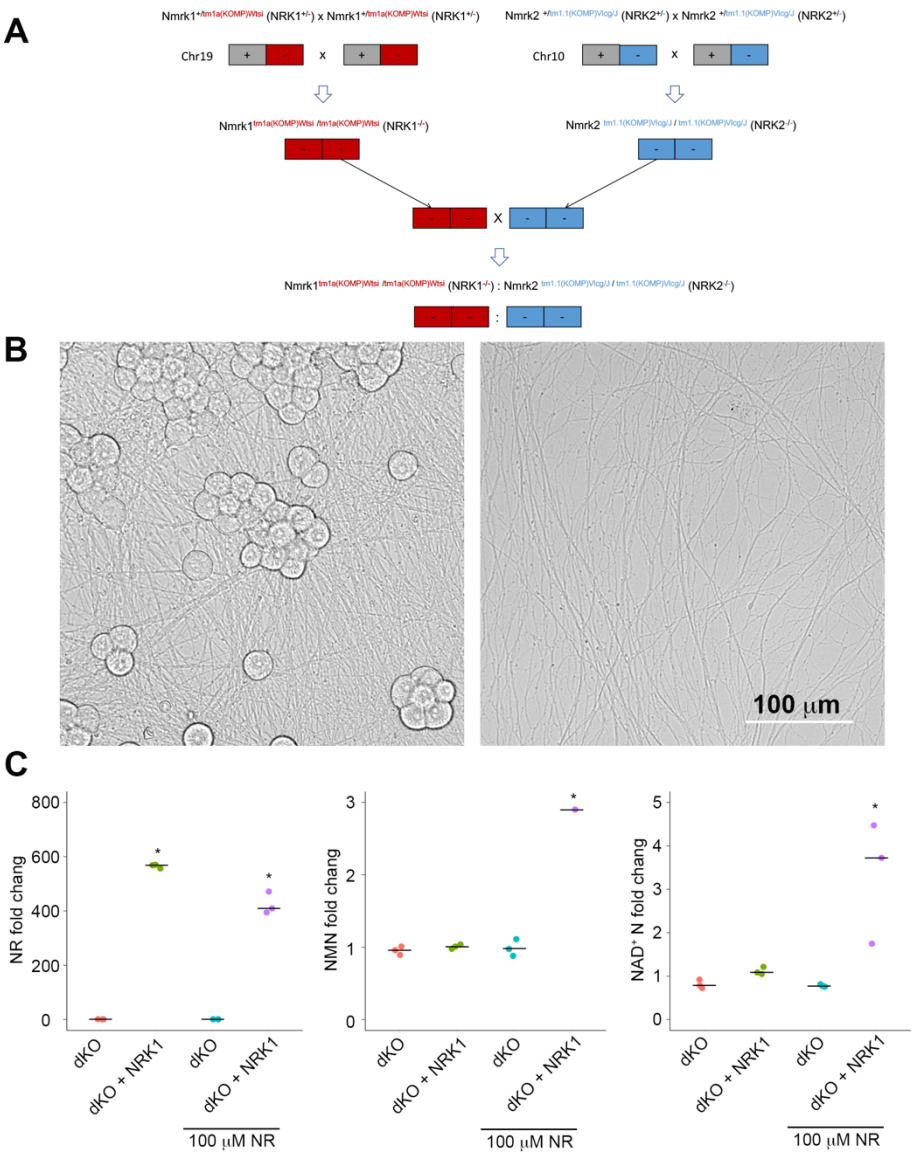



**Figure 6. NMN deamidase supports prolonged axonal protection by decreasing NMN and increasing NaMN.** A) Time scheme showing the timing of axotomy, chemical addition, and axon degeneration assay used in (B). B) Axonal degeneration of wild-type neurons from 0 to 48 h post-axotomy with treatments described in (A) was quantified. NR addition at 24 hours post-axotomy to the NMN deamidase protein transduced axons showed axon degeneration by 48 hours post-axotomy. Statistical analysis was performed by two-way ANOVA with a Tukey post-hoc test. F(3, 256)=946.7,

$p<2.0\times10^{-16}$ among groups. **$p<1x10^{-5}$ denotes the significant difference compared with the degeneration index of control at the same time post-axotomy. N.S. denotes no significant difference. C) Time scheme showing the timing of axotomy, addition of FK866 or NMN deamidase (NMNDD) protein transduction and metabolite measurement. D) Axonal metabolites 0 or 24 h post-axotomy from neurons treated with FK866 or NMNDD protein transduction. NMN levels in the presence of NMNDD protein are not lower than FK866-treated axons with or without axotomy. Statistical analysis was performed by one-way ANOVA with Tukey post-hoc test. $F(4, 40)=27.1$, $p=6.4\times10^{-11}$ for NMN, $F(4, 40)=84$, $p=13.8\times10^{-7}$ for NaMN , $F(4, 40)=340$, $p<2.0\times10^{-16}$ for $NAD^+$ among groups. **$p<1 \times 10^{-5}$ and *$p<0.05$ denote significant difference compared with wild-type control axons. E) Time scheme showing the timing of axotomy, addition of NR, NMN deamidase (NMNDD) protein transduction, and metabolite measurement. F) Axonal metabolites 0 or 30 h post-axotomy with or without NMNDD protein transduction at 24 h post-axotomy and plus/minus NR. NaMN was reduced without changing NMN after NR addition to NMNDD protein-transduced axons. Statistical analysis was performed by one-way ANOVA with a Tukey post-hoc test. $F(3, 32)=7.6$, $p= 5.4 \times10^{-4}$ for NMN, $F(2, 32)=6.6$ , $p=1.2\times10^{-3}$ for NaMN , $F(3, 32)=35.1$, $p<3.1\times10^{-10}$ for $NAD^+$ among groups. **$p<1 \times 10^{-5}$ and *$p<0.05$ denote significant differences compared with wild-type control axons.

Supplemental Figure 1




**Supplemental Figure 1. NRK1/2 dKO mice and neurons are viable.** A) A breeding scheme showing the generation of Nmrk1:Nmrk2 double knockout (NRK1/2 dKO) mice. B) Images showing DIV7 DRG neuronal cell bodies and axons from NRK1/2 dKO mice. Scale: 100 μm. C) Metabolite analysis from NRK1/2 dKO neurons expressing Venus (dKO) or mouse NRK1 (dKO+NRK1) via lentivirus. NMN or $NAD^+$ were low in NRK1/2 dKO neurons with or without NR addition (100 μM, 24 h). Exogenously expressed NRK1 produces high NMN and $NAD^+$ in NRK1/2 dKO neurons. Statistical analysis was performed by one-way ANOVA with a Tukey post-hoc test. $F(3, 8)=31.9$, $p=8.5\times10^{-5}$ for NR, $F(3, 8)=592.4$, $p=9.9\times10^{-10}$ for NMN, and $F(3 ,44)=8.8$ $P=6.4 \times10^{-3}$ for $NAD^+$. *$p<0.05$ denotes the significant difference compared with metabolites of the NRK1/2 dKO control neurons.

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