The Enzymatic Function of the TIR domain: From Axon Degeneration to Innate Immunity

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The Enzymatic Function of the TIR domain: From Axon Degeneration to Innate Immunity
by
Kow Akaa Essuman

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
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Kow Akaa Essuman

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May 2020
Dedicated to my parents.
ABSTRACT OF THE DISSERTATION

The Enzymatic Function of the TIR domain: From Axon Degeneration to Innate Immunity

by

Kow Akaa Essuman

Doctor of Philosophy in Biology and Biomedical Sciences
Molecular Genetics and Genomics

Washington University in St. Louis, 2020

Professor Jeffrey Milbrandt, Chair

The Toll/Interleukin-1 Receptor (TIR) domain is an evolutionarily ancient protein domain conserved from bacteria to eukaryotes, and is an essential signaling component of innate immunity pathways. In animal innate immunity, TIR domains have primarily been described for their scaffolding function in assembling protein complexes in host defense. In plant immunity, TIR domains are key components of the intracellular Nucleotide Binding Leucine rich repeat (NLR) immune receptors that confer resistance to pathogens. These NLR receptors trigger cell death and an immune response upon activation, but their mechanism has remained elusive. In bacteria, TIR domain proteins have been suggested to function as secreted virulence factors against eukaryotic hosts. My dissertation work begins with the study of a TIR domain containing protein, SARM1 (Sterile Alpha and TIR motif containing 1) that is expressed in neurons, and is activated upon axonal injury. SARM1 activation triggers depletion of the essential metabolic cofactor Nicotinamide Adenine Dinucleotide (NAD$^+$), results in energetic failure in the axon, and ultimately axonal death. Since TIR domains were primarily known to function as scaffolds in immune signaling pathways, I embarked on a search for the NAD$^+$ depleting enzyme that was activated by the SARM1 TIR domain, or was binding to the SARM1 TIR scaffold. These studies
aimed not only to advance our understanding of the mechanism of axon degeneration, but sought to identify a therapeutic target for diseases characterized by axon degeneration. In my pursuit of this aim, I surprisingly found that rather than activating a secondary NAD\(^+\) depleting enzyme, the SARM1 TIR domain itself functioned as the enzyme that depleted axons of NAD\(^+\) and caused axonal death. Since TIR domains were not known to possess enzymatic activity, the discovery of the TIR NADase activity in SARM1 placed SARM1 as the founding member of a potentially new class of enzymes. Soon after, I found that the TIR domain enzymatic activity was an ancient enzymatic property, conserved even in bacterial and archaeal TIR domains. Finally, my work has provided significant insights into the biology of plant NLR receptors. We show that TIR domain containing NLR receptors transduce pathogen recognition into cell death via a conserved NAD\(^+\) cleavage activity. These findings establish TIR domain proteins as a new family of enzymes, and carries significant implications for innate immunity, and the treatment of diverse neurodegenerative diseases.
1. Introduction

1.1 TIR Domain Signaling in Animals, Plants, and Bacteria

The innate immune system of living organisms enables the rapid counterattack against invading pathogens, and in certain instances can activate host adaptive immunity to strengthen the immune response (Akira et al., 2006; Medzhitov, 2001). These innate responses involve physiological, cellular, and molecular processes that aim to ultimately ensure host integrity and survival (Akira et al., 2006; O’Neill et al., 2013). Toll-like Receptors (TLRs) and Interleukin-1 Receptors (IL-1R) have long been recognized as integral components of the innate immune system, and their mechanism of action has been the subject of many years of research. (O’Neill et al., 2013; Garlanda et al., 2013). Out of this work has emerged the identification and role of the key, signaling component of these receptors – the Toll/Interleukin-1 Receptor (TIR) domain.

Early insights into the identification of the TIR domain came from studies of IL-1R and the Drosophila Toll receptor. In 1988, IL-1R was cloned, and in 1991, the cytosolic domain of Toll was shown to be homologous to the cytosolic domain of IL-1 (Gay and Keith, 1991). This was quite surprising at the time, since IL-1R was associated with inflammatory and acute phase responses, whereas Drosophila Toll had previously been linked to dorso-ventral patterning in fly development (Anderson et al., 1985). These early studies foreshadowed the notion that this shared cytosolic domain may exert its function beyond immune and inflammatory innate pathways, and involve developmental pathways in animals (O’Neill et al., 2013; Chuang et al., 2005). The functional link between IL-1R and Toll cytosolic domain was eventually solidified
with the discovery that they both activate the same family of transcription factor - Nuclear Factor kappa B (NF-kB), to drive specific responses in the host (Croston et al., 1995; Hetru and Hoffmann, 2009; Steward 1987).

In 1996, studies led by Jules Hoffman also found that the Drosophila Toll gene was important in an antifungal innate immune response by the fly (Lemaitre et al., 1996; Lemaitre, 2004). This seminal work helped earn Hoffman and Bruce Beutler half the 2011 Nobel Prize in Physiology or Medicine. Bruce Beutler and his group identified Toll-Like Receptor (TLR4), as the receptor for lipopolysaccharide (LPS) (Poltorak et al., 1998). In summary, the accepted signaling model for Drosophila Toll, mammalian TLRs, and IL-1R pathways is that the TIR domain of these receptors and of other TIR cytosolic proteins, function scaffolding adaptors to assemble proteins complex to activate downstream pathways. In the innate immune response particularly, these pathways eventually lead to the activation of transcription factors such as NF-kB, and interferon release factors that promote synthesis of pro-inflammatory cytokines and interferons (O’Neill et al., 2013).

A few years after homology was identified between Drosophila Toll and IL-1 Receptor, TIR domain proteins were discovered in plants, with the identification of the N-gene that confers disease resistance against the tobacco mosaic virus (TMV) (Whitham et al., 1994). TMV turns out to be the first pathogen to be described as a virus (Beijerinck, 1898; Mayer, 1886; Ivanowski, 1892; Creager et al., 1996), and the first virus to be chemically purified and crystallized, for which another Nobel Prize was awarded in 1946 to Wendell Stanley (Stanley, 1935). The N-protein also turned out be a prototype of TIR domain-containing proteins in plants that belong to the family of plant receptors called Nucleotide Binding Leucine Rich Repeat (NLR) Receptors (Jones et al., 2016). These NLRs function in plants as immune receptors to
trigger cell death upon pathogen effector recognition (Jones et al., 2016). This response is thought to prevent the spread of the pathogen, although the molecular mechanism has remained a mystery until now.

In 2006, it was also revealed that the evolutionary conservation of TIR domains extends beyond animals and plants, into bacteria (Newman et al., 2006). Early studies on bacterial TIR domains suggested that these TIR domain proteins functioned as virulence factors with examples from *Salmonella enterica*, *uropathogenic E. coli*, and *Staphylococcus aureus* (Newman et al., 2006; Cirl et al., 2008). In this model, bacteria secrete TIR domain proteins that enter eukaryotic cells, and suppress eukaryotic innate immune signaling via molecular mimicry (Waldhuber et al., 2018). This model that bacterial TIR domain-containing proteins primarily function as virulence factors however appears incomplete, and was challenged with reasons such as the abundance of TIR domains in environmental organisms not considered to be pathogens, and the co-occurrence of diverse domains within an individual TIR-containing protein (Spear et al., 2009). While future works will seek to clarify these observations and theories, my dissertation work indeed suggests that the bacterial TIR domains have more to their function than subversion of mammalian innate immune system through TIR-TIR protein interactions.

My dissertation begins with the study of the mammalian TIR domain-containing adaptor, SARM1 (Sterile Alpha and TIR motif containing 1). SARM1 has emerged over the last few years as the central executioner of the injury-induced axon self-destruction program (Gerdts et al., 2016). Loss of SARM1 blocks axon degeneration after traumatic and toxic injuries (Gerdts et al., 2016), and improves functional outcomes in mice after traumatic brain injury and chemotherapy induced peripheral neuropathy (Geisler et al., 2016; Henninger et al., 2016; Ziogas and Koliatsos 2018). Activation of SARM1 signaling on the other hand, via enforced
dimerization of its TIR domain is sufficient to induce axon degeneration without injury through depletion of the essential metabolite Nicotinamide Adenine Dinucleotide (NAD$^+$) (Gerds et al., 2015). Interestingly, SARM1’s TIR domain is phylogenetically closer to prokaryotic TIR domains than to its mammalian counterparts (Zhang et al., 2011). As I detail in the following chapters of my dissertation work, I identify a novel function of the TIR domain in that TIR domains of SARM1, prokaryotes, and plants, possess NAD$^+$ cleavage activity. These findings suggest that the primordial function of the TIR domain is the enzymatic cleavage of NAD$^+$ and that the scaffolding function in TLR signaling could represent a repurposing of this evolutionary ancient enzymatic function.

1.2 SARM1: The Ancestral Animal TIR domain Adaptor in Immune and Neuronal Pathways

There are ten known TLRs and six intracellular TIR adaptor proteins in humans (O’Neill et al., 2013). SARM1 was the fifth animal TLR adaptor to be discovered after MyD88, MAL, TRIF, and TRAM (Mink et al., 2001; O’Neill et al., 2003). Unlike these previously discovered TLR adaptors, SARM1’s TIR domain is more closely related to the TIR domains of bacteria, suggesting it might represent the ancestral TIR domain in eukaryotes (Zhang et al., 2011). Further, the evolutionary conservation of SARM1 in human, mouse, drosophila, and most importantly, C. elegans, suggested that SARM1 likely represented the most evolutionary ancient adaptor, since MyD88, the sole TLR adaptor in drosophila has not been described in the C. elegans genome (Pukkila-Worley and Ausubel, 2012; Jebanathirajah et al., 2002).
In C. elegans, the SARM1 ortholog, tir-1 was originally described to regulate innate immune responses via the synthesis of the antimicrobial peptides NLP-29 and NLP-31 (Couillault et al., 2004). The synthesis of these peptides and activation of this pathway promotes worm defense against bacterial and fungal infections (Couillault et al., 2004). A role for tir-1 was also revealed in neurodevelopment, where tir-1 functions to specify an asymmetric odorant receptor expression pattern in C. elegans neurons (Chuang and Bargmann, 2005). In this developmental program, tir-1 functions downstream of a voltage-gated calcium channel and activates the ASK-1 MAPKKK (MAP kinase kinase kinase) signaling cascade to specify the choice between AWCoff or AWCon neuron (Chuang and Bargann, 2005). More recently, the tir-1/JNK pathway has been shown accelerate olfactory pathways involved in memory and forgetting in C. elegans (Inoue et al., 2013), implicating tir-1 in neuronally controlled behavioral responses. These early studies revealed important insights into the function of SARM1 in worms, yet its role in vertebrates and TLR signaling was still to be determined.

The first report of SARM1’s role in vertebrate TLR signaling suggested that SARM1’s function was different from that of previously identified cytosolic TIR adaptors. While the previous cytosolic TIR adaptors, and TLRs themselves trigger activation of inflammatory and defense signals, SARM1 antagonized the TLR-TRIF pathway (Carty et al., 2006). Subsequent studies further suggested broader inhibition against MyD88 dependent immune signaling (Carty et al., 2006 and Peng et al., 2010). In macrophages lacking SARM1, the production of the cytokine CCL5 was also shown to be blocked due to failure to recruit transcription factors and RNA Polymerase to the CCL5 promoter (Gürtler et al., 2014). In T-cells, SARM1 was shown to promote cell death after T-cell activation, a process that is deemed necessary to ensure immune
homeostasis and prevent lymphoproliferative and autoimmune diseases (Panneerselvam et al., 2013).

Similar to the worm, a role for vertebrate SARM1 was shown to extend beyond the cellular immune responses into the nervous system. In mice, SARM1 expression is highest in the brain compared to other peripheral tissues including thymus, liver, spleen, and lymph node (Szretter et al., 2009). Early studies showed that in mice infected with West Nile virus (WNV), SARM1 was required for the production of tumor necrosis factor (TNF), the activation of microglia in the brainstem, and the restriction of WNV pathogenesis (Szretter et al., 2009). In this model, loss of SARM1 increased neuronal death, which is quite different from the models of La Crosse virus infection or injury induced axon death, where neurons lacking SARM1 showed decreased death (Mukherjee et al., 2013; Gerdts et al., 2016). These differences may be attributed to region-specific roles of SARM1 in the nervous system, or distinct modes of activation and regulation.

Additional roles of SARM1 in cultured mouse neurons have also revealed its requirement for the proper initiation and elongation of dendrites, axonal outgrowth, and neuronal polarity (Chen et al., 2011). These studies of SARM1’s role in the mouse neurons foreshadowed the key discovery that ultimately placed SARM1 as a crucial molecule in neuronal biology. This was the discovery of SARM1 as the central executioner of Wallerian axon degeneration (Osterloh et al., 2012; Gerdts et al., 2016), which I discuss in the subsequent sections. More recently, SARM1’s role in mouse neuronal and immune pathways include SARM1’s regulation of a neuronal intrinsic immune response after traumatic axonal injury (Wang et al., 2018), the regulation of microtubule dynamics in motorneurons (McLaughlin et al., 2016), and its role in glia to promote phagocytosis of dying neurons during development (McLaughlin et al., 2019). Altogether, these
studies indicate that SARM1 influences select neuronal and immune pathways in both vertebrates and invertebrates.

1.3 Wallerian Axon Degeneration and the Wld\textsuperscript{s} mice

More recently, studies on SARM1 have mainly focused on its role in axon degeneration. Axons are long projections off neuronal cell bodies that transmit actions potentials and enable connections to other neurons and targets. Axons can travel long distances, up to a meter in humans; and such distances pose a challenge for the maintenance of metabolic homeostasis. Axonal integrity is also compromised during certain toxic and metabolic injuries, and is observed in a wide number of neurodegenerative diseases (Conforti et al., 2014; Gerdts et al., 2016). Degeneration of axons after injury was actually observed as early as the 1850s by neurophysiologist Augustus Waller who described the fragmentation of nerves in a frog after transection (Waller, 1850). This process came to be known as Wallerian degeneration, referring to the fragmentation of axons distal to the site of injury (Conforti et al., 2014; Gerdts et al., 2016). While this fragmentation was initially thought to be a passive wasting process of axons, studies over the last decade have established that an active, self-destruction program is activated in the axon after injury (Conforti et al., 2014; Gerdts et al., 2016).

An early piece of evidence that challenged the notion of passive degeneration was the discovery of the Wallerian degeneration slow (Wld\textsuperscript{s}) mice (Lunn et al., 1989). The Wld\textsuperscript{s} mice was a spontaneous mutation discovered in the Harlan Olac Laboratories in the Bicester, United Kingdom, where severed sciatic nerves from these mice were noted to have delayed axon
degeneration and myelin clearance (Lunn et al., 1989). The preserved distal axonal segment from these mice was still able to conduct action potentials for up to 2 weeks after the transection (Lunn et al., 1989). These early findings, and later work on Wld³, suggested raised the possibility that axon degeneration could be driven by active process. It turns out the spontaneous mutation in the Wld³ mice was a gain-of-function mutation that encoded a fusion protein between the ubiquitination factor, UBE4B and the NAD⁺ biosynthetic enzyme, Nicotinamide Mononucleotide Adenylyltransferase 1 (NMNAT1) (Coleman et al., 1989; Mack et al., 2001). Several studies now show that it is enzymatic activity of NMNAT1 underlies the axonal protection seen in the Wlds mice (Araki et al., 2004; Gerdts et al., 2016). As I will expound on later, elegant metabolite flux experiments suggest that NMNAT1 exerts its protective effect by inhibiting a SARM1 mediated process of NAD⁺ depletion (Sasaki et al., 2016).

The Wld³ mouse and protein have been a useful tool in the study of mechanism underlying axonal degeneration. Moreover, in several disease models, such as glaucoma, progressive motor neuropathy, toxic neuropathy, Parkinson’s disease, Wld³ mice protect axons, and/or have better functional outcomes compared to wild type mice (Conforti et al., 2014). Wld³ however, being a gain of function mutation was not sufficient to reveal the true biological molecules driving axon degeneration. Loss of function mutation in genes that are required for axon degeneration will solidify the model of an active fragmentation program. Recent discoveries have indeed provided significant insights into the genes involved in the axon self-destruction program, and have further suggested new therapeutic targets.
1.4 SARM1: The Central Executioner of the Axon Self-Destruction Program

The first gene identified to be required for injury induced axon degeneration was the Dual Leucine zipper kinase (DLK), a MAP Kinase Kinase Kinase (MAPKKK) (Miller et al., 2009). Loss of DLK blocked axonal degeneration after transection in both flies and mice, however its duration of protection was short lived (days) compared to that of Wld⁺ (weeks). It was not until two genetic screens, one in flies and one in cultured mouse neurons, identified SARM1 as the molecule that rivaled the protection seen in Wld⁺. In a forward genetics screen in Drosophila, loss of function mutations in drosophila SARM1 (dSarm) were identified in olfactory receptor axons that remained intact after transection (Osterloh et al., 2012). Strikingly, the duration of preservation of some of these olfactory receptor axons extended to 50 days, approaching the lifespan of the fly. In another shRNA genetics screen using cultured mouse dorsal root ganglia, SARM1 was also confirmed to be required for injury-induced axon degeneration (Gerdts et al., 2013). Moreover, in vivo in SARM1 KO mice, both sciatic nerve and optic nerve axons are protected from injury-induced degeneration, with some axons being preserved for up to 2 weeks (Osterloh et al., 2012; Gerdts et al., 2013; Yang et al., 2015; Fernandes et al., 2018). The finding that SARM1 KO mice also show improved functional outcomes compared to wild type mice in disease models of chemotherapy induced peripheral neuropathy, high fat diet-induced peripheral neuropathy, and traumatic brain injury (Geisler et al., 2016; Henninger et al., 2016; Ziogas and Koliatsos, 2016), suggest that SARM1 could represent a therapeutic target for several axonopathies. Additionally, the genetic screens identifying SARM1 as an essential mediator of axon degeneration clearly indicate that an intrinsic axonal self-destruction program is activated after injury.
Recent studies have revealed detailed mechanistic insights into this axon self-destruction program. It turns out the Wld\(^s\) mice, albeit a gain-of-function mutation was not a red herring, and gave us clues into the pathway of axon self-destruction. While the NMNAT1 isoform is not typically localized in the axonal compartment, but rather in the nucleus, the NMNAT2 isoform is localized and distributed in axons. Axonal injury triggers a decline in NMNAT2 due to its short half-life, and the disruption of its anterograde transport (Gilley and Coleman, 2010). Further, very recent work suggests that both MAP kinases and the E3 ubiquitin Ligase system promote the degradation of NMNAT2, and combined inhibition of these two pathways is synergistic, and leads to enhanced accumulation of NMNAT and axonal protection after injury (Summers et al., 2018). Genetic deletion of NMNAT2 is also sufficient to induce spontaneous axon degeneration, causes axonal outgrowth deficits, and NMNAT2 knockout (KO) mice die during the perinatal period (Gilley and Coleman, 2010; Gilley et al., 2015). Interestingly, the loss of SARM1 leads to rescue of the axonal outgrowth deficits, and survival of the NMNAT2/SARM1 double KO mice. Together with other results of axonal protection via overexpression of NMNAT enzymes (Gerdts et al., 2016), these results suggest that NMNAT2 inhibits SARM1 in the axon self-destruction program.

As noted above, inhibition of MAP Kinases have been shown to exert some protective effect on injury-induced axon degeneration, are critical to this pathway as they control the abundance of axon protective factors NMNAT2 and SCG10 (Walker et al., 2017; Summers et al., 2018). Hence, an emerging model of the axon self-destructive program upstream of SARM1 suggests an early activation of MAP Kinases after injury, which leads to the degradation of NMNAT2 and SCG10, and subsequent activation of SARM1. What about the molecular components downstream of SARM1? Again, the Wld\(^s\) mice harbored the clues to the hidden...
secret that would not only elucidate the mechanism of SARM1 in axon degeneration, but would redefine the biology of the Toll/Interleukin-1 Receptor (TIR) domain.

1.5 SARM1 Triggers Axon Degeneration Locally via NAD$^+$ Depletion

A year after initial experiments demonstrated the enzymatic activity of NMNAT1 was responsible for the axonal protection of Wlds mice (Araki et al., 2004), Wang et al., showed that the essential metabolite NAD$^+$ began to decline in wild type axons after transection. This decline occurs before morphological changes of degeneration are visible, and the expression of the Wld$^+$ chimeric protein (cytNMNAT1) blocks this decline (Wang et al., 2005). Interestingly, genetic deletion of SARM1 in neurons also blocks this injury-induced early NAD$^+$ depletion (Gerdts et al., 2015).

To uncover the mechanism underlying SARM1 dependent NAD$^+$ depletion and axon degeneration, Gerdts and others, first performed several essential structure/function experiments in neuronal cultures to identify domains essential for axon degeneration. SARM1 possesses an Armadillo/HEAT motifs in its N-terminal domain, a tandem sterile alpha motif (SAM) domain, and C-terminal Toll/Interleukin-1 (TIR) domain. Deletion of the SARM1 N-terminus (the first 408 amino acids) generates a SAM-SAM-TIR construct that is constitutively active, and kills neurons (Gerdts et al., 2013). The first 408 exert an autonhibitory effect on SARM1 since expression of this full-length SARM1 does not spontaneously kill neurons or cells. (Gerdts et al., 2013; Panneerselvam et al., 2013). To control the activation of the constitutive SAM-SAM-TIR
construct, Gerdts et al., replaced the SAM domains, which are known multimerization domain, with an inducible dimerization/oligomerization domain (Fkbp-Frb or FkbpF36V). Moreover, Toll-like Receptors, which possess TIR domain at their cytosolic surface, dimerize in response to detection of pathogen products, bring TIR domains in close proximity to initiate downstream signaling (Akira et al., 2006; O’Neill et al., 2013). While Expression of Fkbp-TIR in axons does not cause axon degeneration, upon addition of the dimerization analog AP20187 to cells, dimerization is activated and axon undergo fragmentation (Gerdts et al., 2015). Moreover SARM1 could be activated using this system in compartmentalized chambers housing axons only, without cell bodies, further supporting the idea that axons themselves can promote their self-destruction in pathological states. This tool gave investigators temporal control of the axon degeneration process, which allowed probing of mechanisms downstream of TIR dimerization.

To investigate the underlying mechanism of dimerized TIR dependent axon degeneration, Gerdts et al., once again turned to the essential metabolite NAD⁺. As described above, NAD⁺ homeostasis had been implicated through multiple lines of investigation in the axon degeneration pathways. NAD⁺ levels decline after axon injury, and the axon protective factor in the Wldˢ mice was the NAD⁺ synthesizing enzyme NMNAT1. Gerdts et al., found that NAD⁺ levels rapidly declined to 10% of starting levels within 15 minutes of dimerizing SARM1’s TIR domain prior to detecting morphological changes of axon fragmentation. It turns out the molecular machinery responsible for this NAD⁺ decline was also present in mammalian HEK293T cells, as dimerization of SARM1-TIR in 293T also rapidly dropped NAD⁺ before cell death. This decline in NAD⁺ levels could be secondary to a decrease in NAD⁺ synthesis or an increase in NAD⁺ destruction. To distinguish between these two possibilities, Gerdts et al., found that electroporated exogenous NAD⁺ still rapidly declined upon activation of TIR dimerization with
rapalog. Altogether, these finds suggested that the SARM1 TIR domain activated a cascade that rapidly consumed NAD$^+$ prior to axon fragmentation. Further, the ability to rescue axon degeneration via supplementation with NAD$^+$ precursor further supported the notion that NAD$^+$ depletion was an important event in the execution of the axon degeneration pathway.

1.6 NAD$^+$ Metabolism and Signaling

Since its discovery over a century ago as an essential component of fermentation (Harden and Young, 1906), Nicotinamide Adenine Dinucleotide (NAD) has been well known for its role as a cofactor for enzymes involved reduction-oxidation (redox) biology (Canto et al., 2015). NAD$^+$ (oxidized form) is reduced to NADH during redox reactions, and NADH is re-oxidized into NAD$^+$ during oxidative phosphorylation to generate ATP (Canto et al., 2015). The main pathways known to generate NAD$^+$ include the de-novo synthesis pathway, the NAD$^+$ salvage pathway, and NAD$^+$ synthesis from precursors such as Nicotinic Acid (Preiss-Handler Pathway) or Nicotinamide Riboside (Canto et al., 2015). De novo synthesis pathway enables the synthesis of NAD$^+$ from amino acid precursors such as Tryptophan or Aspartate, while the NAD$^+$ salvage pathway reclaims Nicotinamide via the rate-limiting enzyme Nicotinamide Phosphoribosyltransferase (NAMPT). In order to generate NAD$^+$, all synthesis pathways first converge on the synthesis on Nicotinamide Mononucleotide (NMN) or Nicotinic Acid Mononucleotide (NaMN), which is then converted by NMNAT enzymes into NAD$^+$ or Nicotinic Acid Dinucleotide (NaAD). NaAD can then be further converted to NAD$^+$ through the action of NAD$^+$ synthetases (Canto et al., 2015).
As noted above, the NMNAT family of enzymes was key to understanding the molecular underpinning of the axon self-destruction program. However, specific details as to its mechanism of inhibition of the degeneration process remains to be elucidated. Through metabolomic flux analyses, we know NMNAT protects axons by inhibiting the injury-induced SARM1 mediated NAD$^+$ depletion instead of increasing NAD$^+$ synthesis (Sasaki et al., 2016). This finding is consistent with previous observations that axons overexpressing NMNAT or Wld$^s$ gene that were protected from degeneration after severing, did not possess dramatically increased levels of steady state NAD$^+$ when compared to wild type axons (Mack et al., 2001; Sasaki et al., 2009). However, boosting NAD$^+$ levels in injured wild type axons with NAD$^+$ precursors such as Nicotinamide Riboside in the presence of Nicotinamide Riboside Kinase 1 (NRK1), can delay axon degeneration (Sasaki et al., 2016). These findings suggest that distinct mechanisms of axonal protection may be at play here with these genetic and chemical manipulations; however both approaches could be leveraged for therapeutics to combat diseases characterized by axon degeneration.

Beyond the role of NAD$^+$ as a cofactor in redox reactions and energy production, an emerging role of NAD$^+$ is its ability to function as signaling molecule in diverse cellular pathways. Importantly, NAD$^+$ is a substrate for enzymes such as the Poly ADP-Ribose Polymerase (PARP), Sirtuins, and CD38 ADP-Ribosyl cyclase family of enzymes (Canto et al., 2015). PARP enzymes are known primarily for their role in DNA repair where they cleave NAD$^+$, and transfer its ADP-Ribose moiety to other proteins in a process termed ADP-Ribosylation (Canto et al., 2015). Sirtuins like PARPs also cleave NAD$^+$ and couple this cleavage reaction to protein deacetylation, and in certain cases ADP-Ribosylation (Canto et al., 2015). This action of Sirtuin enzymes influences a myriad of cellular and physiological processes
including lifespan, glucose homeostasis, and mitochondria metabolism (Canto et al., 2015). CD38 also cleaves NAD$^+$ into Nicotinamide, ADP-Ribose (ADPR), and cyclic ADPR (cADPR), with cADPR and ADPR known to influence calcium mobilization and signaling (Fliegert et al., 2007). The beneficial impact of NAD$^+$ and its consuming enzymes is more than that described above, with beneficial physiological effects at several tissue and organ levels. Recent reviews on this topic can be found in the following references (Canto et al., 2015; Rajman et al., 2018; Verdin et al., 2015; Yoshino et al., 2018).

With the discovery of NAD$^+$ depletion in axons after SARM1 activation, a search began for the NAD$^+$ consuming enzyme promoting the destruction of this essential metabolites. As noted above, there were several known candidate enzymes in mammals that could be responsible for this process including the Poly ADP Ribose Polymerases, Sirtuins, CD38/CD157, and NUDIX hydrolase. Using exogenous radiolabelled NAD$^+$ electroporated into cells, Gerdts and others revealed that Nicotinamide was produced upon TIR dimerization (Gerdts et al., 2015). The PARP, Sirtuins, and CD38/157 families cleave NAD$^+$ at the nicotinamide-ribosyl bond to generate nicotinamide. Using genetic and chemical approaches, it was shown that PARP1 and CD38 did not mediate the NAD$^+$ depletion seen with SARM1 TIR dimerization (Gerdts et al., 2015). Further, Sasaki and colleagues had also previous shown that axons from CD38 knockout mice, or PARP1/CD38 double knockout still degenerated after transection (Sasaki et al., 2009). The identification of the unknown NADase enzyme responding to SARM1 activation was important, as it could provide a potentially druggable target for diseases characterized by axon degeneration.

It is at this point my dissertation work begins, embarking on a search for the NAD$^+$ consuming enzyme mediating SARM1 dependent NAD$^+$ depletion. In the subsequent chapters, I
describe the surprising fundamental breakthrough that the SARM1 TIR domain itself possesses intrinsic NAD$^+$ cleavage activity, and that this enzymatic activity is required for axon degeneration. This finding identified SARM1 as the founding member of a potentially new family of enzymes of TIR domains. To determine if indeed such a new family exists, we tested multiple TIR domains from different domains of life, including from bacteria, archaea, and plants. We found that indeed multiple TIR domain proteins in these families are also enzymes that cleave NAD$^+$ and NADP$^+$. Many studies over the past two decades have defined the TIR domain as a scaffold promoting assembly of signaling complexes via protein-protein interactions. My dissertation work dramatically extends our understanding of this protein domain, demonstrating that the TIR domain represents an ancient enzyme motif capable of cleaving NAD$^+$, the central constituent of organismal bioenergetics. These studies have identified a therapeutic target for a wide number of neurological diseases, and have opened the door for new lines of investigation in neurodegeneration, innate immunity, the microbiome, cellular signaling, and other disciplines.
2. SARM1: The Founding Member of the TIR domain enzyme family

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2.1 Introduction

Axonal degeneration is a hallmark of several neurological disorders including peripheral neuropathy, traumatic brain injury, and neurodegenerative diseases (Gerdts et al., 2016). In Parkinson’s disease and Amyotrophic Lateral Sclerosis, for example, axonal degeneration is an early event, preceding symptom onset and widespread neuronal loss (Kurowska et al., 2017; Fischer and Glass, 2007). Although these neurological conditions have unique underlying etiologies, blocking axonal degeneration in the early stages of these disorders may slow or perhaps halt their progression by preventing the loss of functional synapses, and maintaining neuronal connectivity. In order to develop effective therapies targeting pathological axonal degeneration, the underlying molecular mechanisms need to be defined and pharmacologically-inhibitable targets identified.

Damaged or unhealthy axons are eliminated via an intrinsic self-destruction program that is distinct from traditional cellular death pathways like apoptosis (Gerdts et al., 2016). This programmed subcellular destructive pathway is triggered in Wallerian degeneration, which was initially thought to be a passive ‘wasting’ process of the severed distal axon segment. However, the discovery of the naturally occurring Wallerian degeneration slow (Wld<sup>s</sup>) mutant mice (Lunn et al., 1989; Mack et al., 2001), whose distal axons degenerate in a delayed fashion after injury, challenged this notion of passive degeneration. Early studies of Wld<sup>s</sup> showed that the Nicotinamide Adenine Dinucleotide (NAD<sup>+</sup>) synthesis enzyme, Nicotinamide Mononucleotide Adenylyltransferase 1 (NMNAT1) (Figure 1A), was the functional moiety of the Wld<sup>s</sup> protein responsible for axonal protection (Araki et al., 2004; Mack et al., 2001). Later, its paralog NMNAT2, was identified as a short-lived axonal enzyme that is required for axon maintenance (Gilley and Coleman, 2010). While the gain-of-function Wld<sup>s</sup> mutation suggested that damaged
axons activate a self-destructive program, the existence of a distinct pathway was secured by the discovery of genes that are required for axonal degeneration after injury (Gerdt et al., 2013; Miller et al., 2009; Osterloh et al., 2012; Walker et al., 2017; Xiong et al., 2012; Yang et al., 2015). Among these pro-degenerative genes, SARM1 (Sterile Alpha and Toll/Interleukin-1 Receptor motif-containing 1) is the central executioner of the degenerative program.

Loss of SARM1 blocks axonal degeneration for weeks after injury (Gerdt et al., 2013; Osterloh et al., 2012) and improves functional outcomes in mice after both traumatic brain injury (Henninger et al., 2016) and vincristine-induced peripheral neuropathy (Geisler et al., 2016). Axonal injury induces NAD\(^+\) loss (Wang et al., 2005), and SARM1 is required for this injury-induced NAD\(^+\) depletion both in vitro and in vivo (Gerdt et al., 2015; Sasaki et al., 2016). Moreover, activation of SARM1 signaling, via enforced dimerization of its TIR domain, is sufficient to induce axonal degeneration in the absence of injury due to a catastrophic depletion of axonal NAD\(^+\) (Gerdt et al., 2015).

NAD\(^+\) is a dinucleotide that is essential for many redox reactions, but it is also consumed by a variety of enzymes (e.g. PARPs, CD38, Sirtuins) where the resulting metabolites influence signaling pathways via their effects on calcium mobilization or protein parylation (Cantó et al., 2015; Fliegert et al., 2007). The identity of the NADase enzyme(s) responding to SARM1 activation and mediating NAD\(^+\) loss in injured axons has been unknown, although PARP1 and CD38 were previously eliminated as candidates (Gerdt et al., 2015; Sasaki et al., 2009). Furthermore, SARM1 is not known to have enzymatic activity, nor have TIR domains from any protein ever been associated with enzymatic activity. TIR domains are rather known for their scaffolding properties in Toll-like Receptor signaling, where they activate downstream enzymes to regulate pro-inflammatory and defense genes (O’Neill et al., 2013).
To further our understanding of this axon death pathway, we sought to identify the responsible NADase enzyme(s). Through a series of experiments, we now show that the TIR domain of SARM1 acts as an enzyme to cleave NAD⁺, and that SARM1 enzymatic activity is necessary to promote axonal NAD⁺ depletion and axon degeneration after both traumatic and vincristine induced axonal injuries. Our findings therefore identify SARM1 enzymatic activity as novel therapeutic target against diseases characterized by axonal degeneration including peripheral neuropathy, traumatic brain injury, and neurodegenerative diseases. More broadly, these findings show that TIR domains can possess intrinsic enzymatic activity.

2.2 Results

2.1.1 SARM1-TIR complex purified from mammalian cells cleaves NAD⁺

SARM1 contains multiple conserved motifs including SAM domains, ARM/HEAT motifs and a Toll/Interleukin-1 Receptor (TIR) domain (Figure 1B) that mediate oligomerization and protein-protein interactions (O’Neill et al., 2013; Tewari et al., 2010; Qiao et al., 2005). Dimerization of SARM1-TIR domains in neurons is sufficient to induce axonal degeneration and to trigger rapid degradation of NAD⁺, demonstrating that the NADase activity is either associated with or induced by dimerized SARM1-TIR domains. TIR domains are common in signaling proteins functioning in innate immunity pathways where they serve as scaffolds for protein complexes (O’Neill et al., 2013). Therefore, we hypothesized that NAD⁺ depletion after SARM1 activation or enforced SARM1-TIR domain dimerization was mediated via an interaction with and activation of an NAD⁺ consuming enzyme like a member of the Sirtuin or Poly ADP Ribose Polymerase (PARP) families. To address this hypothesis, we reasoned that
purification of the native SARM1-TIR complex in a manner that retained NADase activity would be a major step forward in identifying the responsible NAD$^+$ consuming enzyme.

To perform these biochemical experiments, the human SARM1-TIR domain was engineered to include a tandem StrepTag II at the N-terminus and a Venus fluorescent tag at the C-terminus. This protein was expressed transiently in NRK1-HEK293T cells supplemented with Nicotinamide Riboside (NR) to augment cellular NAD$^+$ levels and promote cell viability (Figure 2A). To maintain intact complexes, cell lysates were subsequently prepared by lysing cells under native conditions by sonication, and the recombinant SARM1-TIR protein complexes were affinity purified using MagStrep magnetic beads. We reasoned that extensive purification could disrupt interactions important for detecting NAD$^+$ depletion, so we opted to test directly the magnetic beads loaded with complexes for activity. We incubated beads loaded with SARM1-TIR complexes with NAD$^+$ (5 μM) for up to 30 minutes, extracted the metabolites, and measured NAD$^+$ levels using HPLC (Figure 1C). We found that NAD$^+$ levels dropped precipitously, within 5 minutes, when beads loaded with SARM1-TIR complexes were tested (Figure 1D). In contrast, no decrease in NAD$^+$ was observed if beads exposed to lysates were prepared from either non-transfected NRK1-HEK293T cells or from NRK1-HEK293T cells expressing SARM1-TIR lacking the StrepTag II (Figure 1D). We also tested a non-functional TIR domain mutant [SARM1(E596K)] that we recently identified in a structure/function analysis of SARM1 (Summers et al., 2016). Magnetic beads loaded with complexes assembled on this SARM1-TIR(E596K) mutant failed to degrade NAD$^+$ in this in vitro assay (Figure 1D).
Figure 1: Native SARM1-TIR protein complex cleaves NAD⁺ in an in vitro assay. A) Selected pathways of NAD⁺ synthesis and degradation. Nam–Nicotinamide; NMN–Nicotinamide Mononucleotide; NAD⁺–Nicotinic Acid Adenine Dinucleotide; NAMPT–Nicotinamide Phosphoribosyltransferase; NRK–Nicotinamide Riboside Kinase; NMNAT–Nicotinamide Mononucleotide Adenylyltransferase; NADS–NAD⁺ synthetase; ART–ADP Ribosyltransferase; PARP–Poly ADP-Ribose Polymerase. B) SARM1 domains. MLS–Mitochondrial Localization Signal; ARM–Armadillo/HEAT Motifs; SAM–Sterile Alpha Motif; TIR–Toll/Interleukin 1 Receptor. C) Schematic illustrating the in vitro NADase assay. D) NAD⁺ cleavage reaction timecourse of human SARM1-TIR (wild type and mutant) laden beads in NADase assay (normalized to control at 0 min). E) NaAD reaction timecourse of human SARM1-TIR laden beads in NADase assay (normalized to control at 0 min). F) SYPRO Ruby gel of SARM1-TIR laden beads used in assay. Data for each time point was generated from three independent experiments using purified protein from three independent transfection experiments. Data are presented as mean ± SEM; Error bars: SEM; ***P<0.001 one-way ANOVA.
Figure 2: Purification of TIR domain complexes from NRK1-HEK293T cells. A) NRK1-HEK293T stable line with NR supplementation maintains higher NAD\(^+\) levels than HEK293T with NR, upon SARM1-TIR expression. Data was generated from three independent NAD\(^+\) measurements from three independent transfection experiments, and normalized to data from a non-transfected experiment run concurrently. B) NAD\(^+\) reaction timecourse of human SARM1-TIR G601P, TLR4-TIR, and MyD88-TIR laden beads in in-vitro NAD\(\)ase assay (normalized to control at 0 min). C) Representative SYPRO Ruby gel of SARM1-TIR G601P, TLR4-TIR, and MyD88-TIR laden beads used in assay. Data for each time point was generated from three independent reaction experiments using purified protein from three independent transfection experiments. Data are presented as mean ± SEM; Error bars: SEM; *** P < 0.001 two tailed Student’s t-test.
Another previously identified non-functional mutant SARM1(G601P), also failed to degrade NAD$^+$ in the in vitro assay (Figure 2B and 2C). Finally, we examined the substrate specificity of the SARM1-TIR in vitro NADase reaction. We previously showed that Nicotinic Acid Adenine Dinucleotide (NaAD), a closely related analog of NAD$^+$, was not cleaved after SARM1 activation (Gerdts et al., 2015). Using this in vitro assay, we found that wild type SARM1-TIR complexes do not degrade NaAD (Figure 1E). Together, these results show that the purified SARM1-TIR complex actively degrades NAD$^+$ in a manner consistent with previous characterization of the axonal degeneration process.

We next asked if this enzymatic activity was unique to complexes associated with the SARM1-TIR domain or whether TIR domains from other proteins could also assemble complexes that exhibit NADase activity. We expressed and purified the TIR domains of TLR4, a Toll-like receptor, and MyD88, another member of the TIR adaptor family, from NRK1-HEK293T cells and tested them in the in vitro NAD$^+$ depletion assay. Both TLR4 and MyD88 TIR containing complexes showed no NADase activity (Figure 2B and 2C). These results support the previously reported unique roles of SARM1 among TIR adaptor proteins (Gerdts et al., 2015; O’Neill et al., 2013, Summers et al., 2016) in promoting axonal degeneration and neuronal NAD$^+$ depletion.

2.1.2 Multiple biochemical experiments fail to identify a bona fide NAD$^+$ consuming enzyme in the purified SARM1-TIR complex

To identify SARM1-TIR associated proteins, we first used gel electrophoresis followed by protein staining with the highly sensitive fluorescent stain SYPRO Ruby. The beads loaded with wild type SARM1-TIR complexes isolated from NRK1-HEK293T cells revealed a
prominent band corresponding to SARM1-TIR, and contained few co-purifying proteins (Figure 1F). Furthermore, all bands detected by SYPRO Ruby in wild type SARM1-TIR complex were present in similar abundance in complexes containing inactive SARM1-TIR mutant (E596K), a seemingly unlikely result if one of the bands represented an associated NAD\(^+\) consuming enzyme (Figure 1F). However, to rigorously explore this possibility, we pursued a proteomic strategy using mass spectrometry (LC-MS/MS) to identify potential NAD\(^+\) consuming proteins present in the purified SARM1-TIR complexes. To perform these studies, we further purified wild type and mutant SARM1-TIR complexes by tandem affinity purification (TAP). Wild type SARM1-TIR complexes still robustly degraded NAD\(^+\) and had very few co-purifying proteins detectable by SYPRO Ruby (Figure 3A and 3B). To identify associated proteins, we performed LC-MS/MS analysis on both wild type and mutant TAP complexes. Interestingly, we found no bona fide NAD\(^+\) consuming enzymes (e.g., PARPs, Sirtuins, NUDIX hydrolases, ADP Ribosyl cyclases) in either complex (Essuman et al., 2017). Furthermore, no proteins were significantly enriched in wild type vs. mutant SARM1-TIR complexes, as would be expected if such a protein were responsible for the NAD\(_\text{ase}\) activity (Essuman et al., 2017). In summary, these analyses did not identify a SARM1-TIR associated protein that is a likely source of the NAD\(_\text{ase}\) activity.
Figure 3: SARM1-TIR maintains NAD⁺ cleavage activity after multiple purification schemes from mammalian cells and bacteria. A) Timecourse of NAD⁺ cleavage reaction using tandem affinity purified (TAP) human SARM1-TIR (wild type and mutant) complexes expressed in mammalian cells (normalized to control at 0 min). B) SYPRO Ruby gel of TAP complexes used in assay; representative of three independent experiments. C) Timecourse of NAD⁺ cleavage reaction using bacterially synthesized human SARM1-TIR, purified by TAP, and subjected to 1M and 2M NaCl washes during purification (normalized to control at 0 min). D) Timecourse of NAD⁺ cleavage reaction using bacterially synthesized human SARM1-TIR, purified by TAP, and subjected to either 0.5% Triton X-100 or 0.5% Tween-20 washes during purification (normalized to control at 0 min) Data was generated from two independent reaction experiments and is represented as mean ± SEM. E) Reaction timecourse of purified components of the cell-free protein transcription/translation system incubated with NAD⁺ and non-recombinant plasmid. Data for each time point in A, C, E was generated from three independent reaction experiments. Data are presented as mean ± SEM; Error bars: SEM. *** P < 0.001 one-way ANOVA.
2.1.3 SARM1-TIR domain possesses intrinsic NAD\(^+\) cleavage activity

The failure to identify proteins specific to the wild type vs. mutant SARM1-TIR complexes as well as any bona fide NAD\(^+\) consuming enzymes, suggested that the SARM1-TIR domain itself might possess NADase enzymatic activity. To explore this possibility, we expressed human SARM1-TIR in *E. coli* so that proteins with NADase activity would not be co-purified. We first asked whether the bacteria expressing human SARM1-TIR protein had decreased levels of NAD\(^+\), as this would indicate that it was also cleaving endogenous bacterial NAD\(^+\) pools. SARM1-TIR expression in *E. coli* was induced by IPTG addition, endogenous metabolites were extracted, and NAD\(^+\) levels were assessed by HPLC. We found that bacteria producing wild type SARM1-TIR had remarkably low (almost undetectable) levels of endogenous NAD\(^+\) within 60 minutes after IPTG addition when compared to bacteria harboring non-recombinant vector. Further, bacteria harboring mutant SARM1-TIR (E596K) had NAD\(^+\) levels comparable to those harboring non-recombinant vector or to bacteria in which wild type SARM1 was not induced (Figure 4A). Next, we purified the bacterially expressed SARM1-TIR using TAP and tested for NADase activity. Consistent with our results using SARM1-TIR complexes isolated from mammalian cells, NAD\(^+\) was rapidly consumed by bacterially produced SARM1-TIR protein (Figure 4B). Although it is highly unlikely that human SARM1-TIR would associate with an *E. coli* NADase, we wished to test the intrinsic nature of the SARM1 NADase activity by stringently washing the SARM1 TIR purified complexes with either high salt or detergents to remove potential associated proteins. Using these washed SARM1 TIR beads, we found no decrease in NAD\(^+\) cleavage activity, indicating that SARM1 itself is the NADase (Figure 3C and 3D).
A
- Non-recombinant
- hSARM1-TIR
- hSARM1-TIR E596K

B
- Non-recombinant
- nSARM1-TIR

C
- Mouse
- Zebrafish
- Drosophila

D
- PURExpress system containing purified E-coli Transcription/Translation reagents
- RNase Inhibitor
- Cobalt beads
- pET recombinant plasmid (StrepTag-SARM1-TIR-His)
- newly synthesized StrepTag-SARM1-TIR-His protein

E
- 0 mins
- 5 mins
- 10 mins

F
- Non-recombinant
- hSARM1-TIR

G
- Non-recombinant
- humanSARM1-TIR
- mouseSARM1-TIR
- zebrafish-SARM1-TIR
- drosophila SARM1-TIR
Because the role of SARM1 in axonal degeneration is evolutionarily conserved (Gerdts et al., 2015; Osterloh et al., 2012), we reasoned that SARM1 NADase activity should also be conserved. We expressed and purified mouse, zebrafish, and Drosophila SARM1-TIR domains in E. coli and tested these purified proteins for their ability to cleave NAD⁺. Similar to the human SARM1-TIR domain, bacterially expressed mouse, zebrafish, and Drosophila SARM1-TIR domains also degrade NAD⁺ in vitro (Figure 4C and 4G).

To demonstrate definitively that SARM1-TIR itself possessed the enzymatic activity, we synthesized the human SARM1-TIR in a cell-free protein expression system that utilizes purified E-coli components for transcription and translation. None of the purified E-coli transcription/translation components are known NADases (Shimizu et al., 2001), and we experimentally confirmed that these purified components do not exhibit NADase activity (Figure 3E). To test if SARM1-TIR purified from this in vitro translation system could cleave NAD⁺, we first incubated human SARM1-TIR plasmid DNA with the purified transcription and translation reagents and RNase inhibitor for 2.5 hours at 37°C. Next, we purified the newly synthesized protein from the reaction by TAP, and tested for NADase activity in our assay (Figure 4D). The purified SARM1-TIR from this cell-free protein translation system rapidly cleaved NAD⁺,
consistent with our prior findings with SARM1-TIR purified from both mammalian cells and bacteria (Figure 4E and 4F).

In summary, we find that the SARM1-TIR domain depletes endogenous NAD$^+$ in bacteria, that bacterially synthesized SARM1-TIR from multiple species cleaves NAD$^+$ in vitro, and that SARM1-TIR synthesized and purified from a cell-free protein translation system cleaves NAD$^+$ in vitro. These results demonstrate that the SARM1-TIR domain has intrinsic NADase activity, and, hence, SARM1 itself is responsible for the NAD$^+$ depletion observed after axon injury. Moreover, these findings reveal for the first time that a TIR domain, an evolutionary ancient domain previously demonstrated to function as a protein interaction domain, can also harbor enzymatic activity.

2.1.4 Characterization of the SARM1-TIR enzymatic reaction reveals both cyclase and glycohydrolase activities

To further characterize the SARM1-TIR NADase activity, we sought to identify the NAD$^+$ cleavage products of this enzymatic reaction as well as establish reaction parameters. Our previous study using cultured cells and C$^{14}$-labelled NAD$^+$ identified Nicotinamide (Nam) as a reaction product (Gerdts et al., 2015). However, we were unable to detect other products due to the position of the label on the NAD$^+$ molecule. We therefore performed HPLC and LC-MS/MS analysis of the metabolites produced by human SARM1-TIR, and identified Nam and ADP Ribose (ADPR) as major products, and cyclic ADPR (cADPR) as a minor product (Figure 5A-G and Figure 6A-C). Interestingly, while the mouse and zebrafish orthologs generated a similar ratio of reaction products as the human enzyme (Figure 6D), the Drosophila SARM1-TIR purified either from bacteria or NRK1-293T cells generated more cADPR than ADPR (Figure
This finding is similar to results with the ADP-Ribosyl cyclase family of NADases (Liu et al., 2009), in which the mammalian ADP Ribosyl Cyclase CD38 cleaves NAD$^+$ to generate ADPR as the major product, with minor amounts of cADPR; while the ADP Ribosyl Cyclase isolated from the sea mollusk Aplysia californica cleaves NAD$^+$ into cADPR (Liu et al., 2009). This difference in reaction products between the Drosophila and vertebrate SARM1-TIR NADase may provide insights into the divergent enzymatic activities of the ADP Ribosyl cyclase family of enzymes.

Next, we performed kinetic assays of the SARM1-TIR enzyme, which revealed saturation kinetics (Figure 5H), a distinguishing feature of enzyme catalysts, with an estimated Michaelis constant ($K_m$) of 24 µM, maximum velocity ($V_{max}$) of 3.6 µM/min, and turnover number ($k_{cat}$) of 10.3 min$^{-1}$ (Figure 5H). Although the estimated $k_{cat}$ is lower than the reported values for other ADP-Ribosyl cyclases and NAD$^+$ glycohydrolases (Ghosh et al., 2010), the estimated $K_m$ values are similar (Cantó et al., 2015). In order to obtain information that could be helpful in developing SARM1 inhibitors, we tested whether either of the reaction products could inhibit the enzymatic activity of SARM1-TIR. ADPR did not inhibit SARM1-TIR NADase activity (Figure 6E), however Nam inhibits the enzymatic activity with an IC$_{50}$ of 43.8 µM, which is about 9-fold higher than the starting reaction NAD$^+$ concentration (Figure 5I). Interestingly, Nam also inhibits other NAD$^+$ consuming enzymes such as Sirtuins and PARPs (Avalos et al., 2005; Gibson et al., 2012). Inhibitors of the SARM1-TIR domain modeled after nicotinamide could be useful in preventing the early stages of axonal degeneration (Gerdts et al., 2016; Wang et al., 2005). Ara-2’-F-NAD$^+$, a slow binding inhibitor of CD38 (Berthelier et al., 1998), however did not inhibit SARM1-TIR NADase even at high doses (Figure 6F), so there is likely to be selectivity of inhibitors for different families of NADases.
Figure 5: Characterization of the SARM1-TIR NAD⁺ cleavage reaction. (A-E) HPLC chromatograms showing NAD⁺ cleavage products of human and drosophila SARM1-TIR. Retention time: Nam t~2.40 min; cADPR at t~0.85 min; ADPR at t~1.10 min. (F-G) Quantification of metabolites generated by human (F) and drosophila (G) SARM1-TIR as displayed in A-E (normalized to 0 min NAD⁺). H) Kinetic parameters for human SARM1-TIR cleavage reaction. $V_{\text{max}}$, $K_m$, $k_{\text{cat}}$ were determined by fitting the data to the Michaelis-Menten equation and are presented as mean ± SEM for three independent biological samples. I) Nam dose response inhibition of human SARM1-TIR enzymatic activity. Data was generated from three independent reaction experiments using purified protein from three independent bacteria clones. Data are presented as mean ± SEM; Error bars: SEM; *P<0.05; **P<0.01; ***P<0.001 unpaired two tailed Student’s t-test.
Figure 6: LC-MS/MS profiles of SARM1-TIR NAD⁺ cleavage reaction products and effect of candidate NAD⁺ analogs on enzymatic reaction. A-C) LC-MS/MS spectra of Nam, cADPR, and ADPR from SARM1-TIR NAD⁺ cleavage reaction. D) HPLC chromatograms showing mouse and zebrafish SARM1-TIR NAD⁺ cleavage reaction.
generate Nam and ADPR as major products, and cADPR as a minor product. E) ADPR does not inhibit SARM1-TIR NADase activity (normalized to Nam generated at 5 min). F) ara-2'-F-NAD$$^+$$ does not inhibit SARM1-TIR NADase activity at doses as high as 40 fold the starting concentration of reaction NAD$$^+$$ (5$$\mu$$M). Metabolites were measured by HPLC after 5 minutes of reaction time.

Table 1: Nucleotide hydrolase/transferase enzymes identified via HHpred. SARM1-TIR was used as a query sequence in HHpred to identify candidate enzymes.

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<th>PDB</th>
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<th>Probability</th>
<th>E-value</th>
<th>P-value</th>
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<tr>
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<td>98.2</td>
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2.1.5 Glutamic Acid 642 is a putative catalytic residue in the active site of the SARM1-TIR enzyme

Next, we sought to gain some insight into the structural features enabling SARM1-TIR
NAD$^+$ cleavage. Since there is no reported crystal structure of the SARM1-TIR domain, we used an unbiased template-based prediction (Söding et al., 2005) to identify protein homologs of SARM1-TIR. Interestingly, a recent bioinformatics study showed that some TIR domains share strong structural similarity to nucleotide/nucleoside hydrolases (Burroughs et al., 2015). From our domain prediction analysis using SARM1-TIR, we identified other TIR domains as expected. However, in addition to these TIR domains, we also detected a number of nucleotide hydrolase/transferase enzymes (Table S1). For some of these enzymes, residues that contribute to catalytic activity have been established (Sikowitz et al., 2013; Armstrong et al., 1995). We therefore used structural modeling and sequence alignments to identify putative residues in the SARM1-TIR domain that might contribute to enzymatic activity (Figure 4A, 4B, S4A, S4B). The SARM1-TIR domain was modeled using the crystal structure of two enzymes identified from our prediction: MilB Cytidine 5’ monophosphate (CMP) Hydrolase (PDB: 4JEM) (Figure 4B) and Nucleoside 2-deoxyribosyltransferase (PDB: 1F8Y) (Figure S4B). Importantly, we found that a glutamic acid E642 in the SARM1-TIR domain aligned with both the key catalytic glutamic acid residue in CMP hydrolase (Sikowitz et al., 2013) and the proposed nucleophilic glutamic acid in the active site of nucleoside 2-deoxyribosyltransferase (Armstrong et al., 1995) (Figure 4A, 4B and S4C). Moreover, glutamic acid residues are also known catalytic residues in other NADases (Ghosh et al., 2010). To test if SARM1 TIR E642 had similar catalytic properties, we mutated this residue to an Alanine (E642A) in SARM1-TIR, purified the protein from the cell-free protein translation system, and tested it for NAD$^+$ cleavage activity. We found that purified SARM1-TIR E642A failed to cleave NAD$^+$ in the NADase assay (Figure 4C and 4D). Hence, we suggest that E642 in the SARM1-TIR domain is a key catalytic residue within the active site that is responsible for NAD$^+$ cleavage.
Figure 7: SARM1 enzymatic activity functions in axons to promote pathological axonal degeneration. A) Amino acid sequence alignment of SARM1-TIR with MilB Cytidine 5’ Monophosphate (CMP) Hydrolase. CMP catalytic glutamic acid is highlighted in red box and aligns to glutamic acid 642 in the SARM1-TIR domain. B) Modeling of the SARM1-TIR domain on the crystal structure of CMP Hydrolase bound to CMP. E642 aligns with a catalytic residue of CMP Hydrolase. C) NAD⁺ reaction timecourse of human SARM1-TIR E642A purified from cell-free protein translation system (normalized to control at 0 min). D) SYPRO Ruby gel of SARM1-TIR E642A purified from cell-free protein translation system. E) Axonal NAD⁺ levels after axotomy (normalized to control at 0 hr). NC vector, SARM1 WT, and SARM1 E642A constructs were expressed in SARM1-/- DRG neurons, and levels of NAD⁺ were obtained at indicated timepoints after axotomy. F) Axonal degeneration timecourse after axotomy, quantified as degeneration index (DI) where a DI of 0.35 (indicated by dotted line) or above represents degenerated axons. G) Bright-field micrographs of axons expressing indicated constructs represented in F. H) Axonal degeneration timecourse after vincristine treatment, quantified as DI. I) Bright-field micrographs of axons after vincristine treatment corresponding to selected groups in H. Scale bar, 5 µm. Quantification data were generated from at least three independent biological experiments. Data are presented as mean ± SEM; Error bars: SEM.*P<0.05, **P<0.01, ***P<0.001 one-way ANOVA. J) Selected pathways of NAD⁺ synthesis and degradation including SARM1 as a NAD⁺ consuming enzyme.

2.1.6 SARM1 enzymatic activity functions in axons to promote pathological axonal degeneration

Having demonstrated that the SARM1 TIR domain is an enzyme, and now identified its putative catalytic residue, we next investigated whether this enzymatic activity and, in particular, the identified glutamate, were required for the pro-degenerative functions of full-length SARM1 in neurons. In wild type neurons, axotomy triggers rapid depletion of axonal NAD⁺ and axonal degeneration, while in SARM1-deficient neurons axonal degeneration is blocked and NAD⁺ levels remain significantly higher than in injured wild type axons (Gerdts et al., 2015). First, we tested whether SARM1 NADase activity is necessary for injury-induced axonal NAD⁺ depletion and subsequent axonal degeneration. We expressed either wild type (enzymatically active) full-length SARM1 or mutant (enzymatically disabled) SARM1(E642A) in cultured SARM1-deficient DRG neurons and found both were well expressed in axons (Figure 8D and 8E). Following axotomy, we measured axonal NAD⁺ levels and axonal degeneration. Expression of enzymatically active, wild type SARM1 in SARM1-deficient DRG neurons promotes both axonal NAD⁺ depletion and axonal degeneration after axotomy. In contrast to wild type SARM1,
when the enzymatically disabled SARM1(E642A) mutant is expressed in these neurons, axotomy did not induce axonal degeneration or rapid NAD$^+$ depletion (Figure 7E-G). We also tested the requirement for SARM1 enzyme activity in another injury model – vincristine-induced neurotoxicity. Cultured SARM1-deficient DRG axons are protected from vincristine-induced axonal degeneration (Gerdts et al., 2013). Moreover, we recently demonstrated in vivo that SARM1 is required in mice for the development of vincristine-induced peripheral neuropathy (Geisler et al., 2016). As with axotomy, we expressed either wild type (enzymatically active) full-length SARM1 or mutant (enzymatically disabled) SARM1(E642A) in cultured SARM1-deficient DRG neurons. Enzymatically active SARM1 mediates axon loss in response to the chemotherapeutic vincristine, while enzymatically disabled SARM1 does not promote axon loss following vincristine administration (Figure 7H, and 7I). Altogether, these findings demonstrate that the intrinsic NADase activity of SARM1 (Figure 7J) is necessary to promote axonal degeneration after both traumatic and neurotoxic injuries, and suggest that inhibitors of the SARM1 NADase could block pathological axonal degeneration.
Figure 8: Structural modeling, sequence alignments with SARM1-TIR, and expression of SARM1-Venus constructs in SARM1-deficient DRGs. A) Primary amino acid sequence alignment of SARM1-TIR with ndt (nucleoside 2-deoxyribosyltransferase) (PDB:1F8Y). Nucleoside 2-deoxyribosyltransferase catalytic glutamic acid
is highlighted in red box and aligns to glutamic acid 642 in the SARM1-TIR domain. B) Modeling of the SARM1-TIR domain on the crystal structure of Nucleoside 2-deoxyribosyltransferase complexed with 5-Methyl-2'-deoxyriboseuridine. E642 closely aligns with the catalytic residue of Nucleoside 2-deoxyribosyltransferase in the active site. C) Primary amino acid sequence alignment of SARM1-TIR with candidate enzymes identified from HHpred based analysis (see Table S1). 1. SARM1-TIR 2. MilB CMP hydrolase 3. 2'-deoxynucleoside 5'-monophosphate N-hydrolase 1 4. 2'-deoxynucleoside 5'-monophosphate N-glycosidase 5. BcmB CMP glycosidase 6. Nucleoside 2-deoxyribosyltransferase 7. Uncharacterized protein (P. aeruginosa) 8. Purine 2'-deoxyribosyltransferase 9. uncharacterized protein (E. faecalis) 10. nucleoside 2'-deoxyribosyltransferase 11. nucleoside deoxyribosyltransferase. D) Venus expression of indicated constructs in DRG axons, co-stained for Tuj1 to assess total axon area for each field. E) Venus expression of indicated constructs in DRG cell bodies, co-stained with Hoechst to assess total nuclei in each field.

2.3 Discussion
Here we demonstrate that the TIR domain of SARM1 acts as an enzyme to cleave NAD⁺, and that this enzymatic activity is necessary to promote pathological axonal degeneration. These findings have a number of implications. First, our results describe the first enzymatic activity intrinsic to a TIR domain. Many proteins central to innate immune signaling contain TIR domains (O’Neill et al., 2013), and this finding therefore raises the possibility that these other TIR domains could also be enzymatically active. Second, the SARM1-TIR domain is the closest mammalian relative to the ancestral TIR domains in prokaryotes (Zhang et al., 2011), suggesting that this domain, as an enzyme cleaving NAD⁺, may be a component of an ancient cell death pathway. Third, the generation of cADPR and ADPR by SARM1-TIR may contribute to axonal degeneration. cADPR and ADPR are signals for intracellular calcium mobilization (Fliegert et al., 2007), and calcium is a major stimulus for axonal degeneration (Villegas et al., 2014). However, whether this is a primary mechanism for axonal demise or whether energetic failure secondary to NAD⁺/ATP depletion (Gerdts et al., 2015; Yang et al., 2015) and/or NAD⁺/NADH electron donor activities is most critical is unclear. Fourth, detailed structural studies with SARM1-TIR protein crystallized in complex with its substrate NAD⁺ and/or with analogs could
provide useful information about residues within or outside of the active site that are important for enzymatic catalysis. Finally, NADase activity is integral to the conserved axon death program (Gerdts et al., 2015), and so the discovery that SARM1 is the axonal NADase now provides an identified target for the rational design of inhibitors that will serve as novel therapeutic candidates for the treatment of disorders characterized by axonal degeneration including peripheral neuropathies, traumatic brain injuries, and neurodegenerative diseases.

2.4 Materials and Methods

Recombinant DNA


Bacterial expression constructs cloned into pET30α+: StrepTag-hSARM1-TIR-HisTag, StrepTag-mSARM1-TIR-HisTag, StrepTag-zfSARM1TIR-HisTag.

TIR domain residues

hSARM1-TIR (561-724), mSARM1-TIR (561-724), zfSARM1-TIR (554-713), MyD88-TIR (148-296), TLR4-TIR (670-839). See Supplemental Text for detailed sequence information.
**Cell Culture.** HEK293T and NRK1-HEK293T cells were maintained in 10% FBS in DMEM, supplemented with penicillin/streptomycin and glutamine, and passaged by suspending in 0.05% trypsin. NRK1-HEK293T is a polyclonal cell line we developed that stably expresses Nicotinamide Riboside Kinase 1 (NRK1) so that supplementation with Nicotinamide Riboside (NR), an NAD$^+$ biosynthetic precursor, during protein expression would significantly augment cellular NAD$^+$ levels and maintain cell viability adequate for protein purification.

**Protein Expression and purification from NRK1-HEK293T stable line.** Approximately 10 million cells were plated and transfected the next day with 15 µg of StrepTag SARM1-TIR construct DNA using X-tremeGENE 9 reagent (Roche). Nicotinamide Riboside (NR) was added at a final concentration of 1 mM to improve cell viability. After 2 days the cells were harvested and lysed by sonication in binding buffer (50 mM Sodium Phosphate buffer pH 8, 300 mM Sodium Chloride, 0.01% Tween-20, EDTA-free protease inhibitor tablets). For single step affinity purification, the whole cell lysates were incubated with 20 µL MagStrep (Strep-Tactin) type 3 XT beads suspension (IBA Lifesciences) for 30 min. The beads were then washed three times with binding buffer and resuspended in 100 µL of binding buffer for enzymatic assays and other downstream applications.

**Tandem Affinity purification (TAP) from NRK1-HEK293T stable line.** Dual tagged (Strep-tag and His tag SARM1-TIR) proteins were first purified by Strep Tag affinity methods as described above. For tandem affinity purification, the proteins were then eluted from MagStrep type 3 XT beads with 22.5 mM biotin (Sigma, B4501) for 25 min. Supernatant containing the
eluted protein was separated from MagStrep beads, and then incubated with 10 µL Co²⁺ Dynabead suspension (ThermoFisher) for 30 min to bind SARM1-TIR proteins via the His tag. The beads were then washed at least two times with binding buffer and resuspended in 100 µL of binding buffer for downstream applications.

**Bacterial protein expression and Tandem Affinity Purification (TAP).** The appropriate dual tag (StrepTag and HisTag) SARM1-TIR was cloned into a pET30a+ plasmid. These constructs as well as non-recombinant pET30a+ were transformed into Shuffle T7 Express Competent E-coli (New England BioLabs). Single colonies were grown overnight and the next day, cultures were diluted in LB media, grown at 30°C until they reached $A_{600} = 0.4-0.8$, when IPTG (0.5 mM final concentration) was added. The bacteria were grown for an additional 4 h, pelleted by centrifugation, washed with PBS and stored at -80°C. For protein purification, the frozen bacterial pellet was thawed on ice, resuspended in binding buffer (without protease inhibitors) and incubated with 100 µg/mL lysozyme for 15 min on ice. EDTA-free protease inhibitor cocktail was then added and the cells were lysed by sonication. Tandem affinity purification was carried out as described above.

**NADase assay and metabolite extraction.** Ten microliters of beads incubated with the indicated cell lysate were incubated with 5 µM NAD⁺ in reaction buffer (92.4 mM NaCl and 0.64X PBS). Reactions were carried out at 25°C for the indicated amount of time and stopped by addition of 1M of perchloric acid (HClO₄) and placing the tube on ice. NAD⁺ metabolites were extracted using HClO₄/K₂CO₃ method and quantified by HPLC (see HPLC for metabolite extraction details).
measurement). For LC-MS/MS analysis, the extraction was performed using 50% Methanol in distilled water and chloroform (see LC-MS/MS metabolite measurement for further details).

**HPLC metabolite measurement.** Metabolites were isolated from enzyme reaction mixture by extracting with 1M HClO₄, then neutralized with 3M K₂CO₃, and followed by separation by centrifugation. The supernatant (90 µL) containing the extracted metabolites was mixed with 0.5M Potassium Phosphate buffer (10 µL) and metabolites were analyzed by HPLC (Nexera X2) with Kinetex (100 x 3 mm, 2.6 µm; Phenomenex) column. Internal standards for NAD⁺, Nicotinamide (Nam), Nicotinic Acid Adenine Dinucleotide (NaAD), ADP Ribose (ADPR) were used to generate standard curves for quantification of the respective compounds. The levels for each compound in each experimental sample were normalized to the 0 min time point that was analyzed concurrently.

**LC-MS/MS metabolite measurement.** Samples were prepared by mixing the reactions with 50% methanol in distilled water. The samples were placed on ice, centrifuged, soluble metabolites in the supernatant were extracted with chloroform, and the aqueous phase was lyophilized and stored at -20° C until LC-MS/MS analysis. For LC-MS/MS, the metabolite samples were reconstituted with 5 mM ammonium formate, centrifuged 12,000 x g for 10 min, and the cleared supernatant was applied to the LC-MS/MS for metabolite identification and quantification. Liquid chromatography was performed by HPLC system (1290; Agilent) with Synergi Fusion-RP (4.6 x 150mm, 4 µm; Phenomenex) column. Samples (10 µl) were injected at a flow rate of 0.55 ml/min with 5 mM ammonium formate for mobile phase A and 100%
methanol for mobile phase B and metabolites were eluted with gradients of 0–7 min, 0–70% B; 7-8 min, 70% B; 9-12 min, 0% B. The metabolites were detected with Triple Quad mass spectrometer (6460 MassHunter; Agilent) under positive ESI multiple reaction monitoring (MRM) and parameters for each compound are shown in Table 2. Metabolites were quantified by MassHunter quantitative analysis tool (Agilent) with standard curves. Standard curves for each compound were generated by analyzing NAD$^+$, ADPR, and Nam reconstituted in 5 mM ammonium formate. The levels for each compound in each experimental sample were normalized to the 0 min time point that was analyzed concurrently. Sample identity was blinded to individual performing experiment.

**Endogenous bacterial and mammalian cell NAD$^+$ quantification.** Overnight cultures of E. coli harboring a SARM1-TIR construct were diluted and grown at 30$^\circ$C until they reached $A_{600} =$ 0.4-0.8. IPTG (0.1 mM final concentration) was added to induce protein expression and the cultures were harvested 60 min later. The cultures were normalized to $A_{600} =$ 0.5 ± 0.05 and the pellet from 500 µl of culture suspension was lysed by adding 0.5M HClO$_4$. NAD$^+$ metabolites were extracted using HClO$_4$/K$_2$CO$_3$ method and measured by HPLC. Two hundred thousand NRK1-HEK293T cells grown in presence of NR were transfected with 1 µg SARM1-TIR expression construct. After two days, the NAD$^+$ metabolites were extracted with 0.5M HClO$_4$ and 3M K$_2$CO$_3$ and measured by HPLC.

**SYPRO Ruby Gel Staining.** Purified bead-SARM1-TIR protein complexes were boiled in Laemmli buffer for 10 min and separated on a 10% Bis-Tris Plus gel. After electrophoresis, the
gel was fixed in 50% Methanol/7% acetic acid for 30 min x 2, then incubated overnight in SYPRO Ruby Protein Gel stain (Thermo Fisher). The next day, the gel was washed with 10% methanol/7% acetic acid solution for 30 min, rinsed in distilled water for 5 minutes x 2, and stained proteins were visualized with a UV transilluminator.

**Preparation of peptides for LC-MS.** Purified TAP complexes were eluted by boiling the cobalt magnetic beads for 15 min in Tris-HCl buffer (pH 7.6, 100 mM) (40 µL) containing 4% SDS and dithiothreitol (100 mM). The beads were spun at 16,000 x g for 5 min and the eluted proteins were mixed with 300 µL of Tris-HCl buffer (pH 8.5, 100 mM) containing 8M urea. The SDS was removed using a filter-aided-sample-preparation (FASP) method (Wisniewski et al., 2009). After buffer exchange, 100 µL of buffer (ammonium bicarbonate, pH 7.8, 50 mM) was pipetted into the Microcon® filtration unit (YM-30) and trypsin was added (1 µg in 1 µL). The digest was incubated for 4h at 37°C and then overnight in a humid chamber after the addition of another aliquot of trypsin. The digest was acidified (5 µL of neat formic acid) and the peptides were recovered by centrifugation to the lower chamber. The acidified peptides were treated with ethyl acetate as previously described (Erde et al., 2014). The peptides were desalted by solid phase extraction on a Beckman BioMek NxP robot with C4 and porous graphite carbon Nutips (Glygen) (Chen et al., 2012). The peptides that eluted with acetonitrile (60% in 1% formic acid) were combined, dried in a vacuum centrifuge, dissolved in acetonitrile/formic acid (1%/0.1%) (16 µL). An aliquot (2 µL) was taken for analysis using a fluorescent assay (ThermoFisher Scientific) and the remainder was pipetted into autosampler vials (SUN-SRi), concentrated by vacuum centrifugation and dissolved in aqueous TFA (0.1%) (0.6/µg) for LC-MS analysis (see below).
**Protein Identification by LC-MS Analysis.** LC-ESI/MS/MS analysis was performed using a Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Plus mass spectrometer (ThermoFisher Scientific) coupled to an EASY-nanoLC 1000 system (ThermoFisher Scientific). The samples were loaded (2 µL) onto a 75 µm i.d. × 25 cm Acclaim® PepMap 100 RP column (Thermo-Fisher Scientific). The peptides were eluted at a flow rate of 300 nL/min with an acetonitrile gradient in aqueous formic acid (1%) as mobile phase A. After isocratic elution with A for 5 min the acetonitrile proportion was increased linearly to 30% with solvent B (100% ACN, 0.1% FA) over 180 min, followed by sequential increases in B to 45% in 25 min, 95% B over 5 min and an isocratic wash at 90% B for 7 min. Full-scan mass spectra were acquired by the Orbitrap™ mass analyzer in the mass-to-charge ratio (m/z) of 375 to 1400 and with a mass resolving power set to 70,000. Fifteen data-dependent high-energy collisional dissociations (HCD) were performed with a mass resolving power set to 35,000, a fixed first m/z 100, an isolation width of 0.7 m/z, and the normalized collision energy (NCE) setting of 32. The maximum injection time was 50 ms for parent-ion analysis and 105 ms for product-ion analysis. Target ions already selected for MS/MS were dynamically excluded for 30 sec. An automatic gain control (AGC) target value of 3e6 ions was used for full MS scans and 1e5 ions for MS/MS scans. Peptide ions with charge states of one or greater than six were excluded from MS/MS acquisition. The tandem mass spectra were processed using Matrix Science Distiller version 2.5 without charge state deconvolution and deisotoping. The processed files were used for protein database searches using Mascot (Matrix Science, London, UK; version 2.5.1). The database was UniProt Human Reference database (downloaded May 3, 2014, 69021 entries). A parent ion tolerance and MS2 fragment tolerance were set to 10 ppm and 0.05 Da, respectively. Carbamidomethyl of cysteine
was specified as a fixed modification and oxidation of methionine was set as a variable modification. Protein identifications were performed using Scaffold, version 4.4.8 (Proteome Software Inc., Portland, OR) implementing the Protein and Peptide Prophet algorithms (Keller et al., 2002; Nesvizhskii et al., 2003). Peptide identifications were accepted with > 90.0% probability. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 peptides with unique sequences (a low cut-off to allow for the identification of any low abundant NAD⁺ consuming protein). Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on identification of unique peptide sequences were grouped to satisfy the principles of parsimony. Relative protein quantities were estimated using a normalized total spectrum count generated from two independent transfection experiment (Essuman et al., 2017). Spectral count data from TAP wild type and mutant complexes were each normalized to spectra count of wild type SARM1-TIR lacking Strep Tag II, to account for background non-specific binding.

**Enzyme kinetics studies.** \( V_{max}, K_m, k_{cat} \) were determined from the reaction velocity of NAD⁺ consumption in the first 60 seconds of reaction for increasing substrate (NAD⁺) concentration, and fitting the data to the Michaelis-Menten equation using nonlinear curve fit in GraphPad Prism 7. \( k_{cat} \) was calculated per dimer of purified hSARM1-TIR. Data are presented as Mean ± SEM from three independents biological samples and reaction measurements. Enzyme concentration was determined via densitometry analysis on SYPRO Ruby gel of purified protein, with carbonic anhydrase used as a standard.
**Enzyme inhibition studies.** Purified bacterial hSARM1-TIR was tested in the NADase assay with the addition of 1 mM Nam or 1mM ADPR in the reaction mixture. For dose-response inhibition experiments, varying concentrations of Nam (1, 10, 10², 10³, 10⁴ µM) were added to the reaction mixture. The reaction was stopped after 5 min and NAD⁺ metabolites were extracted by the perchloric acid method and measured by HPLC as indicated above.

**Culture of mouse primary embryonic dorsal root ganglion (DRG) neurons.** DRG neurons were isolated from SARM1⁻/⁻ E13.5 mouse embryos as previously described (Gerdts et al., 2015) and seeded on plates pre-coated with poly-D-Lysine and laminin. DRG neurons were maintained in neurobasal medium supplemented with L-glutamine (Invitrogen), 2% B27 (Invitrogen), 50ng/mL nerve growth factor (Harlan Laboratories), and 1µM 5-fluoro-2’deoxyuridine plus 1µM uridine (Sigma). On DIV 1, neurons were transduced with lentiviral particles generated from HEK293T cells as previously described (Sasaki et al., 2009) expressing Venus alone or the indicated SARM1 construct fused to Venus at the C-terminus. For quantification of Venus expression, DRGs were fixed in paraformaldehyde and Venus fluorescence visualized by microscopy from multiple fields of axons for each experiment. DRGs were co-stained for tubulin (anti-mouse; from Sigma) to assess total axon area for each field. SARM1⁻/⁻ mice (C57/BL6) 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. Approximately equal number of male and female mice were used for the study.
**Analysis of axonal degeneration.** Axons from \textit{SARM1}^{−/−} DRGs expressing the indicated construct were severed with a razor blade or treated with 40 nM vincristine on DIV 7. Axon degeneration was quantified in distal axons from brightfield images using an ImageJ macro (Sasaki et al., 2009) that measures the ratio of fragmented axon area to total axon area. For an individual experiment, six fields were analyzed from 2-3 wells per condition.

**Axonal NAD\textsuperscript{+} measurement.** \textit{SARM1}^{−/−} DRGs were transduced with lentivirus as described above. Cells were supplemented with fresh media every 2 days. On DIV 7, axons were severed with a razor blade. At the indicated timepoint, cell bodies were removed then axonal NAD\textsuperscript{+} was extracted using perchloric acid/sodium carbonate method and separated with high performance liquid chromatography as previously described (Sasaki et al., 2009).

**Statistical Analyses.** Number of n is indicated in each figure legend or appropriate method section. One-way analysis of variance (ANOVA) comparisons were performed for multiple groups and unpaired two-tailed t-tests were used for individual comparisons. All error bars represent SEM. All data analyses were done with Graph Pad Prism 7, Image J macro\textsuperscript{30}, Microsoft Excel, Adobe Illustrator and Photoshop.

**Recombinant DNA sequences:**

**Mammalian expression constructs cloned into FCIV lentiviral vector**

**StrepTag-hSARM1-TIR**

Strep-Tag II  
10..33
Linker 34..69

Strep-Tag II 70..93

Linker 94..123

hSARM1-TIR 124..615

mVenus 625..1344

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**StrepTag-hSARM1-TIR E596K**

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<tr>
<td>Linker</td>
<td>94..123</td>
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<td>hSARM1-TIR E596K</td>
<td>124..615</td>
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<td>mVenus</td>
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52
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agtaa

hSARM1-TIR-Venus

hSARM1-TIR 21..512

Venus 522..1241

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StrepTag-MyD88-TIR
Strep-Tag II 10..33
Linker 34..69
Strep-Tag II 70..93
Linker 94..123
GST 124..777
MyD88-TIR 778..1224
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**StrepTag-TLR4-TIR**

Strep-Tag II 10..33

Linker 34..69

Strep-Tag II 70..93

Linker 94..123

GST 124..777

TLR4-TIR 778..1287

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**StrepTag-hSARM1-TIR-Venus-His**

Strep-Tag II 10..33

Linker 34..69

Strep-Tag II 70..93

Linker 94..123

hSARM1-TIR 124..615

mVenus 625..1341

6xHis 1342..1359

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StrepTag-hSARM1-TIR E596K-Venus-His

Strep-Tag II  10..33

Linker  34..69
Strep-Tag II 70..93
Linker 94..123
hSARM1-TIR E596K 124..615
mVenus 625..1341
6xHis 1342..1359
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SARM1 (full-length)

N-terminus 1..1224

SAM Domain 1 1225..1437

SAM Domain 2 1441..1680

TIR Domain 1681..2175
SARM1 E596K (full-length)

N-terminus 1..1224

SAM Domain 1 1225..1437

SAM Domain 2 1441..1680

TIR Domain E596K 1681..2175
Bacteria Expression constructs cloned into pET30a+

**StrepTag-humanSARM1-TIR-HisTag**

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**StrepTag-mouseSARM1-TIR-HisTag**

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**StrepTag-zebrafishSARM1-TIR-HisTag**

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Table 2: Mass spectroscopy parameters for metabolites measured

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3. TIR Domain Proteins Are an Ancient Family of NAD$^+$ Consuming Enzymes

The work described in this chapter was published in the journal *Current Biology* under the following citation:


**AUTHOR CONTRIBUTIONS:** K.E. identified prokaryotic TIR candidates with intrinsic NAD$^+$ cleavage activity by performing metabolite measurements in *E. coli* lysates, and protein purification from cell-free protein translation system with subsequent enzymatic assays with HPLC measurements. D.W.S. performed structural predictions and modeling of putative prokaryotic TIRs with CMP hydrolase, transient expression of candidate TIRs in mammalian cells, and metabolite measurements by HPLC. Y.S. extracted metabolites, performed LC-MS/MS analysis, including identification of the variant cADPR. X.M. and K.E., tested TIR domains for cleavage of other nucleotide substrates. A.K.Y.Y. helped with the identification of candidate prokaryotic TIR domains. K.E., D.W.S., Y.S., A.D., J.M., designed the research. K.E., D.W.S., Y.S., A.D., and J.M. wrote the paper.
3.1 Introduction

The TIR domain is the signature domain of components of Toll-Like Receptor (TLR) signaling, and is present in numerous receptors and adaptor proteins in innate immune pathways (Akira et al., 2006; Burch-Smith and Dinesh-Kumar, 2007; O’Neill et al., 2013). These TIR domains serve as scaffolds that promote the assembly of signaling complexes to trigger activation of pro-inflammatory cytokines and other defense-related products (O’Neill et al., 2013). In pursuing our work on axon degeneration (Gerdts et al., 2016), we made the surprising discovery that the TIR domain of SARM1 (Sterile Alpha and TIR motif containing 1), a TLR adaptor protein, has enzymatic activity (Essuman et al., 2017). Upon axon injury, the SARM1 TIR domain cleaves Nicotinamide Adenine Dinucleotide (NAD\(^+\)), destroying this essential metabolic co-factor to trigger axon destruction (Essuman et al., 2017; Gerdts et al., 2015). NAD\(^+\) biology, including the function of NAD\(^+\) consuming enzymes like the Poly ADP-Ribose Polymerase (PARP) and Sirtuins, profoundly influences myriad aspects of biology, from metabolism, to aging, to cancer biology, to neurodegeneration (Cantó et al., 2015; Imai et al., 2000; Verdin et al., 2015). Previously, it was thought that the pathways regulating NAD\(^+\) biosynthesis and degradation were mostly known. However, our studies showing that the SARM1 TIR domain is a potent NADase necessitates a re-evaluation of the function of this well-studied canonical scaffolding domain and its impact on NAD\(^+\) metabolism and disease.

Bioinformatic analysis revealed that TIR domains are present in proteins across all domains of life (Spear et al., 2009; Zhang et al., 2011). In plants, these TIR-containing proteins mediate resistance to infection by inducing death of infected cells, yet their molecular mechanism-of-action has remained elusive (Burch-Smith and Dinesh Kumar, 2007; Jones et al., 2016). In bacteria, TIR-domain proteins are associated with virulence, and are thought to do so,
in part, via entry into host mammalian cells, and blockade of mammalian innate immune signaling (Askarian et al., 2014; Cirl et al., 2008; Newman et al., 2006; Patot et al., 2017; Waldhuber et al., 2016). However, TIR domain proteins are also present in non-pathogenic bacteria, as well as archaea (Spear et al., 2009; Zhang et al., 2011), suggesting that additional TIR domain functions beyond virulence are likely to exist.

Current studies of TIR domains focus on their scaffolding function, but our findings with SARM1 inspired us to ask whether this enzymatic activity is the primordial function of the TIR domain, especially since the SARM1 TIR is more closely related to prokaryotic TIR domains than to its eukaryotic counterparts (Zhang et al., 2011). Building on techniques that we pioneered for the study of SARM1, we demonstrate that bacterial and archaeal TIR domains have NAD$^+$ cleavage activity. This finding strongly supports the model that this ancient domain initially served to cleave NAD$^+$ and modulate organismal metabolism, and suggests that this large class of proteins is a central and previously unappreciated regulator of NAD$^+$ biology.

### 3.2 Results

#### 3.2.1 Identification of prokaryotic TIR domains that induce NAD$^+$ loss in host *E. coli*

Phylogenetic studies suggest that the TIR domain of SARM1, the NADase serving as the central executioner of injury-induced axonal death, is closely related to prokaryotic TIR domains (Zhang et al., 2011). This raises the question of whether TIR NADase activity is an evolved function unique to SARM1, or whether such an enzymatic activity may be the primordial function of TIR domains. To distinguish between these two possibilities, we developed an assay to screen for NADase activity in host *E. coli* expressing candidate TIR domains (Figure 9A). We
tested TIR domains from pathogenic bacteria, including those noted for extreme antibiotic resistance, TIR domains from non-pathogenic bacteria, TIR domains from archaea, as well as the SARM1 TIR domain (Figure 9B, 9C; Figure 10A). As previously observed, IPTG protein induction of wild type (enzymatically active) SARM1-TIR induces NAD$^+$ loss in host *E. coli* (Figure 1D; Essuman et al., 2017). In contrast, NAD$^+$ was not depleted after IPTG induction in *E. coli* harboring either a control plasmid or a plasmid encoding enzymatically dead SARM1-TIR (SARM1-TIR(E642A)) (Figure 9D). We therefore reasoned that NAD$^+$ levels would be lower in *E. coli* expressing enzymatically active prokaryotic TIR domains. Consistent with this hypothesis, we found that expression of TIR domains from many pathogenic bacteria like *Staphylococcus aureus* (methicillin sensitive and methicillin resistant), uropathogenic *Escherichia coli*, and *Acinetobacter baumannii* induced NAD$^+$ depletion in the host *E. coli*. Further, TIR domains from bacteria considered non-virulent such as *Paracoccus denitrificans* and *Actinoplanes* species, or from the archaea *Theionarchaea archaeon* and *Methanobrevibacter olleaye*, also caused loss of NAD$^+$ in host *E. coli* (Figure 9D). These observations indicate that, like SARM1, TIR domains from diverse prokaryotes induce losses of NAD$^+$ in *E. coli* host.
Figure 9: Multiple TIR domains from bacteria and archaea induce NAD⁺ loss in host E. coli.

A) E. coli metabolite assay to screen candidate TIR domains for NAD⁺ cleavage activity.

B) SARM1 domain structure and role in axon degeneration. MLS–Mitochondrial Localization Signal; ARM–Armadillo/HEAT Motifs; SAM–Sterile Alpha Motif; TIR–Toll/Interleukin 1 Receptor.

C) Domain structure of candidate prokaryotic TIR containing proteins. CC–Coiled Coil; Ax_dyn_L–Axonemal dynein light chain; TM–transmembrane; ATP_Synth_B–ATP Synthase B/B’ CF(0); MPN–Mpr1p Pad1p N-terminal.

D) Endogenous levels of NAD⁺ in E. coli after 2hr IPTG induction at 30°C, measured by HPLC. Data were generated from at least three independent biological experiments. Data are presented as mean ± SEM; error bars represent SEM. P < 0.001 for SARM1-TIR, ApTir-TIR, AbTir-TIR, TcpA-TIR, TcpO-TIR for individual comparisons (+/− IPTG) using unpaired, two-tailed Student’s t test. P < 0.01 for TcpC-TIR, TcpF-TIR, BtpA-TIR, for individual comparisons (+/− IPTG) using unpaired, two-tailed Student’s t test. P < 0.05 for Pd-TIR for individual comparisons (+/− IPTG) using unpaired, two-tailed Student’s t test. P < 0.001 for TirS-TIR IPTG v.s. NC vector IPTG.
Figure 10: Multiple sequence alignments of prokaryotic TIR domain sequences and SARM1 TIR. A) Sequences of prokaryotic TIRs and SARM1 TIR used in E. coli IPTG experiments. Sequences were aligned using the ClustalO multiple sequence alignment tool in HH Pred (Alva et al., 2016; Sievers et al., 2011). (B-D) Amino acid sequence alignment of TirS-TIR, TcpC-TIR, and TcpA-TIR with MilB cytidine 5’ monophosphate (CMP) hydrolase. CMP catalytic glutamic acid is highlighted by the red asterisk and aligns to a conserved glutamic acid in the prokaryotic TIR domains.

3.2.2 Characterization of an intrinsic NADase activity in prokaryotic TIR domains

The NAD$^+$ depletion observed in E. coli expressing TIR domains from multiple prokaryotic proteins stimulated us to test whether this was due to an intrinsic NAD$^+$ cleavage activity, or was a secondary effect on E. coli metabolism. To test the intrinsic TIR enzymatic activity hypothesis, we adopted a cell-free protein transcription and translation system reconstituted from purified components from E. coli to synthesize recombinant TIR protein (Shimizu et al., 2001). This system does not contain known NADases, and we experimentally verified that it is devoid of NAD$^+$ cleavage activity (Essuman et al., 2017). We programmed this cell-free system with recombinant DNA encoding double tagged (StrepTag and 6xHis) TIR domains of TirS and TcpC, from the Gram-positive bacteria Staphylococcus aureus and Gram-negative bacteria Escherichia coli CFT073 respectively. We also tested a TIR domain from the archaea Theionarchaea archaeon, which we name TcpA-TIR. We purified the synthesized TIR proteins by tandem affinity purification and tested their ability to cleave NAD$^+$. Cobalt beads laden with purified TIR proteins were incubated with NAD$^+$ (5 µM), and metabolites were extracted and analyzed using High Performance Liquid Chromatography (HPLC). We found that all three prokaryotic TIR domains cleave NAD$^+$, with the TIR domain of Staphylococcus aureus showing the most rapid cleavage (Figure 11A).
Since different NAD\(^+\) consuming enzymes display characteristic reaction product profiles, we asked what products were formed by these prokaryotic TIR domains. In previous work, we showed that SARM1 TIR cleaves NAD\(^+\) into Nicotinamide, ADP-Ribose (ADPR), and cyclic ADPR (cADPR) (Essuman et al., 2017). Using HPLC and LC-MS, we found that all three prokaryotic TIR domains cleave NAD\(^+\) into Nicotinamide (Nam) and ADP-Ribose (ADPR) (Figure 11B-11E; Figure 12). However, unlike SARM1, the *Staphylococcus aureus* TirS-TIR and *Escherichia coli* CFT073 TcpC-TIR did not produce cADPR as a product (Figure 12G). A minor trace peak corresponding to cADPR was detected in LC-MS samples of the archaeal protein TcpA-TIR, but was not observed on HPLC chromatograms (Figure 12G and Figure 11D). Hence, we conclude that the prokaryotic TIR domains from *S. aureus* and *E. coli* CFT073 possess a pure NAD\(^+\) glycohydrolase activity, which is distinct from the dual NAD\(^+\) glycohydrolase and cyclase activity of SARM1-TIR.

In characterizing the activity of these prokaryotic TIR domains, we examined their Michaelis-Menten parameters using the purified TIR proteins. The *E. coli* TcpC-TIR exhibited an estimated Km of 196 µM and a Vmax of approximately 1.8 µM/min, while the *S. aureus* TirS-TIR displayed an estimated Km of 490 µM and a Vmax of ~10 µM/min (Figure 11F). The *E. coli* TcpC-TIR Km of 196 µM is very close to the reported 188 µM Km value of the bacterial virulence effector SPN (Streptococcus Pyogenes NAD\(^+\) glycohydrolase) that is implicated in its virulence (Ghosh et al., 2010). Further, the Km values of both TcpC-TIR and TirS-TIR fall within the range of total NAD\(^+\) content in *E. coli* and mammalian cells (~200 - 640 µM) (Cantó et al., 2015; Zhou et al., 2011), suggesting that these TIRs could influence NAD\(^+\) levels in both eukaryotes and prokaryotes.
Figure 11: Characterization of an intrinsic NAD$^+$ glycohydrolase activity in a subset of prokaryotic TIR domains. A) Prokaryotic TIR proteins purified from cell-free translation system cleave NAD$^+$ in NADase assay. B) HPLC chromatogram showing the TIR domain from *Staphylococcus aureus* (TirS-TIR) cleaves NAD$^+$ into Nam and ADPR. C) HPLC chromatogram showing the TIR domain from uropathogenic *Escherichia coli* (TcpC-TIR) cleaves NAD$^+$ into Nam and ADPR. D) HPLC chromatogram showing the TIR domain from the archaea *Theionarchaea archaean* (TcpA-TIR) cleaves NAD$^+$ into Nam and ADPR. Retention time was as follows: NAD$^+$ at t~2.50 min, Nam at t~2.40 min and ADPR at t~1.10 min. E) Quantification of metabolites generated by prokaryotic TIR proteins as displayed in A-D (normalized to 0 min). Data were generated from at least three independent reaction experiments. Data are presented as mean ± SEM; error bars represent SEM. P < 0.001 for all individual comparisons between 0 and 5 min or between 0 and 30 min for each TIR. F) Kinetic parameters for TirS-TIR and TcpC-TIR NAD$^+$ cleavage reaction. Vmax and Km were determined by fitting the data to the Michaelis-Menten equation and are presented as mean ± SEM for two independent purified protein reactions. G) Amino acid sequence alignment of TirS-TIR with MilB cytidine 5’ monophosphate (CMP) hydrolase, and modeling of TirS-TIR domain on the crystal structure of CMP hydrolase bound to CMP. E216 of TirS aligns with the catalytic glutamate residue of CMP hydrolase. H) Amino acid sequence alignment of TcpC-TIR with CMP hydrolase, and modeling of TcpC-TIR domain on the crystal structure of CMP hydrolase bound to CMP. E244 of TcpC aligns with the catalytic glutamate residue of CMP hydrolase. Tan - modeled bacterial TIR; cyan - MilB CMP hydrolase; yellow - cytidine-5'-monophosphate (ligand for MilB). I) Purified TIR proteins with mutation of catalytic glutamate to alanine do not cleave NAD$^+$ in NADase assay (normalized to control at 0 min). These are the glutamate to alanine mutants of the wild type proteins shown in Figure 2A. (J, K) Quantification of metabolites from reactions using purified proteins (TirS-TIR and TcpC-TIR) and substrate analogs (NADP – Nicotinamide Adenine Dinucleotide Phosphate; 3apAD – 3-acetylpyridine Adenine Dinucleotide; sNAD – thionicotinamide Adenine Dinucleotide; NHD – Nicotinamide Hypoxanthine Dinucleotide; NGD – Nicotinamide Guanine Dinucleotide; NaAD – Nicotinic Acid Adenine Dinucleotide; NADH – Nicotinamide Adenine Dinucleotide reduced; NMN – Nicotinamide Mononucleotide; ATP – Adenosine triphosphate; GTP – Guanine triphosphate). Metabolites were measured by either HPLC or LC-MS, and normalized to 0 min controls. L) Heat map summarizing relative levels of substrates after 30 minutes of reaction with the purified TIR proteins indicated. Values range from 0 (green), indicating complete cleavage of substrate within 30 minutes, and 1 (red) indicating relatively unchanged levels of substrates. Data from (I-L) were generated from at least three independent reaction experiments. Data are presented as mean ± SEM; error bars represent SEM.
Figure 12: Mass spectra identifying products of prokaryotic TIR NAD⁺ cleavage reactions. (A-F) Nicotinamide and ADPR were detected by mass spectrometry in reactions catalyzed by TirS-TIR, TcpC-TIR, and TcpA-TIR. G) Trace amount of cADPR were detected in the reactions catalyzed by TcpA-TIR, but not TirS-TIR or TcpC-TIR.
Figure 13: TIR domains cleave other substrates besides NAD⁺

A) SYPRO gel showing purified proteins (wild type and mutant) used in NADase assay timecourse. B) Modeling of the TcpA-TIR domain on the crystal structure of CMP hydrolase bound to CMP. E267 aligns with the catalytic glutamate residue of CMP hydrolase. C) Endogenous levels of NAD⁺ in E. coli after 30 min IPTG induction of wild type and mutant (enzymatically-dead)
TIRs at 30°C, measured by HPLC. Data were generated from three independent biological experiments. Data are presented as mean ± SEM; error bars represent SEM. D) Chemical structures of NAD⁺ and nucleotide analogs tested in NADase assay. Functional groups highlighted in red depict modifications in analogs compared to NAD⁺. Nucleotide analogs include NADP – Nicotinamide Adenine Dinucleotide Phosphate; 3apAD – 3-acetylpyridine Adenine Dinucleotide; sNAD – thionicotinamide Adenine Dinucleotide; NHD – Nicotinamide Hypoxanthine Dinucleotide; NGD – Nicotinamide Guanine Dinucleotide; NaAD – Nicotinic Acid Adenine Dinucleotide; NADH – Nicotinamide Adenine Dinucleotide reduced; NMN – Nicotinamide Mononucleotide; ATP – Adenosine triphosphate; GTP – Guanine triphosphate. E) Quantification of metabolites from reactions using purified SARM1-TIR proteins and nucleotide analogs from D. Metabolites were measured by either HPLC or LC-MS, and normalized to 0 min controls. Data were generated from three independent biological experiments. Data are presented as mean ± SEM; error bars represent SEM. F) HPLC chromatogram showing 3apAD cleavage products by purified SARM1-TIR. G) HPLC chromatogram showing 3apAD cleavage products by purified TirS-TIR. 3ap – 3-acetylpyridine.

Table 3: Nucleotide hydrolase/transferase enzymes identified via HHPred. TirS-TIR, TcpC-TIR, and TcpA-TIR were each used as a query sequence in HHPred to identify candidate enzymes.
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**TcpC-TIR**

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We next investigated structural elements within these prokaryotic TIR domains that contribute to NAD$^+$ cleavage. The SARM1 TIR domain has structural similarities to nucleotide hydrolases, which led us to the identification of a glutamate residue within the proposed active site of SARM1-TIR that is absolutely essential for its catalytic activity (Essuman et al., 2017). Structural motif homology searches revealed that these prokaryotic TIRs share structural similarity with other nucleotide enzymes including cytidine monophosphate (CMP) hydrolase, and nucleoside 2-deoxyribosyltransferase (Table 3). No crystal structures have been reported for SARM1 TIR or the three prokaryotic TIRs, so using SWISS modeling (Arnold et al., 2006) and the known CMP hydrolase structure (PDB:4JEM), we generated structural models of the prokaryotic TIR domains. This analysis demonstrated that the catalytic glutamate of CMP hydrolase aligned with a conserved glutamate in the prokaryotic TIR domains (Figure 11G, 11H and Figure 13B). This glutamate is also the residue in SARM1-TIR that was essential for its catalytic activity (Figure 10). We mutated this glutamate to alanine in each of the prokaryotic TIRs and found, in each case, that this eliminated NADase activity (Figure 11I, Figure 13A-C). These experiments show that these TIR domains share structural homology to a family of nucleotidases, and that a conserved catalytic glutamic acid in the TIR proteins is essential for catalytic cleavage of NAD$^+$. 

| 2F62_B | Nucleoside 2-deoxyribosyltransferase (E.C.2.4.2.6); SGPP, Structural GENOMICS, PSI, PROTEIN; HET: GOL, 12M, SO4; 1.5A {Trypanosoma brucei} SCOP: c.23.14.1 | 96.35 | 0.0048 |
| 4P5E_B | 2'-deoxynucleoside 5'-phosphate N-hydrolase 1 (E.C.3.2.2.-); RCL, DNPH1, Inhibitor, Rossmann Fold; HET: N6P; 1.35A {Homo sapiens} | 96.28 | 0.012 |
In some instances, NADases are capable of cleaving a number of related molecules, such as the NAD$^+$ precursor Nicotinamide Mononucleotide (NMN) in the case of CD38 (Sauve et al., 1998). Further, we wanted to determine whether prokaryotic TIRs could cleave additional substrates, providing additional paths to modifying cell metabolism. To examine the substrate specificity of the prokaryotic TIR domains, we tested their activity using a wide variety of metabolites and analogs. These included biologically important NAD$^+$ analogs such as NADH and Nicotinamide Adenine Dinucleotide Phosphate (NADP$^+$) that help maintain cellular redox state (Ying, 2008), key cellular energetic nucleotides like ATP and GTP, as well as NAD$^+$ analogs like 3-acetylpyridine adenine dinucleotide (3-apAD), Nicotinic Acid Adenine Dinucleotide (NaAD), Nicotinamide Guanine Dinucleotide (NGD), and Nicotinamide Hypoxanthine Dinucleotide (NHD), which possess minor modifications compared to NAD$^+$ that could indicate functional groups important for catalysis (Figure 11J-11L; Figure 13D).

These studies demonstrated that all three prokaryotic TIR domains cleave NADP$^+$, but have little effect on NMN (Figure 11J-11L; Figure 13E). The cleavage of NADP$^+$ suggests that these prokaryotic TIRs could modulate cellular function by altering the cellular NADP$^+$ pools necessary for maintaining levels of NADPH needed for reductive biosynthesis and protection against reactive oxygen species (Ying, 2008). Interestingly, we also found that the SARM1-TIR domain cleaves NADP$^+$, which provides an additional mechanism by which SARM1 could promote axon degeneration.

Next, we found that while all TIRs substantially cleaved 3-acetylpyridine adenine dinucleotide (3-apAD), and thionicotinamide adenine dinucleotide (sNAD), none cleaved Nicotinic Acid Adenine Dinucleotide (NaAD) (Figure 11J-11L; Figure 13E-13G). These three NAD$^+$ analogs possess only a single functional group modification on the NAD$^+$ nicotinamide
ring. For NaAD and 3apAD in particular, the amide (-NH$_2$) group of the nicotinamide ring is substituted for a hydroxyl (-OH) in NaAD or a methyl (-CH$_3$) group in 3apAD (Figure 13D). The differential cleavage pattern suggests that this position on the NAD$^+$ molecule is important for substrate recognition or catalysis by prokaryotic TIR domains.

Additionally, we noted that while prokaryotic TIRs did not cleave NHD or NGD, SARM1-TIR cleaved both of these substrates (Figure 11J-11L; Figure 13E). Interestingly, both NHD and NGD are also cleaved by ADP-Ribosyl cyclases like CD38 (Graeff et al., 1994; Graeff et al., 1996). In particular, NGD has been used to probe the activity of proteins in the ADP-Ribosyl cyclase family that generate cADPR (Graeff et al., 1994; Graeff et al., 1996). These results show clear differences between these prokaryotic TIR domains and their mammalian homolog SARM1, and are consistent with the lack of observed cyclase activity in the prokaryotic TirS-TIR and TcpC-TIR proteins.

Finally, we were unable to detect substantial cleavage of either ATP or GTP by any TIR domain, suggesting the substrate specificity may be limited to nucleotide analogs that more closely resemble the NAD$^+$ molecule. Altogether, our results indicate that the prokaryotic TIRs are positioned to influence various aspects of cellular metabolism through their cleavage of NAD$^+$ or its close analogs, and that functional groups associated with the nicotinamide ring of the NAD$^+$ molecule dictate specificity for this class of enzymes.
3.2.3 Identification of a variant cyclic ADPR generated by a subset of TIR domains

Our findings demonstrate that many prokaryotic TIR domains cleave NAD\(^+\) to produce nicotinamide and ADPR. However, our results with the archaea TcpA-TIR suggested that, like SARM1, some prokaryotic TIR NADases may also be able to generate cADPR (Figure 12G). Moreover, in our initial HPLC metabolite screen using the *E. coli* lysate assay, we observed a distinct and prominent peak (Metabolite X) produced by three different prokaryotic TIRs including the archaea *Methanobrevibacter olleyae* (TcpO-TIR), and the pathogenic bacteria, *Acinetobacter baumannii* (AbTir-TIR) and *Brucella sp.* (BtpA-TIR)(Figure 14A-14D; Figure 15A). The HPLC retention time of this Metabolite X did not correspond to retention times of any known NAD\(^+\) cleavage product such as ADPR, cADPR, or nicotinamide (Figure 11B-11D) suggesting Metabolite X could be a previously unidentified product.

To identify Metabolite X, we asked if it was a direct cleavage product of NAD\(^+\), or if it was an *E.coli* response secondary to TIR signaling. Using our cell-free protein translation NADase assay, we tested the TIR domain from the archaea *Methanobrevibacter olleyae* (TcpO-TIR). We found that it cleaved NAD\(^+\) into Nicotinamide, ADP-Ribose, and a metabolite that had a retention time very close to that of Metabolite X (Figure 14E). These data indicate that Metabolite X is a product of NAD\(^+\) cleavage and raises the intriguing possibility that these TIR domains metabolize NAD\(^+\) to a novel product.

To further characterize Metabolite X, we performed LC-MS analysis on the extracted metabolites from the TcpO-TIR reaction. Examination of the MS spectra (m/z = 70-1200) revealed metabolites produced by the reaction (compare 0 vs. 60 min) with m/z of 664, 123, 560,
and 542, which correspond to NAD$^+$, Nicotinamide, ADP-Ribose, and cADPR respectively (Figure 15B and data not shown).

Figure 14: A variant cyclic ADPR is generated by a subset of prokaryotic TIR domains. (A-D) HPLC chromatogram of metabolites extracted from E. coli lysates expressing candidate prokaryotic TIR domains. Metabolites extracted from TcpO-TIR, and AbTir-TIR reveal a prominent previously unidentified peak (labeled “X”) around retention time 1.7 minutes (C-D), which is not seen in metabolites extracted from SARM1-TIR or TirS-TIR (A,B). E) HPLC chromatogram showing purified TcpO-TIR cleaves NAD$^+$ into Nicotinamide, ADPR, and
Metabolite X is a molecule with an m/z of 542, which corresponds to cADPR; however, the LC retention time of m/z 542 was dramatically different from that of the cADPR standard (Figure 15B). Further, on a separate LC instrument, both commercially available cADPR preparations (Figure 14F and 14G) and cADPR generated from a SARM1-TIR programmed reaction (Essuman et al., 2017), had retention times distinct from Metabolite X indicating it is not cADPR.

MS/MS analysis was then used to further analyze the ion with m/z 542. We found that the major product ion had an m/z of 136, which likely corresponds to the adenine moiety of NAD\(^+\) (Figure 14H). MS/MS spectra of standard cADPR showed a very similar fragmentation pattern to that of Metabolite X (Figure 14H). Moreover, analysis from a high resolution mass spectrometry (LC/Q-TOF) revealed an m/z of 542.0694 for Metabolite X vs 542.0681 for cADPR. To extend this analysis, we collected HPLC fractions corresponding to the Metabolite X peak in lysates prepared from *E.coli* expressing TcpO-TIR, AbTir-TIR, and Btpa-TIR. MS/MS analysis of these fractions showed the signature m/z 542/136 ion pair was produced by each of these TIR domains (Figure 15C-15E).
These analyses indicate that a subset of prokaryotic TIR domains produce a variant of cADPR (Metabolite X) from NAD$^+$ cleavage. The generation of cADPR by ADP-Ribosyl cyclases including the cyclase from *Aplysia californica*, and mammalian CD38 occurs via cyclization at the N1 position of the adenine ring (Graeff et al., 2009; Lee et al., 1994). However, CD38 can cyclize nicotinamide guanine dinucleotide (NGD), an NAD$^+$ analog, using the N7 position of the guanine ring to produce N7-cGDPR (Graeff et al., 1996). Based on this finding and our metabolite analysis, we propose that the variant cADPR (Metabolite X) is N7-cADPR (Figure 15I), a previously unreported molecule. Taken together, our results show that prokaryotic TIR domains are NAD$^+$ consuming enzymes, and that remarkable diversity in the reaction products exists even within TIRs from the same domain of life. Future studies should provide more insights into the structure of the variant cADPR molecule and how prokaryotic TIRs produce this unique reaction product.
3.2.4 Full length TirS from *Staphylococcus aureus* induces NAD\(^+\) loss in mammalian cells

A number of bacterial TIR domain proteins have been identified as secreted virulence factors (Askarian et al., 2014; Cirl et al., 2008; Patot et al., 2017). With the discovery that prokaryotic TIR domains function enzymatically to cleave NAD\(^+\), we tested TIR domain proteins associated with microbial virulence for their ability to degrade NAD\(^+\) in mammalian cells. First, we transiently expressed the TIR domains from *S. aureus* TirS and *E. coli* TcpC in HEK293T cells, extracted metabolites, and measured NAD\(^+\) levels by HPLC. Consistent with results using purified recombinant protein in in vitro assays, we found that both wild type TIR domains induced NAD\(^+\) loss in these cells (Figure 16A). Moreover, this decline in NAD\(^+\) was not observed when catalytically dead (Glu to Ala) mutants of these two TIR were tested, indicating that the intrinsic TIR enzymatic activity was responsible. We confirmed that all of these TIR domains were expressed in HEK293 cells: indeed the mutant TIR domains were expressed at remarkably higher levels (Figure 16B).

With confirmation that these prokaryotic TIR domains are active in mammalian cells, we set out to examine the effects of expressing the full-length *S. aureus* TirS virulence factor (TirS-FL) in HEK293T cells. First, we asked if TirS-FL purified from our cell-free protein translation system was able to cleave NAD\(^+\) in vitro. We found that wild type TirS-FL cleaves NAD\(^+\), whereas the mutant TirS-FL(E216A) was enzymatically inactive (Figure 16C-16E). Similar to the purified TIR-only protein, HPLC analysis showed that TirS-FL cleaves NAD\(^+\) into Nicotinamide and ADP-Ribose (Figure 16C and 16D).
Figure 16: Full-length TirS from *Staphylococcus aureus* induces NAD\(^+\) loss in mammalian HEK293T cells. A) Endogenous NAD\(^+\) levels in HEK293 cells expressing both wild type and mutant TirS-TIR and TcpC-TIR. B) Western blot of Flag-tagged wild type and mutant TirS-TIR and TcpC-TIR proteins expressed in HEK293T cells. C) HPLC chromatogram showing purified full length TirS (TirS-FL) cleaves NAD\(^+\) into Nam and ADPR in NADase
assay. D) Quantification of metabolites generated by TirS-FL NADase reaction as displayed in C (normalized to 0 min NAD⁺). E) SYPRO Ruby gel of purified TirS (wild type and mutant). F) Endogenous NAD⁺ levels in HEK293T cells expressing both wild type and mutant TirS-FL measured 24 hr after transfection. G) Cell viability as measured by resazurin fluorescent assay 48 hr after transfection. TirS-FL expressing HEK293T cells have decreased viability compared to control and TirS FL E216A expressing cells. H) Western blot of Flag-tagged wild type and mutant TirS-FL proteins expressed in HEK293T cells. Data in A, D, F, E were generated from at least three independent experiments. Data are presented as mean ± SEM; error bars represent SEM. *** p < 0.001, unpaired two-tailed Student’s t test.

To test if TirS-FL could induce NAD⁺ loss in mammalian cells, we transiently expressed this protein in HEK293T cells, extracted metabolites, and measured NAD⁺ levels. Consistent with data generated in vitro and in bacteria, we found that wild type TirS-FL induced NAD⁺ loss in mammalian cells within 24 hr of transfection, whereas enzymatically dead TirS-FL E216A did not (Figure 16F). At later times we noticed that some cells expressing wildtype TirS-FL appeared to be dying. We therefore assessed the viability of cells expressing TirS proteins via a resazurin fluorescent assay. We found that HEK293T cells expressing wild type TirS exhibited decreased cell viability, whereas those expressing mutant TirS-FL E216A had viability similar to control (Figure 16G). Again, we observed that the wildtype protein was expressed at much lower levels in HEK293T cells than its catalytically inactive mutant (Figure 16H), suggesting that TirS may be toxic to its mammalian host. These findings suggest that TIR domain proteins identified as virulence factors could promote virulence via their NADase activity and that therapeutic interventions targeting TIR NADases in pathogenic bacteria could restrict infections caused by these organisms.
3.3 Discussion

Many studies over the past two decades have defined the TIR domain as a scaffold promoting assembly of signaling complexes via protein-protein interactions. Here, we dramatically extend our understanding of this protein domain, demonstrating that the TIR domain represents an ancient enzyme motif capable of cleaving NAD\(^+\), the central constituent of organismal bioenergetics. Indeed, TIR domains from proteins present in all three major domains of life possess this enzymatic function. Hence, we postulate that the primordial function of the TIR domain was to regulate metabolic and bioenergetic pathways through modulating NAD\(^+\) levels.

The scaffolding function, which is best characterized in mammalian TIR domains involved in innate immunity, may be a more recent evolutionary adaptation. Moreover, the realization that prokaryotic TIR domains are NADases enables a reevaluation of prior functional studies of these proteins. For example, our finding that the TIR domain protein from *S. aureus* (TirS) reduces NAD\(^+\) levels in mammalian cells suggests that its role as a virulence factor could stem from its NADase activity. Additionally, many of these TIR domains cleave NADP\(^+\) as well, suggesting that perturbations in redox environment could also be influenced by these potential virulence proteins.

The mechanism by which these proteins enter mammalian cells remains unresolved, although cytolysin mediated translocation (CMT), a functional equivalent of type III secretion system in Gram-positive bacteria, is thought to deliver the bacterial effector *Streptococcus pyogenes* NAD\(^+\) glycohydrolase (SPN) (Madden et al., 2001). Identifying how these TIRs are delivered into host cells and how the NAD\(^+\) cleavage activity facilitates virulence may provide additional targets to inhibit disease pathogenesis. Finally, secreted TIR NADases may not only
target eukaryotic hosts, but may also participate in bacterial competition to sculpt diverse microbial communities.

The data demonstrating that purified full-length TirS is enzymatically active suggests that the enzymatic activity of TirS in *S. aureus* must be turned off, attenuated, or regulated in some fashion to prevent the host bacterial cell from consuming its own NAD$^+$. In the case of SARM1, its activity is regulated by unknown mechanisms involving the auto-inhibitory N-terminal domain and by the actions of the NAD$^+$ synthesis enzyme Nicotinamide Mononucleotide Adenylyltransferase 2 (NMNAT2) (Gerds et al., 2013; Gilley and Coleman, 2010; Sasaki et al., 2016). It is likely that similar auto-inhibition or a toxin-antitoxin system (Yamaguchi et al., 2011) is present to regulate TIR domain activity in the host bacterial cell.

Finally, the presence of TIR domain proteins that cleave NAD$^+$ in non-pathogenic bacteria and archaea suggests that these proteins can also play non-virulence related roles, most likely in regulating metabolic pathways. For example, the generation of adenine derived second messengers such as ADPR and cyclic ADPR by this TIR family could control calcium homeostasis, which helps regulate chemotaxis (Dominguez, 2004). It remains to be determined if the variant cADPR molecule (Metabolite X) can substitute for cADPR in inducing calcium release or whether it has completely novel functions. Additionally, these initial studies of TIR domains have focused on NAD$^+$ metabolism; however their structural homology to nucleotide hydrolases (Burroughs et al., 2015; Essuman et al., 2017) opens the possibility that subsets of TIR domains could have even more diverse roles and modulate other aspects of nucleotide metabolism.

In summary, we have discovered that a diverse set of prokaryotic TIR domains regulate levels of the central metabolic regulator NAD$^+$. These results demonstrate that TIR domain
proteins constitute a new family of NADases, and suggest that the TIR domain scaffolding functions in TLR signaling likely represent a ‘repurposing’ of this evolutionary ancient enzymatic domain.

3.4 Materials and Methods

Recombinant DNA and Endogenous NAD$^+$ measurements in host E. coli. Double tagged (N-terminal tandem StrepTag and C-terminal 6xHisTag) bacterial, archaea, and SARM1 TIRs (see sequence in Figure S1) were cloned into a pET30a+ plasmid. These constructs as well as non-recombinant pET30a+ were transformed into Shuffle T7 Express Competent E-coli (New England BioLabs). Single colonies were then grown overnight and the next day, cultures were diluted in LB media, grown at 30°C until they reached $A_{600}$ of approximately 0.4-0.8, when IPTG (0.1 mM final concentration) was added to induce protein expression. The cultures were then harvested at approximately 30 minutes (Figure 13) or 2 hours later (Figure 9). The cultures were normalized to $A_{600}$ of approximately 0.5 ± 0.05 and the pellet from 500 µl of culture suspension was lysed by adding 200 µL 0.5M perchloric acid (HClO$_4$). Samples were then placed on ice for at least 10 minutes, centrifuged, and supernatant collected. 180 µL of supernatant was then added to approximately 67 µL of 3M K$_2$CO$_3$. Samples were placed on ice for at least 10 minutes, and centrifuged. NAD$^+$ metabolites were then measured by HPLC as described in ‘Method Details’ section. Extracted metabolites after perchloric acid or K$_2$CO$_3$ addition can also be stored at -20°C for later processing.
**Cell-free protein transcription and translation.** In vitro cell-free protein transcription and translation was performed using the PURExpress In Vitro Protein Synthesis Kit (New England BioLabs Catalog # E6800S). For a total reaction volume of about 25 µL, the reaction was assembled in the following order: 10µL of Solution A, 7.5µL of Solution B, 3µL of RNase inhibitor (40U/µL), water, and 0.5-1.0 µg of pET30a+ non-recombinant/recombinant DNA. The reaction was incubated at 37°C for 2.5 hours and stopped by placing on ice. Multiple 25 µL reactions can be pooled together to increase protein yield prior to Tandem Affinity purification of proteins, which is described in the ‘Method Details’ section.

**Mammalian Cell Culture and NAD⁺ quantification.** HEK293T cells were maintained in 10% FBS in DMEM, supplemented with penicillin/streptomycin and glutamine, and passaged by suspending in 0.05% trypsin. For NAD⁺ measurements in mammalian cells, HEK293T cells were seeded in 12-well dishes (approximately 2.5 x 10⁵ cells per well) and transiently transfected the following day with 500 ng of the indicated plasmid using polyethylenimine (1mg/mL) in a 3:1 ratio to plasmid DNA. Twenty-four hours later, cells were washed in cold phosphate buffered saline and NAD⁺ extracted using perchloric acid method. NAD⁺ was measured after separation by HPLC.

**Identification of prokaryotic TIR domains and database search.** Candidate pathogenic bacterial TIRs were selected from the published literature where at least seven bacterial TIR domain proteins have been reported to promote virulence [9]. *A. baumannii* was included since carbapenem-resistant *A. baumannii* is classified under ‘Priority 1’ of the 2017 WHO Priority
Pathogens List for R&D of New Antibiotics. *P. dentrificans* TIR domain is not known to promote virulence but was one of the first bacterial TIR domains where the crystal structure was available [9]. The archaeal TIR domains were identified by searching the non-redundant protein database in protein-BLAST, and selecting Archaea (taxaid:2157) under Organism. The blastp (protein-protein blast) algorithm was utilized and the search was performed using the sequence of the bacterial TIR domains TirS-TIR and TcpC-TIR (see sequence in Figure 10). Both *M.olleyae* and *T. archaeon* ranked as the top 2 hits with highest max score, and lowest e-value in the search using TirS-TIR, and *T. archaeon* ranked the highest in the max score and identity, and lowest e-value in the search using TcpC-TIR. The TIR domain from *Actinoplanes* was identified from bioinformatics analyses establishing a phylogenetic relationship between SARM1-TIR and other TIR domains.

**HPLC metabolite measurement.** Supernatant (90 µL) containing the extracted metabolites was mixed with 0.5M Potassium Phosphate buffer (10 µL), and metabolites were analyzed by HPLC (Nexera X2) with Kinetex (100 x 3 mm, 2.6 µm; Phenomenex) column. Internal standards for NAD\(^+\), Nicotinamide (Nam), ADP Ribose (ADPR), and cyclic ADPR were used to generate standard curves for quantification of the respective compounds. Other NAD\(^+\) analogs were quantified and normalized using the area of their corresponding HPLC profile peaks. For enzymatic reactions, the levels for each compound in each experimental sample were normalized to the 0 min time point that was analyzed concurrently.
Native Protein Purification. Cell-free synthesized proteins (two pooled 25 µL reactions) were first purified by Strep Tag affinity methods where synthesized proteins were incubated in binding buffer (50 mM Sodium Phosphate buffer pH 7.6, 300 mM Sodium Chloride, 0.01% Tween-20) and 20 µL MagStrep (Strep-Tactin) type 3 XT beads suspension (IBA Lifesciences) for 30 min. Proteins were then eluted from MagStrep type 3 XT beads with approximately 100µL of 25 mM biotin (Sigma, B4501) for 25 min. TIR-only proteins were further purified by incubating the eluted protein with 10 µL Co²⁺ Dynabead suspension (ThermoFisher) for 30 min to bind TIR proteins via the His tag. The beads were then washed at least two times with binding buffer and resuspended in 100 µL of binding buffer for downstream applications. Multiple 25 µL reactions can be pooled together to increase protein yield.

NADase assay and metabolite extraction. For purified TIR domains, typically, 10-20 µL of cobalt beads laden with purified protein were incubated with 5 µM NAD⁺ and reaction buffer (92.4 mM NaCl and 0.64X PBS), for a total reaction volume of 50 µL. Reactions were carried out at room temperature (25° C) for the indicated amount of time. For biotin eluted TirS-FL, 20 µL (wild type) or 30 µL (mutant) protein was incubated with 5 µM NAD⁺, under the same buffer and temperature conditions. Reaction was stopped by addition of 50 µL 1M of perchloric acid (HClO₄) and placing the tube on ice for at least 10 min. Neutralization was performed with 16.7 µL 3M K₂CO₃. Samples were placed on ice for 10 min, and then separated by centrifugation. NAD⁺ metabolites were quantified by HPLC (see HPLC metabolite measurement). For LC-MS analysis, the extraction was performed using 50% Methanol in water, and chloroform extraction
(see LC-MS metabolite measurement for further details). Extracted metabolites after perchloric acid or K₂CO₃ addition can also be stored at -20°C for later processing.

**Purification of Metabolite X from bacterial lysates.** Purification and separation was performed using HPLC (Prominence; Shimadzu) equipped with C-18 column (150 x 4.6mm, 3 mm; Supelco) at flow rate of 1ml/min with 5mM ammonium formate for mobile phase A and 100% methanol for mobile phase B. Metabolites were eluted with gradients of 0-5 min, 100% A; 5-6 min, 0-5 % B; 6-13 min, 5% B; 13-15 min, 5-15% B; 15-23 min, 15% B; 23-24 min, 15-0% B; 24-30 min, 100% A and eluents between 3-4 min containing compound-X were collected, lyophilized, and stored at -80°C until analysis.

**LC-MS metabolite measurement.** Samples were prepared by mixing the reactions with 50% methanol in water. The samples were placed on ice (or stored at -20°C for later processing), centrifuged, soluble metabolites in the supernatant were purified with chloroform extraction, and the aqueous phase was lyophilized and stored at -20°C until LC-MS analysis. For LC-MS, the metabolite samples were reconstituted with 5 mM ammonium formate, centrifuged 12,000 x g for 10 min, and the cleared supernatant was applied to the LC-MS for metabolite identification and quantification. Liquid chromatography was performed by HPLC system (1290; Agilent) with Atlantis T3 (2.1 x 150 mm, 3 µm; Waters) column. Samples (10 ml) were injected 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 and metabolites were eluted with gradients of 2–6 min, 0–20% B; 6-8 min, 20-50% B; 8 - 10 min 50% B; 10 - 15 min, 50 - 0% B. The metabolites were detected with Triple
Quad mass spectrometer (6460; Agilent) under positive ESI multiple reaction monitoring (MRM). Metabolites were quantified by MassHunter quantitative analysis tool (Agilent) with standard curves. Standard curves for each compound were generated by analyzing NAD+, cADPR, ADPR, and Nam reconstituted in 5 mM ammonium formate. For high resolution Q-TOF analysis of Metabolite X, extracted metabolites from TcpO-TIR reaction were detected by Q-TOF (6545; Agilent) with direct infusion at flow rate of 0.5 ml/min of mobile phase (50% acetonitrile and 50% water) and m/z for compound-X was determined by comparing m/z profiles with that of control.

**Cell Viability Studies.** HEK293T cells were seeded in 96-well plates and transfected with 10ng of the indicated plasmid with polyethylenimine (1mg/mL) (3:1 ratio to plasmid DNA). At 48 hours post-transfection, cells were washed in phosphate buffered saline (PBS) then incubated with 0.01mg/mL resazurin (Sigma Cat# R7017) diluted in PBS for 1 hour at 37°C. Fluorescence intensity was measured with a POLARstar Optima plate reader (BMG Labtech) with 544 excitation and 590 emission filters. At least three wells were assessed per replicate for each condition.

**SYPRO Ruby Gel Staining.** Purified proteins were boiled in Laemmli buffer for 5-10 min and separated on a 4-12% Bis-Tris Plus gel. After electrophoresis, the gel was fixed in 50% Methanol/7% acetic acid for 30 min x 2, then incubated overnight in SYPRO Ruby Protein Gel stain (Thermo Fisher). The next day, the gel was washed with 10% methanol/7% acetic acid
solution for 30 min, rinsed in distilled water for 5 minutes x 2, and stained proteins were visualized with a UV transilluminator.

**Western Blot:** For expression studies in HEK293t cells, the cells were seeded and transfected as described for NAD analysis. One day post transfection, cells were washed with PBS and lysed in Laemmli buffer. Cell extracts were briefly sonicated, centrifuged at 5,000xg for 5min to remove cell debris. The supernatants were then boiled, resolved by SDS-PAGE followed by immunoblotting for Flag (1:1000 Mouse Anti-Flag M2 monoclonal, Sigma F3165) and beta-tubulin (1:1000 Anti-beta-tubulin DSHB Clone E7) and visualization by standard chemiluminescence.

**Enzyme kinetics studies.** $V_{\text{max}}$ and $K_m$ were determined from the initial reaction velocity or reaction velocity in the first 60s of product (ADPR) formation, and fitting the data to the Michaelis-Menten equation using nonlinear curve fit in GraphPad Prism 7. Data are presented as Mean ± SEM from two independents biological samples and a total of four independent reaction measurements.

**Structural Modeling of bacterial TIR domains:** SWISS-Model (Arnold et al., 2006) was used to generate structural models of bacterial TIR domains. The crystal structure of MilB CMP hydrolase (PDB 4JEM) was used as the template. The modeled bacterial TIR domain and MilB crystal structures were visualized and superimposed with Chimera ([www.rbvi.ucsf.edu/chimera](http://www.rbvi.ucsf.edu/chimera)).
tan - modeled bacterial TIR; cyan - MilB CMP hydrolase; yellow - cytidine-5'-monophosphate (ligand for MilB).

**HH Pred Protein Identification.** TirS-TIR, TcpC-TIR, and TcpA-TIR were analyzed for structural homologs using HH Pred (Alva et al., 2016). Only known nucleotide enzymes with an E-value lower than 0.1 and a probability above 95% are shown in Table 3.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Unpaired two-tailed t-tests were used for individual comparisons with an assumption of equal variance between groups. All error bars represent SEM. Other data analyses were done with Graph Pad Prism 7, Microsoft Excel, Adobe Illustrator and Photoshop.
4. **TIR Domains of Plant Immune Receptors are NAD\(^+\) Consuming Enzymes that Promote Cell Death**

The work described in this chapter is published in the journal *Science* under the following citation:

Wan L\(^{†}\), Essuman K\(^{†}\), Anderson RG., Sasaki Y, Monteiro F, Chung EH., Osborne-Nishimura E, DiAntonio A, Milbrandt J, Dangl JL., Nishimura MT. TIR Domains of Plant Immune Receptors are NAD\(^+\) Consuming Enzymes that Promote Cell Death. *Science* 2019. 365 799-803. \(^{†}\)These authors contributed equally to this work.

**AUTHOR CONTRIBUTIONS:** W.L. and R.G.A. performed in planta experiments of transient expression and pathogen effector delivery. K.E. and R.G.A, performed E. coli NADase assay. K.E. performed cell free transcription and translation and tested purified protein for NAD\(^+\), NADP\(^+\) and NaAD cleavage by HPLC. W.L and Y.S. processed plant samples for LC-MS/MS measurement. W.L., K.E., R.G.A, J.M., J.D., M.T.N designed the research. W.L., K.E., F.M., A.D., J.M., J.D., M.T.N wrote the paper.
4.1 Introduction

Plants rely on an innate immune system to detect potential pathogens including fungi, nematodes, and bacteria (Jones and Dangl, 2006). Like animal TLR receptors, plants possess membrane bound receptors that serve as one of the first lines of defense to detect extracellular pathogen/damage associated molecular patterns (Jones and Dangl, 2006). Activation of these receptors mounts an immune response termed PAMP Triggered Immunity (PTI), which sometimes is not enough to control pathogen spread (Jones and Dangl, 2006). Further, in several cases, pathogens employ effector molecules to subvert these PTI responses (Jones and Dangl, 2006). To help combat pathogens that inhibit these PTI responses, plants harbor a second line of defense, termed hypersensitive response (HR), where intracellular receptors mount a rapid, more robust response, that culminates in cell death (Jones and Dangl, 2006; Jones et al., 2016).

Nucleotide binding leucine-rich repeat (NLRs) receptors (Jones et al., 2016) belong to family of receptors responsible for the plant HR. The mechanisms underlying NLR function has however remained elusive since their discovery 25 years ago. One of the major classes of NLRs is the TIR domain containing NLRs. This receptor class possesses a TIR domain on its N-terminal end, and usually a nucleotide binding sequence (NBS) domain, and leucine rich repeat (LRR) domain at the C-terminal end. Structure function studies have suggested that the N-terminal end of TIR-NLRs is required and often sufficient to induce cell death (Monteiro and Nishimura 2018). This finding is similar to SARM1 in Wallerian degeneration, where the truncated SAM-TIR or dimerized TIR domain is sufficient to trigger axon degeneration without injury (Gerdts et al., 2013; Gerdts et al., 2015). With the identification of SARM1 as an NADase, and prokaryotic TIRs as NAD$^+$ cleavage enzymes, we sought to determine if the mechanism of TIR-NLRs in cell death involves an intrinsic NAD$^+$ cleavage activity of the plant TIR domain.
4.1 Results

4.4.1 Plant TIR proteins require a conserved catalytic residue for cell death activity in planta

To determine if plant TIR domains might be mechanistically similar to SARM1, we assessed the extent of conservation of the putative SARM1 catalytic residue (E642, Essuman et al., 2017) in plant TIR domains. A structural homology search with SARM1-TIR using Phyre2 returned multiple plant TIR domains among the highest confidence matches (Table 4). Comparison with plant TIR domains such as RPS4 revealed that both the glutamic acid and neighboring residues are positionally conserved in SARM1 models (Figure 17A). We found that 131 of 146 Arabidopsis TIR domains from the reference Col-0 genome contain a glutamic acid at the corresponding position (Figure 17B and Table 5). The other 15 TIR domains with divergent residues at this position are in proteins that contain unusual domain combinations similar to the cell death-inactive “sensor” NLR RRS1, or are encoded by genes found in a head-to-head genomic locus similar to RPS4/RRS1 (Table 5) (Narusaka et al., 2009; Williams et al., 2014). These trends persist in a larger set of 8,687 plant TIR-domains deposited in public databases (Figure 18). Thus, the putative catalytic residue of the SARM1 TIR NADase is present in potentially active plant executor TIR domains, but notably absent from sensor TNLs likely to lack autonomous activity.

To determine if the conserved putative NADase catalytic residue is required for function, we generated glutamic acid to alanine (E/A) mutations in the Arabidopsis TIR-only immune receptor RBA1 and the TIR domains of the Arabidopsis TNL immune receptors RPS4 and
RPP1_NdA (hereafter RPP1) (Nishimura et al., 2017; Schreiber et al., 2016; Williams et al., 2014). We also tested an uncharacterized TIR-only protein (BdTIR) from the monocot *Brachypodium distachyon*. Monocot genomes have lost all full-length TNL immune receptors (Shao et al., 2016), but BLAST searches identified this TIR-only protein of unknown function. All four tested TIR proteins triggered cell death in *Nicotiana benthamiana* or *Nicotiana tabacum* and this cell death was dependent on the conserved glutamic acid (Figure 17C and 17D).

**Table 4.** Summary of top ten results returned from HsSARM1 Phyre2 search.

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<th>Confidence</th>
<th>%i.d.</th>
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<td>Crystal structure of the tir domain from the flax protein L6</td>
<td>Plant</td>
</tr>
<tr>
<td>c4lzpA</td>
<td>99.9</td>
<td>21</td>
<td>Structure of the tir domain of the immunosuppressor BtpA</td>
<td>Bacteria</td>
</tr>
<tr>
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<td>17</td>
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<tr>
<td>c4lzpB</td>
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<td>21</td>
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<tr>
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Table 5. Arabidopsis thaliana (Col-0) TIR proteins with non-conserved glutamic acid are typically non-canonical.

<table>
<thead>
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<th>Local region of E</th>
<th>Gene type</th>
<th>Notes</th>
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<td>head to head with neighboring TIR</td>
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<td>SSVLCNIQLEKIVNS</td>
<td>TIR-NB</td>
<td>head to head with neighboring TIR</td>
</tr>
<tr>
<td>AT4G16930</td>
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<td>TIR</td>
<td>truncated</td>
</tr>
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<td>in second TIR</td>
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<tr>
<td>AT4G36140</td>
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<td>TIR-NB-LRR</td>
<td>RPS4/RRSI-like (Narusaka, 2009); in second TIR</td>
</tr>
<tr>
<td>AT5G17890</td>
<td>YDNAFPRKLQIVG</td>
<td>TIR-NB-LRR-LIM</td>
<td>RPS4/RRSI-like (Narusaka, 2009)</td>
</tr>
<tr>
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<td>TIR-TIR-NB-LRR</td>
<td>in second TIR</td>
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<tr>
<td>AT5G45050</td>
<td>--TVSLQKVLVLDG</td>
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<td>RPS4/RRSI-like (Narusaka, 2009)</td>
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<tr>
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<td>D-----FDILKKNEN</td>
<td>TIR-PP</td>
<td></td>
</tr>
<tr>
<td>AT5G45210</td>
<td>LSKQCLQTEVEFLER</td>
<td>TIR-NB-LRR</td>
<td>RPS4/RRSI-like (Narusaka, 2009)</td>
</tr>
<tr>
<td>AT5G45220</td>
<td>ESKNLDLVDINKC</td>
<td>TIR-TIR</td>
<td></td>
</tr>
<tr>
<td>AT5G45240</td>
<td>SSVLCLEHLELQ--C</td>
<td>TIR-NB-LRR</td>
<td>head to head with neighboring TIR</td>
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<tr>
<td>AT5G45260</td>
<td>FSEVNLDFAKVLEC</td>
<td>TIR-NB-LRR-WRKY</td>
<td>RRS1; RPS4/RRSI-like (Narusaka, 2009)</td>
</tr>
<tr>
<td>AT5G48780</td>
<td>SSPLCLDSSIKILKF</td>
<td>TIR-NB</td>
<td>head to head with neighboring TIR</td>
</tr>
</tbody>
</table>

Table 6. Summary of published plant TIRs with a mutated SARM1-like putative catalytic glutamate residue.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Context</th>
<th>Mutation</th>
<th>Approach</th>
<th>Reference</th>
</tr>
</thead>
</table>
Figure 17. Plant TIR proteins require a conserved putative catalytic residue for cell death activity in planta. (A) Homology model of the SARM1 TIR (right, blue) indicates that the putative SARM1 catalytic residue (E642, orange) is similarly positioned to E88 in the plant TIR RPS4 (PDB 4C6R; left, yellow). Residues surrounding the putative catalytic glutamic acid are also conserved (green sidechains, black labels). (B) Amino acid sequence pileup of 147 TIR domains from the Arabidopsis Col-0 genome. (C) Schematic of plant proteins expressed in planta. Plant TIR proteins are shown in yellow, human SARM1 domains are shown in blue. BdTIR and RPP1 proteins have an N-terminal extension (N). RPP1 and RPS4 proteins are truncations that delete the NBS and LRR autoinhibitory domains. SAM-RPS4 is a fusion between the SAM oligomerization domain from human SARM1 and the RPS4 TIR
domain. (D) In planta phenotypes of proteins described in (C) after transient expression. RBA1 and SARM1 SAM-TIR were expressed in *Nicotiana benthamiana*, the others in *N. tabacum*. Wild-type TIR proteins trigger autoactive cell death 24-36 hr after inoculation. Putative catalytic residue mutants (E/A) lose the ability to trigger cell death. EV = empty vector negative control. Protein endpoints and epitope tags: myc:RBA1<sub>1-191</sub>, HA-BdTIR<sub>1-224</sub>, RPP1<sub>90-254</sub>:YFP, HA:SAM-RPS4<sub>1-250</sub>, and HA:HsSAM-TIR<sub>409-724</sub>:YFP. Full leaf images and protein accumulation data are in Figure 19.

In our laboratories, we found that the RPS4 TIR required fusion to an orthologous oligomerization domain (SARM1 SAM) to trigger cell death (Figure 17C and 17D, Figure 20). The putative NADase catalytic glutamic acid has been identified in genetic screens as a suppressor of both TIR domain and full-length TNL immune receptor function (Table 6), supporting our results with site-specific TIR mutants. Surprisingly, expression of an autoactive fragment of human SARM1 (HsSAM-TIR) also triggered cell death in planta, and this was dependent on the putative catalytic E residue (Figure 17C). Thus, we demonstrate that the putative HsSARM1 TIR catalytic residue is widely conserved in plant TIRs and is required for cell death activation in planta.

RBA1-triggered cell death and the immune functions of full length RPP1 and RPS4 TNLs are EDS1-dependent (Aarts et al., 1998; Nishimura et al., 2017). The functional mechanism of the lipase-like protein EDS1 is unknown, but it is required for all tested plant TIR protein and TNL phenotypes (Nishimura et al., 2017; Wagner et al., 2013). Consistent with this, we found that autoactivity triggered by the four tested plant TIR domains, including the monocot BdTIR, was EDS1-dependent (Figure 21). HsSAM-TIR cell death in planta did not require EDS1 (Figure 21), suggesting that plant and animal TIR proteins have different mechanisms.
Figure 18. Catalytic ‘E’ abundance in TIRs mined from 106 publicly available genomes (A-C) Alvis’s sequence bundles visualization of the mined TIR domains. The bundle shows amino acid incidence in three sequence groups in different colours (A) black: All 8,687 mined TIRs; (B) blue: 113 RPS4-likes; (C) orange: 141 RRS1-likes. Catalytic ‘E’ residue position is highlighted in light green background. (D) Quantification of amino acids found at position 80 of the MSA.
Figure 19. Phenotypes and protein accumulation of TIR transient expression in *Nicotiana tabacum* or *N. benthamiana*. (A) Protein accumulation of WT and mutant TIR proteins transiently expressed in *Nicotiana* and assayed by western blotting using anti-HA, -MYC or -GFP as appropriate. Ponceau stain indicates protein loading. In the case of multiple bands, caret indicates full-length protein of interest. (B) Visible light images of autoactive cell death phenotypes triggered by transient TIR overexpression. EV is empty vector negative control, E/A is the
putative catalytic dead glutamic acid to alanine mutant. SH/AA and G/R are defined or putative self-association mutants. “Penta” is a SARM1 SAM domain self-association mutant (L442R_I461D_L514D_L531D_V533D). RBA1 and SARM1 are expressed in N. benthamiana, the others are in N. tabacum. (C) UV images of autoactive cell death phenotypes. These images are the source for images in Figure 17.

Figure 20. RPS4 TIR phenotypes. (A) Various RPS4 constructs expressed in N. tabacum. Cell death is evident only for RPS4 aa1-250 and a “core TIR” aa 10-178 construct fused to the SAM domain of SARM1. Cell death was not evident for RPS4 1-250:YFP or for the same construct containing the enhanced self-association mutation R30A. (B) UV image of the leaf in (A). (C) The autoactive SAM:RPS4 aa1-250 construct requires both the putative catalytic residue E88A and the self-association interfaces defined by S33/H34 and G151 (demonstrated and putative, respectively). (D) UV image of leaf in (C). (E-G) Western blot demonstrating protein accumulation for TIR loss of function mutants. Constructs are blotted with either anti-GFP (RPS4) or anti-HA (SAM:RPS4).
Figure 21. Plant TIR autoactivity is EDS1-dependent. (A-F) Transient expression of plant TIRs in either WT or eds1-2 mutant Nicotiana benthamiana. In all cases, plant TIR autoactivity is EDS1-dependent. An autoactive SAM-TIR truncation of Human SARM1 (aa 409-724) retains autoactivity in eds1. EV is an empty vector negative control. MLA10 (CC domain; aa1-160) is used here as a known EDS1-independent positive control. TIR constructs are as described in Fig 1 and fig S3. (G-L) Western blots to confirm protein accumulation of non-active TIRs in eds1 plants. Antibodies used were specific to the various epitope tags (HA:SAM-RPS4, RPP1:YFP, MLA:MYC, SARM1 SAM-TIR:YFP, MYC:RBA1, and HA:BdTIR. Ponceau stain indicates equal loading.
4.4.2 Plant TIR proteins can degrade NAD$^+$ and NADP$^+$

To determine if plant TIR domains are enzymes capable of degrading NAD$^+$, we expressed the four plant TIR domains from Figure 17C in a variety of expression systems. We first assessed the ability of plant TIR domains to degrade endogenous NAD$^+$ after induction in *E. coli*. Similar to the SARM1 TIR domain (Essuman et al., 2017; Essuman et al., 2018), expression of each plant TIR domain resulted in strong NAD$^+$ depletion after two hours of TIR protein induction in *E. coli*, as measured by high performance liquid chromatography (HPLC) (Figure 22A; Figure 23). As with the SARM1-TIR (Essuman et al., 2017; Essuman et al., 2018), activity was dependent on the proposed catalytic glutamic acid residue in all cases (Figure 22, A to D, and Figure 23). Concomitant with NAD$^+$ depletion by all four plant TIR domains, we observed accumulation of breakdown products, including an HPLC peak consistent with a variant of cyclic ADP-Ribose (v-cADPR) (Figure 22, E to H) (Essuman et al., 2018). To further characterize the reaction products and demonstrate definitively the intrinsic enzymatic activity of plant TIR domains, we purified RBA1 and BdTIR after *in-vitro* transcription-translation, and tested the ability of purified proteins to directly degrade NAD$^+$. Both HPLC and mass spectrometry (LC-MS/MS) analysis revealed that purified RBA1 and BdTIR proteins degrade NAD$^+$ into v-cADPR and Nicotinamide (Nam) (Figure 22, I to K; Figure 24, A and B), though more weakly than did SARM1-TIR (Figure 24G). Purified BdTIR additionally produced ADP-Ribose (ADPR) from the NAD$^+$ cleavage reaction. (Figure 22K; Figure 24C). The NAD$^+$ cleavage activity of both RBA1 and BdTIR was dependent on the putative catalytic glutamic acid residue (Figure 22I). v-cADPR produced by plant TIRs in vitro appears identical to v-cADPR previously identified as the product of the archeal TIR protein TcpO (Figure 25, and (Essuman et al., 2018)). Importantly, the accumulation of v-cADPR appears to be a reasonable proxy for plant TIR
NADase enzymatic function, as its appearance is also dependent on the putative catalytic glutamic acid in all cases (Figure 22, E to H).
Figure 22. Plant TIR proteins can degrade NAD$^+$ and NADP$. (A-D) Plant TIR proteins result in glutamic acid-dependent NAD$^+$ depletion in *E. coli* after a 2 hour induction with IPTG when lysates are assayed by HPLC. Error bars represent SEM. Asterisks indicate statistical significance in a one-way ANOVA with Tukey’s multiple comparison test. * p < 0.05, ** p < 0.01, *** p < 0.001. (E-H) HPLC chromatogram of *E. coli* lysates (from A-D) demonstrates glutamic-acid dependent depletion of NAD$^+$, as well as accumulation of a peak consistent with v-cADPR. (I) Plant TIR-only proteins generated by in vitro transcription-translation deplete NAD$^+$ after 60min. Putative catalytic glutamic acid to alanine mutants did not deplete NAD$. Error bars represent SEM Asterisks indicate statistical significance in a one-way ANOVA with Tukey’s multiple comparison test. * p < 0.05, ** p < 0.01, *** p < 0.001. (J) HPLC traces corresponding to (I) indicate that purified RBA1 generated v-cADPR when incubated with NAD$^+$. (K) HPLC traces corresponding to (I) show that purified BdTIR decreases NAD$^+$ and leads to corresponding increases in Nam, ADPR and v-cADPR. (L) Plant TIR-only proteins generated by in vitro transcription-translation degrade NADP$, but not the related molecule NaAD after 60min. Error bars represent SEM. Asterisks indicate statistical significance in a student t-test. * p < 0.05, ** p < 0.01, *** p < 0.001. (M) Mutation of RBA1 putative catalytic residue (E86A) does not affect self-association as measured by co-immunoprecipitation of HA:RBA1 with YFP:RBA1 from *N. benthamiana*.
Figure 23. Protein expression and SARM1-TIR NAD⁺ cleavage reactions (A) Anti-his tag western blots to confirm protein induction in E. coli. Samples correspond to Figure 22A to 22D. (B) SYPRO Ruby Gel stain of WT and E/A mutation of SARM1-TIR, RBA1, and BdTIR to verify protein accumulation for in vitro transcription/translation-based NADase assay in Figure 22I to 22L. (C) SARM1 TIR domain depletes NAD⁺ after 2 hour IPTG induction in E. coli as assayed by HPLC. SARM1 E642A with the putative catalytic glutamic acid mutated to alanine exhibits no depletion. Error bars represent SEM. Asterisks indicate statistical significance in a one-way ANOVA with Tukey’s multiple comparison test (*** p < 0.001). (D) HPLC chromatogram of E. coli lysate expressing WT SARM1-TIR demonstrates depletion of NAD⁺. (E) SARM1 TIR protein generated by in vitro transcription-translation depletes NAD⁺ after 60min. Putative catalytic glutamic acid to alanine mutant does not deplete NAD⁺. Statistics are performed as in (C). (F) HPLC traces corresponding to (E) indicate that purified SARM1-TIR generates Nam, ADPR and cADPR when incubated with NAD⁺, but not v-cADPR. (G) In vitro assays (as in E) showing that SARM1 TIR rapidly depletes NAD⁺. The 0 minute timepoint data are shared in (E) and (G). Statistics are performed as in (C).
While we observed strong NAD$^+$ depletion by the RPS4 and RPP1 TIR domains in the *E. coli* assay, we were unable to confirm NADase activity with these TIR domains as purified proteins from the *in-vitro* assay. We speculate that this assay fails to re-create functionally relevant higher order structures that are generated during expression and accumulation in *E. coli* or lacks necessary factors that are present in *E. coli*. SARM1 has previously been shown to degrade NADP$^+$, but not another NAD$^+$ related molecule, NaAD (Essuman et al., 2018). To test if plant TIRs have similar specificity, we assessed the enzymatic activity of purified RBA1 and BdTIR after *in-vitro* transcription-translation towards NADP$^+$ and NaAD. We found that these plant TIRs degraded NADP$^+$ (Figure 22L) into Nam and ADP-Ribose Phosphate (ADPRP) (Figure 24 D to G). We did not detect significant cleavage of the close analog NaAD (Figure 22L). Altogether, these results support our hypothesis that plant TIR domains are enzymes that can cleave NAD$^+$ (and, in some cases, NADP$^+$) as part of their signaling function.

Plant TIR domains typically require two characterized self-association interfaces for function (Figure 26, and (Zhang et al., 2017)). To determine if self-association is required for NADase activity, we generated mutant TIR domains containing previously demonstrated or proposed loss of self-association mutations (Nishimura et al., 2017; Williams et al., 2014). BdTIR interface(s) have yet to be established. We found that mutation of either The AE- or DE-type self-association interface abolished both cell death in planta (Figure 26B) and NADase activity in our *E. coli* NAD$^+$ depletion assay (Figure 22A and 22C-D). Importantly, the in planta requirement for AE- and DE-interfaces was retained in the functional HsSAM-RPS4 TIR domain fusion (Figure 20), despite fusion to the SAM oligomerization domain (Gerdts et al., 2013). This suggests that plant TIR domains must form a specific multimer for function. Next, we asked if mutation of the putative catalytic glutamic acid residue had an impact on self-association as
measured by co-immunoprecipitation. We found that while the RBA1 SH/AA mutant lost self-
association (Figure 22M; (Nishimura et al., 2017)), the putative NADase catalytic dead RBA1
E86A retained self-association (Figure 22M). This result is consistent with the hypothesis that
TIR enzymatic activity is downstream of, and promoted by, TIR self-association, thus explaining
why plant TIR self-association is a common requirement for function.
Figure 24. LC-MS/MS and HPLC chromatograms of TIR in vitro assay products. (A-C) LC-MS/MS profiles identifying Nam (BdTIR and RBA1) and ADPR (BdTIR) as in vitro NAD+ cleavage products of in vitro transcription/translation produced TIR proteins. (D-E) In vitro transcription/translation produced RBA1 cleaves NADP+ into Nam and ADPRP. (F-G) In vitro transcription/translation produced BdTIR cleaves NADP+ into Nam and ADPRP.
Figure 25. Comparison of v-cADPR and cADPR. (A-D) Comparison of v-cADPR identified as a plant TIR NADase product, v-cADPR from archeal TcpO TIR domain, and commercially available cADPR. v-cADPR and cADPR share the same parent ion, but display different retention times suggestive of alternate cyclization.
Figure 26. Plant TIR enzymatic activity and cell death induction requires self-association. (A) RPP1 structure (PDB 5TEB) indicating self-association interfaces. The AE interface is indicated by purple dashes and purple amino acids S108 H109. The DE interface is indicated by red dashes and the red amino acid G229. The putative catalytic glutamic acid is colored in orange. (B) Autoactivity triggered by RBA1, RPP1 and SAM1-RPS4 (constructs as described in Figure 17C) in Nicotiana spp. is dependent on by AE and DE interfaces as defined by loss of function mutations (SH/AA or G/R). RPP1 and SAM-RPS4 images are from the same experiment presented in Figure 17

4.4.3 Plant TIR protein expression leads to v-cADPR accumulation in planta

Activation of the SARM1 TIR domain in neurons via enforced self-association leads to NAD$^+$ depletion, energetic depletion, and subsequent axonal demise (Gerdts et al., 2015). To date however, we have been unable to detect large scale NAD$^+$ reduction after transient expression of plant TIRs, or after pathogen-mediated delivery of effectors that activate TIR-based immunity. As noted above (Figure 22), NAD$^+$ depletion by plant TIR domains in E. coli and in vitro was accompanied by production of Nam, ADPR and v-cADPR. NAD$^+$ cleavage is a plausible signaling event as both ADPR and cADPR trigger calcium influx to the cytoplasm (Fliegert et al., 2007; Wu et al., 1997), an event strongly correlated with plant NLR function (Grant et al., 2000). To validate that plant TIRs are functional NADase enzymes in planta, we measured the accumulation of v-cADPR by mass spectrometry following transient TIR protein expression in Nicotiana benthamiana leaves, and utilized this as a proxy for TIR enzymatic
function in planta. We found that v-cADPR accumulated in planta in response to RBA1, BdTIR, RPP1 TIR, and SAM-RPS4 TIR expression (Figure 27, A to D). Similar to our *E. coli* NADase assay (Figure 22, E to H), the levels of v-cADPR induced in planta by the TNL TIRs (SAM-RPS4 and RPP1) were lower in comparison to those induced by the TIR-only proteins (RBA1 and BdTIR). Consistent with both the in vitro assays and the in planta cell death phenotypes, the accumulation of v-cADPR in planta following expression of any of the four plant TIR proteins was dependent upon the putative catalytic glutamic acid (Figure 27A to D) and on self-association (Figure 27, A, C, D; the BdTIR interface is uncharacterized). Thus, plant TIR proteins are capable of producing v-cADPR both in vitro and in planta when overexpressed.

We next measured v-cAPDR accumulation in a more biologically-relevant system. Delivery of the type III effector HopBA1 by *Pseudomonas fluorescens* activates RBA1-dependent cell death in Arabidopsis (Nishimura et al., 2017). We found that HopBA1 induced v-cAPDR accumulation in Arabidopsis, while bacteria containing either the empty vector or a loss-of-function allele, HopBA1-H56F (Nishimura et al., 2017), did not (Figure 27E). Cell death itself (when triggered by either the non-TIR immune receptor RPM1 or transient expression of the coiled-coil domain of MLA10), was insufficient to result in accumulation of v-cADPR, or had levels similar to negative control conditions (Figure 28). Thus, we found that accumulation of v-cADPR was correlated with plant TIR function both when overexpressed and when triggered by a bacterial effector protein.
Figure 27. Plant TIR protein expression leads to v-cADPR accumulation in planta. (A-D) cADPR accumulates in *Nicotiana benthamiana* after transient expression of plant TIRs. v-cADPR does not accumulate in untreated plants, or in plants expressing empty vector (EV) or TIR loss of function mutants. Experiments in (C) and (D) were performed together and share the untreated and empty vector data. Error bars represent SEM. Asterisks indicate statistical significance in a one-way ANOVA with Tukey’s multiple comparison test. * p < 0.05, ** p < 0.01, *** p < 0.001. (E) v-cADPR accumulates following delivery of HopBA1 from *Pseudomonas fluorescens* into Arabidopsis (Ag-0) leaves after 24hr. HopBA1 H56F is a loss of function negative control (14). (F-G) v-cADPR accumulates after RBA1 and BdTIR expression in *N. benthamiana* and the eds1-2 and nrg1-1 mutants. Error bars represent SEM. Asterisks indicate statistical significance in a one-way ANOVA with Tukey’s multiple comparison test for (F). Asterisks indicate statistical significance in a Kruskall-Wallis test with Dunn’s multiple comparison test for (G). * p < 0.05, ** p < 0.01, *** p < 0.001. (H) Hypothetical pathway wherein TIR domain enzymatic function is induced by effector recognition and TNL (left) or TIR only (right) activation. This leads to accumulation of potential signaling products derived from NAD+ that accumulate upstream of EDS1 and, subsequently, NRG1 (23). T, Toll-interleukin receptor-like; N, Nucleotide-binding site domain; L, leucine-rich repeat.
Figure 28. In planta NAD+ depletion assays; Cell death triggered by the non-TIR NLR RPM1 or the CC domain of MLA10 is insufficient to trigger v-cADPR accumulation. (A) Transient expression of HsSAM-TIR in *N. benthamiana* reduces NAD+ accumulation relative to the putative catalytic mutant HsSAM-TIR E642A. (B) Plant TIR-only proteins do not measurably deplete NAD+ when transiently expressed in *N. benthamiana*. (C) Plant TIR domains from TNL immune receptors do not measurably deplete NAD+ when expressed in *N. benthamiana*. (D) Delivery of HopBA1 (to trigger TIR-only RBA1) by *P. fluorescens* does not result in measurable NAD+ depletion in Arabidopsis (Ag-0). (E) Bacterial delivery of AvrRpm1 (pathogen effector recognized by the CNL immune receptor RPM1) into Arabidopsis accession Col-0 did not result in accumulation of v-cADPR in excess of empty vector (EV) or the loss of function mutant AvrRpm1 D185A. (F) Overexpression of MLA10 aa1-160 CC domain in *N. benthamiana* does not result in accumulation of v-cADPR.
4.4.4 TIR-NLRs function upstream of EDS1 and NRG1

EDS1 can be found in a complex with TNL proteins (Huh et al., 2017), and is essential for TIR-dependent cell death and disease resistance in planta (Wagner et al., 2013). The four plant TIR proteins investigated in this study all co-immunoprecipitated with EDS1 (Figure 29A) and all four required EDS1 for cell death activity (Figure 21). These findings suggest that EDS1 could be required for TIR function directly, as they are in the same complex, or be required for downstream signaling. To distinguish between these hypotheses, we assayed for the accumulation of the v-cADPR biomarker in WT and eds1 mutant plants. We found that v-cADPR accumulated in the eds1 mutant following expression of RBA1 or BdTIR (Figure 27, F and G). NRG1, a CNL helper NLR immune receptor, was recently proposed to function downstream of EDS1 in TIR signaling pathways (Qi et al., 2018). Consistent with a downstream function, we found that RBA1 and BdTIR expression still induced v-cADPR accumulation in nrg1 mutant plants, despite a lack of cell death (Figure 27, F and G; Figure 30). These findings demonstrate that plant TIR domains are active in the absence of EDS1 and NRG1, but are unable to trigger cell death. This result suggests a model in which TIR enzyme activity, potentially via the liberation of bioactive metabolites such as v-cADPR, signals through EDS1, and then NRG1, to promote cell death (Figure 27H). This raises the intriguing possibility that, similar to other NAD⁺ consuming enzymes like CD38, Sirtuins or Poly ADP-Ribose Polymerases (PARPs) (Canto et al., 2015; Verdin, 2015), plant TIR domains couple NAD⁺ metabolism to cellular signaling to activate downstream components of cell death via an intrinsic enzymatic activity.
Figure 29. Plant TIRs interact with EDS1. (A) Plant TIRs co-immunoprecipitated with EDS1. Proteins were transiently expressed in *N. benthamiana*. EDS1:YFP was immunoprecipitated with anti-GFP beads. Immunoprecipitated samples were then blotted for the presence of HA-tagged plant TIR proteins.
Figure 30. RBA1 and BdTIR cell death requires NRG1. RBA1 autoactive cell death in N. benthamiana (A) is lost in an nrg1 mutant (B). EV is an empty vector negative control. MLA10 (CC domain, aa1-160) is a non-TIR positive control for autoactive cell death in nrg1. BdTIR autoactive cell death in N. benthamiana (C) is lost in an nrg1 mutant (D). (E and F) anti-MYC western blot demonstrates RBA1 and MLA10 protein accumulation in nrg1 plants. (G) anti-HA western blot demonstrates BdTIR protein accumulation in nrg1 plants.
4.3 Discussion

For years, the existence of a signaling pathway downstream of pathogen recognition by TIR-NLRs has been elusive. Our results establish plant TIR domains as enzymes that cleave the essential metabolic cofactors NAD$^+$ or NADP$^+$ to activate cell death. The generation of ADPR and v-cADPR by TIR activity in vitro and in planta suggest that these known calcium mobilization agents could activate immune response pathways subsequent to pathogen recognition and receptor activation. Future studies will focus on the elucidation of the structural requirements for activation of enzymatic function, potential differences in enzymatic function between TIR-only and TNL receptors, and how the products of catalysis might activate downstream signaling. In animal cells, NAD$^+$ is established as a key signaling substrate influencing myriad aspects of biology from metabolism to DNA repair to aging to transcriptional regulation (Demarest et al., 2019). Our data present a new framework for understanding plant NLR biology, and significantly extend the role of NAD$^+$ in immunity across kingdoms.
4.4 Materials and Methods

**RPS4 TIR phenotypes.** In our hands, several RPS4 TIR domain constructs (previously reported as active (Swiderski et al., 2009; Williams et al., 2014)) did not trigger strong phenotypes in either *Nicotiana tabacum* or *Nicotiana benthamiana* (Figure 20). Since plant TIR domain autoactivity phenotypes have been reported to be correlated with the strength of self-association (Schreiber et al., 2016), we generated constructs to enhance self-association of the RPS4-TIR domain. We found that both a minimal (aa 10-178) TIR domain of RPS4 and a longer version (aa 1-250) triggered robust cell death when fused to the C-terminus of the SAM oligomerization domain from human SARM1 (Figure 17C and 17D, and Figure 20; (Gerdts et al., 2013)).

**Plant materials, growth conditions, extract preparation for metabolite identification.** *Nicotiana benthamiana* and *Nicotiana tabacum* were grown in walk-in growth rooms maintained at 24 °C/20 °C with a 16-h/8-h (day/night) cycle. In *Agrobacterium*-mediated transient expressions, relevant strains were grown overnight, and the cell pellet was resuspended in induction buffer containing 10 mM MES (pH 5.6), 10 mM MgCl₂, and 150 µM acetosyringone. *Agrobacteria* (GV3101 pMP90) were hand-injected with 1mL needleless syringes at OD₆₀₀nm of 0.8 (Injections into *N. benthamiana* included 0.1 OD₆₀₀nm of GV3101 carrying 35S:P19, a viral suppressor of gene silencing) into 5- to 6-week-old *N. benthamiana* or *N. tabacum* leaves. Images of cell-death phenotypes were taken 2-5 days post inoculation. For western blots to check protein expression of TIR domains, leaf samples of SARM1, RBA1 and MLA10 CC were collected at 26h post infiltration, while leave samples of BdTIR, SAM-RPS4 and RPP1 were collected 40h post infiltration. For NADase metabolite assays, leaf samples were collected at 26h post infiltration or 40h post infiltration, as above. Nine leaf disks (8 mm in diameter) from at least 4 leaves from 4 different plants were pooled and weighed, and then homogenized into
powder and dissolved in 450 μL 50% (v/v) methanol and stored at -80 °C. 150 μL of supernatant after centrifugation was analyzed by mass spectrometry (see below).

*Arabidopsis thaliana* Ag-0 and Col-0 plants were grown in walk-in rooms maintained at 21° C/18° C with a 9-h/15-h (day/night) cycle. In *Pseudomonas fluorescens* (Pf0-1) or *Pseudomonas syringae pv. tomato* DC3000 (DC3000) mediated effector-delivery assays, Pf0-1 or DC3000 strains were grown overnight, washed once with 10 mM MgCl₂ and then diluted in 10 mM MgCl₂ to an OD₆₀₀nm of 0.2 (Pf0-1) and an OD₆₀₀nm of 0.1 (DC3000). These cultures were hand-injected with needleless syringes into 4- to 6-week old *Arabidopsis* rosette leaves around 10 AM. The cell death phenotypes were recorded 24h and 30h post inoculation. For western blots to check protein levels, leaf samples were collected 24h post inoculation. For NADase metabolite assays, leaf samples were collected at 24h post infiltration. For NADase metabolite assays, leave samples were collected at 24h post infiltration. Six leaf disks (8mm in diameter) from 6 leaves from 6 different plants were pooled and weighed, and then homogenized into powder and dissolved in 300 μL 50% (v/v) methanol and stored at -80 °C. 150 μL of supernatant after centrifugation was analyzed by mass spectrometry (see below).

**LC-MS/MS metabolite measurement.** Plant extracts were prepared as indicated above. For LC-MS/MS analysis, metabolites were extracted in 50% methanol in water and deproteinized with chloroform, and the aqueous phase was lyophilized and stored at -80°C until LC-MS/MS analysis. For LC-MS/MS, the metabolite samples were reconstituted with 5 mM ammonium formate, centrifuged 12,000 x g for 10 min, and the cleared supernatant was applied to the LC-
MS/MS for metabolite identification and quantification. Liquid chromatography was performed by HPLC system (1290; Agilent) with an Atlantis T3 (2.1 x 150 mm, 3 µm; Waters) column. Samples (10 µl) were injected 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 2–6 min, 0–20% B; 6-8 min, 20-50% B; 8 - 10 min 50% B; 10 - 15 min, 50 - 0% B; 15 - 24 min, 0% B. The metabolites were detected with a Triple Quad mass spectrometer (6460; Agilent) under positive ESI multiple reaction monitoring (MRM). Metabolites were quantified by using area under the curve determined by MassHunter quantitative analysis tool (Agilent) and the retention time for each compounds were determined with standard compounds including NAD⁺, cADPR, ADPR, Nam, v-cADPR, ADPRP reconstituted in 5 mM ammonium formate. The following mass-to-charge (m/z) for parent and product ion was used for detection of metabolites on LC-MS/MS: NAD+ (664 > 542); ADPR (560 > 136); Nam (123 > 80); cADPR (542 > 428); v-cADPR (542 >136); ADPRP (640 > 136).

**Bacterial strains and growth conditions.** E. coli Top10 and Agrobacterium tumefaciens strain GV3101/pMP90 were grown in LB medium at 37 °C and 28 °C, respectively. Pseudomonas strains were grown in King’s B medium at 28 °C. The antibiotic concentrations (µg/mL) used for E. coli were ampicillin 100, kanamycin 30, gentamycin 25, and spectinomycin 50. The antibiotic concentrations (µg/mL) used for Agrobacterium were gentamycin 50, kanamycin 100, rifampicin 100, and spectinomycin 100. The antibiotic concentrations (µg/mL) used for Pseudomonas were tetracycline 50, kanamycin 30, and rifampicin 50.

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**Plasmids.** Bacterial expression plasmids were cloned in pET24a+ (RPP1) or pET30a+ (others). Recombinant plasmids include, SARM1 – StrepTag-SARM1-TIR-HisTag (TIR: 561-724); RBA1 – StrepTag-RBA1-HisTag (TIR: 1-191); Bd – StrepTag-Bd-HisTag (TIR: 1-224); RPS4 – StrepTag-RPS4-HisTag (TIR: 1-200); RPP1 – HisTag-RPP1-StrepTag (TIR: 1-254). Plant expression constructs were generated using Gateway-compatible vector systems. Entry clone were generated by BP clonase in pDONR207 or synthesized in pUC57-Kan (Genescript). Site-directed mutants were generated by PCR mutagenesis. Cloning primers are available upon request. Plant expression vectors are from the pGWB600 series. Plant expression clones for BdTIR (NCBI accession XM_003560026) and HsSARM1 SAM-TIR were codon-optimized for Arabidopsis and synthesized by GenScript. The N-terminal HA-SAM vector was constructed by cloning the SAM domain from HsSARM1 (1xHA tag-SARM1\textsubscript{478-578}-GGGGS) into the XbaI site of pGWB602. The RPS4\textsubscript{1-250} entry clone was a gift from Kee Hoon Sohn. The RPP1\textsubscript{NdA 1-254} entry clone was a gift from Brian Staskawicz. The MLA10 CC domain (endpoints: 1-160) entry clone was a gift from Farid El-Kasmi.

**Coimmunoprecipitation and western blotting.** A combination of *Agrobacterium* strains containing relevant constructs were infiltrated into two separate halves of *N. benthamiana* leaves. The leaf samples were collected and flash frozen in liquid nitrogen 36 h post infiltration. Frozen leaf tissue was ground in a mortar and pestle with liquid nitrogen and resuspended in 2 mL of extraction buffer [50 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM EDTA (pH 8.0), 0.2% Triton X-100, 5 mM DTT with 1× plant protease inhibitor mixture (Sigma-Aldrich)]. Soluble supernatants were obtained by centrifugation twice at 10,000 × g for 15 min at 4 °C. 50 μL of α-GFP conjugated magnetic beads (Miltenyi Biotec) was added to each sample and incubated for
2.5 h with constant rotation at 4 °C. Samples were captured using separation columns (Miltenyi Biotec) and were washed with washing buffer (extraction buffer with 0.1% Triton X-100 and 150 mM NaCl) three times. Bound proteins were eluted in 120 µL elution buffer [50 mM Tris·HCl (pH 6.8), 50 mM DTT, 1% SDS, 1 mM EDTA (pH 8.0), 0.005% bromophenol blue, and 10% glycerol]. Samples were resolved by electrophoresis on 12% SDS/PAGE gels and transferred to nitrocellulose membrane. The membrane was blocked for 30 mins in 5% milk dissolved in TBS-T (TBS with 1% Tween) and blotted with HRP-conjugated antibodies overnight at 4 °C in TBS with TBS-T. The following antibody concentrations were used: α-GFP, 1:5,000 (Santa Cruz Biotechnology); α-HA, 1:2,000 (Santa Cruz Biotechnology).

**Endogenous NAD⁺ measurements in E. coli.** Recombinant plasmids in either pET24a+ (RPP1) or pET30a+ (others) were transformed into Shuffle T7 Express Competent *E. coli* (New England BioLabs). Single colonies were grown overnight and the next day, cultures were diluted in LB media, grown at 30°C until they reached an absorbance (*A*₆₀₀) of approximately 0.4-0.8. 0.1 mM IPTG final concentration was added to induce protein expression. Cultures were harvested approximately 2 hours later, and then normalized to *A*₆₀₀ of approximately 0.5 ± 0.05. 500 µl of culture suspension was then aliquoted, and centrifuged. The supernatant was decanted, the pellet washed with PBS, and centrifuged again. Metabolites from the bacterial pellet were extracted by adding 200 µL 0.5M Perchloric acid (HClO₄). Samples were then placed on ice for at least 10 minutes, spun down, and supernatant collected. 180 µL of supernatant was then added to approximately 67 µL of 3M Potassium Carbonate (K₂CO₃). Samples were placed on ice for at least 10 minutes, and centrifuged. NAD⁺ metabolites were then measured by HPLC as described below.
**HPLC metabolite measurement.** Potassium carbonate neutralized reactions were centrifuged, and the supernatant (90 µL) containing the extracted metabolites was mixed with 0.5M Potassium Phosphate buffer (10 µL). Metabolites were analyzed by HPLC (Nexera X2) with a Kinetex (100 x 3 mm, 2.6 µm; Phenomenex) column. Internal standards for NAD$^+$ was used to generate standard curves for quantification of NAD$^+$ concentration.

**Cell-free transcription and translation.** In vitro cell-free protein transcription and translation was performed using the PURExpress In vitro Protein Synthesis Kit (New England BioLabs Catalog # E6800S or E6800L). For a total reaction volume of about 25 µL, the reaction was assembled in the following order: 10µL of Solution A, 7.5µL of Solution B, 3µL of RNase inhibitor (40U/µL), and 0.5-1.0 µg of pET30a+ recombinant DNA. Water can be added to bring volume up to 25 µL, but is not necessary. The reaction was incubated at 37°C for 2.5 hours and stopped by placing on ice for a few minutes. Multiple 25 µL reactions can be pooled together to increase protein yield prior to purification of proteins, described below.

**Native Protein Purification.** Cell-free synthesized proteins were first purified by Strep Tag affinity methods. 20µL MagStrep (Strep-Tactin) type 3 XT magnetic beads suspension (IBA Lifesciences) was first pre-washed (twice) in binding buffer (50 mM Sodium Phosphate buffer pH ~7.6 – 8.1, 300 mM Sodium Chloride, 0.01% Tween-20), and beads separated using a magnet. Cell-free synthesized proteins (four 25 µL reactions for plant TIRs), were then incubated
with the 20µL of MagStrep beads in binding buffer (no more than 500 µL) for 30 min. After 30 min, proteins laden beads were washed three times with binding buffer, and proteins eluted from MagStrep type 3 XT beads with approximately 100µL of 25 mM biotin (Sigma, B4501) for 20-25 min. Biotin eluted proteins were subsequently recaptured via their histidine (His) affinity tag using 10 µL Cobalt (Co^{2+}) Dynabead suspension (pre-washed with binding buffer) for 30 min. Cobalt beads laden with proteins were then washed with binding buffer twice, and re-suspended in binding buffer (usually 100 µL) for further NADase enzymatic assays.

**In vitro NADase Assay with Purified Protein.** For plant TIR proteins, 20 µL of cobalt beads laden with purified protein were incubated with 2.5 µM NAD^{+} (final concentration) and reaction buffer (92.4 mM NaCl and 0.64X PBS), for a total reaction volume of 50 µL. Reactions were carried out at room temperature (25° C) for the indicated amount of time. Reaction was stopped by addition of 50 µL of 1M of perchloric acid (HClO_{4}) and placing the tube on ice for at least 10 min. Neutralization was performed with 16.7 µL 3M K_{2}CO_{3}. Samples were placed on ice for 10 min, and then separated by centrifugation. NAD^{+} metabolites were quantified by HPLC (see HPLC metabolite measurement above). For LC-MS/MS analysis, the extraction was performed using 90 µL of 50% Methanol in water, and chloroform (see LC-MS metabolite measurement for further details). Reactions using NAD^{+} analogs (NaAD, NADP^{+}, NADH, and NADPH) were performed similarly using these analogs as the substrate instead of NAD^{+}. Metabolites from these reactions were extracted using either the perchloric acid method or 50% methanol in water as described above. Metabolites were analyzed by either HPLC or LC-MS/MS.
**SYPRO Ruby Gel Staining.** Purified proteins were resuspended and boiled in Laemmli buffer for 5-10 min and separated on a 4-12% Bis-Tris Plus gel. Gel was then fixed after electrophoresis in 50% Methanol/7% acetic acid for at least 30 min (twice), then incubated overnight in SYPRO Ruby Protein Gel stain (Thermo Fisher). The following day, the gel was washed with 10% methanol/7% acetic acid solution for 30 min, rinsed in distilled water for at least 5 min (twice), and stained proteins were visualized with a UV transilluminator.

**Structural Modeling of SARM1:** SARM1 was modeled using Phyre2. The search was done with the 176aa C-terminus of HsSARM1 (aa 549-724) using the normal mode against the Protein Data Bank’s set of experimentally determined structures.

**Catalytic ‘E’ residue prevalence in TIR-containing proteins across the plant phylogeny:** We investigated the incidence of ‘E’ residue in TIR-containing proteins across the plant phylogeny using a panel of 106 publicly available plant proteomes (Supplemental File 1). Multiple isoforms were detected and only the longest coding sequence was retained for downstream analysis. We used hmmscan from the HMMER package to identify TIR-containing proteins (version 3.1b2 http://hmmer.org/; parameters Pfam-A 31.0 --tblout --domtblout --cut_tc; ). 8,865 TIR domain sequences, delimited by their envelope coordinates, were extracted using awk, grep, sed, sort and cut command lines. We obtained a multiple sequence alignment (MSA) of all TIR-domains with Ultra-large alignments using Phylogeny-aware Profile (UPP; github commit 53242afa7ee844efb30b7035ae1f86a75b3258e2; defaults; ). We then refined the MSA to remove low accuracy sequences (lower than 26% average similarity) and columns with more than 95%
gaps using the seq_reformat program of the T-COFFEE suite. The final alignment contained 8,687 TIR domains. Site-specific amino-acid occurrences in the final alignment were computed using in-house bash scripts. Catalytic ‘E’ incidence in MSA was quantified in R. Alignment visualization as sequence bundles was performed with Alvis.

**Identification of putative RPS4 and RRS1 orthologs:** NLRs from all mined proteomes were identified and used in an all-against-all blastp (version 2.6.0; parameters -dbsize 100000000 -evalue 1e-5 -outfmt 6 -num_threads 8). Putative orthologs were identified using orthogogue (orthogogue version 1.0.3; parameters --taxon_index 0 --protein_index 1 --seperator \| --cpu 8 -A -u -S; ). Cluster structure of the similarity relationships from previous steps was determined using mcl (version 14-137; parameters --abc -I 1.2 -t 8; ). Orthogroups containing the putative orthologs of A. thaliana RPS4 and RRS1 are presented in Supplemental File 2 (Narusaka et al., 2009).

**Statistics:** Figure legends indicate type of statistical test used. Error bars generally represent SEM. For some points where error bars would be shorter than the height of the symbol, Graph Pad Prism software indicates error bars were not drawn.
5. Conclusions and Future Directions

The TIR domain for many years has been described as an adaptor protein in the assembly of signaling protein complexes for host innate immunity. In this dissertation, I redefined the function of the TIR domain, and provide strong evidence across all three Domains of Life that TIR domain proteins are a family of enzymes. These findings allow investigation into new areas of research, and instruct a re-evaluation of TIR domain biology.

I identified SARM1 as the founding member of the TIR domain family of enzymes, and this discovery has several implications for SARM1’s role in axon degeneration, innate immunity, and development. For axon degeneration, others and we have shown that SARM1 is the central executioner of Wallerian axon degeneration (Gerdts et al., 2016). Using a catalytically dead SARM1 in cultured DRG neurons, we showed that the NADase activity of SARM1 is required for axon degeneration (Essuman et al., 2017). The molecular components downstream of SARM1 NADase activation however remain to be described. Previous studies indicated that NAD\(^+\) depletion and energetic failure triggered by SARM1 activation could be rescued with precursors such as nicotinamide ribose (NR) and methylpyruvate (Gerdts et al., 2015; Yang et al., 2015). It is however still not known if NAD\(^+\) cleavage products such as cADPR, and ADPR contribute to the signaling downstream of the NADase enzymatic activity to promote axonal fragmentation. cADPR and ADPR are adenine-containing metabolites that function as calcium mobilizing agents to trigger calcium release in certain biological systems (Fliegert et al., 2007). Further, calcium is known to be a pro-degenerative signal for axon degeneration, and chelation via EGTA inhibits axon degeneration (Villegas et al., 2014). Further, evidence for calcium homeostasis in axon degeneration was recently implicated in a model of multiple sclerosis –
experimental autoimmune encephalitis (EAE), where nanopores in the plasma membrane allow extracellular calcium into axons to promote degeneration (Witte et al., 2019). Future studies, employing both genetic and chemical tools to manipulate cADPR and ADPR might reveal significant insights into the contribution of these metabolites to Wallerian degeneration, axonal calcium homeostasis, and axon loss in other disease models.

In addition to a potential role for calcium mobilizing agents downstream of SARM1, a genetic screen in flies identified the gene Axundead (Axed) as an essential component downstream of SARM1 (Neukomm et al., 2017). Axed is a BTB/BACK domain containing protein, where loss of function mutations in Axed suppresses SARM1 induced axon degeneration in the fly (Neukomm et al., 2017). While genetic epistasis studies place Axed downstream of SARM1, it is not yet known if Axed is conserved in vertebrates. With the axon degeneration program conserved in flies and mice, it is reasonable to believe that if Axed is an essential component of this program, then a conserved vertebrate ortholog is present. Future genetic and/or biochemical screens may help identify this ortholog in vertebrates.

The relationship between the SARM1 NADase activity and Axed also remains to be described. Do metabolites from NAD⁺ cleavage such as ADPR or cADPR, function as activators of Axed to promote axon degeneration? It is interesting to note that overexpression of Axed by itself does not cause axon degeneration (Neukomm et al., 2017). With its BTB/BACK domain, this suggest that Axed may simply be a scaffold that brings essential executioner components together, hence expression of more scaffold will not necessarily drive the axon death program. On the other hand, the absence of degeneration upon expression of Axed also suggests that Axed must be activated by SARM1 or a SARM1 dependent signal. With the discovery of the SARM1 NADase activity, Axed may be activated by an NAD⁺ dependent signal such ADPR or cADPR.
No evidence currently exist that cADPR or ADPR binds to Axed. Freeman and colleagues also suggested that ADP-Ribosylation by SARM1 NADase may be the signaling event in the activation of Axed (Neukomm et al., 2017). Future studies should help clarify the link between the SARM1 NADase activity and Axed.

In addition to the genetic and molecular regulation of axon degeneration, it will be important to consider the sub-cellular processes such as mitochondrial homeostasis, autophagy that are important in the degeneration program (Gerdt et al., 2019; Yang et al., 2013). These studies may reveal significant insights into SARM1 independent alternative pathways that are activated in particular disease processes, but could be targeted for therapeutic benefit. Further, our treatment modalities to target axon breakdown in disease should extend beyond small molecule drugs. Recently, a gene therapy approach in mice using a dominant negative SARM1 was utilized to block axotomy induced axon degeneration (Geisler et al., 2019).

Beyond the role of SARM1 in axon degeneration, SARM1 has been implicated in development of the nervous system in C. elegans and in innate immunity pathways (Carty and Bowie 2019). Early studies in C. elegans revealed that the SARM1 ortholog in C. elegans, tir-1 was required for the asymmetric patterning of odorant receptors (Chuang et al., 2005). Later, it was also determined that tir-1 controls pathways involved in memory and forgetting (Inoue et al., 2013). In innate immunity, tir-1 was shown to control the expression of the antimicrobial peptides NLP-29 and NLP-31 during fungal or bacteria infections (Couillault et al., 2004). Further, a recent paper revealed a role for glial Drosophila SARM in a pathway involved in the clearance of dying neurons during development (McLaughlin et al., 2019). With the identification of the SARM1 NADase activity it now remains to be determined if the NADase
activity is required for any of these immune and developmental programs. These studies will expand our knowledge of the role of NAD$^+$ metabolism, TIR domains, and SARM1 in biology.

In order to better understand the contribution of the SARM1 NADase in the areas noted above, the molecular mechanism of the NAD$^+$ cleavage enzymatic activity needs to be well elucidated. Through structure homology modeling, this dissertation work revealed a conserved glutamic acid residue (E642) that is required for NAD$^+$ cleavage. This residue was also present in bacterial TIR domains, and was widely conserved in plant TIR domains. We posit that this residue is in the NAD$^+$ binding pocket of the SARM1 TIR domain; however, future structural studies including co-crystallization of SARM1 TIR with NAD$^+$ or NADP$^+$ are needed to confirm this. With the identification of TIR domain enzymes from bacteria, archaea, and plants, our findings have provided a broad selection of candidate enzymes to further investigate the catalytic mechanism of NAD$^+$ cleavage. These studies will share insights into commonalities between the TIR enzyme families, and may reveal differences that might explain observations such as the different products produced by different TIR proteins.

After I identified that the SARM1 TIR domain possesses NADase activity, I found that multiple prokaryotic TIR domains also possess NAD$^+$ cleavage activity. Prior to our TIR NADase discovery, bacterial TIR domains were implicated as virulence factors (Newman et al., 2006; Cirl et al., 2008; Walldhuber et al., 2017). Studies revealed that TIR containing proteins such as those from uropathogenic E. coli (E. coli CFT073) and S. aureus could be secreted from host bacteria (Cirl et al., 2008; Askarian et al., 2014). In the case of E. coli CFT073, it was further shown that TIR protein could enter host cells (Cirl et al., 2008). The prevailing model for the mechanism underlying this virulence factor is that of molecular mimicry in that bacteria TIR proteins via protein-protein interaction disrupt eukaryotic host TIR signaling (Walldhuber et al.,
Our studies now showing TirS and TcpC TIR domains from S. aureus and E. coli CFT073 are NAD$^+$ cleavage enzymes, suggests a re-evaluation of this model. Could the NADase activity contribute to the virulence of these bacterial TIR proteins? Or is the NADase activity dispensable? Incorporating the NADase-dead (Glutamic acid to Alanine mutation) of the bacterial TIR proteins into biological assays that examine TIR virulence may help answer such questions.

The bacterial NAD$^+$ cleavage activity has also recently been linked to an antiphage defense program (Doron et al., 2018). This finding opens the door to a myriad of questions. First, what molecular signals activate bacterial TIR NADases? This is an unanswered question not only for bacterial TIR domains, but also for SARM1 in axonal degeneration. It is still not completely clear how SARM1 is activated or kept off in a healthy axon to prevent spurious degeneration. From biochemical and genetic studies, we know NMNAT2 is an axo-protective factor that likely keeps SARM1 off (Gerdts et al., 2016). Metabolic flux analysis revealed that the cytosolic version of the NMNAT1 inhibits the SARM1 dependent NAD$^+$ depletion (Sasaki et al., 2016). Further, deletion of NMNAT can induce spontaneous axonal fragmentation in neuronal cultures, and causes a perinatal lethality in mice (Gilley and Coleman, 2010; Gilley et al., 2015). This embryonic lethality interestingly can be rescued by genetic deletion of SARM1 (Gilley et al., 2015), suggesting that part of the mechanism of SARM1’s activity or activation, involves NMNAT2. A recent study suggested that phosphorylation of SARM1 can enhance its NAD$^+$ cleavage activity, but it is unclear if this post-translational event occurs in axons and its contribution to axon degeneration (Murata et al., 2019). Similar to NMNAT2 keeping SARM1 off, the bacterial TIR NADase has to be regulated in some fashion to prevent the bacteria from
consuming its own NAD\(^+\) in and killing itself. Whether in bacteria this means the presence of an “antitoxin” gene or metabolite remains to be determined.

A second major unanswered question with the discovery of bacterial TIR domains as NAD\(^+\)ases in antiphage response is the downstream mechanism underlying this form of host defense. Studies from Doron and colleagues revealed that engineered host bacteria harboring TIR-like genes and their neighboring domains provide resistance to virus, and display a 10,000-fold reduction in viral titer upon viral infection (Doron et al., 2018). Moreover, deletion of either the TIR-like domain, or mutation of the catalytically glutamate to alanine reverses this viral resistance to control, non-engineered, bacteria levels (Doron et al., 2018). Future work may therefore seek to describe how TIR domains are restricting viral titers. Does the presence of the TIR domain produce effects that directly inhibit aspects of viral replication? Or are bacteria undergoing a form of programmed cell death, which is leading to the decreased production and/or survival of viruses? With the discovery of the SARM1 and plant TIR-NLRs as NAD\(^-\)ase enzymes driving forms of programmed cell death, it is tantalizing to speculate that bacteria are triggering a form of cell death or suicide upon sensing viral infection. Evidence from the literature indicates that bacteria can harbor toxin-antitoxin systems, and can trigger form of cellular suicide in response to various stresses (Allocati et al., 2015). This however raises a teleological point of debate of why a single cell organism will choose the option of suicide and kill itself. Future studies examining the events downstream of bacterial TIR NAD\(^-\)ases in host defense may help address some of these questions.

In this dissertation, we also report the discovery that TIR domains of plant immune receptors are NAD\(^+\) consuming enzymes. This finding addresses a long-standing mystery of how plant TIR-NLRs trigger cell death and disease resistance. Our findings suggest that an intrinsic
NAD\(^+\)/NADP\(^+\) cleavage activity of these receptors is activated upon effector recognition, and that this signals to downstream components to trigger cell death. While the discovery of plant TIR domains as NAD\(^+\) cleavage enzymes is a fundamental breakthrough, future work remains to decipher if NAD\(^+\) cleavage products activate EDS1, which is downstream of the NADase activity in the pathway. Similar to the models proposed above for axon degeneration and Axed, it is plausible that NAD\(^+\) cleavage products such as cADPR, ADPR, and the recently discovered v-cADPR bind to and activate EDS1. Binding assays that determine if NAD\(^+\) derived breakdown products bind to EDS1 may be insightful. The detailed chemical structure of v-cADPR also remains to be determined. We first discovered v-cADPR during our study of prokaryotic TIR domains. We found that 2 bacterial (A. baumannii and B. melitensis) and 1 archaeal (M. olleyae) TIR domains produce v-cADPR from NAD\(^+\) breakdown (Essuman et al., 2018). Subsequently, we found that two plant TIR domains (RBA1 and Bd) produce v-cADPR in large amounts, while small levels of v-cADPR were detected in in-planta lysates of plant RPP1 and SAM-RPS4 TIR domain proteins. Future structural studies should help clarify the chemical structure of v-cADPR, which will enable its synthesis in large quantities for utilization in biological assays. Unlike canonical cADPR it is not known if v-cADPR possesses calcium mobilizing activity in plants, animal, or bacterial cells; so this also remains to be tested.

The cleavage of NADP\(^+\) by plant TIR domains and other TIR domains also suggest that biological pathways mediated by TIR domains could significantly involve redox homeostasis and reactive oxygen species (ROS). NADPH (the reduced form of NADP\(^+\)) is a key component of cellular antioxidation systems (Ying, 2008), and perturbation in NADP\(^+\) levels could result in redox imbalances. TIR domains therefore by the cleavage of NAD\(^+\)/NADP\(^+\) could leave cells vulnerable to redox imbalances that could trigger downstream cell death pathways.
Finally, we were unable to detect NADase activity in TLR4 TIR domains and MyD88 TIR domains (Essuman et al., 2017). To date, it is not know if any of the mammalian TLRs, IL-1Rs, or other mammalian TIR adaptors besides SARM1 possesses TIR domains with enzymatic activity. It is possible that indeed the enzymatic activity is present in evolutionarily ancient TIR domains and been lost or repurposed through evolution. However, the lack of NADase activity with the majority of the mammalian TIR proteins could be related to technical issues of protein folding/solubility, the biological context of enzyme activation, or different substrates other than NAD$^+$ could be involved. The discovery that TLRs or IL-1R are enzymes would further transform our understanding of TIR domain function in innate immunity.

In summary, our discovery that TIR domain proteins represent a new family of NAD$^+$ cleavage enzymes redefined the function of this canonical scaffolding domain. This finding identified a therapeutic target for axonopathies, and opens the door for new investigations in innate immunity, metabolism, and axon degeneration pathways.
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2018 Best Talk/Oral Presentation, Washington University MSTP Retreat
2017 FEBS Journal Poster Prize, FASEB Conference on NAD⁺ Metabolism and Signaling
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2013 DeNardo Summer Research Fellowship
2012 Washington University School of Medicine Distinguished Faculty Scholarship
2010 Alliance for Minority Participation (AMP) Award for Research
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Research Experiences

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Defining TIR domain proteins as a family of NAD\(^+\) consuming enzymes that drive a conserved host defense pathway
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Publications


Degeneration to Innate Immunity and Beyond. *J. Immunol.* 2019. 202 (1 Supplement) 64.1 (Abstract)


**Patents/Intellectual Property**

2016 Inhibitors of SARM1 NADase Activity and Uses Thereof (WO2018057989A1)

2018 Identification of Inhibitors of TcpC and TIR NADase Activity (WO/2019/067625)

**Selected Oral Presentations/Posters**


Essuman K, Summers DW, Sasaki Y, Mao X, DiAntonio A, Milbrandt J. The SARM1 Toll/Interleukin-1 Receptor Domain Possesses Intrinsic NAD⁺ Cleavage Activity that Promotes Pathological Axonal Degeneration. Presented at ASCI-AAP-APSA Joint Meeting 2017; Washington University Medical Scientist Training Program Retreat 2017; and Washington University MGG-CSB-HSG Retreat 2017 (Poster Presentation)

Essuman, K., Gerdts, J., DiAntonio, A., Milbrandt, J. A programmed death pathway in the axon. ASCI-AAP-APSA Joint Meeting 2016 (Poster Presentation)


Teaching Experiences
2018 Washington University School of Medicine
Teaching Assistant /Mentored Teaching Experience
The Human Body: Anatomy, Embryology, and Imaging

2015 – 2018 Washington University School of Medicine
Small Group Leader
Diversity Retreat: First Year Medical Student Orientation

2008 – 2010 Temple University
Math and Science Tutor for undergraduates

Leadership and Service Experiences
2016 – 2017 Washington University School of Medicine
Learning Climate Committee
Student Representative

2015 - 2016 Howard Hughes Medical Institute Medical Fellows Program
Co-Chair, Midwest Group

2013 - 2014 Washington University School of Medicine
Christian Medical Association Chapter
Co-Leader

2012 – 2013 Washington University School of Medicine
Molecular Foundations of Medicine; Genetics
Co-Course Liaison

References
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