DSARM/SARM1 GOVERNS A CONSERVED AXON DEATH PROGRAM

A Dissertation Presented

By

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This work is dedicated to my family, for their unconditional love, support, and encouragement, and for instilling in me a life-long love and passion for learning.

"The roots of education are bitter, but the fruit is sweet." – Aristotle

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Abstract

Axonal and synaptic degeneration is a hallmark of peripheral neuropathy, brain injury, and neurodegenerative disease. Axonal degeneration has been proposed to be mediated by an active autodestruction program, akin to apoptotic cell death; however, loss-of-function mutations capable of potently blocking axon self-destruction have not been described. Using a forward genetic screen in *Drosophila*, we identified that loss of the Toll receptor adaptor dSarm (sterile a/Armadillo/Toll-Interleukin receptor homology domain protein) cell-autonomously suppresses Wallerian degeneration for weeks after axotomy. Severed mouse Sarm1 null axons exhibit remarkable long-term survival both in vivo and in vitro, indicating that Sarm1 prodegenerative signaling is conserved in mammals. Our results provide direct evidence that axons actively promote their own destruction after injury and identify dSarm/Sarm1 as a member of an ancient axon death signaling pathway. This death signaling pathway can be activated without injury by loss of the N-terminal self-inhibitory domain, resulting in spontaneous neurodegeneration. To investigate the role of axon self-destruction in disease, we assessed the effects of Sarm1 loss on neurodegeneration in the SOD1-G93A model of amyotrophic lateral sclerosis (ALS), a lethal condition resulting in progressive motor neuron death and paralysis. Loss of Sarm1 potently protects motor axons and synapses from degeneration, but only extends animal survival by 10%. Thus, there appears to be at least two driving forces in place during ALS disease progression: (1) Sarm1 mediated axon death, and (2) cell body destruction via some unknown mechanism.

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List of Symbols, Abbreviations, or Nomenclature

6-OHDA	6-hydroxydopamine
AAD	acute axonal dieback
AChR	acetylcholine receptor
ALS	amyotrophic lateral sclerosis
AP-1	activator protein-1
APP	amyloid precursor protein
ARM	armadillo/HEAT domain
ASK1	apoptosis signaling kinase-1
ATP	adenosine triphosphate
CaMK	Calcium/calmodulin-dependent protein kinase
CIPN	chemotheraphy induced peripheral neuropathy
CMAP	compound muscle action potential
CMT	Charcot-Marie-Tooth disease
DLK	dual leucine kinase
DRG	dorsal root ganglia
EAE	experimental autoimmune encephalomyelitis
EGTA	ethylene glycol tetraacid acid
EMS	ethyl methane sulfonate
ER	endoplasmic reticulum
ey	Eyeless
FLP	Flippase
FRT	flippase recognition target
GAD	gracile axonal dystrophy
GDC	granular disintegration of the axonal cytoskeleton
GSK3	glycogen synthase kinase 3
IKK	Iκβ kinase
IRF	interferon regulatory factors
JNK	c-Jun kinase
MAP	microtubulin associated protein
MAPK	mitogen-activated protein kinase
MARCM	mosaic analysis with a repressible cell marker
MB	mushroom body
MOG	myelin oligodendracyte glycoprotein
MS	multiple sclerosis
MUNE	motor unit estimate
NAD	nicotinamide adenine dinucleotide

NF	Neurofilament
NFκβ	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
nls	nuclear localization sequence
NMJ	neuromuscular junction
Nmnat	nicotinamide/nicotinic acid mononucleotide adenylyltransferase
OGD	oxygen glucose deprivation
ORN	olfactory receptor neuron
pmn	progressive motorneuronopathy
PMP22	peripheral myelin protein of 22kDa
pttg-1	pituitary tumor transforming-1
ROS	reactive oxygen species
SAM	sterile alpha motif
Sarm	sterile alpha and Armadillo motif
SCG	superior cervical ganglia
SMA	spinal muscular atrophy
SOD	superoxide dismutase
TIR	Toll/Interleukin-1 receptor homology domain
TLR	Toll-like receptor
TRIF	TIR-domain containing adapter-inducing interferon-B
UAS	upstream activating sequence
Ube4B	Ubiquitin conjugation factor E4B
UPS	ubiquitin proteosome system
UTR	untranslated region
VCP	valosin-containing protein
Wlds	slow Wallerian degeneration
WNV	West Nile Virus

Preface

I conducted all of the work presented here, in the laboratory of Marc Freeman, except where noted at the beginning of each chapter.

Some negative results have been excluded from this document, including RNAi and mutant lines that showed no suppression of Wallerian degeneration.

Excluded are data on the gene *progressive motorneuronopathy*, which I cloned into a pUASt vector, because expression of this mutant had no impact on Wallerian degeneration in *Drosophila*; I also cloned *hax-1* into a pUASt vector, but it too had no impact on Wallerian degeneration.

I recombineered a GFP tag just prior to the endogenous stop codon in a BAC containing full length dSarm and 40 kb of surrounding genomic region; we were unsuccessful at recovering successful genomic integrations of this BAC and as such this work is not presented here.

CHAPTER I: Introduction

Why study axons?

Neurons are amazingly complex cells that broadcast information by transmitting electrical and chemical signals. This information is organized by the bipolar nature of neurons, and each compartment of a neuron plays its own exclusive role in the overall activity of the cell. A neuron's dendrites may receive a variety of signals, while the axon delivers the output signal to the connecting neurons, muscles, or glands. An axon is a distinctive and select neuronal process; while most neurons will have many dendrites and many synaptic inputs, not all inputs will result in the neuron firing an action potential. However, once the threshold for signaling is reached, the singular axon, with its many branches, will transmit this signal as one unified output action potential to all cells with which the axon connects.

Axons are incredibly unique, particularly in terms of their size: human motor neurons, for example, have cell bodies that can be up to 80 μm in diameter, while their axons can be over one meter long (Kanning et al., 2010). The sheer size of some axons puts a huge burden on the entire cell, especially axon transport mechanisms that bring proteins from the soma (De Vos et al., 2008) and the trophic support from the surrounding glia (Nave and Trapp, 2008). Repetitive firing and structural maintenance place a huge energetic drain on neurons, and axons have accordingly evolved their own unique biology to keep up with the strenuous demands placed on neurons (Coleman and Freeman, 2010).

Axons fragment after transection

Axon integrity is crucial for a neuron's function; without an intact axon, a neuron is essentially useless. What happens to an axon when its integrity is compromised? Waller first described this phenomenon as he attempted to trace nervous system wiring in the frog tongue (Waller, 1850). Waller noticed that cutting an axon away from its cell body resulted in a stereotyped beading, blebbing, and eventual fragmentation of the severed axon. The axon fragments are eventually "removed by absorption." Axon fragmentation and eventual engulfment after injury together is now known as Wallerian degeneration (Coleman, 2005). Many assumed Wallerian degeneration occurred because the severed axon starved, as it was no longer connected to the presumed singular cell nutrient source, the soma (Lubinska, 1977). This passive wasting away of the severed axon was suspected to have important implications during neurological diseases, as Waller hypothesized that one would see similar "alterations" of nerve fibers, particularly during paralysis (Waller, 1850).

Many years after his initial observations, multiple groups described in detail the biochemical, histological, and ultra-structural changes that occur in severed axons (Johnson et al., 1950): first, there is acute axonal dieback (AAD) on either side of the lesion site, where a short segment of the axon retracts and fragments within minutes after injury (Kerschensteiner et al., 2005); second, the severed axon undergoes a defined latency period, characterized by microtubule depolymerization and a "metabolism failure," with massive decreases in essential metabolic molecules such as adenosine

triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD) (Wang et al., 2005); and finally the severed axon undergoes robust and catastrophic fragmentation, known as granular disintegration of the axonal cytoskeleton (GDC) (Ma et al., 2013), that includes neurofilament breakdown and mitochondrial swelling (Figure 1-1) (Park et al., 2013). This entire process takes between 24-28 hours *in vivo* (Lubinska, 1977; Beirowski et al., 2004; Beirowski et al., 2005) and 12-24 hours *in vitro* (Glass et al., 1993) for mammals.

AAD is a unique part of Wallerian degeneration in that it occurs equally both on the proximal and distal sides of the lesion. The 200-300 μm of axon directly on either side of the lesion site degenerates in less than 5 minutes, occurring within the first hour after lesion (Kerschensteiner et al., 2005). The axon proximal to the injury site may have the ability to regenerate, and AAD is believed to be important for growth cone formation at the remaining axon stump still attached to the soma (Spira et al., 2003). However, a specific role, if any, for AAD in the fragmentation of the distal severed axon remains unclear (Kerschensteiner et al., 2005).

Intra-axonal calcium is required for Wallerian degeneration

Immediately after injury and during AAD, a spike in intra-axonal calcium is observed (Ziv and Spira, 1993; Knoferle et al., 2010; Koch et al., 2010; Adalbert et al., 2012; Avery et al., 2012). The immediate consequence of this calcium spike is the fusion of vesicles at the site of lesion, which seals off the membranes on either side of the lesion (Eddleman et al., 1998). A large calcium spike coincides with AAD (Kerschensteiner et al., 2005), and there is a slow rise in intra-axonal calcium levels in

the distal severed axon during the subsequent latency period (Adalbert et al., 2012). This calcium rise does not appear to be due to ionic leaking or an electrical balance for other ions, as pharmacological blockade of sodium channels does not affect the calcium rise or Wallerian degeneration (George et al., 1995; Press and Milbrandt, 2008).

Where is this calcium coming from? Addition of ethylene glycol tetraacid acid (EGTA) to cell culture media, which chelates extracellular calcium, strongly blocks injury-induced axon degeneration (Schlaepfer and Bunge, 1973), and this blockage is reversed upon the addition of calcium ions back to the media (George et al., 1995). Broad blockade of calcium channels with either cobalt or manganese ions also strongly inhibit axon degeneration after injury *in vitro* (George et al., 1995). Addition of calcium ionophores induces spontaneous axon degeneration in the absence of injury, suggesting that calcium influx is necessary and sufficient to induce axon destruction (George et al., 1995). Voltage gated L-type calcium channels specifically appear to allow calcium into the axon after transection, as pharmacological inhibition of these, but not other calcium channel types, blocks injury-induced degeneration *in vitro* (George et al., 1995) and *in vivo* (Knoferle et al., 2010). Together, these data suggest that extracellular calcium specifically enters the axon via L-type calcium channels to drive Wallerian degeneration.

Calcium entry can activate a variety of intracellular processes, including protein degradation due to the calcium-dependent cysteine proteases calpains. Pharmacological calpain inhibition completely blocks AAD (Kerschensteiner et al., 2005) and weakly suppresses Wallerian degeneration in the distal severed axon *in vitro* (George et al.,

1995; Zhai et al., 2003). Genetically expressed calpain inhibitors also delays fragmentation *in vivo* (Ma et al., 2013). Calpains appear to be involved specifically in the fragmentation phase of Wallerian degeneration, as a genetically encoded calpain inhibitor specifically inhibits neurofilament breakdown but not microtubule disruption (Ma et al., 2013). The weak protection afforded by calpain inhibitors is nowhere near as strong as the protection with EGTA treatment, suggesting that another parallel calcium-dependent mechanism is likely activated during Wallerian degeneration (Park et al., 2013).

Interestingly, axons possess an intrinsic mechanism by which to buffer axonal calcium levels: mitochondria. Addition of cyclosporine A (CsA) to severed mouse sciatic nerve explants robustly blocks degeneration by preventing calcium efflux from mitochondria, effectively increasing the amount of axonal calcium that these mitochondria can buffer (Barrientos et al., 2011). Mitochondrial calcium levels do rise during the latent phase of Wallerian degeneration, although it appears that their normal buffering capacity is not enough to offset the calcium-induced changes that occur after injury (Sievers et al., 2003).

Studies using calcium chelation and channel blockers suggest that calcium activates calpains (see above) to induce neurofilament breakdown and microtubule deterioration. Intriguingly, microtubule depolymerization still occurs in injured neurons cultured in calcium-free media, suggesting that calcium is not required for depolymerization, only for microtubule breakdown (Park et al., 2013). Studies on

microtubule destabilization agents suggest that this sort of cytoskeletal breakdown can induce the variety of downstream changes seen in degenerating neurons, including metabolic failure and eventual fragmentation (Wang et al., 2001; Ikegami and Koike, 2003; Park et al., 2013). It has not been reported if removal of extracellular calcium during the latent phase (i.e., after microtubule breakdown has occurred) can stall degeneration, so it is unclear if calcium is required only for the initial activating steps or throughout Wallerian degeneration.

Wld^{s}: new insights on axon degeneration

Despite the wealth of descriptions on morphologic changes during Wallerian degeneration, it was assumed that this process was passive; therefore no one searched for a genetically encoded pathway that might control Wallerian degeneration (Coleman and Freeman, 2010). The search for an elusive Wallerian degeneration gene inadvertently and serendipitously began by studying the recruitment of macrophages to injured nerves. It had been previously described that a sciatic nerve lesion causes myelinating Schwann cells to undergo mitosis and recruit macrophages to the severed axon. While studying this process, a random mutant strain of mice, C57/Bl6/Ola, was found to have no macrophage recruitment to the injured nerve. Subsequence analysis found that axons in these mice were not degenerating after lesion, and thus no pro-inflammatory signal was being released to draw in macrophages (Lunn et al., 1989). These severed mutant axons remained intact for 2-3 weeks after axotomy, while wild type axons degenerate within 36 hours of lesion (Lunn et al., 1989).

What sort of mutation could block severed axons from degenerating? After several generations of backcrossing, it was found that the mutation (1) acted in a dominant fashion (Perry et al., 1990b) and (2) mapped to mouse chromosome four (Lyon et al., 1993). The mutation in the C57/Bl6/Ola strain was eventually termed slow Wallerian degeneration (*Wld*^{s}) (Perry et al., 1990b) and found to be the result of a tandem triplication (Coleman et al., 1998). This triplication resulted in the overexpression of a novel chimeric fusion protein, Wld^{s}, comprised of: the non-enzymatic first 70 amino acids of Ubiquitin conjugation factor E4B (Ube4B); an 18 amino acid linker from the endogenous 5' untranslated region (UTR) of *nicotinamide/nicotinic acid mononucleotide adenylyltransferase-1* (*Nmnat1*); and full length Nmnat1, a NAD biosynthetic enzyme (Conforti et al., 2000; Mack et al., 2001). Wld^{s} cell autonomously protects severed axons (Perry et al., 1990a; Glass et al., 1993) in a dose-dependent manner (Mack et al., 2001). Expression of Wld^{s} not only affects Wallerian degeneration in mice (Gillingwater et al., 2003; Beirowski et al., 2004; Beirowski et al., 2005) but also protects severed axons from Wallerian degeneration in cell culture (Buckmaster et al., 1995), rats (Adalbert et al., 2005), and *Drosophila* (MacDonald et al., 2006). Interestingly, local protein synthesis does occur in severed axons of both wild type and Wld^{s}-expressing mice (Court et al., 2008), but *de novo* protein synthesis is not required for degeneration or Wld^{s} protection (Gilley and Coleman, 2010). Because Wld^{s} is a chimeric molecule and not itself a normally

occurring gene, it was proposed to act in an unknown gain-of-function manner (Coleman, 2005).

Severed Wld^s nerves show histological preservation of the synapse at both the neuromuscular junction (NMJ) (Wang et al., 2001; Adalbert et al., 2005) as well as the cortex (Gillingwater et al., 2006a; Wright et al., 2010), but does this protection extend past morphology? Do intact severed axons still retain their membrane potential and their functional ability to transmit action potentials? Electrical stimulation of a severed wild type motor axon can evoke normal electrical responses in the muscle, measured as compound muscle action potentials (CMAPs), for 10 hours after lesion, but rapidly decay within the next 8 hours (Moldovan et al., 2009). Wld^s–expressing motor axons showed a substantially slower decay; CMAPs appeared normal for the first 24 hours after lesion, and then deteriorated for the six following days. Unlike wild type animals, Wld^s mice retained some functionally connected synapses a week after axotomy, albeit at a greatly diminished level (Moldovan et al., 2009). Thus, Wld^s protects axons, and to a lesser extent synapses, from degenerating both morphologically and electrically after axotomy.

How and where does Wld^s act to protect axons?

Surprisingly, the Wld^s protein was found enriched in punctae in the nucleus, suggesting that it played a possible role in gene regulation (Jia et al., 2007). When mRNA populations were compared, Wld^s expressing neurons showed a substantial decrease in the mRNA of a single gene, pituitary tumor transforming-1 (*pttg*); however,

pttg1 knockout mice showed normal Wallerian degeneration (Gillingwater et al., 2006b). Proteomic studies have been done on wild type and Wld^s nerves, and there are significant differences in protein expression levels between the two nerves; a few molecules regulating Wallerian degeneration have been identified by this method, but mutants do not show protection at the level of Wld^s (Wishart et al., 2007; Wishart et al., 2012).

The nuclear Wld^s punctae appeared to co-localize with valosin-containing protein (VCP) through the Ub4b portion of Wld^s (Laser et al., 2006; Wilbrey et al., 2008); it has been suggested that this interaction may alter the ubiquitin-proteosome system (UPS) in transected axons (Coleman and Freeman, 2010). Blockade of the UPS alone can delay injury-induced degeneration by a few days (MacInnis and Campenot, 2005; Hoopfer et al., 2006; Zhai et al., 2003). Interestingly, calcium ionophore-induced degeneration is not blocked by UPS inhibition, suggesting that the involvement of the UPS in axon degeneration is calcium independent (Zhai et al., 2003) or that calcium activates a destruction program that is unrelated to protein degradation. However, a connection between VCP and the UPS is not now believed to be critical for Wld^s function (Beirowski et al., 2010).

The nuclear localization of Wld^s is a result of the nuclear localization sequence (nls) in Nmnat1 (Avery et al., 2009; Babetto et al., 2010). However, small amounts of Wld^s can be found out in the synapse (Avery et al., 2012), even though the vast majority of this overexpressed protein is in the nucleus. While the exact mechanism of Wld^s

protection is still unknown, the non-nuclear expression of Nmnat now appears to be crucial for the protective role of Wld^s (Babetto et al., 2010).

The role of Nmnat and the functional site of Wld^s protection from Wallerian degeneration were controversial for quite some time. Multiple groups reported that enzymatic activity of the Nmnat1 portion was essential for the function of Wld^s (Fang et al., 2005; Jia et al., 2007; Avery et al., 2009; Conforti et al., 2009), as were the first 16 amino acids of the Ube4B portion (Avery et al., 2009; Conforti et al., 2009). Some groups reported no protection from Wallerian degeneration with overexpression of Nmnat1 *in vitro* (Watanabe et al., 2007) and *in vivo* (Conforti et al., 2007), while other groups did see protection *in vitro* (Araki et al., 2004).

These discrepancies were eventually rectified when groups looked at the effect of other Nmnat1 homologs on Wallerian degeneration. In mammals, there are 3 homologs of Nmnat: Nmnat1, which is in the nucleus; Nmnat2, which is in the golgi; and Nmnat3, which is mitochondrial (Berger et al., 2005; Lau et al., 2010). Of the homologs, Nmnat2 is enriched specifically in mammalian neurons (Cahoy et al., 2008). Overexpression of a mutated form of Nmnat1 with a disrupted nls, which relocalizes the protein from the nucleus to the cytoplasm, confers robust protection after axotomy *in vivo* (Sasaki et al., 2009). Fusion to a peptide from amyloid precursor protein (APP), which targets the protein out of the nucleus, also confers Wld^s-like protection (Babetto et al., 2010), as does expression of Nmnat3 (Yahata et al., 2009). Mutations in the nls of Nmnat1 within Wld^s (termed "non-nuclear Wld^s") protect severed axons significantly better than the

original Wld^s protein – up to 7 weeks after axotomy (Beirowski et al., 2009). All of these data taken together suggests that overexpression of non-nuclear Nmnat can protect axons from Wallerian degeneration. Thus, the N-terminus (i.e., Ube4B portion) of Wld^s likely helps to direct some of the protein out of the nucleus, where even small amounts of axonal Wld^s protect from Wallerian degeneration (Coleman and Freeman, 2010). Furthermore, addition of cytosolic Nmnat1 protein directly to axons after injury through a lentiviral vector can prevent Wallerian degeneration (Sasaki and Milbrandt, 2010), strongly arguing that Wld^s is acting somewhere in the axon after injury to prevent Wallerian degeneration.

Where within the axon could Wld^s or non-nuclear Nmnat1 be acting? The protection by overexpression of mitochondrial Nmnat3 hinted that mitochondrial Wld^s might be important for its protective abilities (Yahata et al., 2009). Subcellular fractionation has identified Wld^s protein in the mitochondria (Yahata et al., 2009; Babetto et al., 2010), and multiple groups have presented evidence that specifically the levels of Nmnat in the mitochondrial matrix is what correlates with axonal protection (Yahata et al., 2009; Avery et al., 2009; Avery et al., 2012). Furthermore, mitochondria from Wld^s-expressing animals have a fundamental difference compared to wild type mitochondria: they are able to buffer higher levels of calcium (Avery et al., 2012). Wld^s-expressing axons also do not show normal calcium spikes after injury (Avery et al., 2012; Adalbert et al., 2012), supporting the hypothesis that Wld^s protects axons by increasing the calcium buffering capacity of mitochondria (Figure 1-2).

NAD and axon degeneration

How exactly a NAD biosynthesis enzyme is able to potently protect severed axons from degeneration remains unclear. Basal NAD levels are not different in Wld^s nerves when compared to wild type (Mack et al., 2001). However, NAD levels fall quickly after axotomy in wild type neurons but not Wld^s neurons (Wang et al., 2005). The addition of NAD *in vitro* before injury can delay degeneration, suggesting that NAD depletion may be a driving force in the initiation of Wallerian degeneration (Araki et al., 2004). Addition of NAD at very high levels (1-20 mM) a few hours after axotomy can also delay Wallerian degeneration (Wang and He, 2009), as can addition of adenosine (Press and Milbrandt, 2009) or nicotinamide (Wang et al., 2005). This would suggest that NAD depletion alone is responsible for Wallerian degeneration, but mutating genes involved in NAD catabolism is not enough to block axon degeneration, even though the axons have higher levels of NAD before injury (Sasaki et al., 2006). Furthermore, lowering neuronal levels of NAD did not block the ability of non-nuclear Nmnat1 to protect severed axons (Sasaki et al., 2006). This suggests that there is something unique about Nmnat in the context of Wallerian degeneration beyond its ability to synthesize NAD. While enzymatic activity of Nmnat is essential for its protective abilities (Avery et al., 2009; Conforti et al., 2009), it is unknown if Nmnat's enzymatic activity is restricted to producing NAD. There have not been reports on other substrates for Nmnat, but it remains possible that the protective abilities of Wld^s are related to an unknown enzymatic function of Nmnat beyond NAD biosynthesis. This enzymatic activity may be

related to increasing mitochondrial calcium buffering capacity (see above), and it is unknown if addition of NAD directly to mitochondria can increase this buffering capacity.

Three additional models for the role of NAD in Wallerian degeneration have emerged: (1) axotomy triggers the increased activation of NAD-consuming proteins, which deplete the NAD pool and lead to eventual energetic catastrophe (Wang and He, 2009); (2) high levels of NAD are simply required for normal maintenance of the axon, and the axon falls apart when these maintenance needs are unmet (Wang and He, 2009); or (3) NAD can modulate calcium channel openings (Tamsett et al., 2009), and the high levels found in Wld^s axons after injury prevent the influx of calcium and all of the downstream consequences of high axonal calcium levels (Wang and Barres, 2012). However, none of these explanations fully explains the Wld^s phenomenon; nevertheless, both Nmnat and NAD appear to play important, albeit mysterious, roles in protecting axons from Wallerian degeneration.

An endogenous axon survival signal?

Axonal Nmnat has the unique ability to protect severed axons from Wallerian, so it was suggested that endogenous Nmnat may play a role in normal neuronal health. Knock down of *Nmnat1* or *Nmnat3* has no effect on cultured neurons; however, knock down of *Nmnat2* induces spontaneous degeneration. This spontaneous degeneration is blocked with expression of Wld^s, which has been proposed to protect these axons by substituting for the enzymatic activity of Nmant2 (Gilley and Coleman, 2010).

Coincidentally, the half-life of endogenous Nmnat2 after axotomy correlates with the onset of degeneration (i.e., microtubule disruption and neurofilament breakdown) (Gilley and Coleman, 2010). This has been interpreted to mean that Nmnat2 is an endogenous "survival factor" coming from the soma that is required for axon integrity, and that Wallerian degeneration occurs because this survival factor is no longer coming from the nucleus. However, Nmnat2 is a crucial component of metabolism, so it is not surprising that knock down induces spontaneous degeneration. While it is an intriguing observation, half-life correlation with the latent phase is hardly enough to show that Nmnat2 is an endogenous survival factor, and it is unknown how many other molecules show a similar half-life in a degenerating axon.

An endogenous axon death signal?

If there is not a survival factor being actively transported from the soma to the axon, then a "death signal" may somehow be activated in the axon after injury. It has been suggested that this death signal, activated by injury, uncages calcium, allowing for the activation of the calcium-dependent destructive mechanisms associated with Wallerian degeneration (Wang and Barres, 2012). However, there are calcium-independent changes associated with injury-induced degeneration, such as microtubule depolymerization (Park et al., 2013). Nonetheless, there is evidence of calcium-activated kinases, such as calcium/calmodulin-dependent protein kinases (CaMKs), which regulate cellular stress responses (Ding and Nam Ong, 2003; Song, 2013). Could axotomy trigger a pro-degeneration kinase-signaling cascade? Recent studies have

linked c-Jun kinase (JNK) signaling through dual leucine kinase (DLK) to axon degeneration (Miller et al., 2009) as well as glycogen synthase kinase 3 (GSK3) and Iκβ kinase (IKK) (Gerdts et al., 2011). Importantly, pharmacological inhibition of any of these kinases affords axon protection that is far weaker than Wld^{s}, so these kinase cascades are likely not the only regulatory pathway governing Wallerian degeneration.

A clear signaling cascade has not been identified which drives Wallerian degeneration, so axons could be degenerating via a mechanism similar to necrosis. In support of this, severed axons undergo the rapid ATP loss, which is a hallmark of necrosis (Wang and He, 2009); however, another chacteristic of necrosis is the release of calcium from intracellular stores (mainly the endoplasmic reticulum (ER) and mitochondria), and the calcium influx during Wallerian degeneration has been shown to be from an extracellular source (George et al., 1995). If not necrosis, then could severed axons being "dying" via apoptotic cell death pathways?

Apoptosis seemed like a logical candidate as a mechanism for Wallerian degeneration, as fragmenting axons share some features of apoptosis (Coleman, 2005), such as phosphatidylserine residue exposure on the plasma membrane and loss of mitochondrial membrane potential (Sievers et al., 2003). Canonical apoptosis signaling was found to be nonessential for Wallerian degeneration, as *bax-bak* double knock out mice are not protected against Wallerian degeneration despite a broad blockade of cell death (Whitmore et al., 2003). Pharmacological inactivation of caspases (Finn et al., 2000) and serine-proteases (Zhai et al., 2003) were also unable to block Wallerian

degeneration. All of this taken together suggests that if Wallerian degeneration is indeed a genetically encoded program, then it occurs through some unique mechanism independent of known cell death pathways (Coleman and Freeman, 2010).

Axon degeneration beyond injury

Wld^s raised another important question: are all forms of axon degeneration the same? Axons can undergo degeneration not only after physical lesion, but also during (1) developmental pruning; (2) programmed cell death (both induced and developmental); and (3) during neurodegenerative diseases (Luo and O'Leary, 2005). Expression of Wld^s does not block developmental neurite pruning in either mice or *Drosophila* (Hoopfer et al., 2006), although it may offer a slight delay (Schoenmann et al., 2010). Interestingly, the UPS plays a major role in axon degeneration due to developmental pruning, so Wallerian degeneration and pruning may eventually funnel through some common degeneration mechanisms, although the initiating element appears to be unique for each process (Saxena and Caroni, 2007). Developmental cell death occurs normally in Wld^s mice, also suggesting that Wld^s does not affect apoptosis (Deckwerth and Johnson, 1994). Furthermore, Wld^s protection appeared to be axon-specific: during neuronal apoptosis induced by nerve growth factor (NGF) deprivation, Wld^s neurites are spared, but not their cell bodies (Deckwerth and Johnson, 1994). Recent studies highlight that trophic factor-induced degeneration is clearly molecularly distinct from Wallerian degeneration (Nikolaev et al., 2009; Vohra et al., 2010). Thus, there appears to be multiple ways to kill an axon: via apoptosis (i.e., developmental or

NGF withdrawal), developmental pruning, or injury induced Wallerian degeneration (Luo and O'Leary, 2005).

Wallerian-like degeneration during disease

Axon degeneration can also occur when neurons are exposed to toxic agents; for example, peripheral neuropathies are hallmarks of chemotheraphy treatment (Coleman, 2005). Wld^s was found to delay axon degeneration resulting from a variety of chemical insults *in vitro*, including vincristine (Wang et al., 2001), taxol (Wang et al., 2002), and vinblastine (Ikegami and Koike, 2003). These agents cause what is now termed "Wallerian-like degeneration," meaning that the spontaneous degeneration observed is modified by Wld^s, even though there has not been a physical transection. Amazingly, Wld^s protection is specific to the axon, as cell body death is not prevented with any of the above treatments. These drugs act by destabilizing microtubules, to which the axon seems especially sensitive; this suggests that Wld^s may to be acting, either directly or indirectly, by stabilizing microtubules. Overexpression of cytosolic Nmnat1, like Wld^s, can also delay pharmacologically induced axon degeneration: rotenone, a mitochondrial complex 1 inhibitor that causes a catastrophic accumulation of reactive oxygen species (ROS), induces axonal degeneration that is blocked by cytosolic Nmnat1 (Press and Milbrandt, 2008). Thus, Wld^s appears to be broadly protective against chemically induced axon degeneration.

Waller speculated that the degeneration he witnessed after cut may have some connection with neurodegenerative disease (Waller, 1850). Even though physical

transection is clinically rare (Coleman and Freeman, 2010), axon degeneration is a hallmark of essentially every neurodegenerative condition (Raff et al., 2002), and the severity of axon damage is often linked with outcome and the severity of the disease (Medana and Esiri, 2003). In many neurodegenerative diseases, synaptic and axonal loss occurs long before symptoms develop (Saxena and Caroni, 2007). However, it is unknown if the axonal pathology is a driving force in the disease or merely the result of a sick neuron (i.e., the soma) (Cavanagh, 1964). The Wld^{s} mouse allowed for this question to be addressed: if a particular disease model were suppressed in the Wld^{s} background, this would argue that the axonal-die back is a driving force in disease progression (Coleman and Perry, 2002).

This appears to be the case in the progressive motorneuronopathy (pmn) mouse model of motor neuron disease. This disease model, the result of a mutation in a tubulin chaperone, causes severe motor neuron loss and animal death within 6 weeks of birth (Martin et al., 2002) and is used as a model for spinal muscular atrophy (SMA) (Luo and O'Leary, 2005). Wld^{s} strongly protects against both axon and cell body death in pmn mice (Ferri et al., 2003; Simonin et al., 2007), suggesting that in this model of motor neuron disease, axonal degeneration is a initiating factor behind cell body death. Unfortunately, this robust protection was not seen in other mouse models of a motorneuron disease, such as the superoxide dismutase-1 (SOD1) models of amyotrophic lateral sclerosis (ALS) (Vande Velde et al., 2004; Fischer et al., 2005).

Other models of neurodegeneration show some degree of protection with expression of Wld^s; while axon protection appears to delay overall disease progression, this effect is not nearly as robust as in the *pmn* mice. Wld^s is able to modestly protect axons in a mouse model of myelin-related axonopathy, a severe inherited peripheral neuropathy found in humans due to deficiencies in myelin component P0 (Samsam et al., 2003). Injection of another myelin component, myelin oligodendracyte glycoprotein (MOG), causes experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS). Wld^s modestly protects in this EAE model, as does injections with nicotinamide (Kaneko et al., 2006). In a rat model of Charcot-Marie-Tooth disease (CMT) 1A, caused by a duplication of the peripheral myelin protein of 22kDa (PMP22) (Meyer zu Horste et al., 2011), and a mouse model of gracile axonal dystrophy (GAD) (Mi et al., 2005), Wld^s protects against axon and synapse loss, although it does not alter clinical impairments, such as grip strength and rotarod performance. Parkinson's like symptoms can be induced in mice by injection of 6-hydroxydopamine (6-OHDA) into the striatum; Wld^s can protect axons, but not cell bodies, from 6-OHDA induced death (Sajadi et al., 2004). These data taken together suggests that axon death is likely not the only driving force in disease progression, but preserving axon integrity can prolong the lifespan of these neurons to some extent. Furthermore, these studies on Wld^s and neurodegenerative disease models link axon death with injury induced axon degeneration, suggesting that a common axon death pathway may exist.

Unfortunately, because Wld^s is not an endogenous molecule, it does not itself present a druggable-target for pharmaceutical treatment. However, if an endogenous pathway was discovered that mediated Wallerian degeneration, this pathway could potentially be a target for pharmaceutical treatment of a variety of disorders that result in axon death, including chemotherapy-induced peripheral neuropathies (CIPNs). There is a crucial need to identify, if it does indeed exist, this endogenous axon death pathway. Vertebrate and pharmacological studies have been unable to shed any light on this mysterious endogenous pathway so far; thus, a new model needed to be established with which the genetics of Wallerian degeneration could be studied.

Drosophila: an ideal genetic tool for studying Wallerian degeneration

Currently, there are two model organisms well suited for performing genome-wide genetic screens with respect to neuronal function: the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. *C. elegans* severed axons rarely undergo Wallerian degeneration; in fact, regenerating axons fuse with the intact severed axon to restore axonal integrity and function (Neumann et al., 2011). In contrast, *Drosophila* severed axons undergo Wallerian degeneration in a manner that is morphologically and molecularly very similar to vertebrate axons (MacDonald et al., 2006). Thus, the fruit fly appears ideal for the discovery of a unique genetically encoded axon death program.

Recent advances in *Drosophila* genetics have primed the community to perform robust unbiased forward genetic screens. First, specific subsets of cells can be labeled

and manipulated with the binary Gal4/UAS (upstream activating sequence) system. Derived from yeast, expression of the transcription factor Gal4 under the control of a tissue-specific promoter will enhance transcription of a gene downstream of the Gal4 binding sites (Fischer et al., 1988). These sites, referred to as the UAS, enhance the expression of a gene following a UAS promoter and restrict its expression to the subset of cells that express Gal4 (Brand and Perrimon, 1993). This system can be used to label specific sets of cells, for example with a membrane tethered GFP (*UAS-mCD8::GFP*), to distinguish a subset of cells within a tissue, or to drive misexpression of transgenes to determine the cellular impact.

Another important advancement for the use of *Drosophila* genetic screens was the development of the flippase/flippase recognition target (FLP/FRT) system, also using genes derived from yeast. This system allows for FLP-mediated site-specific recombination at transgenic FRT sites on homologous chromosomal arms (Golic and Lindquist, 1989). If a parent cell expresses FLP during mitosis and carries FRT sites at a homologous region of a specific chromosomal arm, then a recombination event can occur at the FRT sites. During chromosomal segregation, there is a 25% chance of the daughter cell becoming homozygous specifically at the region distal to the FRT site while remaining homozygous throughout the rest of the chromosomes (Theodosiou and Xu, 1998). This technique allows for analysis of mutants distal to FRT sites in a tissue-specific subset of cells instead of the entire animal, allowing for examination of mutants that might otherwise be lethal if homozygous. While traditional screening techniques

only allow for the "first essential function of a gene" to be analyzed (St Johnston, 2002), this development permits analysis of mutations at a variety of developmental stages in the given tissue type of choice.

One issue remained with the FLP-mediated recombination technique: how do you know which cell is mutant and which is not? This technique was greatly enhanced and optimized by the development of a system with which you could specifically label the mutant mitotic clone generated with FLP-mediated recombination: mosaic analysis with a repressible cell marker (MARCM). MARCM combines the FLP/FRT system with the Gal4/UAS system, including Gal80, another yeast protein that binds to Gal4 and represses its ability to bind to a UAS. Now, specific clones are made by expression of tissue-specific *flp* and subsequently labeled in a tissue-specific manner by Gal4-mediated expression of a cellular marker under UAS control (Lee and Luo, 1999; Lee and Luo, 2001). Because of the transgenic placement of a ubiquitously-expressed Gal80 distal to a FRT on the chromosome of choice, this system specifically labels only the daughter cells that are homozygous and without the Gal80-induced Gal4 repression. Thus, we are able to specifically label our homozygous mutant cells. This mosaic system allows for the analysis of normal cells during development with single-cell resolution, as well mutant cells within an otherwise phenotypically normal tissue (Lee and Luo, 2001).

All of the above genetic tools allow for rapid analysis of potential Wallerian degeneration mutants, even those that may cause lethality. One additional tool is missing: the mutagen. While many studies have used P-element transposons because of

the ease of insertion identification (Salzberg et al., 1997), these insertions are not random and rarely cause stops or deletions. With the advances in genome-wide sequencing (Gonzalez et al., 2012), use of another mutagen, ethyl methane sulphonate (EMS), has become advantageous. EMS-induced mutagenesis acts randomly and causes a high rate of single point mutations (St Johnston, 2002). Many EMS-mutations result in protein coding changes, which increases the chances for identifying mutants that have altered function. Therefore, we have assembled a genetic system that can be randomly mutagenized with a high rate of protein coding changes.

The final detail remaining is to decide which *Drosophila* neurons to use in the study of Wallerian degeneration. These neurons must exist within a defined subset that can be labeled with the Gal4/UAS system and be accessible for axotomy. Strong candidates are peripheral neurons, where axotomy may be performed with minimal damage to the surrounding tissue. *Drosophila* olfactory neurons (ORNs) thus are an excellent tool with which to study Wallerian degeneration using MARCM. First, ORNs house their cell bodies and dendrites in the antennae and project their axons to the antennal lobe of the brain (Couto et al., 2005); injury can be induced by simple non-lethal surgical ablation of the antennae, leaving the axons in the brain behind to undergo Wallerian degeneration. This also removes any possible interference from regenerating axons because the cell bodies are no longer attached to the animal. Second, small subsets of ORNs have been identified based on their olfactory receptor expression (Clyne et al., 1999) and their axonal projections to the antennal lobe of the *Drosophila* brain have

been mapped (Couto et al., 2005). One subset, the approximately 20 OR22a+ ORNs in each antenna (Dobritsa et al., 2003), can be identified with near single-axon resolution when labeled with a membrane tethered GFP by *OR22a-Gal4, UAS-mCD8::GFP* (MacDonald et al., 2006), as opposed to the large axon bundles found with many other subsets of ORNs. ORNs are derived from sensory organ precursor (SOP)-like progenitors that express the transcription factor *eyeless* (*ey*); when *flp* expression is driven under the control of the *ey* promoter, then FLP activity is induced in ORN progenitors (Newsome et al., 2000), allowing for the production of mosaic ORN clones. Importantly, *ey-flp* activity occurs only in the eye-antennal disc, so (1) all resulting mosaic clones are clearly of neuronal, not glial, origin (Hummel et al., 2003), and (2) ablation of *ey*+ progenitors does not cause lethality. Mosaic clones induced with *ey-flp*, even if completely ablated, will have little or no effect on the rest of the animal. Thus, *Drosophila* ORNs are an excellent model with which to study Wallerian degeneration: they are easily labeled with the Gal4/UAS system; we have tools in place to reliably generate mosaic clones with MARCM; and we have a method to easily and quickly induce Wallerian degeneration by simply removing the antennae.

Using this system, we performed the first forward genetic screen to identify mutants that potently block Wallerian degeneration after injury. In this thesis, I describe the identification and initial characterization of *sarm* (sterile alpha and Armadillo motif), the first gene required for severed axons to undergo Wallerian degeneration. In Chapter 2, I describe the genetic screen used to isolate three loss of function mutations in dSarm

(*Drosophila* Sarm) that potently protected severed axons from degeneration. The role of Sarm in promoting axon self-destruction after injury is highly conserved, as axons from Sarm KO mice are also robustly protected from Wallerian degeneration. In all assays to date, protection from Wallerian degeneration by loss of Sarm phenocopies the protection of axons by expression of Wld^{s}. I present my initial characterization of Sarm function in Chapter 3, where I have performed a structure-function analysis to determine the roles of each of Sarm's predicted subdomains in its function. In Chapter 4, I address the role of axon death in a mouse model of ALS and show that loss of Sarm can block axonal degeneration in SOD1-G93A ALS mice. Finally, I present a model for the molecular action of Sarm in promoting Wallerian degeneration in Chapter 5 and suggest future experiments to further understand these molecular mechanisms. First, however, I will present a summary of the previous work from other groups on Sarm and its potential function(s) beyond promoting Wallerian degeneration.

Identification of mammalian *Sarm1*

Sarm1 was first identified in a genome-wide screen of highly conserved regions among mammals. The human gene is located on chromosome 17q11 and occurs in a region syntenic with mouse chromosome 11 (Mink et al., 2001). Interestingly, human, but not mouse, cDNA libraries revealed the presence of a 350 bp antisense transcript that overlaps with the 5'end of *sarm1*, indicating that there may be endogenous RNAi regulatory mechanisms in place to control Sarm1 levels (Mink et al., 2001). In humans, *Sarm1* mRNA transcripts are found mainly in the kidney, liver and placenta (Mink et al.,

2001), and in the mouse, transcripts occur almost exclusively in the brain (Kim et al., 2007). Both human and mouse forms of *sarm* contain 9 nearly identical exons, with first exon being larger in the mouse (Mink et al., 2001). Notably, this gene is highly conserved across phyla, as *C. elegans* and *Drosophila sarm* (*tir-1* and *dsarm*, respectively) share over 40% identity with human *Sarm1* (Mink et al., 2001). Endogenous expression in both the worm and fly is very low, although it is detected in neurons (Chuang and Bargmann, 2005; Osterloh et al., 2012). Interestingly, *tir-1* is also controlled by an endogenous microRNA, *mir71*, although it is unknown if this regulation occurs in flies or mice (Hsieh et al., 2012).

Sarm and neural development

Sarm loss-of-function mutants do not broadly disrupt neural development (Kim et al., 2007; Osterloh et al., 2012); however, there are finer roles for Sarm during neural patterning (Chuang and Bargmann, 2005) and dendritic outgrowth (Chen et al., 2011). *C. elegans* has two bilateral olfactory neurons, AWCL (left) and AWCR (right), that are essential for chemotaxis. While born identical, one neuron will switch to AWC^{ON}, which expresses the receptor *str-2* and detects butanone, while the other will remain in the default AWC^{OFF}, which detects pentanedione and does not express *str-2* (Chuang and Bargmann, 2005). It is random which AWC (left or right) will become AWC^{ON}, but in wild type animals there is always one AWC^{ON} and one AWC^{OFF}. In *tir-1* loss-of-function mutants, however, both neurons become AWC^{ON}, indicating that during development, tir-1 activity falls in the neuron that is fated to become AWC^{ON}, altering transcription of

str-2 (and other possible unidentified genes) (Chuang and Bargmann, 2005). Epitasis experiments ultimately put AWC fate under control of a calcium-dependent mitogen-activated protein kinase (MAPK) pathway, requiring: *unc-43* (calcium/calmodulin-dependent protein kinase-II, CaMKII), *tir-1*, and *nsy-1* (the MAPKKK apoptosis signaling kinase-1 (ASK1)). Interestingly, this pathway is also controlled by the endogenous microRNA *mir-71*, which acts to downregulate levels of *tir-1* specifically in the AWC^{ON} neuron (Hsieh et al., 2012). Thus, there are two parallel pathways regulating *tir-1* during neural development in the worm: calcium-dependent activation, and microRNA-dependent control of protein expression.

tir-1 also plays a role in serotonin-based learning and behavior in *C. elegans*. During olfactory adaptation, the serotonin biosynthesis gene *tph-1* is upregulated, resulting in changes in calcium adaptation, and ultimately behavior, causing the worm to avoid certain odors (Inoue et al., 2013) or pathogens (Xie et al., 2013). Behavioral plasticity due to odor adaptation signals through the CaMKII-TIR-ASK1 pathway (Inoue et al., 2013), while pathogen avoidance plasticity signals only through CaMKII-TIR to activate transcription factors unrelated to AWC fate (Xie et al., 2013). Thus, *tir-1* plays multiple roles in mediating neuronal transcription factor activation, depending on context, and, intriguingly, all of the roles described thus far require CaMKII activity, suggesting Sarm is intimately coupled to calcium-dependent events.

Sarm1 also plays a role in mammalian neural development. Sarm1 is partially regulated by Syndecan-2, a heparan sulfate proteoglycan that activates dendritic spine

formation during development (Chen et al., 2011). Endogenous mouse Sarm1 localizes in puncta through neurites and near the synaptic density marker PSD-95 (Chen et al., 2011). Knockdown of *Sarm1* in hippocampal neurons results in decreased dendritic length and arborization, however developmental pruning still occurs normally (Chen et al., 2011). Conversely, overexpression of Sarm1 results in longer dendrites; this effect is suppressed with a JNK inhibitor, suggesting Sarm is regulating dendritic morphology in a JNK-dependent manner. Notably, these cultured neurons fail to establish polarity, as 40% never develop a dominant axon (compared to 10% in wild type cultures) (Chen et al., 2011). *Sarm1* knockdown *in vivo* results in a slight reduction in brain size (10%) and shorter branches in cortical CA1 neurons (Chen et al., 2011). However, Sarm KO mice are viable and show no overt behavioral defect (Kim et al., 2007), indicating that gross neural function remains intact despite loss of Sarm. Extensive behavioral tests have not been performed on these mice however, and it remains possible that the above alterations in morphology may result in learning and memory defects.

Sarm plays mixed roles in innate immunity

Sarm's function in the brain was tested because mouse Sarm is expressed almost exclusively there; however, Sarm's protein structure (see below) predicts that it would have a role in innate immunity (O'Neill et al., 2003), and as such much of the previous work on Sarm has been in this context. The *Sarm* protein product was predicted to have three domains: armadillo/HEAT domain (ARM), sterile alpha motif (SAM), and Toll/Interleukin-1 receptor homology domain (TIR) (O'Neill et al., 2003). Toll-like

receptors (TLRs), which recognize specific pathogens, signal through their intercellular TIR domain via downstream adaptor proteins, which also contain a TIR domain, to activate gene expression (Yamamoto and Takeda, 2010). In mammals, TLR activation leads to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon regulatory factors (IRF), activator protein-1 (AP-1) and MAPKs to stimulate expression of pro-inflammatory genes in order to eliminate a pathogenic threat (Jenkins and Mansell, 2010; Yamamoto and Takeda, 2010).

The roll of Sarm in immunity was first investigated in *C. elegans*. Worms have only two TIR-containing proteins: Tol-1, the ortholog of TLRs, and Tir-1, the Sarm ortholog and presumed cytosolic adaptor for Tol-1. Knockdown of *tir-1* with RNAi or genetic ablation of *tir-1* resulted in increased susceptibility to death by fungal and bacterial infections (Liberati et al., 2004; Couillault et al., 2004), as well as noxious chemical insults, such as heavy metals (Kurz et al., 2007). *tir-1* mutants showed no major defects in development or longevity, suggesting that the role of *tir-1* is involved in stress-induced responses (Liberati et al., 2004; Couillault et al., 2004). Interestingly, Tir-1 signals independently of Tol-1 through a p38-MAPK pathway to activate expression of antimicrobial genes (Liberati et al., 2004; Couillault et al., 2004) (Kurz et al., 2007); the signaling complex downstream of Tol-1 remains a mystery (Fekonja et al., 2012).

This transcriptional antimicrobial response appears to have evolutionarily diverged in animals that contain multiple TIR-containing adaptor proteins. Unlike in worms, Sarm acts as a negative regulator of antimicrobial responses in shrimp (Wang et

al., 2013) and *Drosophila*, downstream of tollo/toll-8 activation (Akhouayri et al., 2011). In mammals, overexpression of TIR adaptor proteins *in vitro* leads to activation of NF-κB, with the notable exception of Sarm, which has no effect on this activation (Carty et al., 2006). Instead, Sarm appears to negatively regulate innate immune responses specifically through another TIR-domain containing protein, TIR-domain-containing adapter-inducing interferon-β (TRIF): *Sarm1* knockdown increases TRIF-induced gene expression, while *Sarm1* overexpression decreases this response in a dose-dependent manner (Carty et al., 2006; Peng et al., 2010; Yuan et al., 2010). Interestingly, *Sarm1* overexpression also results in a decrease of p38 activation (Peng et al., 2010). As in worms, Sarm seems to interact with the MAPK pathway; unlike *C. elegans*, however, *Drosophila* and mammalian Sarm appear to negatively regulate innate immunity. This is perhaps to prevent excess inflammatory responses, which can possibly be lethal, as is the case with septic shock (O'Neill, 2006). *Sarm* transcript levels rise after infection, supporting this theory (Belinda et al., 2008; Peng et al., 2010). However, immune cells from Sarm KO mice show normal responses to a variety of insults, and the animals have no obvious defect in immunity (Kim et al., 2007), so there are likely other redundant mechanisms in place that work together to fine-tune inflammatory responses (Fekonja et al., 2012).

Sarm plays an additional, unique role during melioidosis, a condition in which bacteria specifically infect macrophages. Macrophages, which do not normally express detectable levels of Sarm, upregulate Sarm within 30 minutes of infection; it appears that

the bacteria's machinery has purposefully induced this Sarm expression so as to dampen the host's inflammatory response. Knockdown of *Sarm* increases macrophage resistance to infection (Pudla et al., 2011), supporting Sarm's role as a negative regulatory of innate immunity.

Intracellular localization of Sarm

The mechanism(s) of Sarm function, whether it is in neural development, innate immunity, or Wallerian degeneration, may be dependent on a specific subcellular localization. In COS-1 and HEK-293 cells, used because human Sarm is expressed in the kidney (Mink et al., 2001), overexpression of Sarm::EGFP localizes to mitochondria (Kim et al., 2007; Panneerselvam et al., 2012); however, high expression levels cause mitochondria to swell into large, round clusters that center around microtubule organizing centers (Kim et al., 2007). Sarm expression also seems to increase stabilizing tubulin acetylation, even in neuronal cell lines (Chen et al., 2011), suggesting that Sarm may mediate mitochondrial localization relative to microtubules or microtubule stability directly (Kim et al., 2007). In HEK-293 cells, the first 27 amino acids of mammalian Sarm are responsible for the *in vitro* mitochondrial localization, as confirmed with transmission electron microscopy (Panneerselvam et al., 2012).

In vitro localization studies have all used overexpression in kidney-derived cell lines, not neurons; interestingly, *in vivo* studies have only investigated Sarm localization in neurons, where Sarm has not been found to localize with mitochondria in any organism (Chuang and Bargmann, 2005; Chen et al., 2011; Osterloh et al., 2012).

Preliminary *in vitro* work suggests that Sarm1::GFP, when highly overexpressed in neuronal cultures, will eventually co-localize to mitochondria (M. Coleman, personal communication), suggesting that mitochondrial localization may be an artifact of cell culture and/or overexpression. While endogenous expression was not detectable, overexpression of Tir-1::EGFP in worm olfactory neurons showed a punctate localization in the axon, with exclusion from the dendrite and cell body (Chuang and Bargmann, 2005). This fusion protein co-localized with post-synaptic sites in the axon (Chuang and Bargmann, 2005). Chemical disruption of microtubule polymerization causes a *tir-1* loss of function like phenotype in AWC neurons; likewise, this chemical disruption diminishes the activity of *tir-1* gain of function mutants (Chang et al., 2011). Tir-1 puncta in the axon are also greatly disrupted with this treatment, suggesting that Sarm function and localization requires microtubule stability in this context (Chang et al., 2011). Tir-1 localization is regulated by Unc-104, the transport motor for presynaptic vesicles along microtubules. Intriguingly, Unc-104 acts non-cell autonomously to regulate AWC fate, suggesting that some other presynaptic factor regulated by Unc-104 is impacting Tir-1 localization and function in worms *in vivo* (Chang et al., 2011). Together, this suggests that Sarm localization, and perhaps function, involves interactions with microtubules, but the importance of this interaction in Wallerian degeneration is unknown.

Suggested function of Sarm domains

Because the mechanism of action for Sarm is so mysterious, investigating the potential function of the individual domains of Sarm may shed some light into Sarm's mode of action. Loss of the TIR or ARM domains showed normal localization of tir-1::EGFP, while loss of the SAM domain resulted in broad axonal distribution (Chuang and Bargmann, 2005), suggesting that the SAM domain controls subcellular localization. Loss of the ARM domain resulted in a gain-of-function phenotype in AWC development (Chuang and Bargmann, 2005). Loss of the TIR or SAM domain generally yielded a non-functional molecule (Carty et al., 2006; Peng et al., 2010; Panneerselvam et al., 2012; Panneerselvam et al., 2013). Thus, the ARM domain appears to be involved in auto-regulation, the SAM domain in localization, and the TIR domain in signaling.

Possible signaling partners

Tir-1 acts in multiple roles through a CaMKII-TIR-ASK1 pathway (Chang et al., 2011), but there are likely more binding partners that (1) signal though Sarm and (2) confer downstream specificity. Overexpression of Sarm in COS-1 cells pulls JNK3 from its normally cytoplasmic distribution to mitochondrial clusters (Kim et al., 2007), further supporting a role for Sarm in MAPK signaling. A yeast two hybrid screen on individual domains of horse shoe crab Sarm found four interactors with the TIR domain (HAX-1, involved in apoptosis regulation; SUMO-1, involved in sumoylation; Proteosome Subunit α; and DnaJ/Hsp40, a chaperone) and one with the SAM domain (CaMKI) (Belinda et al., 2008), but the importance of these interactions, if they are indeed real, is

unknown in any context. Overexpression of a constitutively active CaMKI with Sarm1 and TRIF increased Sarm's inhibitory effect on TRIF-signaling (Belinda et al., 2008), suggesting that Sarm's activity may be regulated by a calcium-dependent protein kinase. Coaffinity purification from *Drosophila* cells yielded over 40 potential binding partners for dSarm (Guruharsha et al., 2011). Together, these data indicate that Sarm may have multiple binding partners, depending on the experimental context, and it remains unclear which if any are involved in Wallerian degeneration. However, these data are consistent with Sarm acting as a kinase scaffold, potentially bringing together calcium-activated kinases with their downstream effectors to induce Wallerian degeneration. Immunoprecipation experiments with domains of Tir-1 support this hypothesis, as the ARM domain specifically interacts with the calcium activated CaMKII and the TIR domain interacts with the kinase ASK1 (Chuang and Bargmann, 2005).

Sarm and cell death

Wallerian degeneration is not cell death, and Sarm KO mice have defects in Wallerian degeneration but not developmental cell death (Osterloh et al., 2012). However, new mechanisms of caspase-independent cell death are beginning to emerge, and Sarm may play a role in this process. *C. elegans* linker cell death, which is developmental but caspase independent, is blocked in *tir-1* mutants (Blum et al., 2012). *tir-1* mutants also show an increase in survival under anoxic, but not hypoxic conditions; this survival is mediated by MAPK signaling but not p38 nor JNK (Hayakawa et al., 2011). In mice, Sarm KOs are protected from oxygen-glucose-deprivation (OGD)-

induced cell death, a hypoxia model, at twice the rate of wild type animals (Kim et al., 2007). Thus, Sarm appears to function normally to promote neurodegeneration. Interestingly, this function also extends to infected neurons, where Sarm-mediated axon destruction prevents pathogenic spread. During West Nile Virus (WNV) infection, which broadly affects neurons, Sarm1 KO mice are twice as succeptable to death after WNV infection, despite comparable viral loads and normal T and B cell responses (Szretter et al., 2009). This suggests that the ability to promote axon destruction may actually be advantageous in some situations, as it may allow an infected neuron to destroy contaminated processes to prevent further viral spreading between neurons. How or why a pathway that responds to viral attacks also responds to physical injury is unclear, but suggests a common link between axon degeneration and innate immunity.

A role for Sarm in mediating stress-induced cell death appears to be coupled with levels of Sarm protein. Overexpression of full length Sarm induces death, without any upstream signal, in ~25% of cultured HEK cells (Panneerselvam et al., 2012). This death appears dependent on that 1-27aa stretch that confers mitochondrial localization in kidney cells (Panneerselvam et al., 2012). When Sarm is expressed in primary T cells, over 80% of the cells undergo classical apoptosis, resulting in sequential activation of caspase -9, then -3, then -8 (Panneerselvam et al., 2013). Sarm overexpression also induces a decrease in mitochondrial membrane potential and an increase in ROS (Panneerselvam et al., 2013). In these cells, this apoptosis could be partially blocked by overexpression of the anti-apoptotic gene *bcl-xl* and enhanced by addition of a MAPK

inhibitor (Panneerselvam et al., 2013). Knock down of Sarm in T-cells leads to increases in survival and proliferation, supporting the role for Sarm in moderating and negatively regulating immune responses (Panneerselvam et al., 2013). Together, this suggests that Sarm functions in regulating stress-induced cell death, possibly by mediating interactions between kinases. Notably, in COS-1 cells, where overexpression of Sarm causes mitochondrial clumping and JNK redistribution, no activation of cell death has been reported. Thus, Sarm appears to play many cellular roles under a variety of circumstances, highlighting the importance of studying Sarm's mechanism of action within the specific context of Wallerian degeneration.

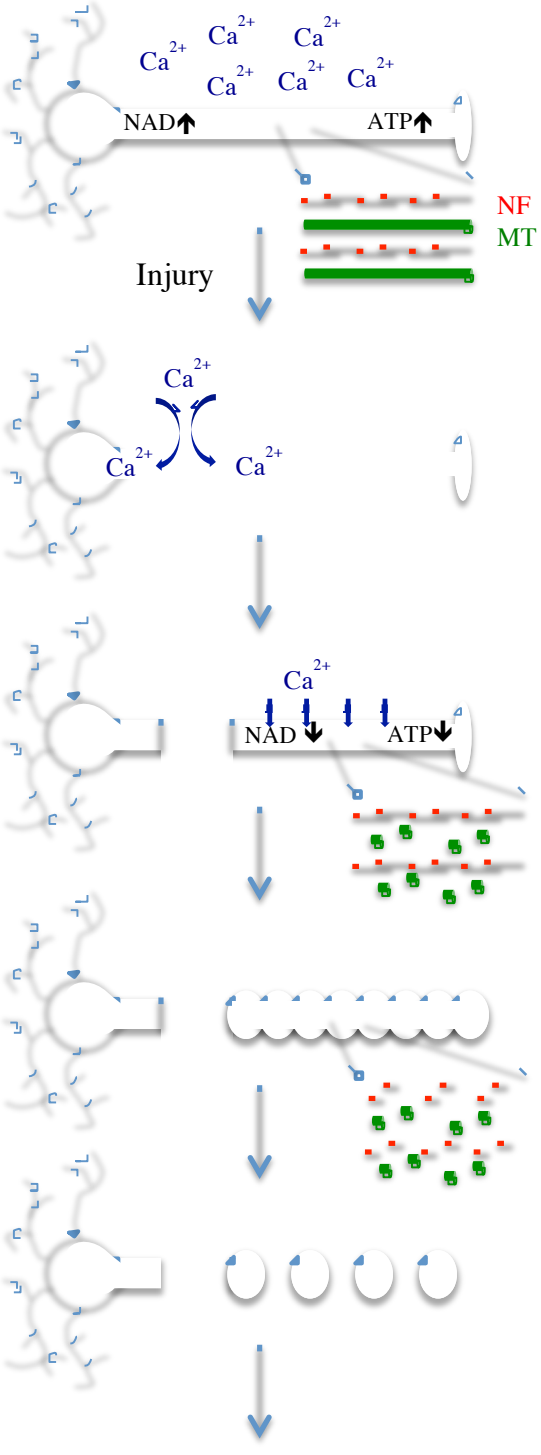


Figure 1-1. Progression of Wallerian degeneration in wild type nerves
Healthy axons (top) maintain an intact cytoskeleton comprised of neurofilaments (red) and microtubules (green). Examples of changes during Wallerian degeneration are presented: AAD (second from top), the latency phase (middle), and GDC (bottom two).

<u>AAD</u>
- Initial calcium spike
- Vesicle fusion to reseal the membrane
- Degeneration of 200-300 μM of axon on either side of the lesion

<u>Latency Phase</u>
- Slow intra-axonal calcium rise through L-type calcium channels
- Microtubule depolymerization
- Energetic failure

<u>GDC</u>
- Neurofilament and microtubule breakdown
- Mitochondrial swelling
- Membrane fragmentation

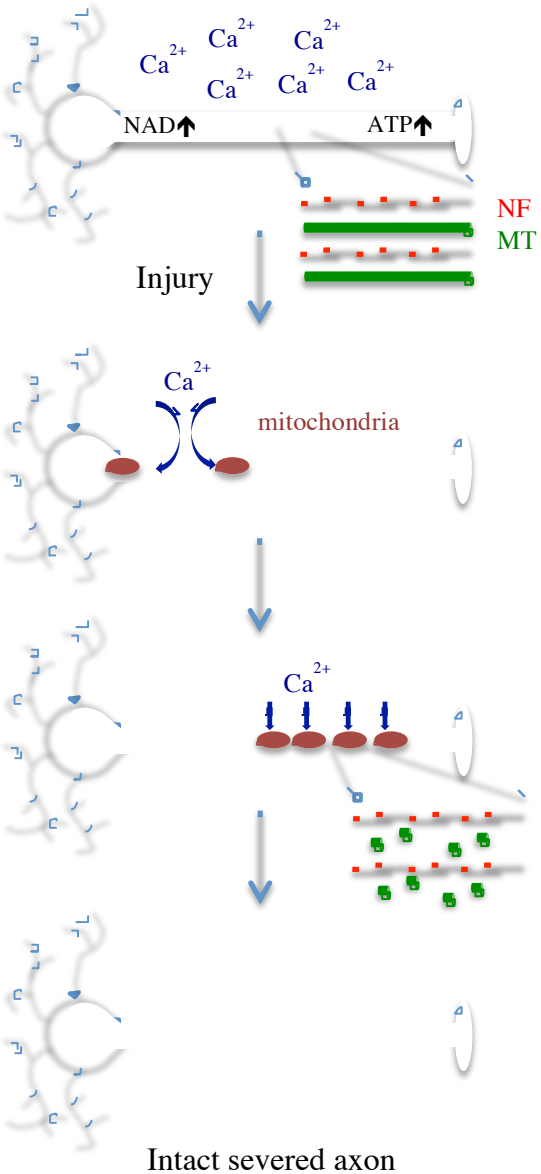


Figure 1-2. Proposed mechanism of Wld^s—mediated protection of severed axons from Wallerian degeneration
Recent evidence suggest that mitochondria from Wld^s-expressing animals are able to buffer larger concentrations of calcium than their wild type counterparts (Avery et al., 2012), blocking the following processes:

<u>AAD</u>

- ~~Initial calcium spike~~
- Vesicle fusion to reseal the membrane
- ~~Degeneration of 200-300 µM of axon on either side of the lesion~~

<u>Latency Phase</u>

- ~~Slow intra-axonal calcium rise through L-type calcium channels~~ *Mitochondria take up excess axonal calcium*
- Microtubule depolymerization
- ~~Energetic failure~~ *ATP and NAD levels remain high*

<u>GDC</u>

- ~~Neurofilament and microtubule breakdown~~
- ~~Mitochondrial swelling~~
- ~~Membrane fragmentation~~

CHAPTER II: dSarm/Sarm1 is required for activation of an injury-induced axon death pathway

The following work is the final author manuscript; all supplemental figures have been incorporated into the manuscript and all figures subsequently renumbered and reformatted. This work appears in the *Science* article of the same name, published as:

Osterloh JM, Yang J, Rooney TM, Fox AN, Adalbert R, Powell EH, Sheehan AE, Avery MA, Hackett R, Logan MA, MacDonald JM, Ziegenfuss JS, Milde S, Hou YJ, Nathan C, Ding A, Brown RH Jr, Conforti L, Coleman M, Tessier-Lavigne M, Zuchner S, Freeman MR. (2012). Science *337*, 481-484.

This work was conducted in the laboratory of Marc Freeman. I performed the *Drosophila* screen described, along with Michelle Avery, Rachel Hackett, Mary Logan, Jennifer MacDonald, and Jennifer Ziegenfuss. I performed all of the described *Drosophila* work, with the exception of: the pruning and tdc neuron experiments, which were performed by Tim Rooney; and the mRNA *in situ* and L1 dMP2 experiments, which were performed by Nicki Fox. Michelle Avery counted the axons in Table 2-2. Amy Sheehan cloned *dSarm*. Mammalian primary culture experiments were performed by Jing Yang, in the laboratory of Marc Tessier-Levigne, with the exception of the SGC explants. Ying-Ju Hou, Carl Nathan, and Aihao Ding provided primary cultures for Marc Tessier-Lavigne's group. For *Sarm1* -/- experiments, I dissected the SGCs, which were subsequently injured and imaged by Robert Adalbert in the laboratory of Michael Coleman. Wld^{s} SCG experiments were performed by Laura Conforti, and Steven Milde stained wild type nerves for Sarm localization, both in the laboratory of Michael Coleman. I performed all animal handling, surgeries and dissections; Robert Adalbert performed the sciatic nerve preparation and imaging for EM; Jing Yang performed the immunohistochemistry. Eric Powell was responsible for the *Drosophila* sequencing in the laboratory of Stephan Zuchner.

Abstract

Axonal and synaptic degeneration is a hallmark of peripheral neuropathy, brain injury, and neurodegenerative disease. Axonal degeneration has been proposed to be mediated by an active auto-destruction program, akin to apoptotic cell death; however, loss-of-function mutations capable of potently blocking axon self-destruction have not been described. Here, we show that loss of the Drosophila Toll receptor adaptor dSarm (sterile α/Armadillo/Toll-Interleukin receptor homology domain protein) cell-autonomously suppresses Wallerian degeneration for weeks after axotomy. Severed mouse Sarm1 null axons exhibit remarkable long-term survival both in vivo and in vitro, indicating that Sarm1 prodegenerative signaling is conserved in mammals. Our results provide direct evidence that axons actively promote their own destruction after injury and identify dSarm/Sarm1 as a member of an ancient axon death signaling pathway.

Results and Discussion

Axons have traditionally been thought to be strictly dependent upon the cell body for survival, as axons robustly degenerate upon separation from the soma (Waller, 1850). However, this notion was directly challenged by the identification of the *slow Wallerian degeneration* (*Wld*^{s}) mutant mouse in which the distal portion of severed axons remained morphologically intact for 2-3 weeks after axotomy (Lunn et al., 1989; Glass et al., 1993). The remarkable long-term survival of severed axons in the *Wld*^{S} mouse also raised the intriguing possibility that Wallerian degeneration is driven by an active molecular program akin to apoptotic cell death signaling (Raff et al., 2002; Coleman and Perry, 2002). However numerous studies have demonstrated that *Wld*^{S} is a gain-of-function mutation that results in the neuronal overexpression of a chimeric fusion protein containing the NAD^{+} biosynthetic enzyme Nmnat1 (Mack et al., 2001; Coleman and Freeman, 2010). As such, the Wld^{S} phenotype may be unrelated to normal Nmnat1 function and NAD^{+} metabolism, despite its ability to inhibit endogenous axon death pathways. Wallerian degeneration appears to be molecularly distinct from apoptosis since potent genetic or chemical inhibitors of cell death (Deckwerth and Johnson, 1994; Finn et al., 2000; Whitmore et al., 2003) or the ubiquitin proteasome pathway ; Hoopfer et al., 2006, Neuron, 50, 883-95} do not block Wallerian degeneration. Mutants reported to affect Wallerian degeneration, such as *wnd/DLK*, delay the clearance of degenerating axons in *Drosophila* for only ~1-2 days, and mouse axons for several hours (Miller et al., 2009) – an extremely weak degree of suppression when compared to Wld^{S}.

Thus the existence of axon death pathways has remained only speculative.

If Wallerian degeneration is indeed an active process, we reasoned that loss of function mutants that exhibit Wld^{s}-like protection of severed axons should exist. We first revisited the question of whether Wallerian degeneration might be related to previously described cell destruction programs. We performed a comprehensive screen of existing mutants and dominant negative constructs for *Drosophila* genes affecting apoptosis, autophagy, or other defined cell degradative pathways, but these failed to suppress Wallerian degeneration *in vivo* at any level (Table 2-1).

To identify novel genes required to promote Wallerian degeneration, we performed a F_2 forward genetic screen for mutants that exhibited long-term survival of distal severed axons (Figure 2-1). Because genes required for Wallerian degeneration may cause lethality when mutated, we designed our screen to allow for characterization of both viable and lethal mutants though MARCM clonal analysis (Lee and Luo, 1999). In control animals, severed olfactory receptor neuron (ORN) axons degenerated and were completely cleared from the right antennal lobe 7 days after axotomy. We identified three lines, *l(3)896*, *l(3)4621*, and *l(3)4705*, where severed homozygous mutant axons generated by MARCM remained fully intact 1 week after axotomy (Figure 2-2A). While the number of uninjured axons is slightly reduced in each mutant, 100% of GFP-labeled axons exhibited long-term preservation after injury (Table 2-2). Remarkably, mutant axons remained fully intact 30 days after injury (Figure 2-2B) and a significant but reduced number remained morphologically intact even 50 days after

injury (Figure 2-2C). *l(3)896*, *l(3)4621*, and *l(3)4705* therefore provide axonal preservation that rivals that of Wld^{s} in *Drosophila*, and which lasts essentially for the lifespan of the fly. Neuroprotection in these mutants extended to synapses: synaptobrevin punctae localized to synaptic terminals even 30 days after axotomy (Figure 2-2D,E).

All three mutants are lethal and recessive in their axon degeneration phenotype (Figure 2-3A), and each failed to complement one another for lethality; thus each line represented an independently isolated lethal mutation in the same gene. Neuronal morphology appears normal in mutant animals (Figure 2-2; Figure 2-3B), indicating these mutations do not grossly affect neuronal development.

Previous work (Finn et al., 2000; Whitmore et al., 2003; Hoopfer et al., 2006) and the above data argue that Wallerian degeneration is molecularly distinct from apoptosis and developmental neurite pruning. We next asked whether *l(3)896* is broadly required for neuron pruning or apoptotic cell death. We examined MARCM clones in *Drosophila* mushroom body (MB) γ neurons, as these neurons undergo both dendritic and axonal pruning during metamorphosis (Luo and O'Leary, 2005). In both control and *l(3)896* animals, mushroom body γ neuron axons and dendrites were pruned normally (Figure 2-4A). During early embryogenesis, dMP2 neurons are present in each segment, but by late embryogenesis, all but the posterior 3 pairs undergo developmentally-programmed apoptosis (Miguel-Aliaga et al., 2008). We found that dMP2 neurons were generated normally in *l(3)4621* animals, and the appropriate subset of neurons

underwent apoptosis (Figure 2-4B). Finally, we expressed the pro-apoptotic gene *hid* in the *Drosophila* visual system (Grether et al., 1995) to induce widespread apoptotic death in cells of the developing eye disc. We found that *l(3)896* mutant clones failed to suppress activation of cell death (Figure 2-4C,D).

To identify the gene mutated in *l(3)896*, *l(3)4621*, and *l(3)4705*, we took two complementary approaches. First, we mapped the lethality of each mutant using small chromosome deficiencies, and identified a single gene at cytogenetic region 66B (Figure 2-5A). At the same time we utilized next-generation sequencing technology to re-sequence the entire genome of each mutant (along with our unmutagenized control line) to an average read depth of 70x (Table 2-3). We identified a single gene affected in all three mutants, which resided in cytogenetic region 66B: *ect4*, which we refer to as *dsarm* (*Drosophila* sterile alpha and Armadillo motif). The *dsarm* gene encodes a protein with an Armadillo/HEAT (ARM) domain, two sterile alpha motifs (SAM), and a Toll/Interleukin-1 Receptor homology (TIR) domain. Each identified *dsarm* allele contained a unique premature stop codon in *dsarm* open reading frame (Figure 2-5B,C). From these data we conclude that *l(3)896*, *l(3)4621*, and *l(3)4705* are loss-of-function alleles of *dsarm*. Consistent with this interpretation, expression of full length *dsarm* cDNA using the postmitotic *OR22a-Gal4* driver in *l(3)896* mutant clones was sufficient to fully revert the suppression of axonal degeneration observed in *dsarm* mutants (Figure 2-5D). In addition, we rescued both the lethality and suppression of Wallerian degeneration phenotypes of *l(3)896/l(3)4621* and *l(3)896/Df(3L)BSC795* trans-

heterozygous animals with a *BAC* clone containing the *dsarm* gene (Figure 2-5D). Together these data indicate that *dsarm* is necessary in post-mitotic neurons for axonal destruction after axotomy, and loss of *dsarm* function is sufficient to provide long-term preservation of severed axons.

Based on RNA *in situ* hyridizations to embryos, larval brains, and adult brains, RT-PCR from dissected neural tissues, and analysis of a *dsarm-Gal4* driver line, *dsarm* appears to be widely expressed in the *Drosophila* nervous system (Figure 2-6). This observation suggests that dSarm is broadly required to promote Wallerian degeneration in the nervous system.

We next assayed Wallerian degeneration in null mutants for the mouse ortholog of *dsarm*, *Sarm1*. We first grew 5 day cultures of superior cervical ganglia (SCG) from wild type, *Sarm1* -/- , and *Wld*^{s} mice, severed axons, and scored axonal integrity over the next week. *Sarm1* -/- SCG explants exhibited robust protection from degeneration up to 72 hours after axotomy, similar to what is observed with Wld^{S} SCG neurons, while wild type axons degenerated within 8 hours (Figure 2-7). This robust protection was also seen in cultured *Sarm1* -/- cortical neuron axons (Figure 2-8A,B) and dorsal root ganglia (DRG) (Figure 2-8C,D). Notably, *Sarm1* -/- DRG explants were not protected from nerve growth factor (NGF) deprivation (Figure 2-9), a mouse model for developmental axon pruning (Raff et al., 2002; Luo and O'Leary, 2005; MacInnis and Campenot, 2005; Buss et al., 2006), suggesting that in mammals Sarm1 protection is specific to injury-induced axon degeneration. Thus Sarm1 loss of function robustly suppresses Wallerian

degeneration in multiple types of mammalian axons *in vitro*. In addition, based on our highly selective growth conditions, our data argue that Sarm1 is required cell autonomously for programmed axonal death.

To test whether Sarm1 is required *in vivo* for Wallerian degeneration we performed sciatic nerve lesions of the right hind limb of *Sarm1* -/- mice and their heterozygous littermate controls. Impressively, while *Sarm1* +/- controls exhibited a dramatic breakdown of the axon and myelin sheath within 3 days of injury, 61.2% of lesioned *Sarm1* -/- axons were protected from degeneration at least 14 days after injury ($p = 0.002$) (Figure 2-10). Analysis of sciatic nerve ultrastructure revealed a remarkable structural preservation at 14 days after axotomy of the Schwann cell and myelin sheath, axonal neurofilaments, and axonal mitochondria (Figure 2-10). We performed Western blots of sciatic nerve and probed them with antibodies to neurofilament-M (NF-M), α-Internexin, and β-tubulin class III (TUJI). While we observed a dramatic breakdown of these proteins in wild type nerves, they remained largely intact in *Sarm1* -/- axons (Figure 2-11A). We also observed preservation of the NF-M signal by immunofluorescent staining of the nerve in *Sarm1* mutants (Figure 2-11B,C).

We next examined neuromuscular junctions (NMJs) in tibialis anterior muscles after sciatic nerve transection. Synaptic integrity was scored by co-localization of presynaptic marker (NF-M/synaptophysin) with the post-synaptic acetylcholine receptor (AChR). In wild type animals, motor endplate denervation was complete by 2 days after axotomy. However in *Sarm1* -/- animals, the majority of synaptic of terminals were

partially innervated even at 6 days after injury (Figure 2-12). We also note that macrophage/monocyte infiltration of lesioned nerves was suppressed in *Sarm1* knockout animals (Figure 2-13). Taken together, our results indicate that *Sarm1*-/- mutations provide a dramatic preservation of sciatic nerves *in vivo* from initial axonal cytoskeletal breakdown to recruitment of macrophages for myelin clearance.

Finally, we assayed *in vivo* localization of dSarm and Sarm1. Expression of dSarm::GFP in larval neurons resulted in punctate localization in neuronal cell bodies, and broad localization to neurites (Figure 2-14). Similarly, stains with anti-Sarm1 antibodies to *in vitro* cultured mammalian neurons showed a broad, punctate pattern in neurites, and we note that endogenous Sarm1 did not show preferential localization with a mitochondrial marker (Figure 2-14).

Molecular pathways that mediate axon self-destruction have long been elusive. Our identification of *dsarm*/*Sarm1* provides direct evidence that Wallerian degeneration is driven by an ancient, conserved axonal death program. How is dSarm activated after axotomy? Influx of extracellular calcium is known to be necessary and sufficient to trigger Wallerian degeneration (George et al., 1995). Intriguingly, Tir-1 functions downstream of the Ca^{2+}-CaM kinase signaling cascade in *C. elegans* (Chuang and Bargmann, 2005; Chang et al., 2011), therefore dSarm/Sarm1 may respond directly to axonal calcium increases after axotomy. Intriguingly, *Sarm1*-/- neurons in slice cultures have been found to exhibit reduced cell death in response to oxygen/glucose deprivation (OGD) (Kim et al., 2007), whether this is an indirect result of axonal preservation

remains untested, but is an exciting possibility. Pharmacological inhibition of Sarm1 now represents a promising therapeutic avenue for patients with axonal loss, particularly if Sarm1 deletion is shown to benefit animal models of neurodegenerative disease. Based on the functional conservation we report for dSarm/Sarm1, it seems likely that similar forward genetic screens in invertebrates will uncover additional proteins that potently influence axon survival in mammals.

Materials and Methods

Drosophila stocks, transgenics, and injury protocol

The following Drosophila strains were used in this study: *OR22a-Gal4*; *dMP2-Gal4* (Miguel-Aliaga et al., 2008); *pUAST-mCD8::GFP*; *pUAST-nSynaptobrevin::GFP*; *201y-Gal4*; *FRT2A/FRT82B*; *GMR-hid*; *Ubi-GFP::nls*; *FRT2A, tub-Gal80; ey-flp*; usp^2 (*Tp(3;1)KA21*); *Ect4-Gal4* (Drosophila Genetic Resource Center); and the 3(L) Deficiency Kit (all from Bloomington Stock Center unless noted). Mutants listed in Supplementary Table 2-1 are all from Bloomington Stock Center or generous gifts from: E. Baehrecke; E. Arama; R. Tanguay; M. Guo; N. Tapon; and K. McCall. Lethal mutants were recombined onto a chromosome harboring a *flippase recognition target* (FRT) sequence and screened using MARCM clonal analysis with *ey-flp*. To establish mutant stocks for screening, we used the mutagen ethyl methane sulfonate (EMS) and established ~2000 individual third chromosome F_2 mutant stocks containing FRT sites on both chromosomal arms (2A and 82B). We induced antennal injury using a modification of a previously described protocol (MacDonald et al., 2006). Adult flies

were aged for 7 days at 25°C after ablating the right third antennal segment only. Both antennae were ablated for synaptic preservation studies, as ORNs from each antenna synapse on both glomeruli. Injured flies were aged at 25°C for the indicated time (7, 30, or 50 days) before dissecting and fixing the brain. Axonal integrity was scored as previously described (MacDonald et al., 2006; Avery et al., 2009).

Cloning

The first 630-4828 nucleotides of the partial *dsarm* cDNA GH07037 (DGRC) was cloned into the pCashsp40-LacZ vector with BglII/XhoI. The remaining sequence was obtained by PCR amplifying a fragment from cDNA IP03452 (DGRC) using 5'ECT4-D NotI (ATATATATGCGGCCGCAAAACATGGGCAATCGTTTGAGCGGC) and PM001 (CGTTAGAACGCGGCTACAAT), then cut with NotI/BglII and ligated into the pCashsp40-LacZ vector. The resulting full length *dsarm* was then PCR amplified off the pCashsp40-LacZ vector using the above 5'ECT4-D NotI and 3' Ect4-D aa1610-1637 (AGATACTCGAGTTACCAAAATATCATGCGCCCGGCATTGGGGGAGGTGGCC TTGGACAGAATGATGCCCGAAAGTTCCTCGTCCTCCATTTCGTTGTTTTTTAT CAGCGAGCGGACCTTCTTCATCG), cut with NotI/XhoI, and ligated into pUAST vector (generating *pUASt-dsarm*). *pUASt-dsarm::GFP* was generated by cloning into the NotI/SpeI sites of pUASt-CT EGFP with PCR-amplification off the *pUASt-dsarm* construct using 5'ECT4-D NotI and 3' ECT4-D SpeI (GATCACTAGTCCAAAATATCATGCGCCCGGCATTGG).

The mito-tagRFP construct was created by PCR amplification of the mitochondrial targeting sequence (aa 1-24) of *Mus musculus* cytochrome c oxidase subunit VIIIb (GenBank AK003116) and insertion into the MCS of the pTagRFP-N vector (Evrogen).

Drosophila immunohistochemistry

Eye-imaginal discs from third instar larvae were dissected and TUNEL stained using In Situ Cell Death Detection Kit (Roche) as previous described (Klein, 2008). Embryos expressing *dMP2-Gal4, UAS-mCD8::GFP* were fixed as previously described (Miguel-Aliaga et al., 2008). 1st instar larvae expressing *dMP2-Gal4, UAS-mCD8::GFP* were imaged live. Whole brains from either pre-pupae or pupae 18 hrs APF were dissected and staining with anti-FasII as described (Lee and Luo, 2001). Embryos from *yw* or mutant stocks were fixed and staining with anti-FasII as preciously described (Lee and Luo, 2001). Fas II antibody was used at a 1:10 dilution (Developmental Studies Hybridoma Bank). For *dsarm* rescue experiments, *22aGal4* was recombined with *UAS-dsarm* using standard fly techniques, and MARCM clones were generated using a line containing *ey-flp, UAS-mCD8::GFP*. *Tdc-Gal4* neurons were imaged using a live fillet preparation (Ataman et al., 2008). Secondary antibodies were obtained from Jackson Immunolabs and used at 1:200.

Drosophila confocal microscopy

Samples were mounted in Vectashield antifade reagent and viewed on a III Everest Spinning disk confocal microscope. The entire antennal lobe was imaged in 0.27 μm

steps for each sample for scoring axonal integrity. TUNEL-stained eye-imaginal discs were imaged on a Zeiss LSM5 Pascal confocal microscope.

Drosophila sequencing

We utilized next-generation sequencing technology to re-sequence the entire genome of each mutant (along with our unmutagenized control line) to an average read depth of 70x. We identified between ~92,000 and ~140,000 variants, with ~14,000 to ~20,000 of these being non-synonymous or splice site changes genome wide. By filtering these variants for unique and coding changing lesions, only six genes were shared genome-wide among all three mutants (Supplementary Table 2-3). Since we sequenced heterozygous adult animals, we then narrowed these criteria to heterozygous and non-synonymous or splice site changes on chromosome 3L.

Additional details regarding this genome-wide resequencing can be found here:

Gonzalez MA, Van Booven D, Hulme W, Ulloa RH, Lebrigio RFA, Osterloh J, Logan M, Freeman M, Zuchner S. Whole Genome Sequencing and a New Bioinformatics Platform Allow for Rapid Gene Identification in *D. melanogaster* EMS Screens. *Biology*. 2012; 1(3):766-777.

Drosophila in situs

Standard methods were used for collection, fixation, and immunohistochemistry of *Drosophila yw* animals. dSARM cDNA corresponding to exon 5 in *dsarm* transcript RD was PCR-cloned into pCRII (Invitrogen). Digoxigenin-labeled RNA probes were generated according to the manufacturer's instructions (Roche). RNA in situ hybridization to embryos was carried out as described previously (Broadus et al., 1998). *T*hird-instar larvae were decapitated in 1× PBS and fixed in 9% formaldehyde in PBS for

45 mins. Larval heads were hybridized in hybridization buffer (50% formamide, 5X SSC, 5X Denhardts, 250 ug/ml yeast tRNA, 500 ug/ml herring sperm DNA, 50 ug/ml heparin, 2.5 mM EDTA, and 0.1% Tween-20). Adult heads were decapitated on CO_2 and transferred to plastic embedding molds containing Tissue-Tek OCT. The samples were frozen on dry ice, and 15 μm frozen sections were processed for in situ hybridization as previously described (Vosshall et al., 1999), with digoxigenin-labeled riboprobes and detected with TSA-Plus Fluorescein System (Perkin Elmer). Anti-digoxigenin-POD was diluted 1:500 (Roche).

Antibodies and reagents for mammalian studies

Antibodies used in this study were: mouse monoclonal anti-Tau-1 (clone PC1C6, #MAB3420), and rabbit anti-neurofilament-M (#AB1987) from Millipore; rabbit monoclonal anti-b-tubulin class III (#MRB-435P), and rabbit anti-a-internexin (#PRB-572C) from Covance; mouse monoclonal anti-b-actin (clone AC-15, #A5441) from Sigma; rat monoclonal anti-CD11b (clone M1/70.15, #MCA74EL) from AbD Serotec; rabbit anti-synaptophysin (#08-0130) from Invitrogen. Monoclonal neutralizing antibody against mouse NGF was previously described (Nikolaev et al., 2009). Secondary detecting antibodies conjugated with indicated Alexa dyes, and Alexa-594 conjugated a-bungarotoxin were from Invitrogen. Goat serum, donkey serum, and horseradish-peroxidase conjugated donkey anti-rabbit IgG and donkey anti-mouse IgG were from Jackson ImmunoResearch. All other chemicals were from Sigma unless otherwise specified.

Neuronal cultures

SCG explants were dissected from 0-2 day old pups and cultured as previously described (Gilley and Coleman, 2010). Axons were allowed to extend for 5 days before separation from the cell body mass using a scalpel. The degeneration of the isolated axons was followed at different time points for 72 h after cut. Bright field images were acquired on a microscope (IX8I; Olympus) coupled to a digital camera (U-TV 0.5XC; Olympus) using AnalySIS software (Soft Imaging Systems GmbH, Germany). Axonal protection was quantified as described (Gilley and Coleman, 2010). Typically an image of intact axons has a protection index (PI) value around 1. A PI around 0 occurs when axons detach from the dish. A two-way repeated measures analysis of variance (ANOVA) was used to show the difference in axonal protection between wild-type and *Sarm-/-*. For dissociated SCG cultures, cells were microinjected as previously described (Gilley and Coleman, 2010) with 50ng/µl of mito-tagRFP construct. 24 hours after microinjection cultures were immunostained using 0.3μg/ml mouse monoclonal anti-SARMI antibody (Chen et al., 2011) and Alexa-488 secondary antibody. Cultures were visualized on an Olympus FV1000 point scanning confocal microscope using a 60x 1.35NA apochromat objective.

For cortical neuron cultures, Campenot dividers (Tyler Research) were set up in poly-D-lysine/laminin-coated 2-well chamber culture slides. On DIV 0 (Day In Vitro 0), neocortices were dissected from six E16.5 *Sarm1* +/+ or *Sarm1* -/- embryos, pooled, and dissociated in Hank's Balanced Salt Solution (withouth Ca^{2+} and Mg^{2+}, Invitrogen)

containing 0.05 % (w/v) trypsin / EDTA at 37ºC for 10 min. After adding a final concentration of 10 % (v/v) heat-inactivated fetal bovine serum (HI-FBS), the tissues were spun down at 500 *g* for 3 min. The tissues were then suspended and triturated in Neurobasal / B-27 medium [Neurobasal medium supplemented with 2 % (v/v) B-27, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin] containing 10 % HI-FBS. The cells were plated in the cell-body compartment of Campenot dividers at a density of 2.5 x 10^5 per well. To facilitate the axotomy later, Campenot dividers were removed after 6 hours, when the cells already attached to the slides. From DIV 3, half of the culture medium was replaced every other day by fresh Neurobasal / B-27 medium containing 5 mM 5'-flourouridine and 5 mM uridine to suppress the proliferation of non-neuronal cells. On DIV 10, the cultures were subjected to axotomy.

For the dorsal root ganglion (DRG) cultures, DRGs were dissected from four E13.5 *Sarm1* +/+ or *Sarm1* -/- embryos on DIV 0. The explants from each embryo were individually plated in poly-D-lysine / laminin-coated 4-well chamber culture slides in F-12 / N-3 medium [Ham's F-12 medium supplemented with 40 mM glucose, 4 % (v/v) N-3 supplement, and 50 ng/ml mouse NGF]. On DIV 1, the cultures were changed to fresh F-12 / N-3 medium containing 1 μM cytosine β-D-arabinofuranoside to suppress the proliferation of non-neuronal cells. On DIV 3, the explants were subjected to either the axotomy, or the NGF deprivation with a final concentration of 50 mg/ml anti-NGF antibody.

To visualize the axons, the cultures were fixed in 4 % paraformaldehyde (w/v)/ phosphate-buffered saline (PBS) at room temperature for 30 min, followed by the permeabilization with 0.1% (v/v) Triton X-100 / PBS for 30 min. The axons were then immunostained with the indicated primary antibodies in 0.1% Triton X-100 / PBS containing 2 % (w/v) bovine serum albumin, and 2 % (v/v) goat serum at 4°C overnight, followed by the corresponding Alexa-488 or Alexa-568 conjugated secondary antibodies. To quantify axon degeneration, the images of distal axons were taken from 2 random fields per well, and 40 to 80 singly distinguishable axons in each field were examined, with any sign of fragmentation scored as degeneration. For each time point, 4 wells of *Sarm1* +/+ or *Sarm1* -/- cortical neuron cultures, or the DRG explants from 4 embryos of *Sarm1* +/+ or *Sarm1* -/-, were included.

Mouse surgery and immunohistochemistry

All surgical and experimental procedures in mice were performed in compliance with the protocols approved by the Institutional Animal Care and Use Committee of The Rockefeller University, Cornell Weill Medical College, and The University of Massachusetts Medical School. Mice were anesthetized with isoflurane, and the skin on their right hind limb was shaved and prepared with iodine and alcohol. An incision was made between the knee and the hip joint, and the gluteal muscles were separated carefully with a pair of forceps. The sciatic nerve was transected as close to the thigh with a pair of sterile surgical scissors, and 1- to 2-mm of nerve segment was removed to prevent the regeneration of axons into the distal stump. The gluteal muscles were then

brought back into their original anatomical position, and the overlying skin was re-approximated by surgical staples or sutures.

For light microscopy, the animals were euthanized at indicated time points post-surgery and nerve segments 3-6 mm distal to the lesion fixed with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4 (72h at 4ºC). After an extensive wash in 0.1 M PBS, 2 h of postfixation (1% osmium tetroxide) and dehydration in graded ethanol and propylene oxide, nerve segments were embedded in Durcupan resin (Fluka Chemie). After polymerization for 48 h at 60ºC, transverse semithin sections (0.5μm) were cut on a Leica ultramicrotome, stained with toluidine blue and photomicrographed. To quantify survival, 500 randomly chosen axons were counted. Survival criteria were normal myelin sheaths, uniform axoplasm and intact, unswollen mitochondria. A two-tailed one sample t-test was performed using GraphPad Prism 5.

For the biochemical analysis of sciatic nerves, the animals were euthanized at indicated time points post-surgery. A 10-mm segment of the nerve distal to the transection site was harvested, and immediately homogenized in 200 ml Urea / SDS buffer [50 mM Tris-Cl (pH 6.8), 8.0 M urea, 10 % (w/v) SDS, 10 mM sodium EDTA, and 50 mM DTT]. The nerve samples from two *Sarm1* +/+ or *Sarm1* -/- mice were processed for each time point. After heating at 95ºC for 10 min, 10-ml aliquot of each nerve homogenate was subjected to 4 - 15 % gradient Tris-glycine SDS-PAGE (Bio-Rad), and transferred to Immobilon-P PVDF membranes (Millipore) for immunoblot analysis. The membranes were immunoblotted with the indicated primary antibodies,

and then the corresponding secondary antibodies in PBS/Tween-20. The bound antibodies were visualized by SuperSignal chemiluminescence reagents (Pierce). All membranes were exposed to Phoenix Blue X-ray film for 5 to 10 sec.

For immunohistochemistry, the mice were lethally anesthetized at the indicated time points post-surgery, and transcardially perfused with 4 % paraformaldehyde/PBS. The sciatic nerves distal to the transection site was dissected, and post-fixed in 4 % paraformaldehyde/PBS at 4ºC overnight. After washing three times in PBS, the nerves were cryoprotected in 30 % (w/v) sucrose / PBS at 4ºC overnight, and then frozen in the 2:1 mixture of 30 % sucrose/PBS:OCT (Tissue Tek) for 12-mm longitudinal cryosections. The nerves were permeabilized in 0.5 % Triton X-100 / PBS for 1 hour, and blocked in 0.5 % Triton X-100/PBS containing 2 % bovine serum albumin, and 4 % goat serum at 4ºC overnight. Immunohistochemistry was carried out with either rabbit anti-neurofilament-M (1:500) or rat anti-CD11b (1:500) in the same blocking buffer at 4ºC overnight, followed by washing for 1 hour in 0.5 % Triton X-100/PBS three times. The sections were then labeled with the corresponding Alexa-488 or Alexa-568 conjugated secondary antibodies for 2 hours, washed in PBS, and mounted in fluoromount-G. Images were taken at 2-mm distal to the transection sites, and 3 nonadjacent sections of each nerve sample were examined. Four (neurofilament-M axons) or three (CD11b macrophages / monocytes) mice of *Sarm1*+/+ or *Sarm1*-/- were included per time point.

To examine denervation at neuromuscular junctions, the tibialis anterior muscles were dissected from perfused animals, and post-fixed in 1% paraformaldehyde/PBS at 4ºC overnight. After washing three times in PBS, muscles were cryoprotected in 30% sucrose/PBS at 4ºC overnight, and embedded in OCT for 80-mm longitudinal cryosections. The tissues were permeabilized in 0.5 % Triton X-100/PBS for 1 hour, and blocked in 1% Triton X-100/PBS containing 4% bovine serum albumin, and 4% donkey serum at 4ºC overnight. To label axons and neuromuscular junctions, muscles were stained with rabbit anti-neurofilament-M (1:500) and rabbit anti-synaptophysin (1:5) in the same blocking buffer at 4ºC for at least 24 hours. After washing for 1 hour in 0.5% Triton X-100/PBS three times, the muscles were incubated with Alexa-647 conjugated donkey anti-rabbit antibody (1:500) and Alexa-594 conjugated a-bungarotoxin (1:1000) overnight. After washing in PBS for 4 hours, sections were mounted in fluoromount-G, and imaged by LSM 510 laser scanning confocal microscope. To analyze presynaptic structures, maximum-intensity projections of z-stack images from 6 to 8 nonadjacent sections of each muscle sample were generated by AutoQuant X. Partial or full denervation was determined as the postsynaptic AChR sites not apposed by the presynaptic marker (colored in green for better visualization). About 100 neuromuscular junctions were examined for each muscle, and four *Sarm1* +/+ or *Sarm1* -/- mice were included per time point.

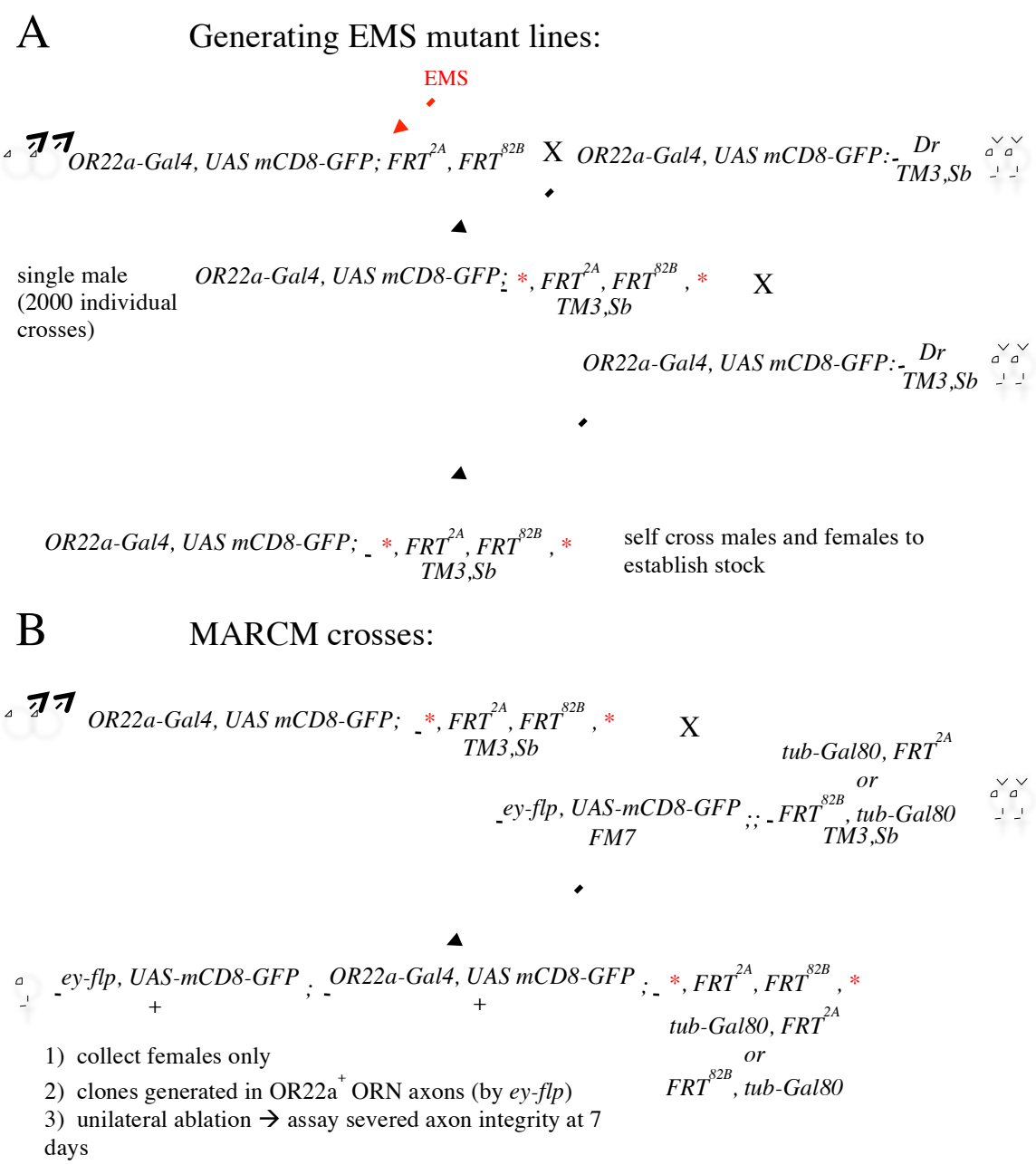


Figure 2-1. Crossing schemes for EMS mutant lines and generating MARCM lines for screening

A. Crossing scheme for generating a collection of ~2000 mutant stocks, each with a unique EMS-mutagenized third chromosome containing FRT sites on each arm.
B. Scheme for generating MARCM clones and screening mutants for phenotypes in OR22a-positive neurons using *ey-flp*.

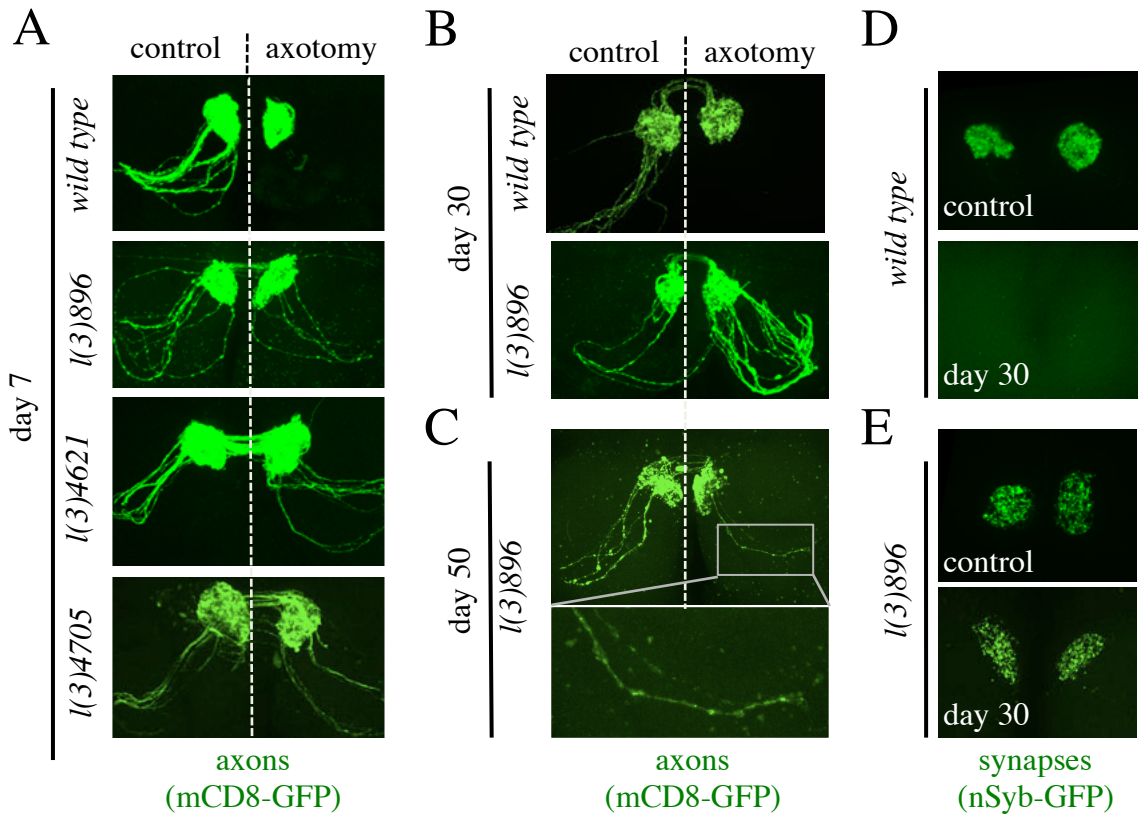


Figure 2-2. Identification of three mutations that suppress Wallerian degeneration *in vivo*

A. ORN MARCM clones in control, *l(3)896*, *l(3)4621*, and *l(3)4705*. Right, axotomy; left, uninjured control. Boxed regions are enlarged at right. n≥15.
B. Control and *l(3)896* brains 30 days after injury. n≥10.
C. *l(3)896* clones 50 days after injury. n=11.
D. Control ORN MARCM clones labeled with *UAS-nSyb::GFP*, uninjured (top) and 30 days after axotomy (bottom). n≥15.
E. *l(3)896* MARCM clones labeled with *UAS-nSyb::GFP*, uninjured (top) and 30 days after axotomy (bottom). n≥15.

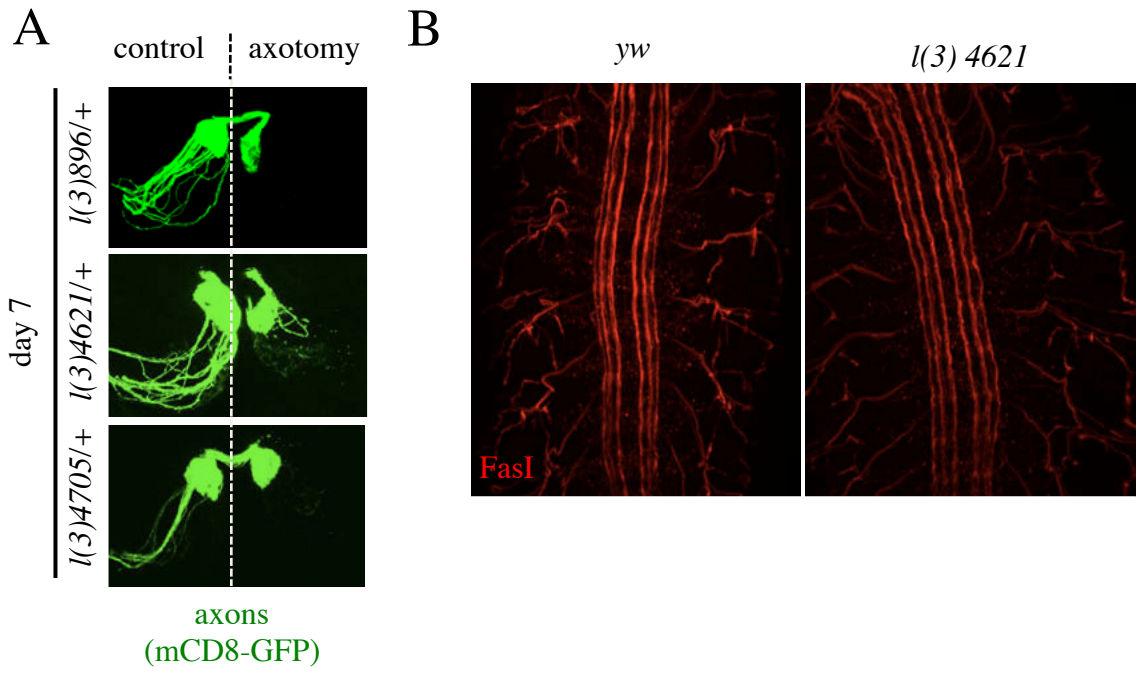


Figure 2-3. Mutants that suppress Wallerian degeneration are recessive and do not affect neuronal development

A. Axon integrity was assayed in heterozygous animals of the indicated genotypes 7 days after axotomy. Axons are labeled with *OR22aGal4, UAS-mCD8::GFP* in a non-MARCM background. n≥7 antennal lobes.

B. Embryonic motorneurons and interneurons were labeled using anti-FasII staining (anti-1D4, red) in control and *l(3)4621* backgrounds. Motor neuron innervation of muscles in the body wall appeared unaffected in mutants (not shown). n≥10 for both.

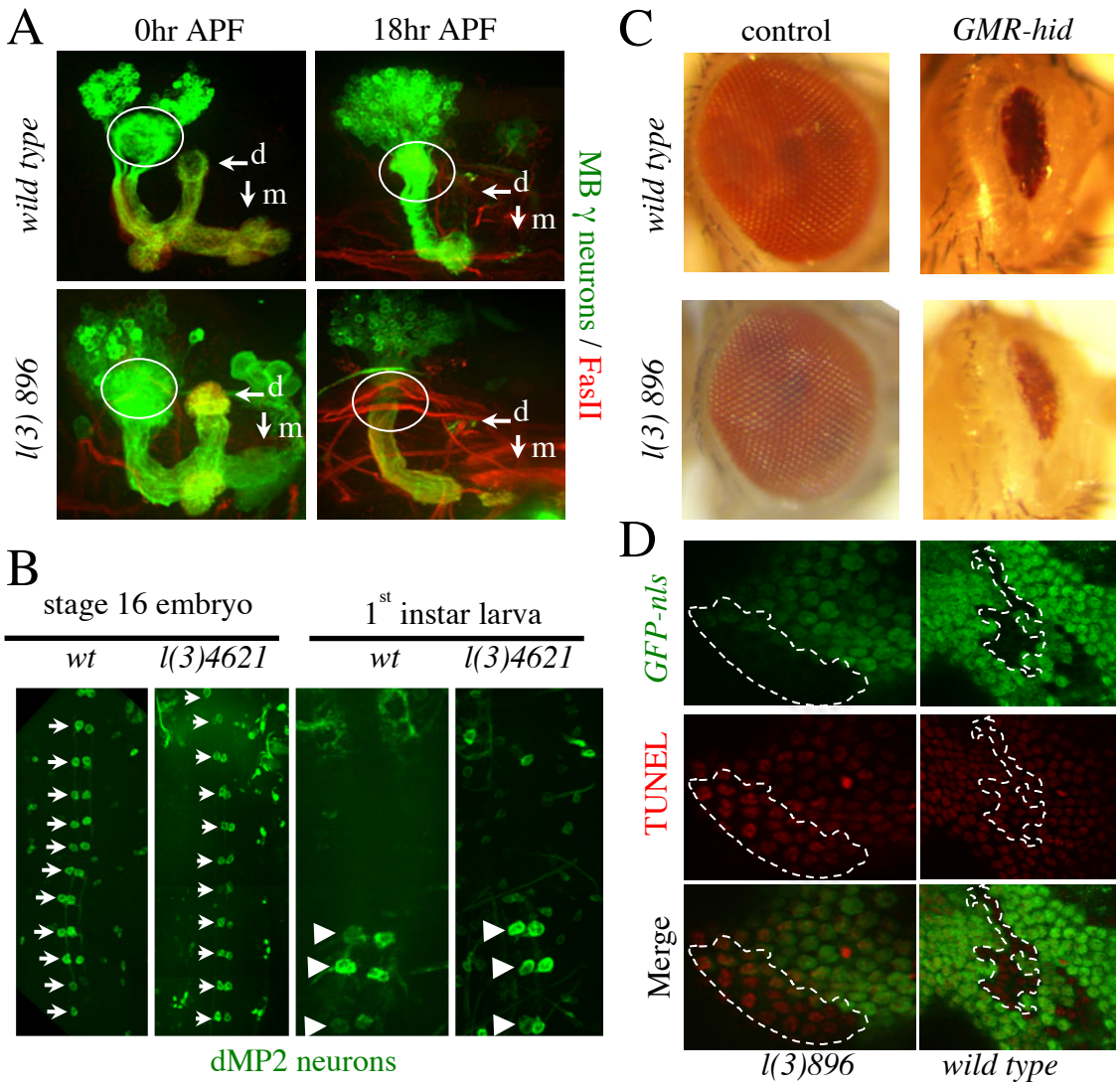
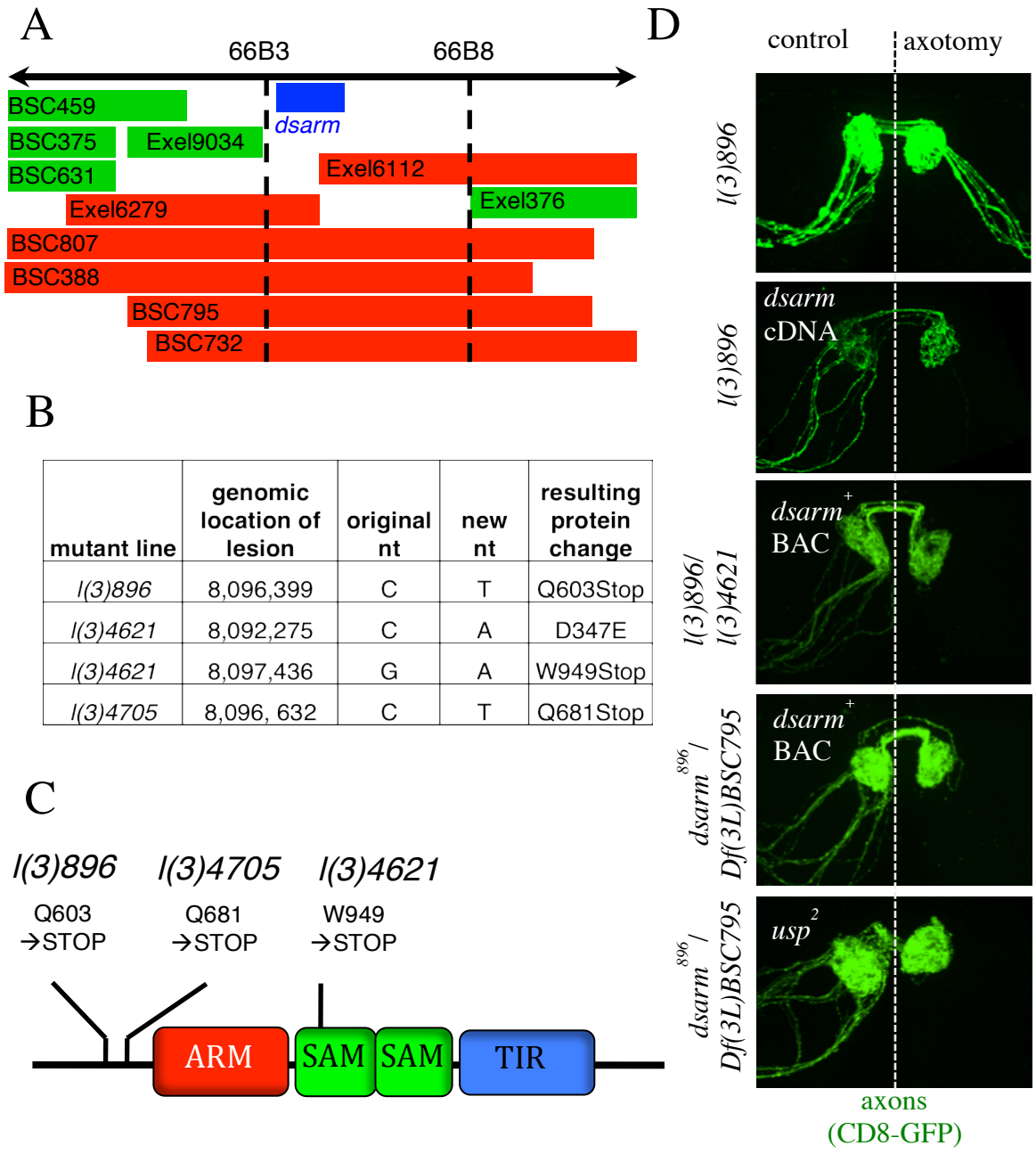


Figure 2-4. Wallerian degeneration mutants do not block other forms of neural degeneration

Figure 2-4. Wallerian degeneration mutants do not block other forms of neural degeneration

A. MARCM clones MB ϒ neurons in control and *l(3)896* backgrounds at the indicated developmental stages. dorsal (d) and medial (m) axonal branches (arrows), and dendrites (circled). n≥15 for all.
B. dMP2 neurons with *GFP*. Ventral views (anterior up) of stage 16 embryos (left) and 1st instar larvae. dMP2 neurons before (arrows) and after (arrowheads) segment-specific apoptosis. n≥20 at each time point.
C. Wild type and *l(3)896* mutant clones with (right) or without (left) ectopic expression of *hid*. n≥20.
D. Wild type and *l(3)896* mutant clones in the eye-antennal disc of 3rd instar larvae. Homozygous mutant clones are labeled as GFP-negative (circled). Red, TUNEL staining. n≥10.



mutant line	genomic location of lesion	original nt	new nt	resulting protein change
l(3)896	8,096,399	C	T	Q603Stop
l(3)4621	8,092,275	C	A	D347E
l(3)4621	8,097,436	G	A	W949Stop
l(3)4705	8,096, 632	C	T	Q681Stop

Figure 2-5. Mutations in *dsarm* block Wallerian degeneration

Figure 2-5. Mutations in *dsarm* block Wallerian degeneration

A. The lethality of *l(3)896*, *l(3)4621*, and *l(3)4705* was mapped to region 66B. Red boxes, deficiency/mutants is lethal; green boxes, deficiency complements lethality.
B. The locations of the point mutations in *dsarm* that block axon degeneration and their corresponding resulting protein change. nt, nucleotide
C. Dsarm protein domains, positions and effect of predicted point mutations.
D. *UAS-dsarm*, a *dsarm* transgenic BAC, or a translocation of *dsarm* to the X chromosome (*usp*^{2}*) rescue* axonal degeneration defects 7 days after injury in the listed mutants. n=12.

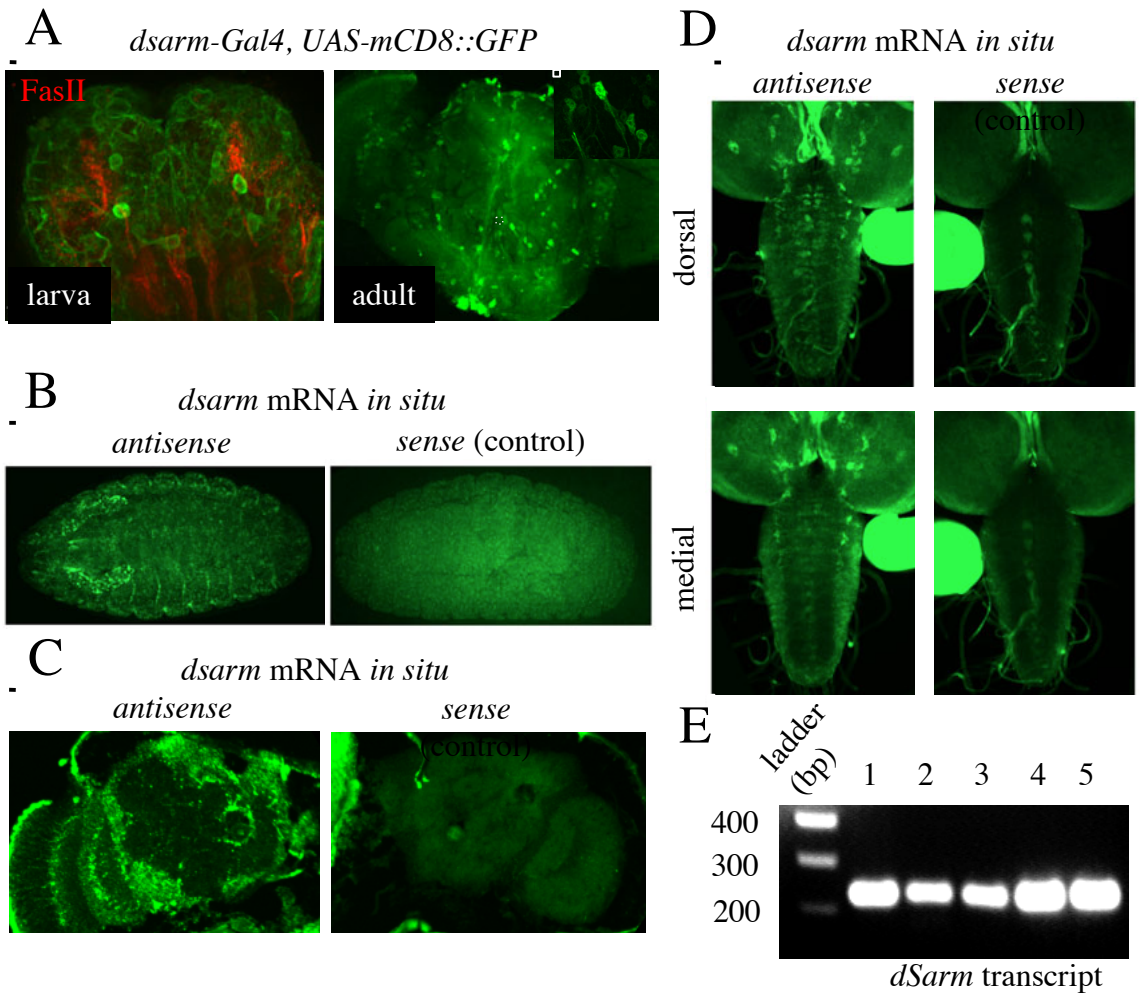


Figure 2-6. dsarm has broad endogenous expression

Figure 2-6. dsarm has broad endogenous expression

A. A *P[GawB]* insertion in the *dsarm* locus (*dsarm-Gal4*) drives *UAS-mCD8::GFP* in the larval (left) and adult (right) brain. Larval brains were co-stained for FasII, n>5 brains each.
B. RNA *in situ* hybridization to embryos using *dsarm* probes. Note low level, but broad expression throughout embryo with antisense probes.
C. RNA *in situ* hybridization to adult brain sections. Note broad expression throughout cell cortex and in visual system, and low signal in the neuropil (consistent with cell body localization of transcripts).
D. RNA *in situ* hybridization to larval brain sections. Note low level, but broad expression throughout larval brain in dorsal cell body-rich regions, in lateral regions of the ventral nerve cord in more medial regions, and low expression in the neuropil with antisense probes.
E. Non-quantitative RT-PCR for the *dsarm* transcript. Lane 1: yw embryos; lane 2: unmutagenized control strain, L1 larvae; lane 3: *l(3)896/Df(3L)BSC796* L1 larvae; lane 4: unmutagenized control strain, adult brain; and lane 5: canton S adult brain.

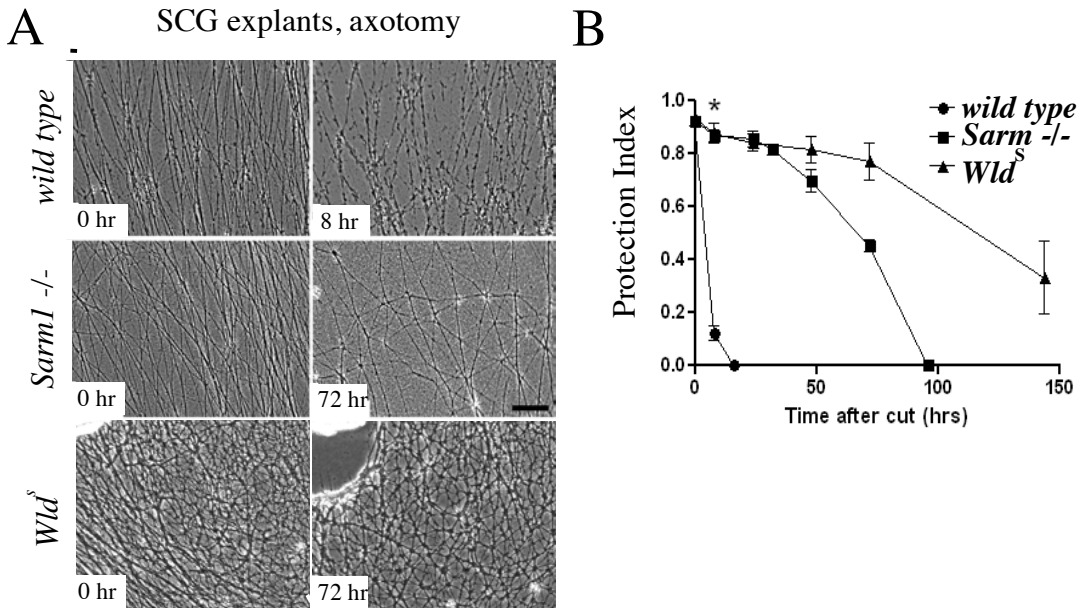


Figure 2-7. *Sarm1 -/-* SCG cultures are protected from Wallerian degeneration

A. Phase contrast images of SCG explant cultures from wild type (top), *Sarm1 -/-* (middle), and Wld^s expressing (bottom) animals at the indicated time after axotomy.

B. Quantification from A. Mean ± SEM, *$p<0.01$.

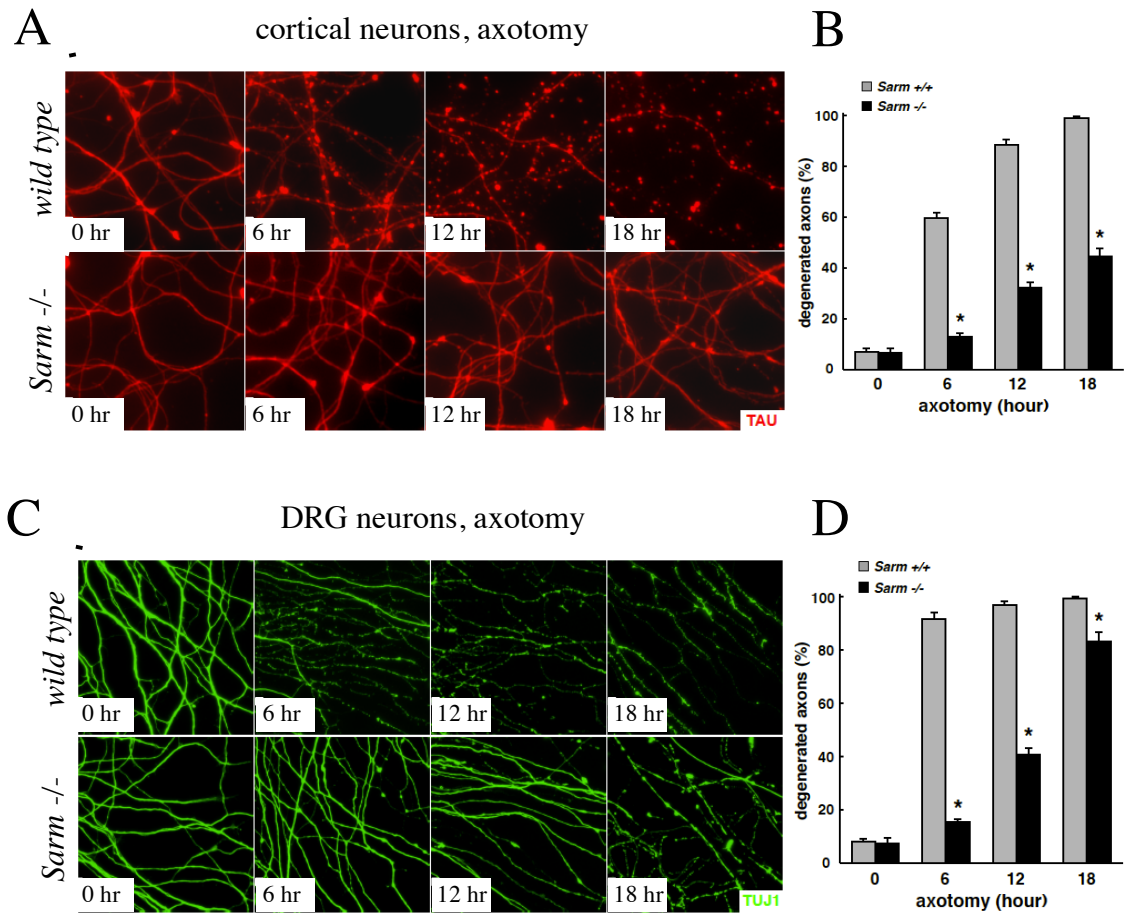


Figure 2-8. *Sarm1 -/-* primary cultures are protected from Wallerian degeneration

A. Axon preservation at the indicated time points in cortical neuron cultures from E16.5 mouse embryos. a-Tau, red.
B. Quantification from C. Mean ± SEM, *$p<0.01$.
C. Axon preservation at the indicated time points in DRG cultures from E13.5 mouse embryos. a-TUJI, green.
D. Quantification from E. Mean ± SEM, *$p<0.01$.

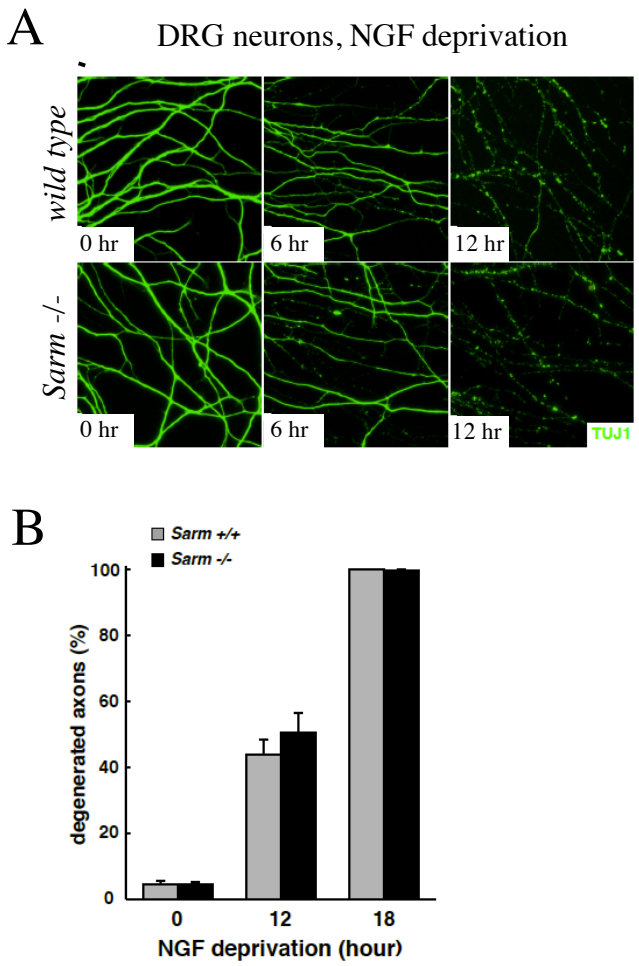


Figure 2-9. *Sarm1* -/- primary cultures are not protected from NGF withdrawal-induced axonal degeneration

A. DRG explant cultures from E13.5 mouse embryos after NGF withdrawal at the indicated time points. a-TUJI, green.

B. Quantification from G. Mean ± SEM, *$p<0.01$.

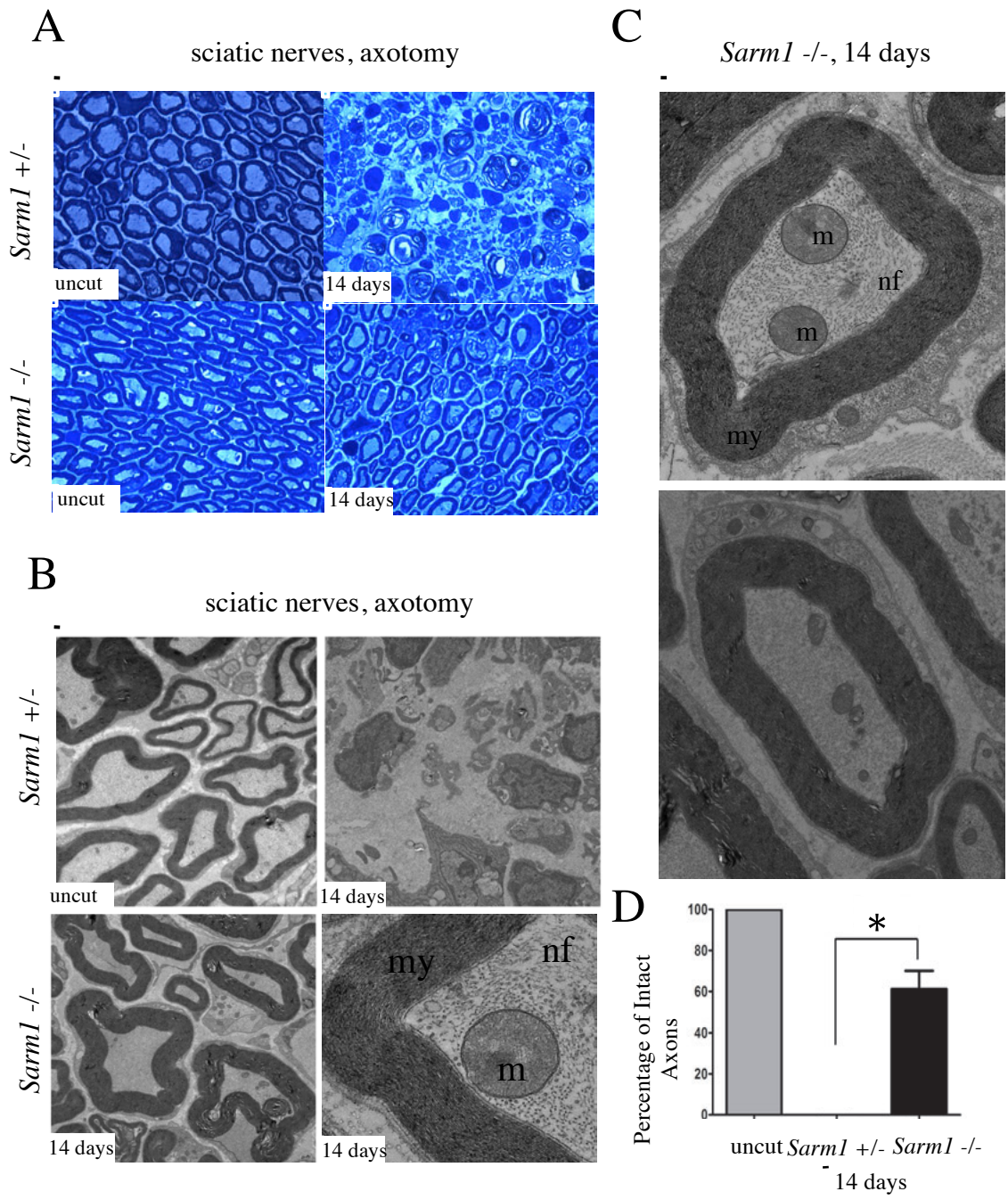


Figure 2-10. *Sarm1* is required for Wallerian degeneration in mice *in vivo*

Figure 2-10. *Sarm1* is required for Wallerian degeneration in mice *in vivo*

A. Sciatic nerve distal to the injury site stained with Toludine blue. Time points and genotypes as indicated.
B. Ultra-structural analysis of *Sarm1*+/- and *Sarm1* -/- axons before or 14 days after axotomy. my, myelin; nf, neurofilaments, m, mitochondrion.
C. Additional images of *Sarm1* -/- axons 14 days after injury.
D. Quantification from A. n=5 for all. ($p = 0.0002$).

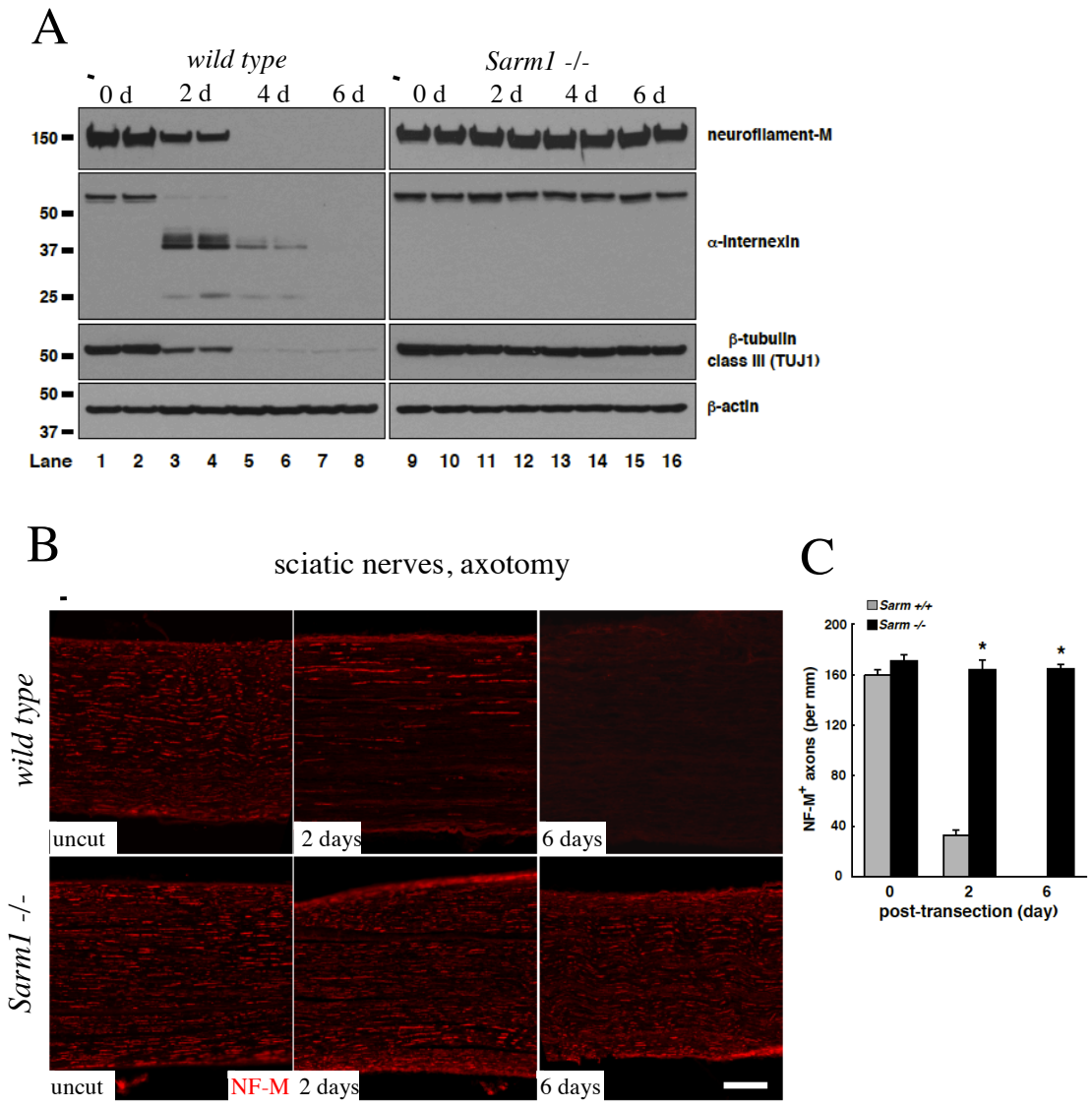


Figure 2-11. Cytoskeletal markers are persevered after injury in *Sarm1 -/-* axons

A. Immunoblot analysis of distal injured nerve segment. n=4 at each time point and genotype.

B. Transected nerves were stained for neurofilament-M as a marker of structure integrity of the injured axon at the indicated time points. n=4 mice for all.

C. Quantification of B. Values are presented as mean ± SEM, *$p<0.01$.

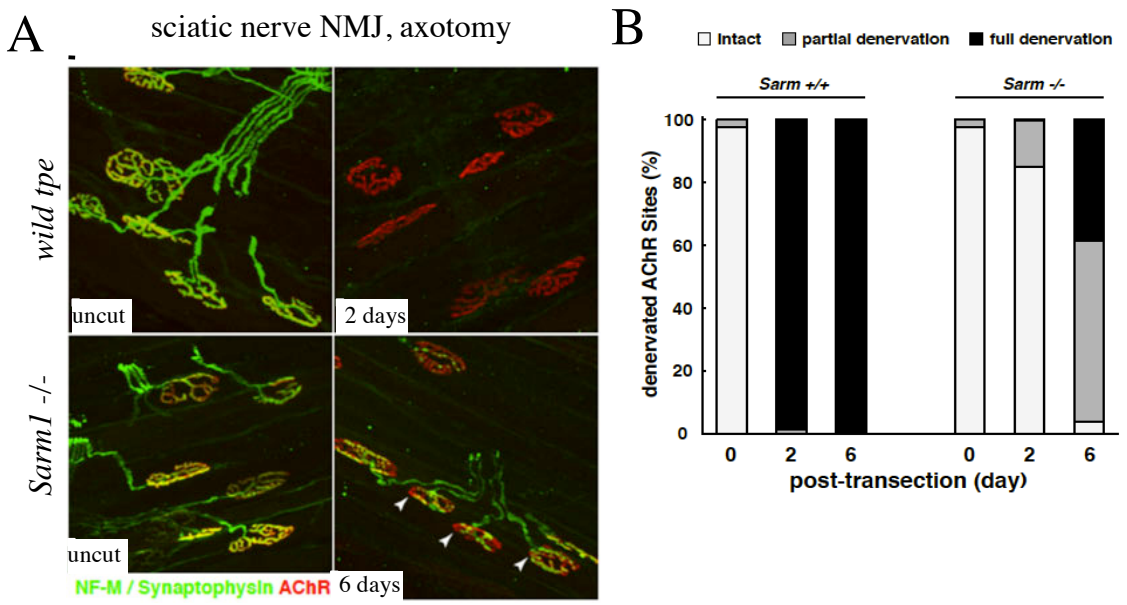


Figure 2-12. NMJs are preserved in *Sarm1* -/- transected nerves

A. NMJ preservation at tibialis anterior muscles. red, AChR (post synapse/muscle); green, NF-M / synpatophysin (presynapse).

B. Quantification from A. n >200 synapses for each genotype and time point.

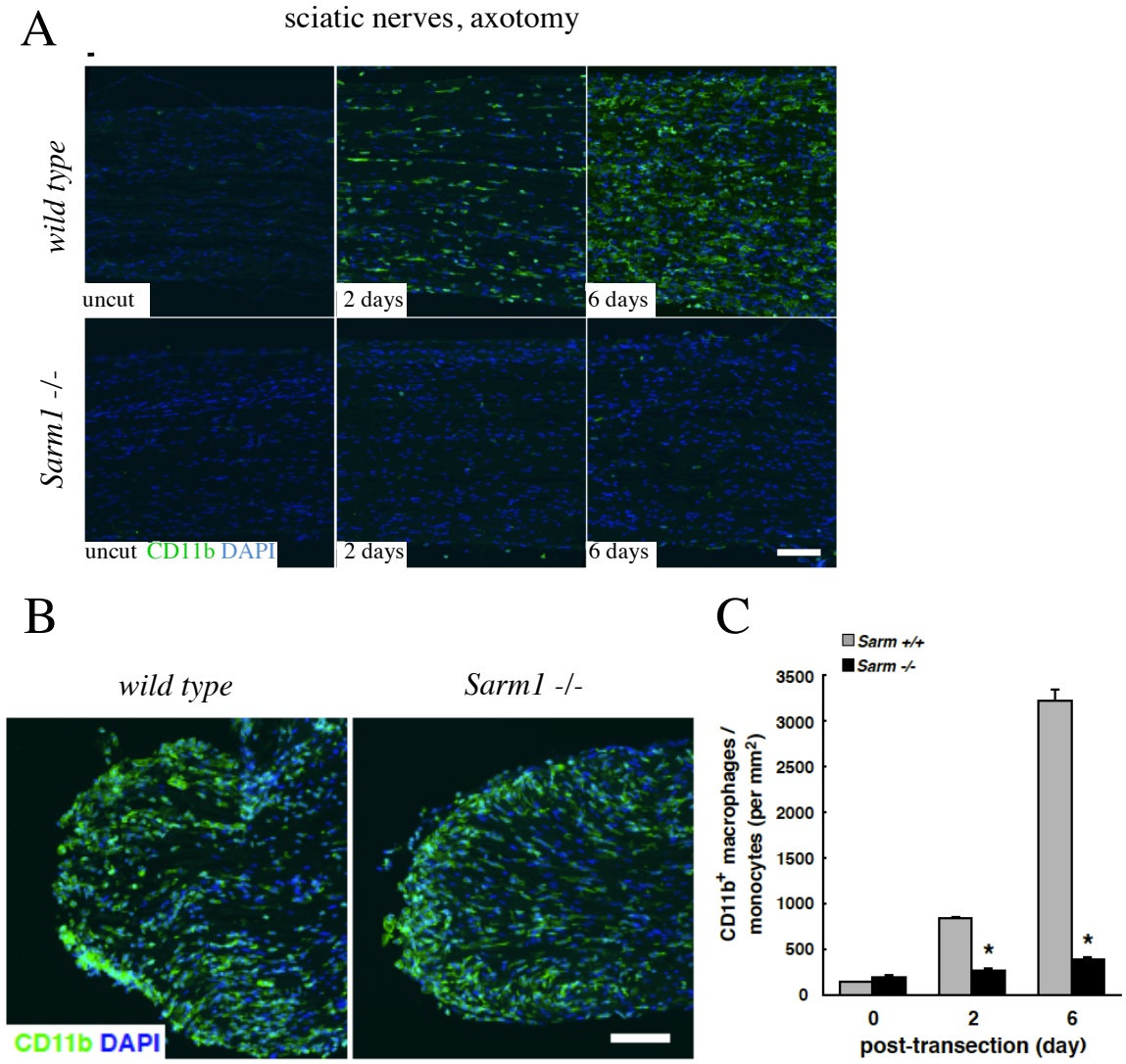


Figure 2-13. Macrophages do not infiltrate severed axons in *Sarm1* -/- animals

A. Macrophage/monocyte infiltration into transected nerves was assayed by staining for CD11b (macrophages) and DAPI (all cells) at the indicated time points after lesion. n=4 mice for all.

B. Macrophage infiltration at the primary injury site was assayed using CD11b staining. scale bar is 100 μm. n=4 mice for all.

C. Quantification of A. Values are presented as mean ± SEM, *$p<0.01$.

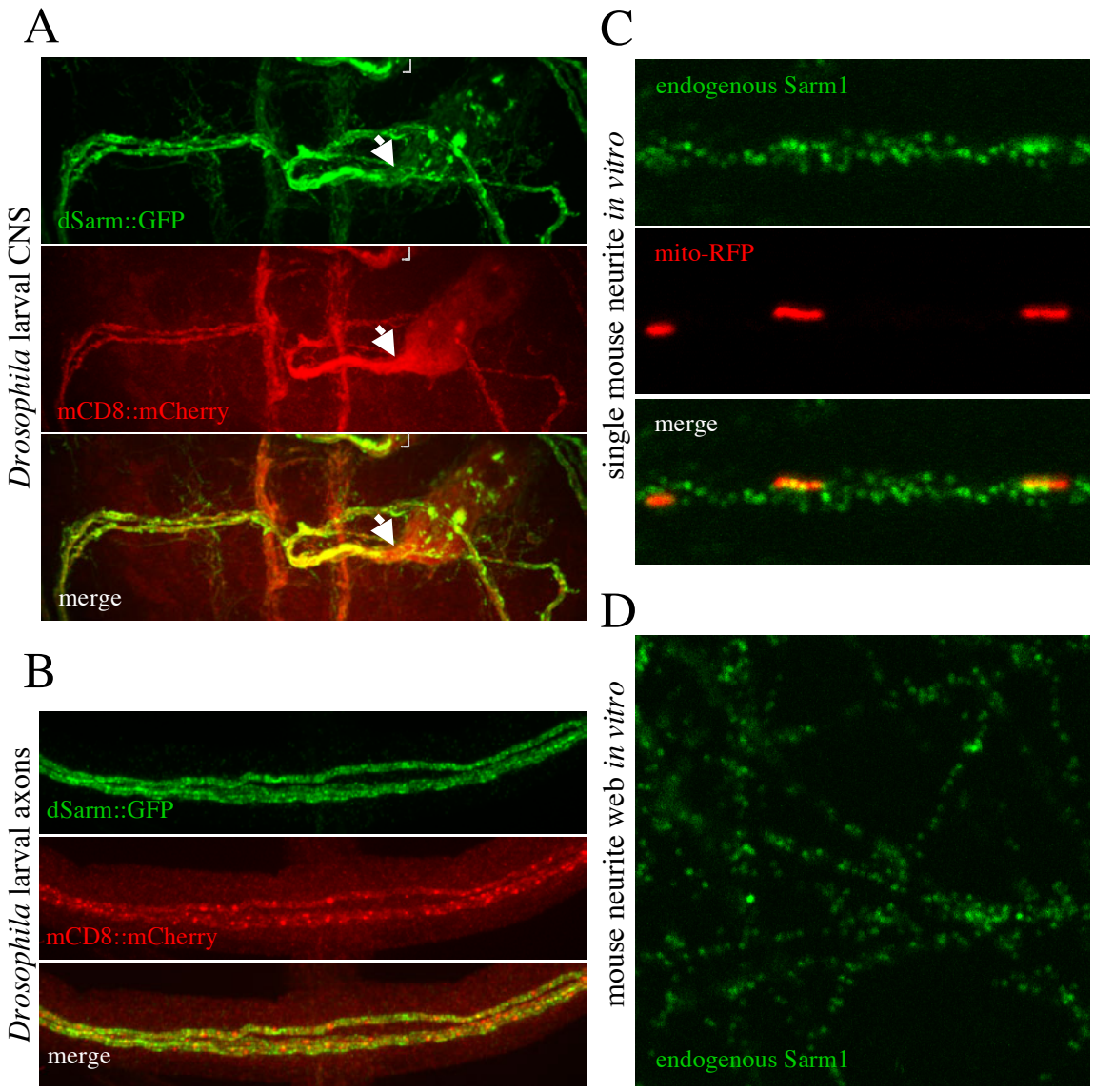


Figure 2-14. dSarm and Sarm1 localize to neurites *in vivo*

A. *UAS-dsarm::GFP* and *UAS-mCD8::mCherry* were driven with *tdc-Gal4* in third instar larvae. Arrows: cell bodies. n >5 larvae.

B. More distal axons from animals in A.

C. A single neurite from a wild type dissociated SCG cultured neuron expressing mito::RFP was stained for endogenous Sarm1 (green).

D. A web of multiple neurites from a wild type dissociated SCG cultured neuron was stained for endogenous Sarm1 (green).

Gene	Mutant Allele	Allele Type
ask1/pk92B	DN	OE
atg1	Δ3D	LOF
atg1	KQ #5B	OE of DN
atg1	68	OE of DN
atg1	EP3009	LOF
atg1	KG03098	LOF
atg1	EP3348	LOF
atg2	EP3697	LOF
atg6	0.00096	LOF
atg7	d77	LOF
atg7	d14	LOF
atg18	KG03098	LOF
bsk/Jnk	DN	OE
bsk/Jnk	flp147E	LOF
buffy	H37	LOF
calcineurin A1	ED6346	DEF
calcineurin A1	BSC749	DEF
calx	UAS	OE
CaMKII	UAS	OE of DN
cullin-3	mds1	LOF
cullin-3	UAS	OE
cyt-c d	bin1	LOF
damm	f02209	LOF
dark	CD4	hypomorph
dark	CD8	hypomorph
debcl	E36	LOF

Gene	Mutant Allele	Allele Type
debcl	W105	truncated 5'
dIAP/thread	th1	LOF
dIAP/thread	UAS	OE
dIAP2	G2326	OE
dredd	B118	Null
dronc	CG	OE of DN
dronc	51	LOF
drp1	KG03815	LOF
dunce	1	Null
hsp22	EP(3)3247	OE
ik2	KAIA	OE of DN
imd	1	Null
mstprox/toll-3	Exel 6146	Def
omi/htra2	UAS	OE
omi/htra2	Δ07	LOF
p35	UAS	OE
pmn	UAS	OE
puckered	UAS	OE
roc1b	dc3	LOF
sod1	UAS	OE
strica	4	Null
toll-6	ex13	Null
toll-7	g1.1	Null
tollo	59	Null
tor	TED	UAS of DN

Table 2-1. Mutants tested that fail to suppress Wallerian degeneration
All mutants or misexpression constructs were crossed into a background where a subset of ORNs were labeled with GFP (*22a-Gal4, UAS-mCD8::GFP*). ORN axons were severed, and degeneration was scored 5 days after axotomy. LOF = reported amorphic loss of function allele; OE = over expression of construct under UAS-promoter control; DN = dominant negative; FL = full length of unmutagenized gene. n ≥10 antennal lobes for all.

mosaic chromosome	**number of axons, uninjured***	**number of axons, 7 days after axotomy***
wild type	11.08±1.52	0
l(3)896	4.71±1.76	5.43±2.12
l(3)4621	7.44±0.66	6.13±0.74
l(3)4705	4.94±0.85	5.00±1.61

Table 2-2. Production of MARCM clones and persistence of severed axons in mutant backgrounds

*Represents number of individual axon fibers identified in z-stacks from confocal imaging of entire antennal lobe. n≥10 antennal lobes for all.

	One mutant line (# of genes)	Any two mutant lines (maximum # of genes)	All three mutant lines (# of genes)
Unique coding variants; genome wide	92 (*l(3)896*) 997 (*l(3)46210*) 272 (*l(3)4705*)	166	6
Unique coding variants; chromosome 3L	34 (*l(3)896*) 132 (*l(3)46210*) 46 (*l(3)4705*)	8	3
+ nonsynonymous or splice site and heterozygous changes	17 (*l(3)896*) 66 (*l(3)46210*) 23 (*l(3)4705*)	2	1

Table 2-3. Identification of *dsarm* through re-sequencing of mutant genomes
The number of identified variants are indicated within and across mutant lines. Filtering strategy is as indicated, see methods for details.

CHAPTER III: Identification of key domains in dSarm for promotion of Wallerian degeneration

The following work was performed in the laboratory of Dr. Marc Freeman. Amy Sheehan cloned all of the constructs used in this study, and I performed all experiments presented here. Lukas Neukomm has confirmed many of these experiments in the *Drosophila* wing sensory neurons, which are not presented here.

The following publication is in preparation as follows:

Osterloh, JM, Neukomm, L, Sheehan A, and Freeman MR. Removal of auto-inhibitory domain of dSarm induces spontaneous Wallerian-like degeneration.

Abstract

dSarm/Sarm1 is required for severed axons to undergo Wallerian degeneration, although the mechanism by which dSarm/Sarm1 promotes axon self destruction is unknown. To investigate this mechanism, we made truncated dSarm constructs lacking predicted protein domain(s). dSarm function requires both the sterile alpha motif (SAM) and Toll/Interluekin-1 receptor homology domain (TIR) to promote Wallerian degeneration, and this SAM domain is required for localization. Interestingly, the third domain, Armadillo/HEAT domain (ARM), appears to normally auto-inhibit dSarm activity, as loss of the ARM domain results in spontaneous Wallerian-like degeneration in uninjured adult neurons. *dSarmΔARM* expression induces not only degeneration of axons, but also of dendrites and cell bodies that occurs independently of caspases. This suggests that the ARM domain in dSarm may be a crucial regulator of Wallerian degeneration activation and axon-death machinery can also promote the destruction of other neuronal parts. Amazingly, Wld^s co-expression potently protects neurons from this induced degeneration, indicating that Wld^s functions downstream of dSarm to protect severed axons.

Introduction

Axon degeneration is a hallmark of most neurodegenerative disorders (Coleman, 2005). Axonal pathology during disease states often resembles that of an axon transected from its cell body (Coleman and Perry, 2002). Such a transection induces Wallerian degeneration, where the severed axon fragments and is engulfed by surrounding tissue (Waller, 1850). While long believed to be a passive wasting way, Wallerian degeneration is now known to be controlled by a genetically encoded axon self-destruction program, akin to but molecularly distinct from apoptosis (Miller et al., 2009; Osterloh et al., 2012; Xiong et al., 2012).

Wallerian degeneration can be inhibited with multiple genetic mutations. The first discovered was an overexpression of a chimeric fusion protein, termed slow Wallerian degeneration (Wld^{s}), in which enzymatically active Nmnat is redistributed from the nucleus to the axon, conferring protection via a mechanism(s) of action that remains controversial (Lunn et al., 1989; Conforti et al., 2000; Mack et al., 2001; Beirowski et al., 2010). To investigate further pathway(s) involved in axon degeneration, we recently reported the first forward genetic screen for mutants defective in Wallerian degeneration (Osterloh et al., 2012). With this screen in *Drosophila,* we identified *dSarm* (*Drosophila* sterile alpha and armadillo motif containing protein) as a gene required for severed axons to undergo Wallerian degeneration. Its highly conserved mammalian ortholog, *Sarm1*, is also required for Wallerian degeneration, as severed axons are protected from degeneration for weeks *in vivo* in *Sarm1* null mice (Osterloh et al., 2012). dSarm/Sarm1 plays a vital role in regulating an axon's response to injury.

The cellular function of Sarm is beginning to emerge as a scaffold for signaling kinases that together promote cellular destruction. The *C. elegans* ortholog TIR-1 interacts with the calcium-activated CaMKII and the MAPK ASK1 to regulate downstream transcription (Chuang and Bargmann, 2005). All predicted domains of Sarm are anticipated to be involved in protein-protein interactions and without enzymatic activity. Together, this suggests that Sarm may act as a framework to bring together and direct pro-Wallerian degeneration kinase activation. Interestingly, in mammalian T-cells, overexpression of *Sarm1* can induce apoptosis and *Sarm1* knock down increases T-cell survival and proliferation (Panneerselvam et al., 2013), indicating a potential role for Sarm in cell survival. Interestingly, Sarm appears to localize to mitochondria with overexpression in human kidney cell lines (Kim et al., 2007; Panneerselvam et al., 2012), but not in neurons either *in vitro* or *in vivo* (Chuang and Bargmann, 2005; Chen et al., 2011; Osterloh et al., 2012). The reason for this variance remains unknown, although it may be due to an artifact of tissue culture. A role for Sarm has also recently been described in regulating neuronal polarity and dendritic outgrowth (Chen et al., 2011), further suggesting that Sarm has multiple responsibilities depending on cell type and context.

To investigate how dSarm regulates Wallerian degeneration, we turned to its predicted protein structure. Sarm was predicted to have three domains: armadillo/HEAT domains (ARM), sterile alpha motif (SAM), and Toll/Interleukin-1 receptor homology domain (TIR) (O'Neill et al., 2003). Both the ARM and SAM domains are predicted to play a role in protein-protein interactions, although there is no clear target for these

interactions. The SAM domain has been implicated in localization (Chuang and Bargmann, 2005), while the ARM domain may be auto-regulatory (Chuang and Bargmann, 2005; Carty et al., 2006; Peng et al., 2010). TIR domains traditionally mediate signaling between Toll-like receptors (TLRs) and their downstream signaling molecules (Brikos and O'Neill, 2008), although what part, if any, TLR signaling plays in Wallerian degeneration remains to be seen.

To investigate the function of dSarm in Wallerian degeneration, we created truncated deletion constructs and assayed their ability to promote or inhibit axon degeneration *in vivo*. We show here that the SAM and TIR domains are critical for dSarm function. While the SAM domain dictates dSarm localization, the ARM domain acts as an auto-inhibitory domain. Removal of the ARM domain results in an activated form of dSarm that induces spontaneous Wallerian-like degeneration in the absence of an injury signal. This suggests that regulation of the ARM domain is a critical step in activating Wallerian degeneration. Furthermore, dSarmΔARM induced degeneration occurs independently of caspases but is blocked by co-expression with Wld^{s}. This argues further that Wld^{s} normally functions downstream of dSarm to block Wallerian degeneration.

Materials and Methods

Drosophila stocks, transgenics, and injury protocol

The following *Drosophila* stocks were used in this study: *OR22a-Gal4; Elav-Gal4; GH146-Gal4; PDF-Gal4; 109(2)80-Gal4; ey-flp; FRT2A, tubulin-Gal80; Gal80^{ts};UAS-mCD8::GFP; UAS-myr::tdt::tomato; FRT2A, dSarm^{896}* (Osterloh et al., 2012)*;* and *UAS-*

dSarm::EGFP (Osterloh et al., 2012), all from the Bloomington Stock Center unless otherwise noted. We induced antennal injury using a modification of a previously described protocol (MacDonald et al., 2006). Adult flies were aged for 7 days at 25°C after ablating the right third antennal segment only. Axonal integrity was scored as previously described (MacDonald et al., 2006; Avery et al., 2009) and injured axon counts were normalized to the mean axon number in uninjured samples to generation percentage of intact axons after injury. Data sets were compared using a paired t-test with Graph Pad Prism 5 software. Larval sensory neurons were dissected as previously described (Grueber et al., 2007)

Cloning

UAS-dSarm::myc was cloned by amplifying the full length *dSarm isoform D* (Osterloh et al., 2012) with 5'Ect4-D NotI (atatatatgcggccgcaaaacATGGGCAATCGTTTGAGCGGC) and 3'Ect4-D NotI (atatatatagcggccgCCAAAATATCATGCGCCCGGCATTGG) and inserted into *pUASt-myc* using Not1.

UAS-dSarmΔARM::GFP was cloned by amplifying nucleotides 1-2091 (amino acids 1-697) with 5'Ect4-D NotI and 3'Ect4-D Xho/Spe/NotI nt 2091 (aattactaagcggccgcccactagtgatcctcgagATCCAGACCATTATCCACCACATGTTTGC) and nucleotides 2437-4911 (amino acids 813-1637) with 5'Ect4-D XhoI nt 2437 (gattctcgagAACAAGGAGATTGAAGCCGAGG) and 3'Ect4-D SpeI from *UAS-dSarm*. Both products were inserted into *pUASt-CT EGFP* with Not1/Xho1 and Xho1/SpeI, respectively.

UAS-dSarmΔARM::myc was made by cutting out *dSarmΔARM* from the above construct with Not1/Spe1 and inserting it *into pUASt-CT myc*.

UAS-dSarmΔSAM::GFP was made by PCR amplifying nucleotides 1-2820 (amino aicds 1-940) with 5'Ect4-D NotI and 3'Ect4-D Xho/Spe/NotI nt 2820 (aattatatatagcggccgcccactagtgatcctcgagCAAGGGAACCTGTTGGGACAGCTTGTGC) and nucleotides 3217-4911 (amino acids 1073-1637) with 5'Ect4-D XhoI nt 3217 (gatcctcgagAACTTGGAGAATTCGC) and 3'Ect4-D SpeI from *UAS-dSarm*. Both products where inserted into *pUASt-CT EGFP* with NotI/XhoI and XhoI/SpeI, respectively.

UAS-dSarmΔSAM::myc was made by cutting out *dSarmΔSAM* from the above construct with Not1/Spe1 and inserting it into *pUASt-CT myc*.

UAS-dSarmΔTIR::EGFP was made by PCR amplifying nucleotides 1-3270 (amino acids 1-1090) with 5' Ect4-D NotI and 3'Ect4-D Xho/Spe/Not nt 3270 (aattatatatagcggccgcccactagtgatcctcgagTAGCGTCTTGGCCATGTTCTCCTCC) and nucleotides 3685-4911 (amino acids 1229-1637) with 5'Ect4-D XhoI nt 3685 (tgatactcgagCGCGGCGAAAAGAATATCGATCGCATTGC) and 3'Ect4-D SpeI from *UAS-dSarm*. Both were inserted into *UAS-CT EGFP* with NotI/XhoI and XhoI/SpeI, respectively.

UAS-dSarmΔTIR::myc was made by cutting out *dSarmΔTIR* from the above construct with Not1/Spe1 and inserting it into *pUASt-CT myc*.

UAS-ARM::EGFP was made by PCR amplifying nucleotides 1-2820 (amino acids 1-940) with 5'Ect4-D NotI and 3'Ect4-D Xho/Spe/NotI nt 2820 and nucleotides 3685-4911(amino acids 1229-1637) with 5'Ect4-D XhoI nt 3685 and 3'Ect4-D SpeI from *UAS-dSarm*. Both were inserted into *UAS-CT EGFP* with NotI/XhoI and XhoI/SpeI, respectively.

UAS-ARM::myc was made by cutting out ARM from the above construct with Not1/Spe1 and inserting it into *pUASt-CT myc*.

UAS-SAM::HA was made by PCR amplifying nucleotides 1-2091 (amino acids 1-697) with 5'Ect4-D NotI and 3'Ect4-D Xho/Spe/NotI nt 2091, nucleotides 2437-3271 (amino acids 813-1090) with 5'Ect4-PD Xho 2437 and 3'Ect4 PacI/AgeI/XbaI 3271 (ggacctcgtctagagatcaccggtgatcttaattaaTAGCGTCTTGGCCATGTTCTCCTCCG), and nucleotides 3685-4911 (amino acids 1229-1637) with 5'Ect4 PacI 3685 (ccgtccgggatcttaattaagCGCGGCGAAAAGAATATCGATCGCATTGC) and 3'Ect4 stop SpeI from *UAS-dSarm*. All three were inserted into *UAS-CT HA* with NotI/XhoI, XhoI/AgeI, and AgeI/SpeI, respectively.

Immunohistochemistry

Drosophila ORNs were not antibody stained, except where noted (Figure 3D), and instead were visualized by their expression of either a membrane tethered GFP (*UAS-mCD8::GFP*) or tomato (*UAS-myr-tdt-tomato*). When antibody staining, adult brains were fixed as previously described (MacDonald et al., 2006) and incubated with 1:200 of mouse anti-GFP (Invitrogen) overnight and then with 1:100 Alexa488-anti-mouse (Jackson ImmunoResearch) for 1 hour at room temperature. Anti-PDF (Developmental

Studies Hybridoma Bank) was used at 1:10 under the same conditions as above. *Drosophila* embryos were fixed and stained as previously described (Broadus et al., 1995) using anti-eve at 1:10 (Developmental Studies Hybridoma Bank). Anti-HRP conjugated with Cy5 was used at 1:200 (Jackson ImmunoResearch).

Confocal microscopy

Samples were mounted in Vectashield antifade reagent and viewed on an Intelligent Imaging Innovations (3i) Everest Spinning disk confocal microscope. The entire antennal lobe was imaged in 0.27 μm steps for each sample for scoring axonal integrity. Abdominal segments of the ventral nerve cord from embryos and body wall neurons in 3rd instar larvae were also imaged in 0.27 μm steps. PDF neurons and projection neurons in the adult were imaged in 1.0 μm steps when viewed with the 10x objective lens and 0.27 μm steps when viewed with the 63x objective.

Results

Expression of dSarm truncation constructs does not interfere with Wallerian degeneration

We first sought to identify key functional domains required for dSarm to induce Wallerian degeneration. We created truncated dSarm constructs lacking either the SAM or TIR domain, or both (Figure 3-1), and fused them with either a C-terminal green florescent protein (GFP) or myc tag. First, we expressed these constructs in wild type olfactory receptor neurons (ORNs) to determine if expression of any of these constructs could interfere with the function of endogenous, wild type dSarm. Expression of these constructs with either C-terminal tag had no effect on Wallerian degeneration 7 days

after unilateral antennal ablation in wild type nerves (Figure 3-1), indicating these constructs do not act in a dominant-negative manner to block Wallerian degeneration.

dSarm requires both the SAM and TIR domains to promote Wallerian degeneration

Loss-of-function mutations in *dsarm* are able to potently block axon degeneration in *Drosophila* ORNs up to 50 days after injury (Osterloh et al., 2012). We next assayed if any of the deletion constructs could functionally rescue Wallerian degeneration in a *dsarm* mutant ($dsarm^{896}$) background. While both GFP- and myc-tagged full-length dSarm were able to restore degeneration in *dsarm* mutant axons, all of the truncated constructs were unable to rescue the mutant phenotype (Figure 3-2). Thus, dSarm requires both the SAM and TIR domains to induce Wallerian degeneration.

dSarm localization varies in different neurons but requires the SAM domain

We next wanted to determine where dSarm localized *in vivo*. Studies in cultured human kidney cells have suggested a role for Sarm1 in mitochondria *in vitro* (Dalod, 2007; Panneerselvam et al., 2012), although neuronal Sarm1 shows no localization to mitochondria (Osterloh et al., 2012). To assay dSarm localization in *Drosophila in vivo*, we turned to PDF neurons, where a small subset of neurons can be visualized in their entirety in the adult *Drosophila* brain with both a PDF antibody and expression of *pdf-Gal4* (Kaneko et al., 2000). The fully functional GFP-tagged dSarm localizes primarily to the cell body and main neurite branches of PDF neurons, but not throughout the entire axon or the dendritic field (Figure 3-3A-C, top row). Removal of the SAM domain redistributes the molecule throughout the entire neuron (Figure 3-3A-C, second row

from top), while loss of the TIR domain does not appear to affect localization (Figure 3-3A-C, second row from bottom). The ARM domain alone localizes throughout the neuron (Figure 3-3A-C, bottom row). To determine if this localization was consistent in multiple neuron types, we also expressed these constructs in larval sensory neurons (Figure 3-4A) and adult projection neurons (Figure 3-4B), where a similar pattern emerges: dSarm appears to be actively excluded from some neurites, and this specific localization requires the SAM domain. However, localization appears to vary among neurons. In adult projection neurons, dSarm::GFP is mainly in the cell body and dendrites while excluded from axons (arrows) (Figure 3-4B); however, in adult PDF neurons, dSarm::GFP is mainly in the cell body and part of the axon while excluded from dendrites (Figure 3-3A-C). Interestingly, in OR22a+ ORNs, dSarm::GFP is only detectable in the glomeruli with anti-GFP staining (Figure 3-3D); even with signal amplification, dSarm is barely detectable. The reason for this variety in localization remains unknown; there are likely other functions of dSarm beyond promoting Wallerian degeneration, possibly in neural development (Chen et al., 2011), and variances in localization may be due to unknown differences in the function of dSarm in these individual neurons. Nevertheless, in all neuron cell types tested, loss of the SAM domain resulted in widespread redistribution of the protein throughout the cytosol, indicating that the SAM domain is required for localization of dSarm.

Loss of the ARM domain induces spontaneous degeneration of adult neurons

In *C. elegans*, the ARM domain of TIR-1 acts an auto-inhibitory domain, suggesting that loss of the ARM domain in dSarm may induce Wallerian-like

degeneration in the absence of injury. To test this, we first expressed *dSarmΔARM* in OR22a+ ORNs without injury. Normally, these axons are robustly labeled with a membrane-tethered GFP (*OR22aGal4, UAS-mCD8::GFP)*. However, when the dSarmΔARM construct was co-expressed, OR22a+ ORNs were barely visible within two days of eclosion (Figure 3-5A), indicating that these ORNs had either (1) spontaneously degenerated or (2) a drastic developmental defect. To test the impact of adult-specific expression, we expressed either full length or dSarmΔARM specifically in adult OR22a+ ORNs using a temperature sensitive mutant of the Gal4-repressor Gal80. Within 5 days of activation, ORNs expressing dSarmΔARM showed dramatic axonal defects, including fragmentation (Figure 3-5B, bottom), while expression of full-length dSarm had no obvious effect on these axons (Figure 3-5B, top). Together, this suggests that dSarmΔARM is inducing axon degeneration in the absence of injury.

To further investigate the effect of dSarmΔARM, we expressed this molecule specifically in adult PDF neurons, again using a temperature sensitive Gal4-repressor, $Gal80^{ts}$. Within one day of expression, PDF neurons expressing dSarmΔARM undergo robust fragmentation, with most neuronal debris undetectable within 5 days of dSarmΔARM expression (Fig 3-5C, left column). Remarkably, dSarmΔARM is able to kill not only the axon, but also the cell body and dendrites of adult neurons, suggesting that activation of an axon-death pathway can also trigger somal and dendritic death.

dSarmΔARM-induced degeneration is blocked by Wld^{s} but not p35

To determine if dSarmΔARM was inducing neuronal apoptosis, we co-expressed dSarmΔARM with p35, a baculovirus protein that broadly inhibits caspase-dependent

cell death in *Drosophila*. Expression of p35 had no effect on dSarmΔARM-induced destruction of PDF neurons (Figure 3-5C, middle column). However, co-expression of Wld^s potently blocked the ability of dSarmΔARM to destroy these neurons (Figure 3-5C, right column), suggesting that (1) dSarmΔARM is able to induce Wallerian-like degeneration independent of axonal injury and (2) Wld^s acts downstream of dSarm to protect against axonal degeneration.

dSarmΔARM expression does not grossly alter neuronal patterning or differentiation in the embryo

We next sought to determine whether dSarmΔARM could kill embryonic neurons, or if its killing ability was restricted to adult neurons. To address this, we drove dSarmΔARM expression in embryonic neurons with *elav-Gal4* and assayed the presence of *eve+* neuronal nuclei. Expression of full-length *dSarm* in all embryonic neurons results in the expected number and organization of eve+ neurons per hemi-segment (13.06 ± 0.18), as did expression of *dSarmΔARM* (12.93 ± 0.19) (Figure 3-6). While expression of *dSarmΔARM* in embryonic neurons lead to head involution defects, the pattern of *eve+* neurons in abdominal segments remained unchanged, suggesting that *dSarmΔARM* does not grossly affect embryonic patterning of the nervous system. However, there appeared to be disruptions in the anti-HRP staining, which labels of all neuronal processes (blue, Figure 3-6A). This suggests that either dSarmΔARM expression is not high enough or turned on for long enough to induce cell death in embryonic neurons, or embryonic neurons are more resistant than adult neurons to dSarmΔARM-induced cell death.

Discussion

dSarm/Sarm1 plays a crucial role in activating Wallerian degeneration following injury to an axon, although the details of Sarm signaling during this process are unknown. We sought to investigate these details by performing a structure function analysis on dSarm. Deletion of the SAM or TIR domains renders the molecule dysfunctional. Interestingly, the SAM domain is required for both dSarm localization and function, suggesting that sub-cellular localization may be critical for dSarm activity. While others have reported Sarm1 localization to mitochondria, staining of endogenous Sarm1 in mouse neurons argues against mitochondrial localization (Chen et al., 2011; Osterloh et al., 2012). Nevertheless, this localization appears to correlate with dSarm's function in promoting axon self-destruction.

The TIR domain is crucial for dSarm's function, although its signaling partners remain unknown. In *C. elegans*, the TIR domain interacts with apoptosis signaling kinase-1 (ASK1) to alter transcription; unfortunately available *Drosophila* ASK1 mutants have no effect on Wallerian degeneration (Osterloh et al., 2012). Thus, it remains essential to uncover what downstream molecules interact with dSarm's TIR domain, as these are likely key molecules in executing axon destruction. Screening for suppressors of auto-activated dSarm may unearth these critical downstream signaling molecules.

Deletion of the ARM domain in dSarm results in spontaneous Wallerian-like degeneration in adult neurons. Work in *C. elegans* suggests that the calcium-activated CaMKII interacts with the ARM domain (Chuang and Bargmann, 2005; Chang et al.,

2011), however, as with ASK1, available mutations in CaMKII have no effect on Wallerian degeneration (Osterloh et al., 2012). It has been well documented that increases in intra-axonal calcium occur after injury and are required for Wallerian degeneration (Schlaepfer and Bunge, 1973; George et al., 1995; Adalbert et al., 2012; Avery et al., 2012), so it remains an intriguing possibility that a calcium-activated protein interacts with the ARM domain in dSarm to remove its auto-inhibitory properties. What distinguishes the intra-axonal calcium spike after injury from other calcium spikes, such as during action potential propagation, remains entirely unclear, and there are likely other "stress" signal(s) involved that together activate dSarm to induce Wallerian degeneration. Further screening to identify other Wallerian degeneration mutants may illuminate this activation step. It also remains possible that dSarm affects transcription before injury to "prime" axons for injury-induced death, and no transcriptome or proteomic studies to date have analyzed differences in wild type versus *Sarm* null axons.

dSarmΔARM expression induces degeneration of not only axons, but also dendrites and the soma. This suggests that activating axon death mechanisms can also result in degeneration of other neuronal parts. Remarkably, it does so in a p35-insensitive manner, arguing that this process is caspase-independent. Interestingly, immature embryonic neurons may be partially resistant to dSarmΔARM-induced death, but young axons appear to still be vulnerable to destruction. This suggests that possibly adult neurons are fundamentally more vulnerable to destruction than their younger counterparts, or all of the required downstream machinery to execute degeneration is not expressed or active until later developmental stage. It remains to be seen what effect

expression of a well-established pro-apoptotic gene, such as grim or reaper, has on neuronal processes and Eve staining of embryos at this point. It is also unknown if degenerating dSarmΔARM neurons show hallmarks of classical apoptosis, such as positive TUNEL labeling, nuclear condensation, and phosphatidylserine exposure.

Auto-activation of Wallerian-like degeneration with dSarmΔARM is another tool with which to investigate how Wld^s blocks axon degeneration. Co-expression of *Wld^s* with *dSarmΔARM* potently blocks degeneration of all neuronal parts, arguing that Wld^s acts downstream of dSarm to block Wallerian degeneration. Previously, Wld^s was thought to only protect axons and not cell bodies from death (Coleman and Freeman, 2010); however, Wld^s appears to block somal destruction from dSarmΔARM, indicating that in this instance axon death precedes cell body death as a driving force in overall cellular destruction; that is, the axon has to commit to self-destruction before the entire neuron dies, and Wld^s blocks this commitment.

The molecular pathways facilitate Wallerian degeneration have long been elusive, but recent mutants (Osterloh et al., 2012; Xiong et al., 2012) are beginning to bring this process to light. We present here that the ARM domain within dSarm is a crucial self-regulatory domain, and it is imperative to uncover relevant interactions and regulators of this domain and their importance in promoting Wallerian degeneration. The SAM domain appears to regulate localization, although the importance and consequences of this localization remains to be determined. Finally, the TIR domain likely interacts with downstream effectors that promote the morphological changes associated with Wallerian

degeneration; further understanding of these interactions should further illuminate the molecular mechanisms activated during Wallerian degeneration.

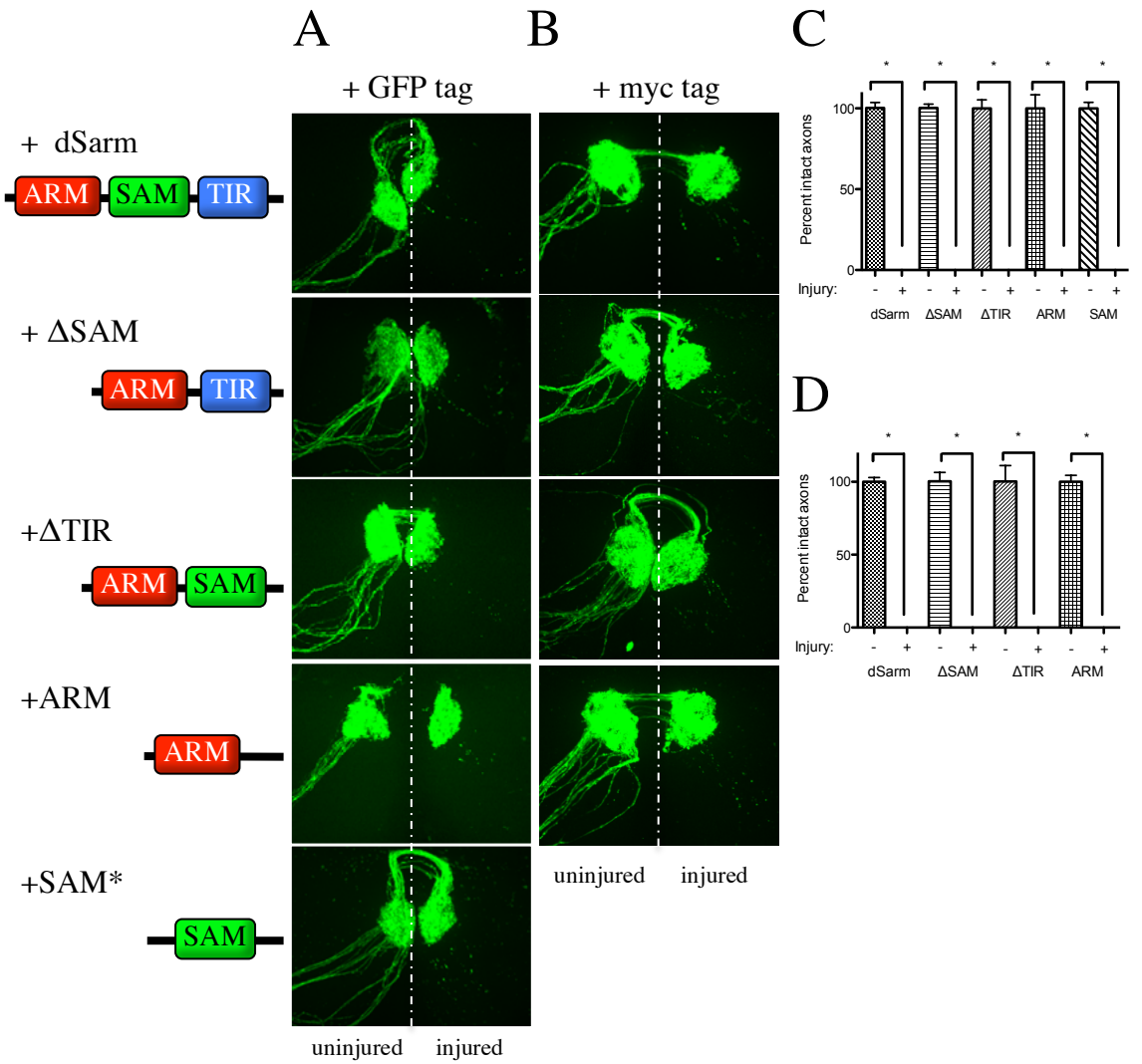


Figure 3-1. Expression of dSarm constructs does not interfere with nor induce Wallerian degeneration

A, B. Wild type ORNs expressing indicated dSarm construct (left) were unilaterally injured and degeneration was scored 7 days later. dSarm constructs were tagged with either a C-terminal GFP (A) or a C-terminal myc (B) tag. *SAM construct was tagged with a C-terminal HA epitope only (no GFP or myc construct).

C, D. Quantification of A and B, respectively. Axon counts for injured samples were normalized to mean axon number in uninjured samples. Values presented as mean ±SEM, $p<0.0001$. $n\geq6$ for all.

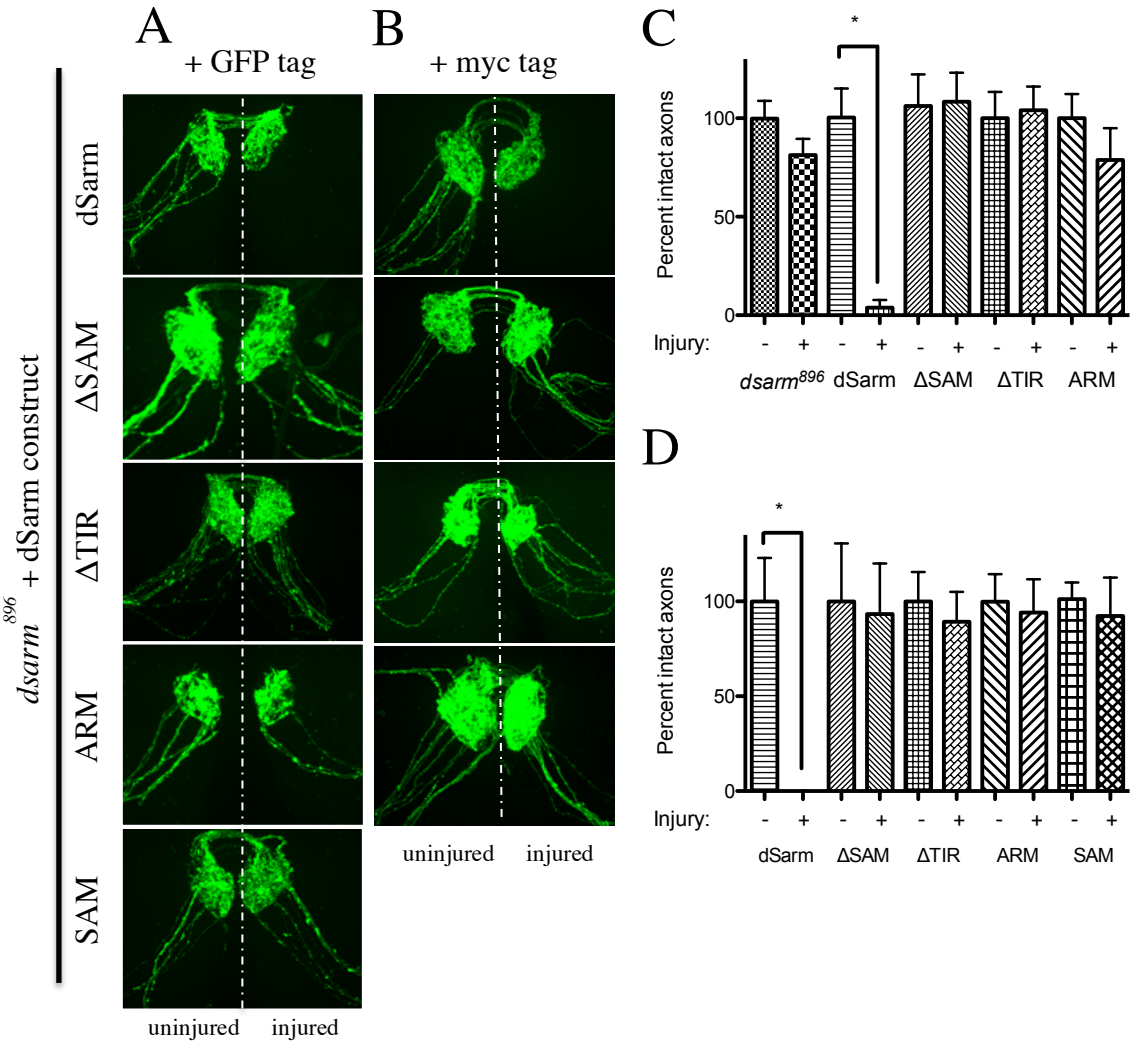


Figure 3-2. dSarm requires both the SAM and TIR domains to promote Wallerian degeneration

A, B. *dsarm*^{896} ORNs expressing indicated dSarm construct were unilaterally injured and degeneration was scored 7 days later. dSarm constructs were tagged with either a C-terminal GFP (A) or a C-terminal myc (B) tag. *SAM construct was tagged with a C-terminal HA tag only (no GFP or myc construct). Wild type ORNs expressing indicated dSarm construct (left) were unilaterally

C, D. Quantification of A and B, respectively. Axon counts for injured samples were normalized to mean axon number in uninjured samples. Axon counts for *dsarm*^{896} alone are included in quantification (C). Values presented as mean ±SEM, $p<0.0001$. $n\geq6$ for all.

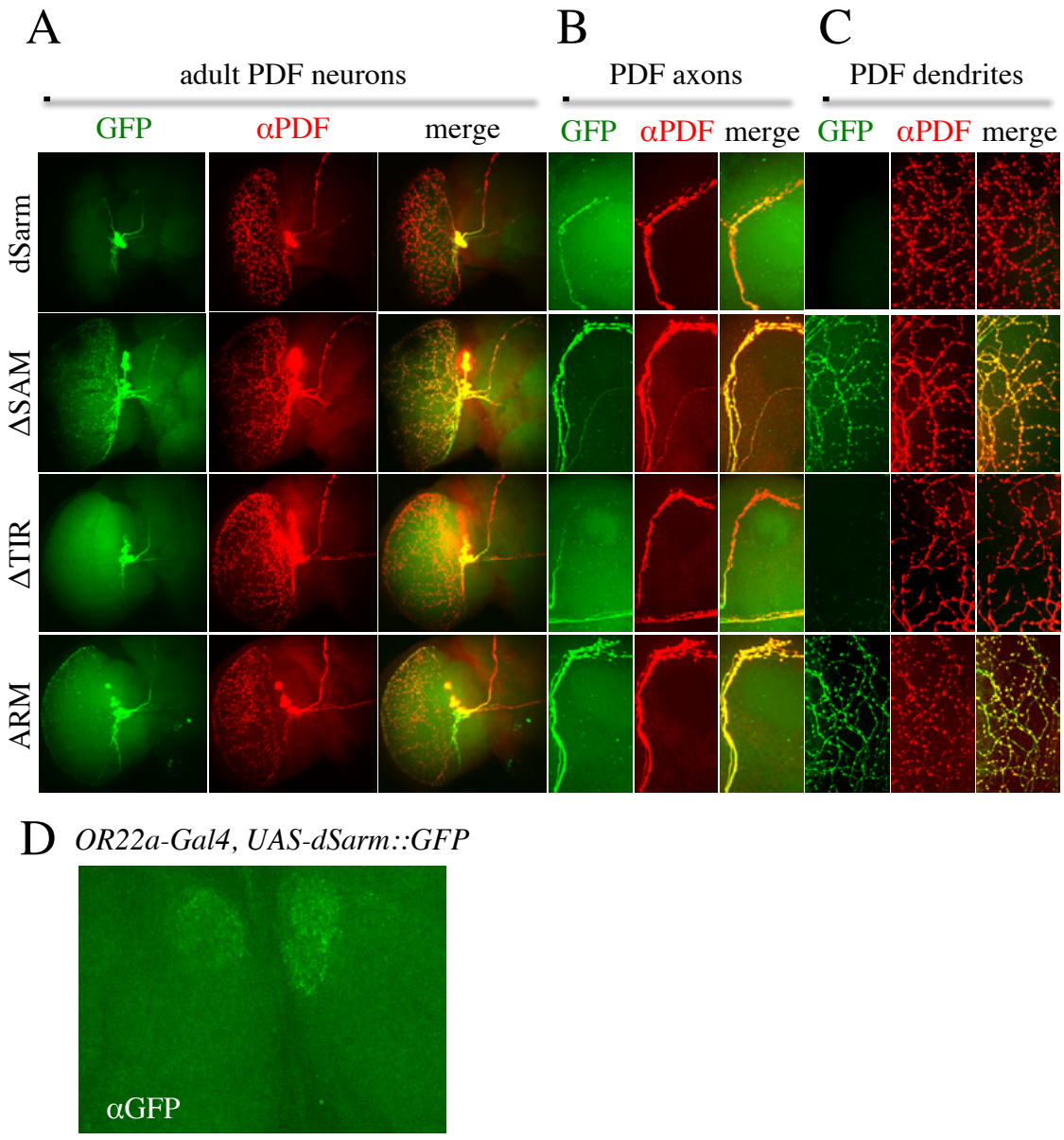


Figure 3-3. dSarm localization requires the SAM domain in adult neurons

A-C. Expression of indicated GFP-tagged dSarm constructs in PDF neurons (red, anti-PDF). (A) Adult PDF neurons in one hemi-brain (8 neurons total). (B) Zoomed in view of adult PDF axons. (C) Zoomed in view of adult PDF dendrites. n≥10 animals for all.

D. dSarm::GFP in adult OR22a+ neurons stained with anti-GFP. n=5 animals.

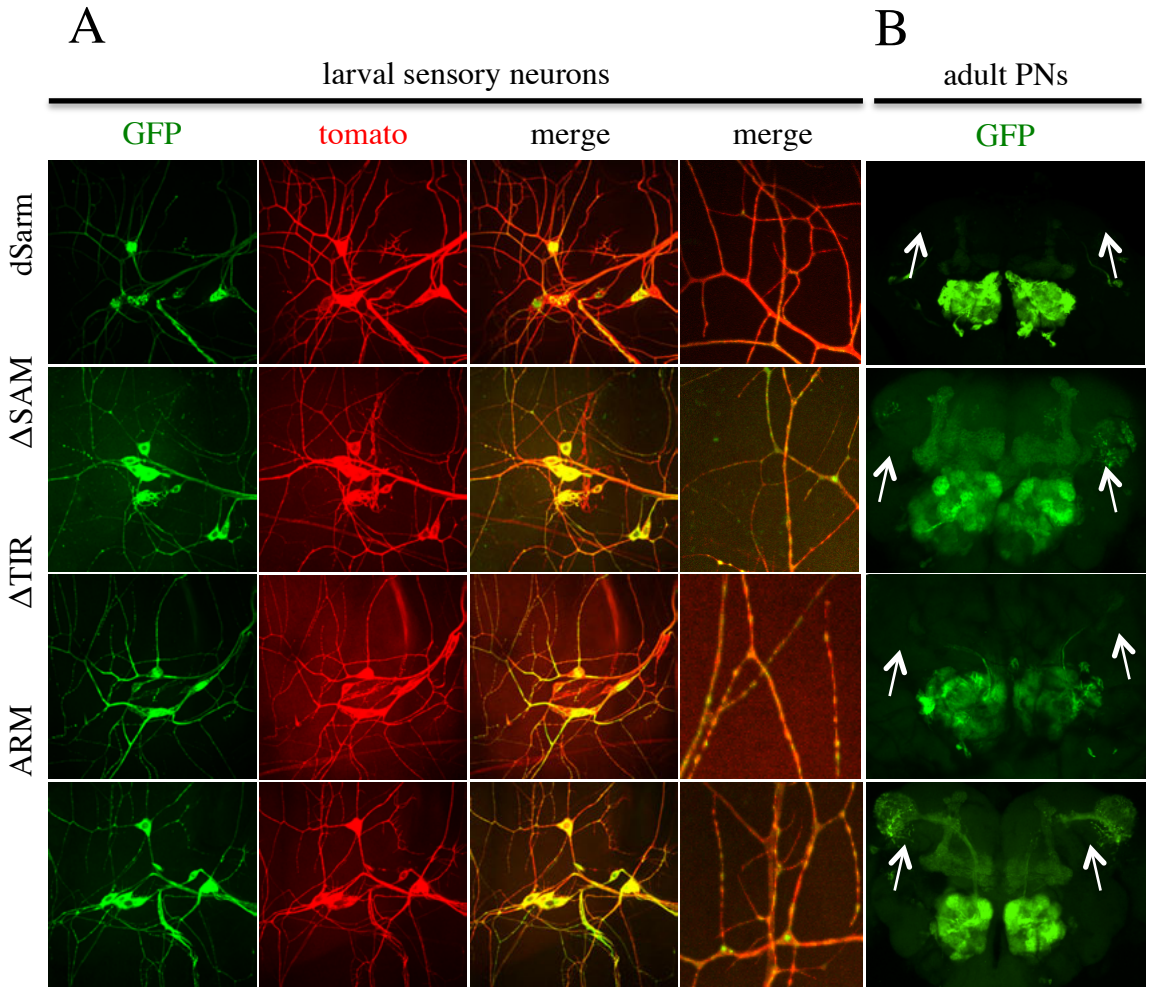


Figure 3-4. dSarm localization requires the SAM domain

Expression of indicated GFP-tagged dSarm constructs. n≥6 animals for all.

A. Larval body wall sensory neurons, labeled with *109(2)80-Gal4*. Constructs are co-expressed with myristoylated tomato, which labels cell membranes. A zoomed in merge view of the dendrites is shown on the far right.

B. Adult projection neurons (PNs) labeled with *GH146-Gal4*.Axons are indicated with arrows.

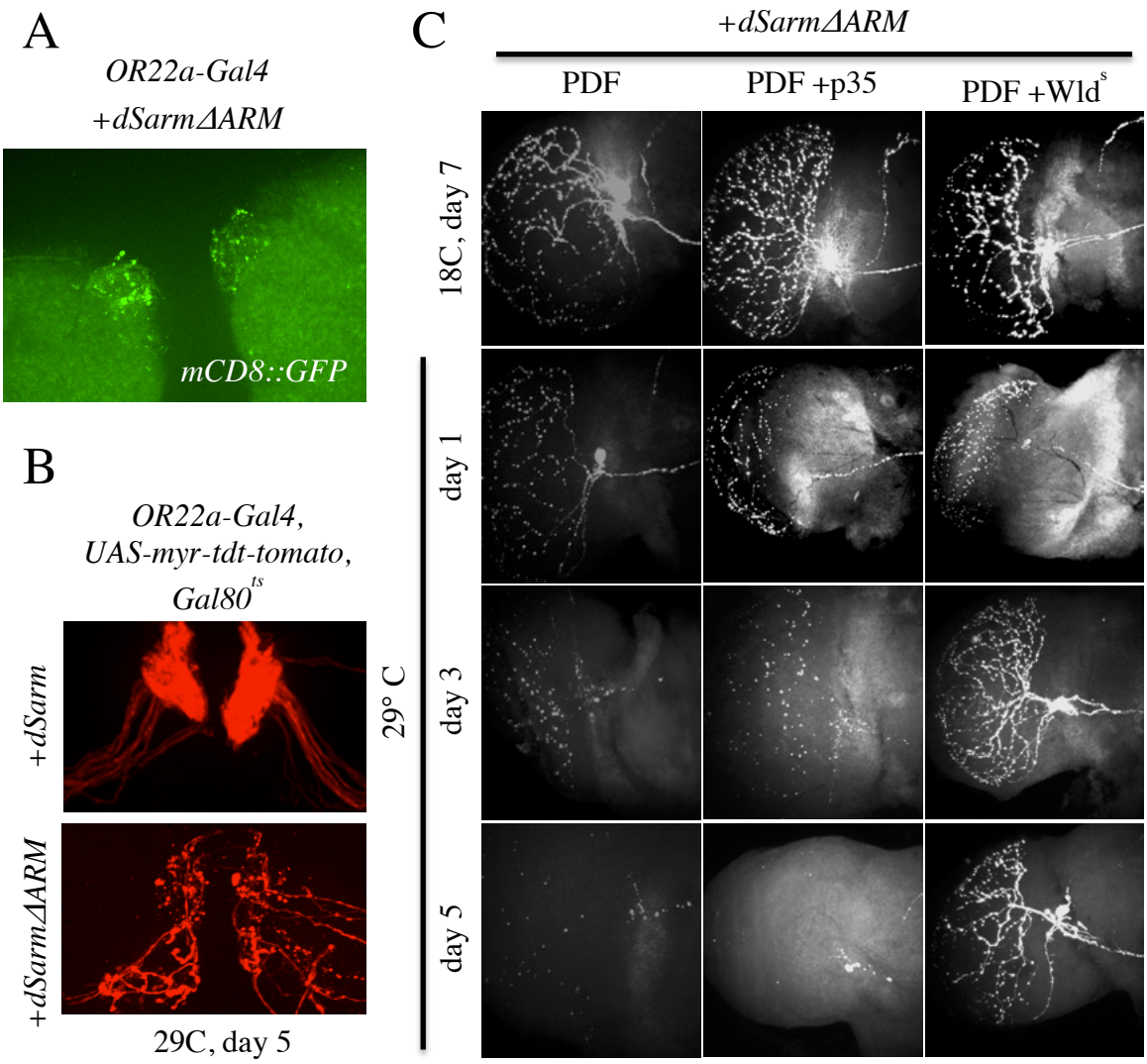


Figure 3-5. Expression of dSarmΔARM induces spontaneous Wallerian-like degeneration in adult neurons

A. *UAS-dSarmΔARM::GFP* was expressed with *UAS-mCD8::GFP* by *OR22aGal4*. Adult brains were dissected without injury within 48 hours of eclosion. n=12.

B. Either *UAS-dSarm::GFP* (top) or UAS-*dSarmΔARM::GFP (bottom)* was expressed with *OR22aGal4* specifically in the adult in a temperature sensitive manner for 5 days. Membranes are labeled with a myrstoylated tomato. n=10 each.

C. dSarmΔARM::GFP was expressed specifically in adult PDF neurons in a temperature sensitive manner either alone (left), with the anti-apoptotic protein p35 (middle), or with Wld^s (right) for the indicated time points. White, anti-PDF to label all neurons expressing the construct(s). n≥25 brains for all.

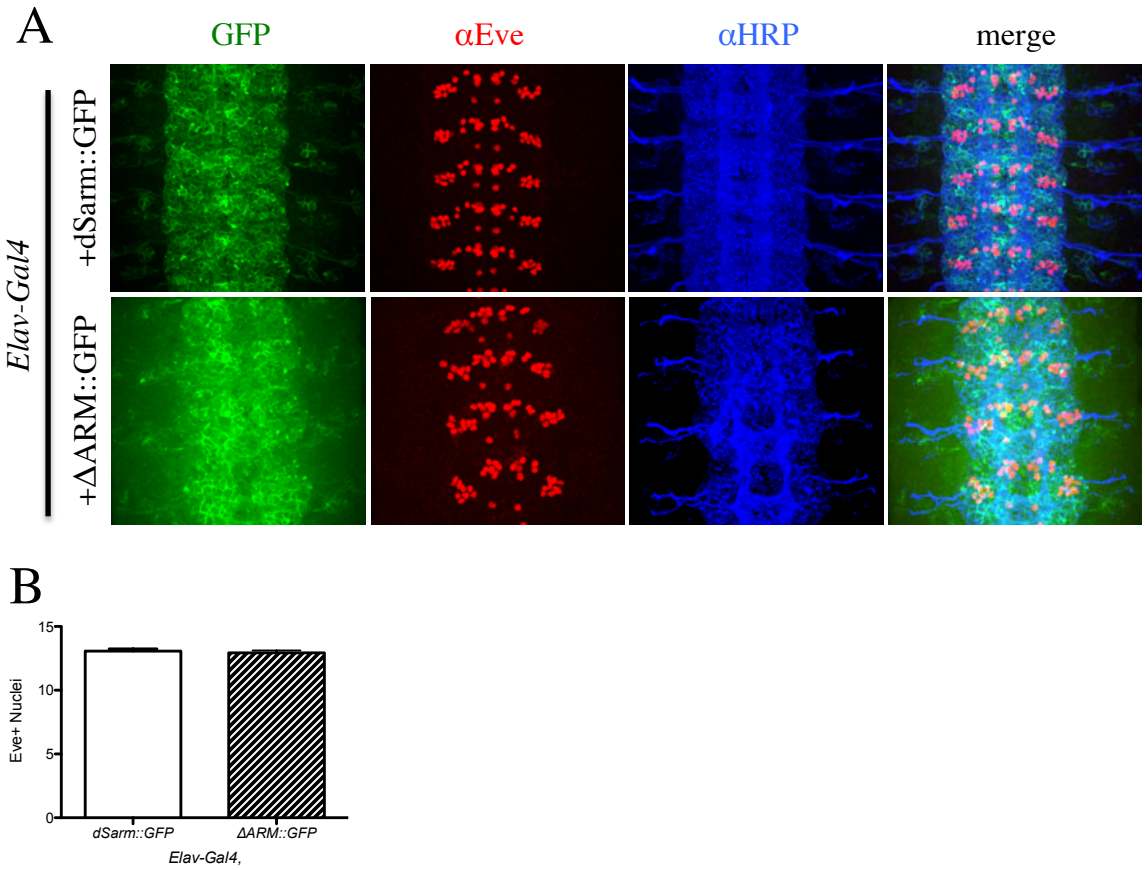


Figure 3-6. Expression of dSarmΔARM induces axon loss but not cell body death in embryonic neurons

A. Either dSarm::GFP (top) or SarmΔARM::GFP (bottom) was broadly expressed in all embryonic neurons with *elav-Gal4*. Anti-eve (red) labels a small, specific set of differentiated neuronal nuclei, while anti-hrp (blue) labels all neuronal processes. n= 12 embryos.

B. Eve-positive nuclei from (A) were counted per hemisegment. Values presented as mean ±SEM, n≥40 hemisegments.

CHAPTER IV: *Sarm1* loss prevents axon degeneration in a mouse model of Amyotrophic Lateral Sclerosis (ALS)

The work described in this chapter was performed in the lab of Dr. Marc Freeman. I performed all of the animal handling, behavioral experiments, and tissue dissections. Dr. Johnny Salameh performed the electrophysiology recordings. Dr. Owen Peters is currently staining tissue from the animals used here (data not presented). The following publication is in process:

Osterloh JM, Peters, O, Salameh J, Brown RH, and Freeman, MR. *Sarm1* loss prevents axon degeneration in a mouse model of ALS

Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal, progressive neurological disease that specifically targets motor neurons and affects 25,000 Americans at any given time. Because disease progression is so rapid and devastating, it is essential to understand the underlying causes of motor neuron death in ALS. Genetic abolishment of cell death pathways has only provided some extension of lifespan in mouse models of ALS. To determine if programmed axon death is involved in ALS progression, we bred *Sarm1* knockouts, which are robustly protected from injury induced Wallerian degeneration, to the SOD1-G93A mouse model of ALS. We found loss of Sarm1 robustly preserves axons and synapses and modestly prolongs lifespan in these ALS mice, suggesting that Sarm1 regulates a common axon death pathway that is activated both after injury and during the course of this neurodegenerative disease. This new insight into ALS pathogenesis may be important for future human therapies.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressively fatal motor neuron disease and manifests clinically as progressive muscle weakness and atrophy, ultimately resulting in fatal respiratory failure. Pathologically, dying motor neurons exhibit swellings in the axon and soma, as well as aggregates of phosphorylated neurofilaments and ubiquitinated proteins. Activated microglia and astrocytes are also present (Pasinelli and Brown, 2006). Unfortunately, there are few available treatments for this disease, and the symptomatic measures provide little temporary relief.

There are approximately 25,000 people living with ALS in the USA, with a lifetime risk between 1:800 and 1:2,000 (Cleveland and Rothstein, 2001; Rothstein, 2009). Approximately 90% of all cases are sporadic; the remaining 10% of ALS cases are autosomal dominant and inherited (Rothstein, 2009). Of the familial ALS patients, 20% have mutations in Cu/Zn superoxide dismutase-1 (SOD1), a ubiquitous cytosolic protein. (Pasinelli and Brown, 2006). There have been multiple loci linked to familial ALS, but SOD1, the first ALS-linked gene discovered, is by far the most heavily studied causative gene (Pasinelli and Brown, 2006). Over 150 mutations in SOD1 have been linked to ALS, with mutations spanning all 5 exons. Enzymatic activity appears to play no role in disease progression, and SOD1 mutants are believed to act via some unknown, gain-of-function toxic mechanism (Pasinelli and Brown, 2006). SOD-1 inclusions have been found in sporadic ALS patient tissue, suggesting a possible role for this molecule outside of familial ALS . In order to better understand the mechanisms of human disease, several animal models of ALS have been generated; many of these experimental models

involve expression of human mutant SOD1 (McGoldrick et al., 2013). The first of these animal models contains a glycine to alanine substitution (SOD1-G93A), which is expressed under control of the human promoter and with over 20 copies of the coding sequence. In mice, this results in progressive motor neuron death in the spinal cord, leading to paralysis and ultimately death by 6 months of age (Gurney et al., 1994). Subsequent analysis has revealed that these animals share many features seen in post-mortem human samples, including axon transport defects, mitochondrial dysfunction, progressive neuromuscular junction (NMJ) denervation, and reactive gliosis (McGoldrick et al., 2013). Many other transgenic ALS mice have since been created, including lines expressing other SOD1 mutations, but the SOD1-G93A mice remain the most highly studied experimental model of ALS (McGoldrick et al., 2013).

Transgenic ALS mice allow for the investigation of not only disease pathogenesis, but also for testing potential therapeutic treatments. There have been many suggestions regarding the nature of motor neuron stress and death in ALS, including: oxidative damage, toxic protein aggregates, mitochondrial dysfunction, apoptosis, axon transport blocks, growth factor defects, toxic glia, glutamate excitotoxicty, and altered RNA metabolism (Rothstein, 2009). A wide variety of transgenic and pharmaceutical therapies have been tested in an attempt to alleviate the above conditions; however, none to date have proven to be effective in the treatment of human disease. These results highlight the complexity surrounding ALS pathogenesis and treatment studies.

ALS is not merely a disease where motor neurons undergo apoptosis, as transgenic animals that potentially block cell death offer only modest protection

(Vukosavic et al., 1999; Gould et al., 2006; Reyes et al., 2010). Axon degeneration is also a hallmark of ALS (Cleveland and Rothstein, 2001), although it is unknown if this is a driving force behind disease progression. Apoptosis inhibitors are able to block motor neuron cell body death, but not axonal loss, suggesting that another mechanism may be activated in degenerating neurons. Because axonal dieback during ALS looks morphologically similar to Wallerian degeneration, it is possible that same genetically encoded program controls both axon death after injury and axon death during ALS. Unfortunately, expression of the chimeric *slow Wallerian degeneration* (*Wld*^{s}) gene, which potentially blocks injury induced degeneration (Coleman and Freeman, 2010), showed little effect on transgenic ALS models (Vande Velde et al., 2004; Fischer et al., 2005). However, the protective effects of Wld^{s} may decline with age, and the dose of Wld^{s} may be altered in motor neurons expressing such high levels of SOD1 (Gillingwater et al., 2002; Wright et al., 2010). We recently described the first loss of function mutants, in *dsarm*/*Sarm1,* that potently block Wallerian degeneration to the same extent as Wld^{s} expression (Osterloh et al., 2012). *Sarm1* null neurons are also protected from cell death due to oxygen-glucose deprivation (Kim et al., 2007), excitotoxicity (Massoll et al., 2013), and some neurological viruses (Mukherjee et al., 2013). With the discovery of *Sarm1* and its role in axon death, we sought to determine if injury-induced axon death is related to axon degeneration during ALS progression. Here, report that loss of *Sarm1* remarkably preserves axons and the NMJ and also modestly prolongs lifespan in SOD1-G93A mice.

Materials and Methods

All experimental procedures in mice were performed in compliance with the protocols approved by the Institutional Animal Care and Use Committee of The University of Massachusetts Medical School.

Animal breeding

Sarm1 null females (Osterloh et al., 2012) were bred with SOD1-G93A males on the C57/Bl6 background (Jackson Laboratories). Sarm +/-, huSOD+ males were bred to Sarm1 null females. The resulting huSOD+ mice were used in the studies below, and the huSOD- littermates were kept for at least 250 days as lifespan controls. Only mice containing at least 25 copies of huSOD, as confirmed by QRT-PCR as previously described (Caraganis et al., 2008) were used for this study. Wild type C57/Bl6 mice were purchased from Jackson Laboratories.

Behavioral studies

Beginning at 50 days old, huSOD+ mice were trained twice weekly on a rotarod (Omnitech Electronics) set at a constant speed of 20 revolutions per minute. Beginning at 70 days of age, the mice were placed on the moving rotarod (20 revolutions per second) and required to stay on the rotarod for 600 seconds. Testing sessions were performed twice weekly in the afternoon with 2-3 days between testing. Before each rotarod session, each mouse was weighed. During each session, each animal had up to three trials on the rotarod, without resting between trials. The longest performance for each animal was recorded. Mice were no longer tested when unable to stay on the rotarod for at least 10 seconds. These mice were then housed with food and gel packs on

the cage floor; these animals were euthanized at endpoint, when unable to right themselves after 10 seconds on one side. The animals were euthanized with a lethal dose of isoflurane and then perfused with phosphate buffered saline, followed by 4%paraformaldehyde, 4% sucrose in 0.1M phosphate buffered saline. After incubating for 24 hours at 4°C in fixative, mice were placed in phosphate buffered saline for another 24 hours at 4°C. Spinal cords, ventral roots, and sciatic nerves were then dissected and housed in 30% sucrose at 4°C. Lifespan was compared using a Kaplan-Meyer survival analysis on Graph Pad Prism 5 software. Weight and rotarod performance were compared using average area under the curve, with at least 3 data points per time point, followed by a student's t-test on Graph Pad Prism 5 software,

Electrophysiological recordings

Electrophysiological recordings were done as previously described (Xia et al., 2010) on 130-day-old mice. Mice were deeply anesthetized with isoflurane, and their rear legs were shaved with an eye brow trimmer (Remington) and wiped with alcohol pads. Mice were restrained on a Styrofoam platform on top of a heating pad with adhesive tape. A pair of monopolar disposable 28G needle electrodes and ring electrodes (CareFusion) were lightly coated with electrode gel (SignaGel) and used for stimulation and recording, respectively. The active recording electrode was placed over the gastrocnemius muscle, with the reference electrode over the tendon. The stimulating cathode was placed 5 mm proximal to the recording electrode in the midline of the posterior thigh. The anode was placed subcutaneously in the midline over the sacrum. A surface electrode (CareFusion) was grounded on the mouse's tail. Motor conduction studies and motor unit estimate

(MUNE) were performed using a portable electrodiagnostic system (Cardinal Synergy). For the motor nerve conduction studies, the low-pass filter was set at 30 HZ, and the high-pass filter was set at 10 kHZ. The nerve was stimulated with single square-wave pulses of 0.1-ms duration. Supramaximal responses were gradually generated, and maximal responses were obtained with stimulus currents <20 mA (most often <10 mA). The distance between distal and proximal stimulation sites was measured with a millimeter-graduated tape measure. Data were acquired with a sensitivity of 20 mV/division and sweep speed of 3 ms/division. The distal latency, distal and proximal compound motor action potential (CMAP) amplitudes, and distal and proximal CMAP durations (measured from onset of initial negative deflection to initial return to baseline) were determined for each nerve studied.

For the MUNE recordings, the incremental technique was used (Shefner et al., 2002; Shefner et al., 2006). The setting was the same as for the motor nerve conduction test reported above. The low pass filter was set at 20 HZ, and the high-pass filter was set at 10 kHZ. Data were acquired with a sensitivity of 100 μV/division and sweep speed of 1 ms/division. Using a repetition rate of 1/s, stimulus intensity was slowly increased from subthreshold levels until a small all or none response was produced. Thus the sciatic nerve was stimulated with single pulses of gradually increased intensity until the first response appeared, representing the first motor unit recruited. With the next stimuli, quantal increases in the response were recorded. Increments >25 μV were considered as likely the recruitment of an additional motor unit. The amplitude of a single motor unit

was calculated as the mean of 5 consistent increases, with the overall MUNE calculated as follows:

$$\text{MUNE} = \frac{\text{CMAP}_{\text{maximum amplitude}}}{\text{mean surface detected motor unit action potential}}$$

MUNE values were compated using an unpaired t-test on Graph Pad Prism 5 software. After recording from each side, the animals were euthanized with a lethal dose of inhaled isoflurane and perfused as described above.

Results

To study the role of axon death genes in ALS, we bred *Sarm1* null mice with SOD1-G93A mice and then backcrossed *Sarm1* +/-, *huSOD1-G93A*+ males with *Sarm1* null females. The resulting *huSOD1-G93A*+ littermates with at least 25 copies of the human mutant SOD1 were compared in the subsequent experiments.

Loss of *Sarm1* does not delay disease onset

First, animal weight was measured twice a week starting at day 75. Both *Sarm1* +/- and *Sarm1* null animals reached their peak weight at day 99, and showed progressive weight loss for the rest of the animals' life span (Figure 4-1A). There was no statistical difference between the two groups. *huSOD1-G93A*- littermates showed no decline in weight over time (Figure 4-1B), indicating weight loss was a result of the *huSOD1-G93A*+ transgene and not due to the loss of *Sarm1*. Peak weight is generally considered to be the time of onset in this model of ALS, and the above data indicates that *Sarm1* loss does not alter disease onset.

Gross motor performance is not preserved *in Sarm1* null mice

Next, motor performance was scored twice weekly by assessing latency to fall on a constant speed rotarod. Both *Sarm1* +/- and *Sarm1* null *huSOD1-G93A*+ mice show progressive decline in rotarod performance overtime with no difference between groups (Figure 4-1C). There was no significant difference between groups when comparing the mean age at which functional paralysis (time on rotarod ≤10 seconds) set in, which was 146 days for *Sarm1* nulls and 150 days for *Sarm1* +/- animals (Figure 4-1D). *Sarm1* null animals alone show no defects in rotarod performance with age (Figure 4-1E). Thus, there appears to be no gross preservation of motor circuit function at this test speed with loss of *Sarm1*.

Loss of *Sarm1* modestly prolongs lifespan

Notably, loss of *Sarm1* extends lifespan of SOD1-G93A mice by approximately 10%; *Sarm1* null *huSOD1-G93A*+ mice lived an average of 184.5 days, while their *Sarm1* +/- *huSOD1-G93A*+ littermates lived only 169.0 days (Figure 4-2). *Sarm1* null animals alone live at least 250 days (Figure 4-2). Loss of *Sarm1* extends lifespan in SOD1-G93A mice, suggesting that axon death may play a role in disease progression of ALS.

Axons and synapses are preserved with loss of *Sarm1*

We next assayed the direct ability of *Sarm1* loss to preserve both axons and synaptic terminals in these ALS mice. CMAPs from the gastrocnemius muscle after stimulation of the sciatic nerve were recorded from 130-day-old mice (Figure 3). *Sarm1* +/-, *huSOD1-G93A*+ mice at day 130 showed remarkably low MUNE scores: 23.8± 2.9.

These mice are undergoing mid-stage paralysis; they are still able to stay on the rotarod for some time, but they are showing appreciable signs of muscle atrophy and weakness. Interestingly, *Sarm1* null*, huSOD1-G93A*+ mice at day 130 showed similar decline on the rotarod, and no observable behavioral difference between littermates was noted; however, their MUNE scores show remarkable preservation at the NMJ: 94.1± 17.0, indicating that loss of *Sarm1* robustly preserves axons and synapses in SOD1-G93A mice. While there was variability in the MUNE score, much of this is due to asymmetric innervation from leg to leg of the same animal (i.e., one leg has a higher score than the other); intriguingly, this uneven distribution is also found in human patients (Baumann et al., 2012).

Discussion

The role of axon self-destruction genes in neurodegenerative disease has long been speculated (Raff et al., 2002) but until the discovery of dSarm/Sarm1 there were few tools available to investigate this phenomenon. ALS appeared as a clear first stepping stone for investigating the roll of axon death genes in neurodegeneration, as many studies have clearly shown that preventing cell body death does not block axon loss and ultimate animal death (Vukosavic et al., 1999; Gould et al., 2006; Reyes et al., 2010). To investigate this possibility, we bred axon self-destruction deficient mice into the SOD1-G93A mouse model of ALS. While *Sarm1* loss does not appear to delay disease onset or gross motor decline, lifespan is modestly extended (10%). Electrophysiological recordings from gastrocnemius muscle show that axons and synapses are remarkably well preserved in late stages of ALS progression in *Sarm1* null

mice, indicating that axon loss is prevented and arguing that a genetically encoded axon death program may be activated during disease progression to destroy axons.

The work here enhances earlier studies on axon death and disease pathogenesis in mouse ALS models. Expression of the chimeric protein Wld^s in multiple ALS models provided few changes in disease progression, although NMJs did remain innervated at a slightly higher (~10%) rate during pre- and early-symptomatic stages (Vande Velde et al., 2004; Fischer et al., 2005). At the time, it was concluded that axon death was not a factor in ALS progression; however, electrophysiological recordings were not performed. Wld^s protection, particularly at the synapses, may be age and is definitely dose-dependent (Mack et al., 2001; Wright et al., 2010), and these variables may account for the lack of axonal protection in Wld^s-SOD1 mice. Fortunately, these variables are removed by studying axon degeneration with *Sarm1 null* animals, and this may explain the differences in preservation between Wld^s-SOD1 and Sarm1 KO-SOD1 results. There may also be unknown differences in the mechanism of axon protection between Wld^s and Sarm1 KO mice, especially because one strain is the result of gene overexpression (Wld^s) and the other a knock out (Sarm1).

Curiously, axons and synapses are preserved but there was no improvement in rotarod score. This is not necessarily surprising, given that the animals were tested at a constant speed; this test cannot pick up the subtle differences that an accelerating rotarod test would have. Alternatively, there is known to be cell death in the motor cortex of ALS mice and patients; if the motor cortex is not properly wired to the corresponding motor neurons in the spinal cord, then NMJ preservation would be not be detectable with

a behavioral assay. The motor neuron cell bodies could also not be functioning correctly, thus depriving the axon and synapses of necessary materials for producing endogenous action potentials; however, because the machinery to fire an action potential is still in place, we may be detecting intact NMJs in a situation where the neuron is no longer firing normally itself. It would be interesting to directly stimulate other neurons in the motor circuit, such as the motor cortex or upper motor neurons, in these mice to test if this circuit is intact. Spontaneous amplitudes could also be recorded from the muscle to determine if the motor unit is still able to fire normally. However, dendrites may not be intact or functional in these animals. It is likely that all of these possibilities play some role in functional motor neuron output during ALS progression, as any disruption in the complex motor circuitry would result in a behavioral defect, even if one component is highly preserved. The fact that we still observe functional NMJs (even if they are not actually functioning endogenously) is quite remarkable.

Even if these motor units are not working by propagating endogenous action potentials, their functional preservation is still exciting and novel. This suggests that axon death genes do indeed get turned on during disease progression to drive destruction of the axon. It is unknown if Sarm mRNA or protein levels rise in the axon during ALS progression, and we unfortunately currently have no biochemical read out for "activated" Sarm (i.e., phosphorylation or cleavage at a specific site). However, because the *Sarm1* null animals show only slight lifespan extension, there are likely distinct somal-death mechanisms in place to destroy the cell body. Together with data on cell death-deficient mice (Gould et al., 2006; Reyes et al., 2010), this argues that during disease both axon-

specific and soma-specific death programs are initiated. However, histological studies are still needed to determine if cell bodies are still dying in *Sarm1-G93A* mice and to confirm that axons are morphologically preserved. Also, while loss of *Sarm1* is not predicted to alter expression levels of transgenic SOD1, testing for even expression levels of mutant SOD1 remains an important control remaining to be done. How a gain-of-function, toxic molecule can activate both death programs remains unknown but highlights some of the limitations of the SOD1 mouse in modeling ALS.

ALS mouse models have provided a remarkable tool with which to study the potential mechanism(s) of disease progression; however, there are limitations with the model used in this study. Because SOD1 is normally involved in removing ROS, it was assumed that ALS might be a disease of oxidative stress. However, work done on several models of ALS with various gain-of-function SOD1 mutations has shown that neurodegeneration is induced independently of ROS production (Van Den Bosch, 2011). Unfortunately, loss-of- function SOD1 mutants do not show ALS-like neurodegeneration, and it is still unclear how these mutants induce neuronal death. Disease progression is heavily dependent on the number of transgenic copies of mutant SOD1 present. The model tested here is considered a very aggressive form of ALS, and thus may not represent most human cases of the disease (Van Den Bosch, 2011). While over-expression of a mutant protein is a major limitation of this model, at this time it is one of the few available tools to investigate pathogenic mechanisms of ALS. Even recent models from familial-ALS-linked genes, such as TDP-43, encounter the same limitations: the disease models are a result of protein overexpression well beyond

"normal" levels, not due to the loss of function of a gene. These limitations make the above *Sarm1* results more promising: there is a difference with loss of *Sarm1* even when using a "sledgehammer" model such as SOD1-G93A. Loss of Sarm in a less aggressive model of ALS may highlight larger differences in disease progression than the model used here. This makes *Sarm1* a potential pharmaceutical target for neuroprotection in human disease, which is arguably less aggressive than the model used here.

It remains to be seen how well *Sarm1* loss can preserve axons in a variety of neurodegenerative disease models. Studies on Wld^s indicate that Sarm1 loss should protect against a variety of chemically induced axon degeneration and models of peripheral neuropathies. However, Wld^s is not universally protective, and it is unlikely that Sarm1 loss will be. Thus, it remains important to understand the specific underlying mechanisms of a variety of neurodegenerative diseases, as some may be linked specifically with Sarm1-induced death, and others not. Wld^s protection suggests that there is likely not a universal axon self-destruction program that always gets turned on to induce axon degeneration; in fact, we already know that Sarm1-mediated axon degeneration is genetically separate from developmental axon and dendrite pruning (Osterloh et al., 2012). However, the possibility of a universal "stress"-activated (i.e., non-developmental) self-destruction program remains an intriguing possibility that warrants further study.

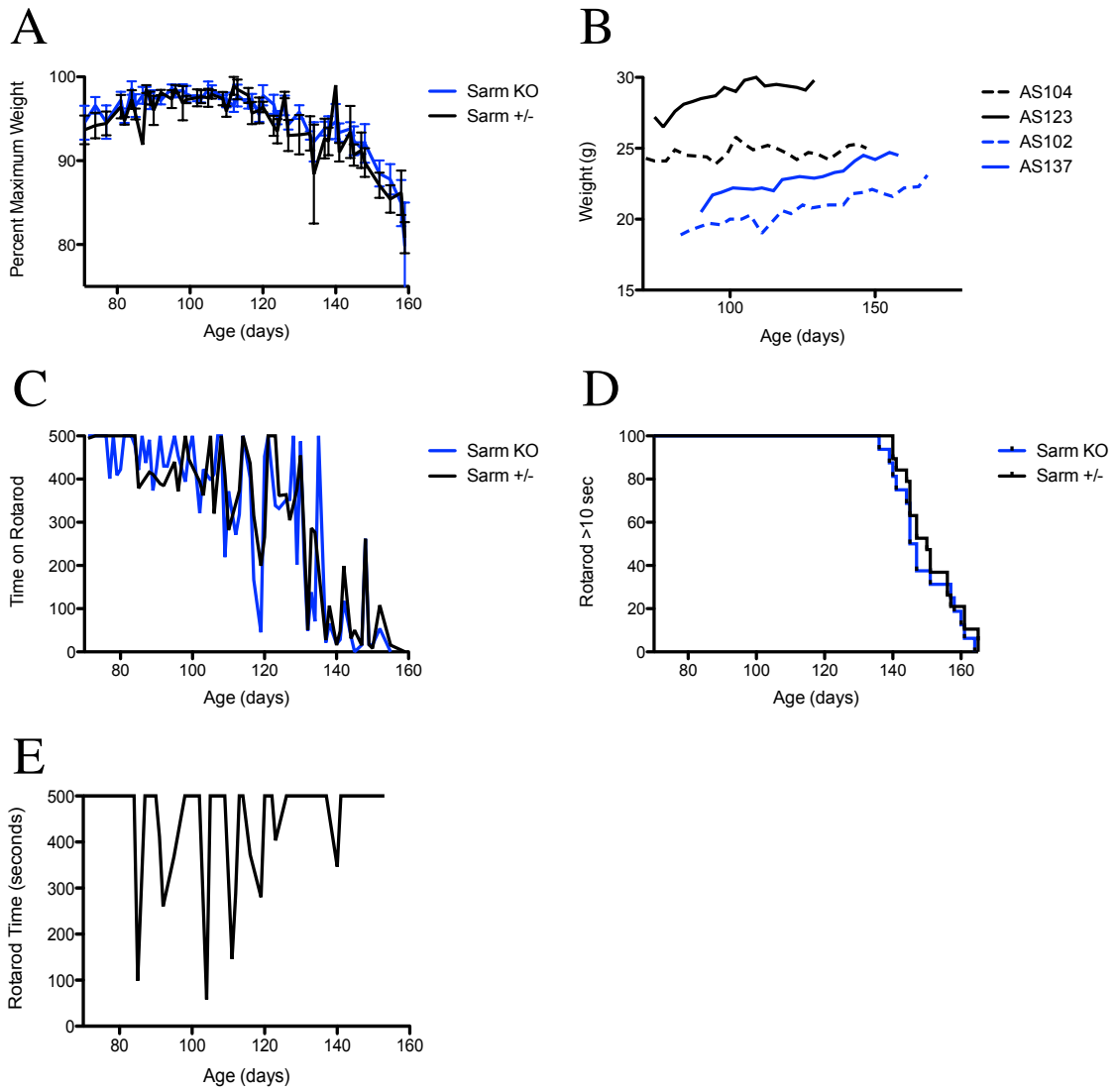


Figure 4-1. *Sarm* loss does not rescue weight loss or motor performance decline in the SOD1-G93A mouse model of ALS

n= 14 *Sarm1 KO huSOD1-G93A+*, n=12 *Sarm1+/- huSOD1-G93A+*, n=4 *Sarm1 KO*

A. Mean percent of maximum weight verses age in *Sarm1+/-* and *Sarm1* KO littermates with *huSOD1-G93A*, ± SEM. ns between cohorts.
B. Weight (in g) of individual *Sarm1 KO*, *huSOD-* animals with age.
C. Mean time on rotatod (maximum = 500s) verses age in *Sarm1 +/-* and *Sarm1* KO littermates with *huSOD1-G93A*. ns between cohorts.
D. Kaplan-Meyer plot of age when *Sarm1+/-* and *Sarm1* KO littermates with *huSOD1-G93A* are unable to remain on the rotarod for at least 10 seconds (functional paralysis). ns between cohorts.
E. Mean time on rotarod for *Sarm1 KO*, *huSOD-* animals.

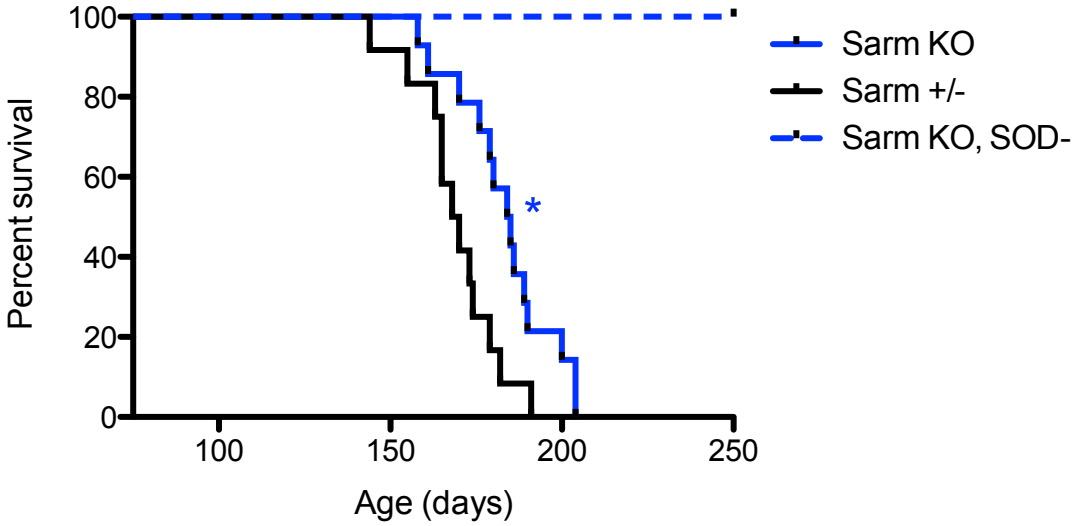


Figure 4-2. *Sarm* loss modestly extends lifespan in the SOD1-G93A model of ALS. Kaplan-Meyer plot of survival of *Sarm1 KO*, *Sarm1 +/-*, *huSOD1-G93A+*, and *Sarm1 KO, huSOD1-G93A+* littermates. Median survival for *Sarm1 +/-, huSOD1-G93A+* is 169.0 days, while median survival for *Sarm1 KO, huSOD1-G93A+* is 184.5 days. p=0.0136. All *Sarm1 KO, huSOD-* controls were alive for at least 250 days. n= 14 *Sarm1 KO huSOD1-G93A+*, n=12 *Sarm1+/- huSOD1-G93A+*, n=15 *Sarm1 KO*

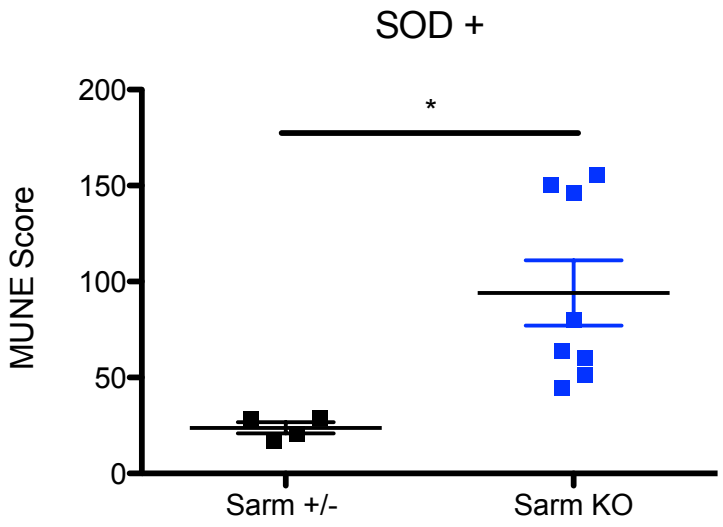


Figure 4-3. Sarm loss protects axons and synapses in the SOD1-G93A mouse
MUNE scores for 130 day old *Sarm1 +/-, huSOD1-G93A+* mice (■, 23.8± 2.9; n=4 legs) and *Sarm1 KO, huSOD1-G93A+ mice* (■,94.1± 17.0; n=8 legs). *, p=0.0174

CHAPTER V: Discussion

Wallerian degeneration was long assumed to be a passive wasting away of the severed axon (Lubinska, 1977). The discovery of Wld^{s} (Lunn et al., 1989) induced a paradigm sift in which many began to suspect Wallerian degeneration to be a genetically encoded axon death program. However, the identity of this death program remained an elusive target as it became clear that Wallerian degeneration is unrelated to known cellular destruction programs such as apoptosis and pruning (Coleman and Freeman, 2010). Using a forward genetic screen in *Drosophila*, we identified the first endogenous gene, *Sarm*, required for severed axons to undergo Wallerian degeneration. While many experiments remain to test the potential molecular mechanisms of Sarm activity, there is finally strong evidence that Wallerian degeneration is in fact a unique, genetically encoded death program. I will propose a model in this chapter for Sarm function in governing axon death.

Model for Sarm-mediated destruction of severed axons

Sarm is highly conserved at both the protein and genomic level between invertebrates and vertebrates (Mink et al., 2001); thus, the well-described calcium-sensitive MAPK signaling activity of TIR-1 appears as a foundation with which to model Sarm function in promoting Wallerian degeneration. In *C. elegans*, TIR-1 is activated by the calcium sensitive CaMKII during development. This interaction between CaMKII and TIR-1 subsequently activates an ASK1-dependent MAPK pathway to alter transcription of odorant receptor genes in olfactory neurons (see Chapter 1). While the context described in *C. elegans* is very different from axon death, the connection between calcium-dependence in both TIR-1 signaling and Wallerian

degeneration is difficult to ignore. I propose that Sarm is activated by dual signals, including calcium, after injury to induce downstream effector kinase activity to promote Wallerian degeneration. During AAD, a sharp spike in intra-axonal calcium is observed (Avery et al., 2009); this calcium spike activates a calcium-dependent kinase that binds the ARM domain of Sarm. A decrease in axonal ATP levels activates a second kinase complex that also binds the ARM domain; when both kinases bind the ARM domain, the auto-inhibition of Sarm is removed. This conformational change allows for the TIR domain to bind with and activate a downstream effector kinase(s), which ultimately contributes to promoting many of the morphological and molecular changes associated with Wallerian degeneration. One of the main effects of Sarm activation maybe the opening of L-type calcium channels, which allows for axonal calcium influx to devastating levels. Based on the localization and rescue data presented in Chapter 4, the SAM domain likely dictates a unique subcellular localization that is critical for executing this pathway after axonal injury (Figure 5-1).

Upstream activator(s) of Sarm

Work in the worm (Chuang and Bargmann, 2005; Chang et al., 2011) indicates that TIR-1 acts downstream of CaMKII, suggesting that CaMKII, or some other calcium-activated kinase, may specifically activate Sarm after injury. Unfortunately, in *Drosophila* overexpression of a dominant negative CaMKII mutant, which may or may not be fully functional, has no affect on Wallerian degeneration (Table 2-1), and loss-of-function mutants are not available because of the few existing genetic tools for work

with genes on the fourth chromosome. If the above model for Sarm function is correct, then calcium-sensitive kinase-binding sites should exist in the ARM domain of Sarm.

Using the group prediction site (GPS) software (http://gps.biocuckoo.org) to predict kinase-binding sites, dSarm is expected to have a variety of potential kinase interaction positions (Xue et al., 2011). Very few of these sites rest in the ARM domain; most are in the SAM and TIR domains. While there are no predicted CaMKII binding sites in dSarm, there are however two predicted adenosine monophosphate (AMP) kinase binding sites in the ARM domain, and AMPK has previously been shown to be activated by high levels of AMP (i.e., low levels of ATP relative to AMP levels) to help regulate cellular metabolism (Hawley et al., 2005). This suggests that low ATP levels, which occur after injury in the severed axon, could be one factor that activates Sarm to promote Wallerian degeneration.

There are no predicted CaMKII binding sites in Sarm; however, there is evidence that AMPK can be activated by calcium-calmodulin kinase kinase (CaMKK)-2, another calcium-sensitive signaling kinase (Ng et al., 2012). AMPK functions as a trimer, with three subunits, all of which have multiple splice forms: α, which contains the catalytic kinase site; β, which is regulatory and uses glycogen as a co-factor; and γ, which is also regulatory and responds to AMP:ATP ratios (Amato and Man, 2011). Recently, multiple groups have found that CaMKK2 can replace the AMPK γ-subunit to form a calcium, but not AMP, sensitive complex (Racioppi and Means, 2012). These different forms of AMPK (i.e., with and without CaMKK2) show different regulatory activities *in vitro* in a

variety of contexts (Amato and Man, 2011). If these predicted AMPK binding sites in the ARM domain are real and functional, together this suggests a two-part activation of Sarm: (1) a drop in cellular ATP levels activates calcium-independent AMPK signaling, possibly involving Sarm and microtubules (see below), upon which the AMPK α-β-γ complex binds one of the AMPK binding sites in the ARM domain of Sarm; and (2) increases in intra-axonal calcium activate a CaMKK2-AMPK complex that binds the second predicted AMPK-binding site in the ARM domain of Sarm.

I would predict based on this model that both calcium influx and low ATP levels in the severed axon act as activation steps and are both required for Sarm to promote Wallerian degeneration, which is why Sarm is not activated (and therefore Wallerian-like degeneration does not occur) during normal calcium influxes (i.e., cellular ATP levels are high, so AMPK is not activated) or during low energetic states, such as starvation (calcium levels are low, so CaMKK is not activated). Interestingly, NAD and ATP levels fall after injury in wild type but not Wld^{s} axons (Wang et al., 2005); however, NAD and ATP levels fall in severed Sarm1 KO axons (J. Gilley and M. Coleman, personal communication), suggesting that metabolic failure (and therefore AMPK activation) occurs upstream of Sarm. This also suggests that energetic failure alone is not enough to kill an axon or even induce necrosis, further highlighting the importance of Sarm in promoting Wallerian degeneration. Additional bioenergetics studies on Sarm1 KO severed axons are needed to investigate this further, but these initial results are consistent with a model in which Sarm is activated downstream of a metabolic failure after injury.

The above two-step activation model can also explain the calcium-insensitive features of Wallerian degeneration. Sarm has been shown previously to regulate microtubule stability (i.e., knock-down of Sarm reduces tubulin acetylation (Chen et al., 2011)), and microtubule depolymerization after injury is calcium-independent (Park et al., 2013). AMPK could be partially activated after injury by low ATP levels, and this pulls Sarm away from microtubules, destabilizing them and causing depolymerization. However, Sarm mislocalization and microtubule depolymerization is not enough to initiate all of the features of Wallerian degeneration; for this, a calcium-sensitive kinase is also needed to further activate Sarm. With dual-activation from both calcium and ATP sensitive kinases, the ARM domain could release Sarm auto-inhibition after increases in calcium levels to induce the calcium-dependent features of Wallerian degeneration, such as cytoskeletal fragmentation and eventual GDC. This two-step activation not only explains the calcium dependent and independent part of Wallerian degeneration but also helps to explain a potential role for Sarm during development: if local ATP levels fall, then Sarm could destabilize microtubules in a manner that would assist with neurite remodeling, which is suggested by *in vitro* work in hippocampal neurons (Chen et al., 2011). However, if calcium levels are not high enough to activate CaMKK2, then Wallerian-like degeneration does not proceed and instead neuronal processes can be refined and altered during development.

Further study is needed on these potential AMPK and CaMKK2 binding sites, particularly in terms of their affinity for the various forms of activated AMPK (i.e., α-β-γ AMPK verses CAMKK2-α-β AMPK). It remains a possibility that these binding sites

are slightly promiscuous and can be phosphorylated by either kinase, where extreme levels of either activating factor can induce Sarm-dependent death. For example, knockdown of Nmnat2, which induces spontaneous Wallerian-like degeneration (Gilley and Coleman, 2010), may create such an energetic crisis that α-β-γ AMPK levels are high enough to cross-activate the normally calcium-sensitive binding site on Sarm. In support of this, Sarm1 KO axons are protected from Nmnat2-RNAi induced spontaneous degeneration (J. Gilley and M. Coleman, personal communication), suggesting that metabolic devastation signals for axon degeneration through a Sarm-dependent mechanism. It will be interesting to test if increases in axonal calcium with normal ATP levels can also cause spontaneous degeneration in a Sarm-dependent manner.

This dual-activation model with both CaMKK2 and AMPK predicts that there are two essential "injury signals:" high calcium levels and a depletion of cellular energy levels. This model therefore suggests an additional function for how Wld^s protects severed axons: Wld^s keeps cellular energy levels high, preventing AMPK (and then Sarm) activation. However, because Wld^s blocks auto-activated *dSarmΔARM*, Wld^s must be functioning in a capacity beyond inhibiting AMPK activity, as the above model puts AMPK upstream of Sarm activation. Alternatively, AMPK could potentially bind another domain in Sarm, and Wld^s would protect by preventing AMPK activation. However, this model would suggest that expression of *dSarmΔARM* could somehow lower ATP to a level that would activate AMPK signaling, and then this activated AMPK signaling is required to further activate Sarm to induce Wallerian-like degeneration. However, there are no other predicted AMPK binding sites in Sarm

outside of the ARM domain (Xue et al., 2011); while this does not exclude this alternative model, it would predict a self-activation loop for Sarm. This alternative model suggests that the ARM domain is the "calcium sensitive" part of Sarm, while another domain is the "ATP-loss" sensitive section; this would predict that loss of the SAM or TIR domain might be able to functionally rescue loss-of-function mutants in the presence of an injury-induced calcium rise, which is not the case (Figure 4-2).

Further studies should clarify the importance of AMPK and CaMKK2 in Wallerian degeneration. Biochemical studies on AMPK binding sites in Sarm are needed, as are subsequent mutagenesis of these sites and the effect on AMPK-Sarm binding. Work on dAMPK mutants and their effect on Wallerian degeneration will also help this mechanism; the model suggested here predicts that these mutants will block Wallerian degeneration, but not *dSarmΔARM*-induced degeneration. There are also a variety of biochemical and immunohistochemical tools available to test AMPK activation following axon injury. There is a *Drosophila* ortholog of CaMKK2, *CG17698*, however no mutants have been published. A targeted deletion may be a feasible method to produce a loss-of-function allele; alternatively, expression of transgenic constructs with mutated catalytic domains (based on the high degree of homology between mammalian and *Drosophila* CaMKK2) may alter the normal function of CaMKK2 in Wallerian degeneration. Together, these experiments may help decode the role of AMPK and CaMKK2 activity upstream of Sarm. In addition, there are a multitude of other predicted kinase binding sites in Sarm, and it remains important to discover which of these sites are actually responsible for producing a biologically relevant interaction in the context of

axon degeneration. The important kinase binding sites may actually reside outside of the ARM domain. Currently, we have no data to indicate what importance, if any, these predicted sites have in mediating Wallerian degeneration.

Calcium influx and Sarm

There are many morphological and molecular changes downstream of Sarm during Wallerian degeneration, and it remains unknown what processes Sarm directly initiates. However, an important step in axon degeneration appears to be the influx of calcium through L-type calcium channels in the severed axon, as pharmalogical blockade of these channels potently blocks Wallerian degeneration (Deckwerth and Johnson, 1994). One of the ways in which Sarm activity promotes Wallerian degeneration may involve these voltage-gated channels. Effector kinase activity downstream of Sarm may open these channels; alternatively, Sarm may bind L-type calcium channels directly through its SAM domain. However, direct opening of these channels would likely result in a rapid increase in intra-axonal calcium, similar to what is observed during an action potential, which is not seen after axotomy (Avery et al., 2012; Adalbert et al., 2012). It is more likely therefore that the depletion of energy levels after injury prevents the maintenance of the membrane potential in the injured axon, and these intra-axonal energetic changes cause ion leaking through the L-type calcium channels. Why this ionic leak occurs specifically through L-type channels is unknown, but it is well established that this channel type specifically is at fault (George et al., 1994). Alternatively, Sarm activity may directly contribute to this leaking by binding the

channels directly. In support of this, data from *C. elegans* suggests that TIR-1 directly binds the L-type calcium channel UNC-2 (see below) (Chang et al., 2011).

Experiments with L-type calcium channel agonists and antagonists in both wild type and Sarm KO neurons should illuminate the relationship between channel opening and Sarm activity, as well as electrophysiological recordings and calcium imaging from Sarm KO severed axons. If Sarm is acting upstream of channel opening, then channel agonists should induce spontaneous degeneration in wild type and Sarm KO axons; however, if Sarm is downstream of L-type calcium channels, then Sarm KO axons would be protected from agonist-induced degeneration. Alternatively, agonist could be added to severed Sarm KO axons, and if Sarm is upstream of calcium channels then the agonist should restore Wallerian degeneration to severed Sarm KO axons. In addition, if Sarm acts upstream of L-type calcium channels, then channel antagonists should block *dSarmΔARM*-induced degeneration; however, if antagonists have no effect on this construct, then Sarm likely acts downstream of channel opening. Measuring calcium levels in severed Sarm KO axons is also needed; if slow leakage through these channels is required for full Sarm activation (i.e., via CaMKK2-AMPK), then the normal calcium rise after injury should still occur in Sarm KO axons; alternatively, if Sarm activity is required for the calcium rise, then it should not occur in Sarm KO axons.

Based on the interaction in *C. elegans*, I predict that AMPK partially activates Sarm, which dissociates it from microtubules (destabilizing them, see above) and re-distributes Sarm to L-type calcium channels. Following redistribution, CaMKK2 (having been activated by the calcium spike during AAD) binds to and activates Sarm. This

interaction, potentially in conjunction with changes in axonal metabolism, causes L-type calcium channels to slowly leak calcium into the severed axon. Over time, this raise in intra-axonal calcium reaches a threshold level at which calpains become activated and mitochondrial swell and rupture. This model suggests that it is the calcium influx that causes cytoskeletal disintegration and mitochondrial bursting, instead of Sarm acting on a signal cascade that would activate these two events separately; however, we currently have little data to distinguish these two possibilities. Sarm may also regulate neurofilament or microtubule degradation; *in vitro* biochemical assays are needed to determine if a direct interaction between Sarm and cytoskeleton elements exists, as neurofilaments behave differently in the presence of Wld^{s} *in vitro* (Bernier et al., 1999). This degradation however may be a result of the increases in calcium levels, not by direct association with Sarm.

The above model predicts that Sarm is required upstream of L-type channels. While *Sarm*-null axons will undergo normal AAD, the slow intra-axonal calcium rise will not occur during the latency period because Sarm is required for L-type channels to leak after injury (Figure 5-1). If this is true, then a biochemical interaction between Sarm and a subunit of L-type calcium channels should be detectable. TIR-1 has been shown to interact genetically and biochemically with the L-type channel UNC-2 (Chang et al., 2011); unfortunately, loss-of-function clones in ORNs of the *unc-2* ortholog *cacophony* appear to degenerate spontaneously, and thus far we have not been able to test its role in Wallerian degeneration (work not presented here). It remains to be tested if *cacophony* mutant neurons undergo Wallerian-like degeneration (i.e., degeneration

can be blocked by expression of Wld^{S}), or if they are developmentally highly irregular. I would predict that Sarm co-localizes with cacophony after injury, but not before, and this interaction is necessary for the calcium-dependent processes of Wallerian degeneration, although this remains to be tested. I would also predict that a calcium ionophore would restore Wallerian degeneration to cut Sarm KO axons and that chelating calcium from wild type axons during the late stages of the latency period would block degeneration. It is possible however that calcium influx occurs before Sarm activation after injury; if this is the case, then an interaction with a calcium channel such as Cacophony may or may not be required before injury to promote Sarm-dependent signaling. Again, more genetic and biochemical experiments are needed to test the link, if it does exist, between Sarm and L-type calcium channels directly.

Molecules downstream of Sarm activation

Following increases in intra-axonal calcium in a severed axon, a variety of calcium-dependent processes occur: microtubules and neurofilaments are degraded by activated calpains; mitochondria swell and rupture; and the membrane eventually beads and fragments (Wang and Barres, 2012). While all of these processes are blocked in severed Sarm KO axons, it is unknown which of these processes (possibly all) are due to direct interactions with Sarm or if they are a byproduct of Sarm-dependent processes. For example, effector kinase signaling downstream of Sarm may be responsible for many aspects of Wallerian degeneration. It is unknown what effector kinase acts downstream of Sarm; an obvious candidate, based on *C. elegans* data, is dAsk1 (Chuang and Bargmann, 2005; Chang et al., 2011; Hayakawa et al., 2011); however, the only

available tool in *Drosophila*, a dominant negative transgene, has no impact on Wallerian degeneration (Table 2-1), and attempts at generating a mutant through imprecise excision of an existing Minos element and FLP-mediated recombination between FRT-bearing transposon insertions flanking dASK1 have not been fruitful (N. Fox, personal communication). Thus, we cannot rule out the involvement of this molecule in Wallerian degeneration, especially because ASK1 has a well-established role in mediating a variety of cellular stress responses, including calcium influx and oxidative stress (Hattori et al., 2009; Shiizaki et al., 2013).

If not ASK1, then what other molecules are downstream of Sarm? Based on Sarm's structure, it is predicted that the TIR domain plays an important role in signal transduction. There are a variety of predicted kinase binding sites in the TIR domain, and it remains to be seen if these potential sites are biologically relevant in terms of Wallerian degeneration. TIR domains are traditionally found in TLRs and their downstream adaptors and are responsible for innate immune signaling (O'Neil et al., 2006). However, we have found no evidence for Toll-signaling in Wallerian degeneration, as loss of function mutants in Tolls 6-9 do not impact Wallerian degeneration in *Drosophila* (Table 2-1; work not presented here) nor do TLR3 or UNC93B mutants in mice (T. Rooney, personal communication). TIR domains tend to be "sticky" biochemically, so pull-downs with this domain are likely to produce large sets of potential interacting proteins, which may or may not be relevant for Wallerian degeneration. This interaction may also be dependent on "activation" of Sarm, and at the moment this is not possible *in vitro*; furthermore, dSarm::GFP, when expressed in

sensory neurons, does not localize to axons (Figure 4-3), so there is no way to injure these neurons and then pull down dSarm::GFP from the injured axons. A more targeted approach would be to screen for suppressors of dSarmΔARM-induced degeneration; mutants in downstream pathways should block or delay this cell death.

Five candidate binding partners can be found from a yeast two-hydrid screen with horseshoe crab Sarm: Hax-1, SUMO-1, proteasome subunit alpha, Hsp40, and CaMKI (Belinda et al., 2008). Interestingly, CaMKI is predicted to interact with the TIR domain, while the other is predicted to bind the SAM domain. Unfortunately, CaMKI is on the forth chromosome, next to CaMKII, in *Drosophila*, making it difficult to test and no reagents are available currently. However, this possible interaction is worth studying *in vitro* in mammals. In work not presented here, I cloned *Drosophila hax-1* into a UAS-vector, as it is predicted to be anti-apoptotic (Simmen, 2011). Unfortunately, overexpression of Hax-1 had no affect on Wallerian degeneration and loss-of-function mutants are not available. The other molecules have not been tested to date but remain candidates in Wallerian degeneration.

It remains possible that the main function of Sarm is to activate the intra-axonal calcium influx, and it is this influx of calcium that directly causes the morphological changes associated with Wallerian degeneration. For example, high levels of axonal calcium during excitotoxicity in the retina can lead to degeneration that looks very similar to Wallerian degeneration (Saggu et al., 2010) and is blocked by expression of Wld^{s} (Bull et al., 2012). This would predict that the intra-axonal calcium rise after axotomy does not happen in Sarm KO axons, but addition of a calcium ionophore (or a

L-type channel agonist) to these severed axons would restore degeneration to Sarm KO axons. Thus, L-type calcium channels could be the only molecule to act directly downstream of Sarm. Channel mutants that block an interaction with Sarm, but not normal channel function, should block Wallerian degeneration. If this is true, and the earlier model is correct, then CaMKK2 becomes activated during AAD, not the latent phase, to promote Sarm activity.

Despite the predicted connection between Sarm activity and calcium, it is unknown if calcium-dependent processes during Wallerian degeneration are changed in severed Sarm KO axons. Ultrastructural analysis by electron microscopy of severed wild type axons treated with EGTA showed intact neurofilament, but not microtubule, fibers (Park et al., 2013). Interestingly, microtubule "clumps" were still detected with immunohistochemistry, suggesting that calcium is required for neurofilament breakdown and microtubule degradation, but not for microtubule strand disruption. It remains to be seen if microtubules depolymerize in severed *Sarm1* KO axons, as this process appears to be calcium independent (Park et al., 2013). However, thus far *Sarm1* KO axons have pheno-copied both Wld^{s}-expressing and EGTA-treated severed axons, suggesting that Sarm regulates the calcium-dependent changes that occur during Wallerian degeneration, but this remains to be tested.

Calcium influx during Wallerian degeneration may activate catastrophic changes in mitochondria. After injury, mitochondria in wild type severed axon eventually undergo mitochondrial permeability transition pore (MPTP) opening, causing them to swell and burst (Barrientos et al., 2011). *Sarm1 KO* mitochondria do not swell after

injury (Chapter 2), although it is unclear if this is due a fundamental change in the MPTP in Sarm KO neurons or due to the loss of an upstream activator of pore opening. High calcium levels are known to cause pore opening (Ikegami and Koike, 2003), so it will be interesting to see if mitochondria swell and rupture in Sarm KO axons after addition of a calcium ionophore. I would predict this to be the case, as Sarm in this model is upstream of an intra-axonal calcium rise that would cause MPTP opening. It will also be interesting to see if loss of Sarm changes mitochondrial responses to other stressors, such as uncouplers and electron transport change complex inhibitors; the model here predicts that loss of Sarm would have no affect on these toxins, but further work on the molecular differences in Sarm KO mitochondria compared to wild type and Wld^{s} mitochondria is needed.

It is also possible that the calcium leak through L-type channels occurs independently of Sarm activity, but high intra-axonal calcium levels "fully" activate Sarm through CaMKK2 to promote Wallerian degeneration. What this active form of Sarm interacts with is unknown, but this highlights the importance of screening for suppressors of *dSarmΔARM*-induced spontaneous degeneration. If Sarm acts only on L-type channels, then L-type channel antagonists should block *dSarmΔARM*-induced degeneration. However, a suppressor screen may identify a variety of cellular targets, such as factors regulating cytoskeletal degradation and mitochondrial homeostasis, that would argue for a wide-spread role for Sarm in promoting Wallerian degeneration beyond increasing intra-axonal calcium.

Wld^s and Sarm

Where does Wld^s fit in with Sarm and Wallerian degeneration? In our initial observations on the robust protection from Wallerian degeneration in Sarm KO axons, we were unable to distinguish any differences between Sarm KO and Wld^s-expressing axons (Chapter 2). If *Sarm1 KO* axons are phenol-copying Wld^s-expressing axons, then Wld^s may act by directly offsetting normal Sarm function. Recent evidence suggests that Wld^s may be acting by increasing mitochondrial buffering capacity (Avery et al., 2012). Calcium dynamics have not been tested in Sarm-null axons, but initial experiments show that Sarm1 KO mitochondria have normal levels of calcium buffering (P. Sullivan, personal communication). This suggests that *Sarm1 KO* axons are not identical to Wld^s-expressing axons, and implies that the slow intra-axonal calcium rise during the latency period, which does not occur in Wld^s-expressing axons (Adalbert et al., 2012; Avery et al., 2012), may occur in Sarm-null axons. If true, this would suggest that Sarm-null axons can survive even with persistently high calcium levels. However, calcium-imaging studies have not been done on Sarm KO axons, and work presented in Chapter 4 using the *dSarmΔARM* construct argues that Wld^s acts downstream of this construct of to block axon degeneration.

I propose that Wld^s can prevent Sarm-mediated axon destruction in multiple ways (Figure 5-2). First, Wld^s could act upstream of Sarm by buffering calcium rises during AAD, which would prevent CaMKK2 activity. Without an activated calcium-dependent kinase, Sarm remains in its auto-inhibited state and thus cannot promote Wallerian degeneration. In support of this, it has been shown previously that Wld^s-

expressing axons also do not undergo AAD (Kerschensteiner et al., 2005) and Wld^s-expressing mitochondria can buffer more calcium than their wild type counterparts (Avery et al., 2009); however these studies need to be repeated in Sarm KO neurons and mitochondria.

An alternative way by which Wld^s can protect severed axons from Sarm-mediated Wallerian degeneration may be through the enzymatic function of Wld^s. Wld^s contains an active Nmnat1 that is required for protection (Avery et al., 2009; Conforti et al., 2009), which may help stabilize axonal energetics after injury. This activity may prevent AMPK activation, which would prevent Sarm activation independent of calcium levels. Both of these explanations are feasible, and immunohistochemical studies on severed Wld^s and wild type axons may show differences in CaMKK2 and AMPK activation or phosphorylation levels.

In wild type severed axons, the above model predicts that Wld^s acts upstream of Sarm and prevents its activation by buffering calcium and maintaining ATP levels, and I predict that this same activity can also work downstream of Sarm to block Wallerian degeneration. Based on data presented in Chapter 3, the spontaneous degeneration that occurs in uninjured neurons expressing the *dSarmΔARM* construct may be due to the activation of L-type calcium channels by "auto-activated" Sarm. Wld^s-expressing mitochondria can buffer these increases in intra-axonal calcium due to L-type calcium channel activation. Without increases in intra-axonal calcium, mitochondria stay healthy and calpains are not activated, resulting in maintenance of neuronal integrity. However, calcium-imaging studies need to be done on *dSarmΔARM*-expressing neurons. This

model would predict that *dSarmΔARM* expression causes increases in neuronal calcium levels, and these increases should be blocked by co-expression of Wld^s. Expression of a calcium-chelating agent with the ability to prevent axonal calcium rises should also mimic Wld^s. This explanation also supports a model in which Sarm acts only upstream of L-type calcium channels; furthermore, treatment of *dSarmΔARM*-expressing axons with a L-type calcium channel antagonist should prevent spontaneous degeneration.

Notably, no one has published experiments regarding calcium-ionophore induced spontaneous axon degeneration in Wld^s axons, and it would be interesting to determine if Sarm-null axons do not spontaneously degenerate due to this ionophore, even in the presence of very high calcium levels. Furthermore, work on wild type axons has shown that L-type calcium channels are required for severed axons to undergo Wallerian degeneration, and it remains to be seen if treatment with a L-type calcium agonist, such as FPL-64176 (Liu et al., 2003) can activate Wallerian-like degeneration in wild type or Sarm1 KO axons. If Sarm is downstream of this calcium signal, than Sarm1 KO axons should be resistant to this agonist-induced axon degeneration. It would also be interesting to look at cytoskeletal changes in wild type axons with agonist treatment in order to better understand the role of calcium in cytoskeletal changes during axon degeneration.

Molecular Injury Signal(s)

The above model predicts that decreases in ATP levels after injury and a calcium spike during AAD are the two molecular injury signals that are required for Wallerian

degeneration. However, it is unknown if these (or other) intrinsic injury signal(s) persist after injury; they could be a momentary "SOS" to the axon that is lost over time. This suggests that Sarm could be re-introduced to a severed Sarm KO axon but may not be able to restore Wallerian degeneration unless the calcium-sensitive signal (i.e., CaMKK2) is still active. No reports have been published to date regarding the effect of a second lesion within a previously severed axon. For example, if functional Sarm protein were supplied to severed Sarm-null axon, would the additional Sarm initiate degeneration? Or, would a second lesion be needed to supply the activating injury signal? It has been shown previously through virally-delivered particles that cytosolic Nmnat can protect axons when delivered to the severed axon after injury (Sasaki and Milbrandt, 2010). Resupplying Sarm1 to severed Sarm1-KO axons may induce degeneration, suggesting that the injury signal persists even after the severed axon has resealed. However, if resupplied Sarm1 does not rescue the blockade of axon degeneration, then a second lesion may be needed to activate Sarm1. If this is the case, then blocking the initial signal alone may be enough to prevent axon degeneration. If not, then the "SOS" signal, indicating that the axon has been severed, likely persists throughout the rest of the latency period.

AAD and regeneration without Sarm

AAD is believed to be an important first step in activating axon regeneration in the proximal injured axon (Kerschensteiner et al., 2005; Knoferle et al., 2010). It remains to be tested if regeneration is indeed inhibited in transected Sarm1 KO axons; however, if Sarm1 KO axons behave similar to Wld^{s} axons (Kerschensteiner et al., 2005),

AAD will be severely inhibited and regeneration will be stalled. Live-imaging of severed Sarm1 KO axons will help to address this question, as will looking at long-term *in vivo* regeneration. However, the model presented here predicts that AAD will occur normally, so regeneration initiation should not be hindered. However, the regenerating axon is likely to run into a large roadblock (i.e., the remaining severed axon stump). Could a regenerating axon fuse with an intact severed axon? This is found in *C. elegans* (Neumann et al., 2011), where severed axons do not undergo Wallerian degeneration. This would be an exciting possibility for treatment of nerve injuries, particularly if a method for allowing CNS regeneration were established. A regenerating axon could theoretically rejoin the old axon and restore function through the original connections without having to completely rewire the circuit, which can take years in the human PNS and does not occur in the CNS. Together, these techniques are exciting possibilities for treating a variety of traumatic neuronal injuries, including spinal cord damage.

Sarm and Calpains

Calpains and calcium are the driving force behind AAD, and the impact of loss of Sarm on calpain activation remains to be seen. Addition of calcium to nerve extracts will induce neurofilament degeneration in a dose-dependent manner by calpain activation; this occurs in Wld^s extracts but requires a higher calcium concentration (Glass et al., 1994). Interestingly, Wld^s nerve extracts were resistant to degradation *in vitro* by added activated calpains (Bernier et al., 1999), suggesting that there is (1) an intrinsic, calcium-activated process that makes neurofilaments succeptable to degeneration or (2) axons possess a protective mechanism that normally inhibits

neurofilaments breakdown. It will be interesting to test Sarm1 KO axon extracts to see if there is a difference in calpain activity and activation, as it remains possible that Sarm's normal function is to aide in activation of calpains or to make cytoskeletal proteins such as neurofilaments more succeptable to calpain-mediated proteolysis. This would imply that Sarm functions beyond opening L-type calcium channels and plays a more direct role in axonal destruction and degradation. It would be interesting to add recombinant Sarm to neurofilaments or microtubules, followed by activated calpains, to determine if Sarm can mediate calpain cleavage of the cytoskeleton directly. This more direct role may instead be executed by downstream effector kinases that interact with the TIR domain of activated Sarm.

Microtubules and Sarm

Recent results have suggested that calcium is required for neurofilament breakdown but not microtubule disruption (Park et al., 2013), suggesting that two parallel pathways exist in severed axons to execute GCD. There is evidence that calpains are responsible for neurofilament degeneration during GCD (Ma et al., 2013), but less is known about microtubule disruption. Wld^{s} and Nmnat2 overexpression block microtubule fragmentation (Feng et al., 2010) but fibrous integrity has not been assayed. Unlike in wild type nerves, microtubules do not fragment in cut Sarm1 KO axons (Figure 2-11); however, microtubule integrity at the ultrastructural level was not assayed. This fiberous integrity is crucial for axon health, as addition of microtubule-destabilizing agents such as vinblastine induce spontaneous Wallerian-like degeneration (Ikegami and Koike, 2003). Interestingly, while mitochondrial failure and ATP loss are consequences

of vinblastine-induced degeneration, treatment with a mitochondrial uncoupler, which recapitulates the same effects without disrupting microtubules, induced degeneration in both wild type and Wld^s axons (Ikegami and Koike, 2003), suggesting that microtubule integrity specifically is a key component of Wld^s protection. *In vitro* Sarm1 appears to co-localize with microtubules (Kim et al., 2007) and promotes stabilizing tubulin acetylation when overexpressed in neurons (Chen et al., 2011), supporting the idea that Sarm1 is interacting with, and possibly stabilizing, microtubule fibers. If this interaction is calcium dependent or changes with calcium fluctuation remains to be tested.

Sarm1's role in neuronal development and polarity (Chen et al., 2011) further supports this idea, and biochemical interactions with tubulin may classify Sarm as a microtubule-associated protein (MAP). Studying the effect of microtubule disrupting drugs on Sarm1 KO axons may provide further insights into this interaction. This is particularly intriguing because dysfunction of another MAP, Tau, has been implicated in neurodegenerative disease, and stabilization of microtubules can offset disease progression (Zhang et al., 2012). While microtubule inhibitors can prevent axon degeneration after stretch injury (Tang-Schomer et al., 2012), long-term treatment with microtubule stabilizers, such as during chemotherapy, induce peripheral neuropathies (Lee and Swain, 2006). Thus, even though microtubule-disrupting drugs induce Wallerian-like degeneration, microtubule stability is not the only factor contributing to axon degeneration after injury. However, microtubule destabilizing agents may provide

a potential means by which we can "cut" axons without physically lesioning them and help to decipher different components of Wallerian degeneration.

It is also unclear if microtubule binding is essential for Sarm function; the fact that the dSarmΔSAM construct is unable to rescue loss-of-function mutants suggests this, but specific point mutants that block microtubule association directly without grossly altering protein folding should clarify this. It would also be interesting to see if knock-in of a mutant with microtubule localization disruption renders a loss-of-function phenotype. It may alter developmental functions of Sarm potentially without blocking Wallerian degeneration; according to the model presented here, disrupting the microtubule binding itself should not alter the AMPK or CaMKK2 binding (and activating) sites. This localization however may be important for interactions with downstream signaling molecules.

MAPK activity and Wallerian degeneration

MAPKs are known to regulate some components of TIR-1 signaling (Chuang and Bargmann, 2005; Chang et al., 2011) and their inhibition can slightly delay but not completely inhibit axon degeneration (MacInnis and Campenot, 2005; Miller et al., 2009; Gerdts et al., 2011). Interestingly, the protective effect of proteasome inhibitors has been suggested to be the result of increased levels of MAPKs in severed axons (MacInnis and Campenot, 2005). In support of this, loss-of-function JNK mutant neurons exhibit spontaneous Wallerian-like degeneration that is blocked with Wld^{s} expression (Rallis et al., 2013) but not p35 expression (J. Osterloh and M. Freeman, unpublished observation), suggesting that in this case loss of JNK does not simply

induce caspase-dependent cell death. It is unknown what during injury activates these stress-induced pro-survival kinases, and because chemical inhibition only delays degeneration, MAPK activation may be a result of, but not a causative agent in, Wallerian degeneration. As with all kinase cascades, there is likely context-dependent fine-tuning; further assaying activation and interactions after injury may illuminate potential roles for these molecules during axon degeneration. Nevertheless, calcium is a known activator of some kinase signaling pathways (Hattori et al., 2009; Song, 2013), and it remains possible that calcium activates a MAPK pathway through Sarm in a manner or combination that has not previously been tested.

The potential involvement of CaMKK2 in Wallerian degeneration could link Sarm with previous *in vitro* results in which knock down of some MAPKs delay Wallerian degeneration. The MAPK GSK3 can phosphorylate CaMKK2, and this phosphorylation is a crucial factor in regulating CaMKK2 protein stability and protecting it from degeneration. Thus, knockdown of GSK3 would decrease levels of CaMKK2, which could delay Sarm activation; this would explain why knockdown of GSK3 delays, but does not block, Wallerian degeneration (Gerdts et al., 2011). Further proteomic studies are needed to determine the relationships between molecules like GSK3 and the stability of potential Sarm-interacting proteins, such as AMPK and CaMKK2. There are likely other proteins that regulate stability of Sarm and its upstream partners, and this stability may explain why UPS inhibitors delay but not block Wallerian degeneration: proteins that normally negatively regulate Sarm (or upstream

molecules like AMPK and CaMKK2) activity or levels are more abundant with UPS inhibition, which slows Sarm activation and delays Wallerian degeneration.

Mechanism of Sarm regulation

UPS regulation (see above) likely plays an important role in regulation of Sarm levels and activation, as UPS inhibition strongly delays Wallerian degeneration (Zhai et al., 2003). Intriguingly, loss-of-function mutants in the E3 ubiquitin ligase *highwire* (*hiw)* have recently been found to potently block injury induced Wallerian degeneration (Xiong et al., 2012). *highwire* acts by directing protein targets for ubiquitination, suggesting that there is a specific protein(s) that either (1) block Wallerian degeneration themselves, and loss of *hiw* increases these to a threshold that blocks axon destruction or (2) promote Wallerian degeneration, and these are regulated by proteins that are regulated by *hiw*. The model presented here would suggest that *hiw* acts by regulating proteins that control Sarm, CaMKK2, and/or AMPK levels; loss of *hiw* increases the levels of proteins that ultimately result in Sarm suppression. CaMKK2 and AMPK activity in *hiw* mutants should be tested, and I would predict that protein levels of these kinases would be lower or their activity would be severely inhibited. If the model presented here is correct, then overexpression of Sarm, CaMKK2, and/or AMPK together or alone may be able to restore Wallerian degeneration in *hiw* mutants.

The regulation of Sarm by UPS degradation remains unknown, but there is evidence for Sarm regulation by another method: RNA interference. Human cDNA studies have detected the presence of an endogenous microRNA targeted to Sarm1 (Mink et al., 2001), as well as one in worms (Hsieh et al., 2012) although no such

microRNA has been reported in mice or *Drosophila*. Because endogenous expression of dSarm and Sarm1 are very low (Kim et al., 2007; Osterloh et al., 2012), it is possible that an endogenous RNA mechanism exists to control and limit Sarm levels. It would be interesting to determine if loss of interfering RNA machinery induces spontaneous Wallerian-like degeneration.

It is also possible that Sarm protein is expressed at minimal levels before injury, and injury induces local axonal translation of Sarm protein. This is option is not likely however, as addition of ribosomal inhibitors just before injury do not block Wallerian degeneration (Gilley and Coleman, 2010). It is possible, however, that these inhibitors are not complete, and some small amount of Sarm is still translated. mRNA levels in the axon before and after injury should be assayed to address this.

When and where is Sarm required to promote axon death?

Is Sarm required before, after, or during injury to signal for axon degeneration? While the simplest explanation is that injury induces some sort of cascade through Sarm, it remains unknown if Sarm-null neurons are fundamentally changed before injury in such a manner that would prevent degeneration. Sarm could be acting by altering transcription or translation in a way that primes axons for death after injury. Proteomic or transcriptome profiling would address this question: are there any differences pre-injury, other than a loss of Sarm, in Sarm1 KO verses wild type neurons? Could Sarm be involved in pro-Wallerian degeneration gene regulation? If so, are these changes relevant to axon degeneration, or are they related to the potential developmental function of Sarm?

Another possibility is that Sarm is required after injury. To test this, one could resupply Sarm into Sarm1 KO axons through a viral vector, similar to the Nmnat transduction experiments (Sasaki and Milbrandt, 2010). If this alone is enough to activate Wallerian degeneration, then Sarm is required after injury. Sarm could also be required during the injury event itself; if this is the case, then the Sarm1 KO axon, after being re-supplied with Sarm1, would have to be cut a second time to activate Sarm-induced Wallerian degeneration. These experiments would also shed light on the persistence of the injury signal (see above). While we favor the hypothesis that resupplying Sarm1 to a severed Sarm1 KO axon will activate Wallerian degeneration for the sake of simplicity, there is no evidence to date that suggests one possibility is more likely over the others.

Is Sarm required in the axon to promote Wallerian degeneration? This question is to be approached with caution, as much time was spent on understanding the predominantly nuclear localization of Wld^{s} (Coleman, 2005) even though we know now that small amounts of Wld^{s} act in the axon to prevent Wallerian degeneration (Coleman and Freeman, 2010). One approach to this question would be to resupply Sarm1 to uncut Sarm1 KO axons in the presence of an axon transport inhibitor, similar to the Nmnat2 expression study (Gilley and Coleman, 2010). If Sarm1 was re-expressed but excluded from the axon, and then did not promote axon degeneration after lesion, Sarm is likely (1) required in the axon to promote degeneration and (2) not acting outside the axon (i.e., in the soma) to “prime” axons for degeneration.

Work presented here unfortunately does not clarify the localization of Sarm. While it localizes to mitochondria in kidney cell lines *in vitro*, Sarm does not localize to mitochondria in neurons and instead appears in a punctate pattern (Figure 2-14). Expression of a dSarm::GFP construct varies slightly *in vivo* in different neuron sub-types, and no clear sub-cellular localization pattern has emerged. What could account for the differences in dSarm localization? This may be indicative of the variety of other functions of dSarm beyond controlling Wallerian degeneration. It could also be due to a variety of levels of regulatory factors or binding partners in these difference neurons. At this time, it is unknown if varying localization of Sarm confers different sensitivities or timing to Wallerian degeneration. In sensory neurons, dSarm::GFP is excluded from the axon, so we are unfortunately unable to assay localization changes after injury. Live imaging studies in *Drosophila* larvae of dSarm::GFP does not appear to be moving throughout the axon (T. Rooney, personal communication), and instead appears to be localized primarily around the cell body and main neurite branches. However, these experiments were done with an overexpression system and may not represent endogenous localization. Studies of endogenous localization with a recombineered GFP-tag (not presented here) may shed some light on any injury-induced changes. Sarm expression may be upregulated after injury; if this is the case, then more GFP+ signal should be detected with this tool. The GFP-tagged BAC may also help determine Sarm expression regulators. Treatment with a UPS inhibitor may increase Sarm-GFP levels, and this may be a device with which to screen for Sarm managers.

The *dSarmΔARM* construct also allows us to investigate the necessity of subcellular localization and dSarm activity. While the SAM domain does direct localization, it remains unclear if this is important for function. By targeting dSarmΔARM to a variety of areas, such as the mitochondria matrix, the nucleus, or the cell membrane, we can determine if a discrete location is necessary for dSarmΔARM-induced death. In addition, we may be able to restore function (i.e., the ability to rescue a loss-of-function mutant) to the dSarmΔSAM construct by targeting this mislocalized molecule to a specific subcellular localization.

Studying dSarm activation in cell culture

Further work with the *dSarmΔARM* construct *in vitro* may elucidate a method by which to study axon degeneration in cell culture. A current limitation of using cell culture to study Sarm and Wallerian degeneration is that there is no way to "injure" and subsequently look at Sarm activation in an S2 cell, and overexpression of dSarm does not induce cell death. Thus, expression in S2 cells may provide us with downstream markers of dSarm activation that could be used as an *in vitro* cell culture read out for Wallerian degeneration, even though these cells have no axons. Possible targets include phosphorylation or ubiquitination changes in these cells, as well as microtubule stability..

Sarm beyond Wallerian degeneration

The above models only address the role of Sarm in promoting Wallerian degeneration. Sarm likely has other functions in cells, possibly as a negative regulator of innate immunity and a microtubule stabilizer. Sarm overexpression in hippocampal cultures increases stabilizing tubulin acetylation (Chen et al., 2011). However, if Sarm is

involved in microtubule stability, this function may also be important during Wallerian degeneration. Activation of Sarm, possibly by AMPK during local decreases in ATP, may remove Sarm-microtubule interactions, thereby destabilizing microtubules independent of the proposed kinase scaffolding function of Sarm. This is consistent with the observation that microtubule depolymerization occurs very early in Wallerian degeneration. Interestingly, AMPK is not required for cortical development, but activation does inhibit axonal outgrowth and polarization (Williams et al., 2011). AMPK does have many targets in different contexts, and it remains unclear if AMPK interacts with Sarm under any condition.

Is Sarm required in non-neuronal tissues? dSarm loss-of-function mutants are lethal at L1 larval stages; surprisingly, dSarm expression only in neurons is not enough to rescue lethality, indicating that dSarm plays essential functions in another, non-neuronal tissue. However, broad misexpression with an abundant driver, such as *tubulin-Gal4, ubiquitin-Gal4,* or *actin-Gal4*, also does not rescue lethality. Together, this suggests that dSarm is normally required in low levels in small set of non-neuronal cells. Interestingly, too much dSarm is likely toxic, and we have not identified a specific subset of cells (neuronal or otherwise) that require Sarm function for animal viability. dSarm loss-of-function mutants show reduced numbers of clones in ORNs with MARCM, indicating that dSarm may play an unknown function in neuronal development or maintenance. Clonal induction numbers seem too decline with age, suggesting an important role for dSarm in neuronal, or at least axonal, maintenance. It is unknown if this sensitivity is restricted to ORNs however, as similar observations have

not been seen in wing sensory neurons (L. Neukomm, personal communication). There is a recent report of dSarm being involved in tollo/toll-8 –dependent innate immunity in the *Drosophila* gut; however, the mutants tested were not loss-of-function but instead weak hypomorphs (Akhouayri et al., 2011), so it is difficult to interpret the role of dSarm in the *Drosophila* gut and immune system. Whatever this function of dSarm may be, it is either not required in mammals, or other gene(s) have assumed this role(s), as Sarm loss-of-function mice are viable and seemingly normal.

How do severed axons survive without somal support?

The protection of axons by loss of Sarm (and by expression of Wld^{s}) is remarkable for many reasons, and raises an important question about basic axonal biology: how can axons survive without trophic support from the soma for weeks? There are many examples in invertebrates where severed axons survive and potentially function for extended periods of time (months after lesion) (Parnas et al., 1991; Stengl, 1995). There are also examples in invertebrates where neuronal nuclei lyse during the final stages of development, leaving behind functional, anucleated neurons (Polilov, 2012). This suggests that axons are not dependent on soma-derived nutrient support, and instead may function metabolically independently of other cellular processes.

At first it may seem advantageous for cells to forgo their cell bodies once post-mitotically differentiated with all connections formed; this could prevent tumors and other detrimental consequences of unregulated mitosis. However, cell body-derived growths or regeneration would also be prevented. This would substantially limit an organism's ability to recover from a neuronal injury, assuming of course that an axon

would be unable to induce regeneration from itself without somal support. It is unknown at this time if severing a Sarm KO axon would destroy the ability for the severed axon or the synapse to remodel; while it may be assumed that somal support is required for synaptic plasticity, there have not been genetic tools to test this until now. If axons can indeed grow without a soma, then this would highlight the importance of axonal biology while diminishing the role of the nucleus and soma in the function of adult neurons.

There is evidence of protein transfer from surrounding glia (Lasek et al., 1977; Sheller et al., 1995; Fruhbeis et al., 2012), even to cut axons (Court et al., 2008), suggesting that glia may be able to adopt the traditional roles of the soma for the axon. While it is likely that glial support is necessary for long-term survival of severed axons, the mechanisms of this trophic support remain unknown. Glia are known to be involved in phagocytosis of axonal debris (MacDonald et al., 2006; Doherty et al., 2009; Ziegenfuss et al., 2012), but this process is not believed to be an initiating factor in axon degeneration. However, a lack of glial support can negatively affect axons (Yamanaka et al., 2008; Ilieva et al., 2009). Further studies are needed to understand the molecular mechanisms regarding glial support of severed axons. It is likely that many nutrient transporters are needed to pass metabolites from glia to neurons, but the nature of this transfer, if it does indeed occur, remains a mystery. This relationship may explain why cut axons undergo Wallerian degeneration so much more quickly *in vitro* versus *in vivo* after axotomy (approximately 6-8 hours compared to 24-28 hours). Glia may also contribute to the functional preservation of severed Sarm and Wld^{s} axons by providing

factors that assist in maintaining the resting membrane potential despite the lack of input signals after injury.

Functional preservation of severed axons

A major area of interest surrounding protected severed axons is their preservation of functionality: could they fire an action potential if stimulated, even though there are no longer any "fire" signals from the rest of the neuron? Wld^s motor neurons show some conservation of axonal function after injury, as their synapses are able to contract connecting muscle even a week after axotomy, albeit at a greatly diminished force compared to uncut nerves (Moldovan et al., 2009). Severed Wld^s axons even maintain bidirectional axon transport at speeds similar to uncut axons at least 8 days after lesion (Smith and Bisby, 1993). While we have not assayed organelle transport in severed Sarm KO axons, they do exhibit similar declines in CMAP levels after injury as Wld^s mice (work not presented here). Together, this suggests that either (1) synapses need somal-derived support factors for maintenance, so axotomy removes the source of trophic support causing synaptic degeneration; (2) regular firing of action potentials are required for maintenance of synaptic architecture; or (3) synapses possess their own self-destruction program that is independent of Sarm or Wld^s. In support of the later model, preliminary experiments with double *Bax KO-Sarm1 KO* mice, which are deficient in axon death and most cell death mechanisms, show substantial increases in CMAP protection: 40-80% of uninjured CMAP levels remain 6 days after injury, compared with 5% in Sarm1 KO alone or Wld^s mice. This suggests that synapses, like axons, have their own self-destruct program that may be triggered by similar initiating events. More work

is needed on synapse preservation in the absence of axon degeneration to understand this phenomenon further.

Why undergo Wallerian degeneration?

The relationship been axons and surrounding glia may hint at the importance of Wallerian degeneration in biology: the injured limb needs to be able to recover as much as functionally possible. Wallerian degeneration allows for a sick nerve to be removed while activating the surrounding tissue to do so. This allows for theoretically unhindered regrowth of a new axon from the soma in the PNS. There have been many reported cases where presence of the severed axon obstructs axonal regrowth. While the initiation of axon sprouting is unhindered in Wld^s nerves (Lunn et al., 1989), axon regeneration is functionally highly impaired (Brown et al., 1992; Martin et al., 2010), as it appears the fragments act as a physical blockade to the regenerating axon. Similarly during development, new axon growth is not impeded by a severed axon; however, the new axon recognizes the severed axon as a "sibling" outgrowth and overlaps (Wang and Macagno, 1998), which potentially alters functionality of that neuron. Thus, it appears that severed axons fragment to allow a passageway for the regenerating axon to reinnervate the same targets. However, some studies have found that regenerating axons can actually fuse with the severed axon, resulting in a functional re-connection in the leech (Deriemer et al., 1983), *Aplysia californica* (Bedi and Glanzman, 2001), and *C. elegans* (Ghosh-Roy and Chisholm, 2010; Neumann et al., 2011). Unfortunately, this capability may not be conserved in vertebrates: Wld^s mice show minimal functional fusions in motor neurons, and a highly reduced number in sensory neurons (Brown et al.,

1992). However, with a better understanding of cellular regeneration mechanisms and developmental tiling, it may be possible to block Sarm (and axon degeneration) after injury, followed by regeneration induction and fusion of the new axon to the old. This would greatly speed up functional recovery after PNS injuries and may even be a way to recover from CNS insults, such as strokes and traumatic brain injuries. Sarm seems poised as a key initial player in uncovering this area of therapeutic importance.

The biological importance of Wallerian degeneration may also be embodied within this first key gene: a supposed "innate immunity" molecule is required for axons to degeneration after physical lesion. It is possible that Wallerian degeneration exists to aid in the removal of cellular debris, as this could potentially become a target of autoimmune activity. Or, perhaps mammalian nerves have evolved to degenerate to remove any sort of "corrupt" or toxic component, and the neuron would rather start over than try to repair.

This self-destruction process may have initially evolved for more chronic insults, as axons self-destruct in order to conserve resources for the animal (Raff et al., 2002) while preventing any sort of pathogenic spread. The broad range of animal neurodegeneration models that are delayed by Wld^{s} expression supports this hypothesis (Coleman, 2011), as do recent studies on Sarm's role in immunology. Sarm1 KO mice show increases in viral load and mortality after WNV infection (Szretter et al., 2009), suggesting that axons may initiate their own self-destruction to prevent further viral spreading. Perhaps this mechanism of auto-activation during infection will shed light on how Sarm is activated after physical lesion, as they might overlap significantly. It

appears that axons either (1) have a common self-destruct pathway that is activated by multiple stress signaling pathways, including lesion, or (2) a common overreaching "axon-health" signal that can be diminished by multiple insults. Further work on Sarm-null animals and suppression of axon degeneration via multiple mechanisms, including injury, neurodegenerative disease models, and infection, is needed to elucidate this further.

Axon death in disease

Work presented here (Chapter 4) suggests that Sarm-regulated axon death pathways are activated during neuronal death in a mouse model of ALS. While blocking axon death does not substantially prolong lifespan (Figure 4-2), Sarm1 presents itself as a candidate therapeutic to potentially delay symptoms in this disease. Cell body protection is likely also required to attenuate motor neuron loss in this disease, so a combinatorial approach of blocking axon death by inhibiting Sarm while also blocking cell body death (potentially with Bax or Bak inhibitors) may synergistically improve neuronal function and survival. Further studies on other neurodegenerative disease models in the Sarm-null background are needed to determine if axon death pathways are activated in other diseases.

It is also unknown if Sarm-dependent axon death is activated by the same mechanisms that promote somal death, or if axon death is at the beginning of a whole cell destruction cascade. Is there a universal "stress signal" or response within a neuron that activates multiple death programs, or is there an overall threshold that sums various types of cellular stress that eventually signals death programs once a threshold is

reached? The model presented here suggests that low cellular energy levels coupled with influxes in calcium may contribute to disease progression. Detailed bioenergetics and calcium level studies have not been done in ALS mice, although they present challenges experimentally for studying such transient changes over the long course of disease progression. It remains possible that cellular stresses related to the diseased state triggers energetic fluxes that in conjunction with an inability to maintain proper membrane potential can slowly activate Sarm and other destructive mechanisms. More work is needed on pre-disease states in mutant neurons to determine what axonal changes could potentially trigger Sarm-mediated destruction. It is unknown if a few "regulatory" triggers, such as energy or calcium levels, control degeneration directly, or instead are part of many factors that contribute to an overall cellular stress state.

Despite the wealth of research being done on neurodegenerative disease, it is still unknown what ultimately fates certain adult neuron populations to die during these diseases. While the ultimate result (i.e., cell death) is the same, the commonality among disease pathways remains unclear, as many animal models of multiple diseases show similar intracellular irregularities and disruptions (Gilley et al., 2011). For example, ALS models have increases in oxidative stress, ER stress, and mitochondrial disruption (Rothstein, 2009), but it is unclear if any of these catalyze cell death or are merely the result of a sick motor neuron trying to stay alive under the dire circumstances at hand. Regardless, the ability to genetically "save" a part of a neuron from death and destruction is exciting and may be the tipping point for discovering ways to save a sick neuron from dying.

Sarm modification presents a potential therapeutic target for diseases of axon loss, particularly CIPNs (Lee and Swain, 2006). Assaying conditional loss of Sarm in adult neurons is an important next step, as it is important to determine if loss of Sarm1 specifically in adult neurons will fundamentally change them, either morphologically or electrophysiologically. However, Sarm1 KO mice are viable, and there are no known behavioral defects in these animals, making Sarm inhibition an even more promising target. In addition, blocking Sarm function in adult hood would eliminate any potential developmental issues, as suggested by the *in vitro* hippocampal work (Chen et al., 2011). Axon death components may be important for plasticity, or even the brain's response to infection so care must be used. This potential conflict highlights the importance of ultimately uncovering more elements that initiate cellular destruction.

Because loss of Sarm potently protects axons from Wallerian degeneration, it would be interesting to look at Sarm mutations in human genomic studies to determine if mutations altering Sarm function can decrease susceptibility for or the severity of neurological diseases. For example, there is a well-studied ALS-linked SOD mutation, D90A, which causes a slowly progressing form of ALS and is recessive in Scandinavian populations; however, this same mutation is dominant and causes classical ALS pathogenesis is non-Scandinavians (Pasinelli and Brown, 2006). It is possible that a resistant population may have mutations in Sarm that hinder its function; or, groups that are more susceptible to degeneration may have defects in Sarm regulation that produces higher levels of Sarm expression. Further bioinformatics studies are needed to determine what link, if any, exists between Sarm mutations and disease susceptibility.

It is unlikely that a single mutation will greatly increase susceptibility to a disease; it is more likely that Sarm will be one of many genes that together either weaken a neuron or strengthen its cellular defenses against the onslaught of aging. It remains important therefore to assemble large amounts of genetic data, including epigenetic regulators, on a diverse group of both healthy and diseased populations. As with work on regenerative and axon-death therapies, Sarm's importance may ultimately be its position as the initial gene to catalyze discovery of many factors that function in balancing axonal health with death.

Conclusions

Work presented here provides the first direct evidence that a conserved axon death program exists and is activated in some forms of neurodegenerative disease. We have shown strong evidence that Wallerian degeneration is indeed a unique genetically encoded destructive program, joining developmental pruning and apoptosis as ways in which neurons can execute the demise of their processes. Sarm provides an exciting target for not only understanding axon death under a variety of circumstances, but also for potentially treating neuropathies. While many questions regarding axon death and Sarm function remain unanswered, it is clear that axons have a unique biology separate from the neuron as a whole, and this unique biology has a large importance in the understanding and treatment of neurological disease.

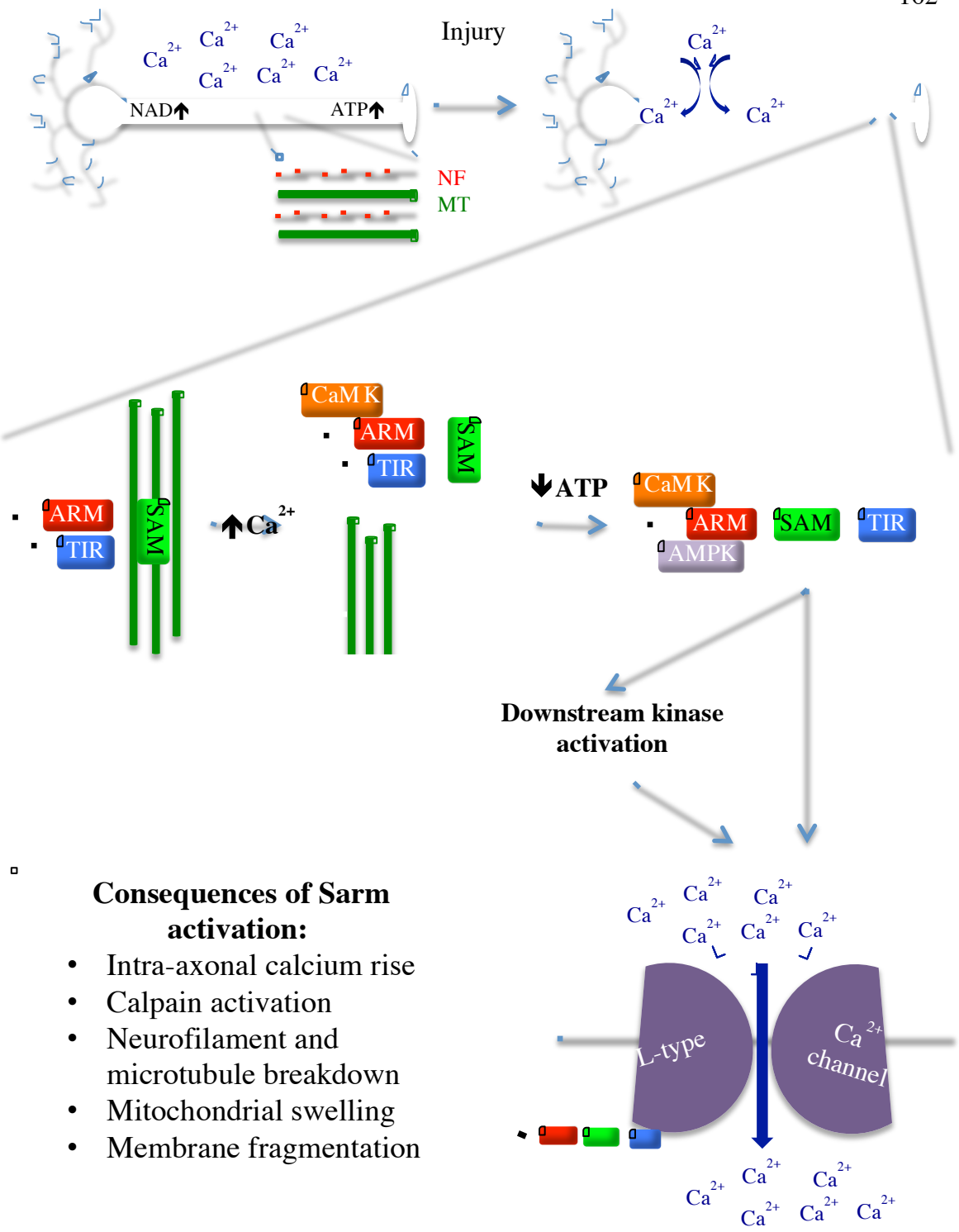


Figure 5-1. Proposed mechanism of Sarm-mediated Wallerian degeneration

Figure 5-1. Proposed mechanism of Sarm-mediated Wallerian degeneration
Calcium enters the severed axon during AAD and activates a calcium-sensitive kinase, represented here as CaMK. CaMK binds to the auto-inhibitory ARM domain, induces a conformation change within the SAM domain that releases Sarm from microtubules. This release de-stabilizes microtubules. ATP levels in the severed axon fall, which activates AMPK to bind to the ARM domain. This dual-activation induces activated Sarm to either directly associate with L-type calcium channels or signals through the TIR domain to induce kinase activity; the result of either of these steps is that L-type calcium channels gradually open to increase axonal levels of calcium. Once a threshold is reached, high intra-axonal calcium induces a variety of changes associated with Wallerian degeneration: calpain activation; cytoskeletal breakdown; mitochondrial swelling and rupture and eventual membrane fragmentation.

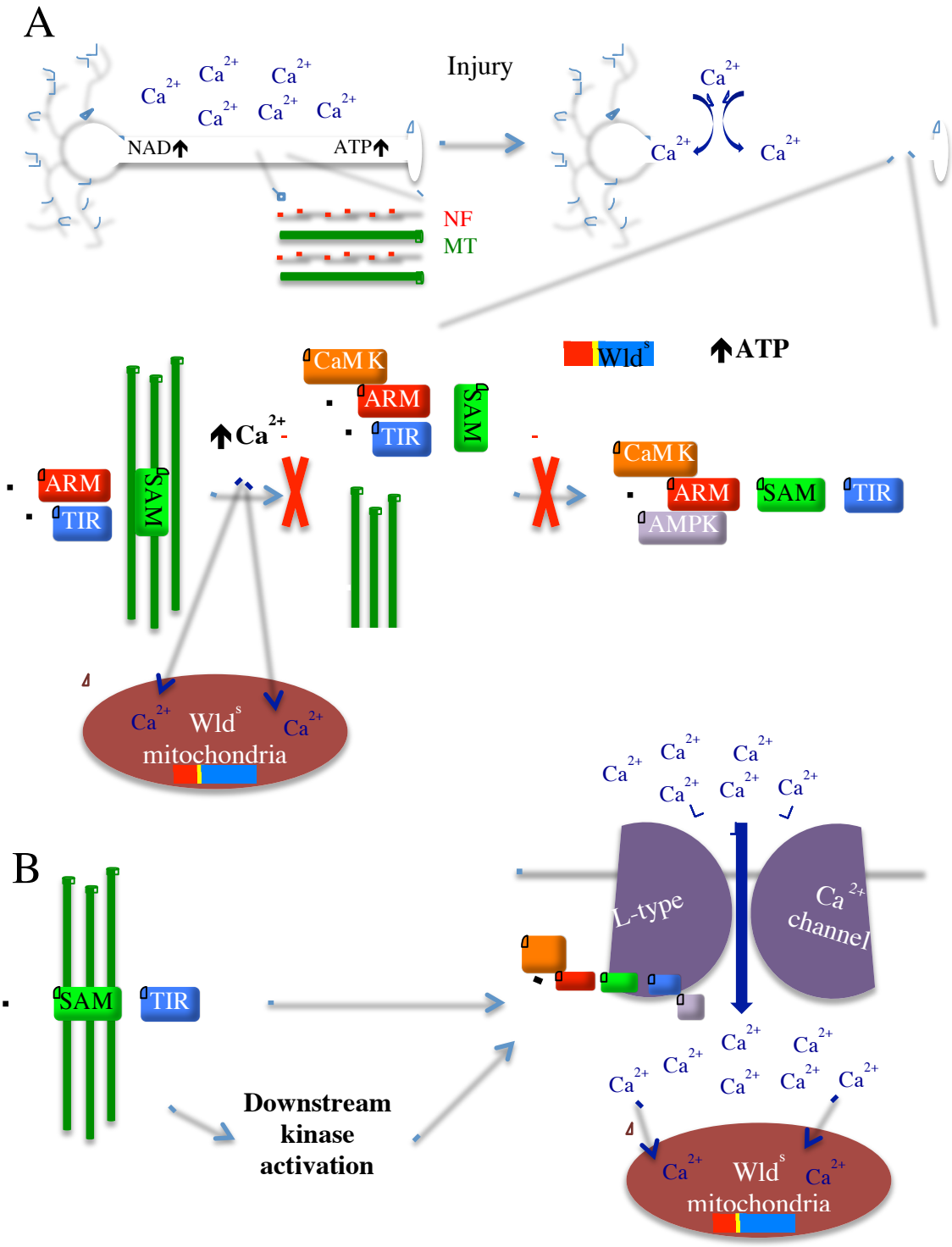


Figure 5-2. Revised mechanism for Wld^s-mediated protection

Figure 5-2. Revised mechanism for Wld^s-mediated protection

A. Calcium enters the severed axon during AAD; instead of activating a calcium-dependent kinase (CaMK), the calcium is taken up by the mitochondira containing Wld^s. This prevents CaMK from binding the ARM domain of Sarm. Wld^s also prevents AMPK activation by keeping axonal ATP levels high through the enzymatic activity of Nmnat. Without this dual-activating signaling complex, Sarm remains bound to microtubules and is unable to associate with L-type calcium channels. This prevents the slow intra-axonal calcium rise.

B. dSarmΔARM-induces degeneration independent of upstream calcium and energy homeostasis sensors. This molecule, either directly of through association with downstream kinases, opens L-type calcium channels and causes rises in calcium levels through the entire neuron. This increase would normally cause mitochondrial rupture and calpain activation; however, Wld^s-expressing mitochondria buffer any rises in calcium, which prevents mitochondrial rupture and calpain activation.

References

Adalbert, R., Gillingwater, T. H., Haley, J. E., Bridge, K., Beirowski, B., Berek, L., Wagner, D., Grumme, D., Thomson, D., Celik, A., Addicks, K., Ribchester, R. R., and Coleman, M. P. (2005). A rat model of slow Wallerian degeneration (WldS) with improved preservation of neuromuscular synapses. Eur J Neurosci *21*, 271-277.

Adalbert, R., Morreale, G., Paizs, M., Conforti, L., Walker, S. A., Roderick, H. L., Bootman, M. D., Siklos, L., and Coleman, M. P. (2012). Intra-axonal calcium changes after axotomy in wild-type and slow Wallerian degeneration axons. Neuroscience *225*, 44-54.

Akhouayri, I., Turc, C., Royet, J., and Charroux, B. (2011). Toll-8/Tollo negatively regulates antimicrobial response in the Drosophila respiratory epithelium. PLoS Pathog *7*, e1002319.

Amato, S., and Man, H. Y. (2011). Bioenergy sensing in the brain: the role of AMP-activated protein kinase in neuronal metabolism, development and neurological diseases. Cell Cycle *10*, 3452-3460.

Araki, T., Sasaki, Y., and Milbrandt, J. (2004). Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. Science *305*, 1010-1013.

Ataman, B., Ashley, J., Gorczyca, M., Ramachandran, P., Fouquet, W., Sigrist, S. J., and Budnik, V. (2008). Rapid activity-dependent modifications in synaptic structure and function require bidirectional Wnt signaling. Neuron *57*, 705-718.

Avery, M. A., Rooney, T. M., Pandya, J. D., Wishart, T. M., Gillingwater, T. H., Geddes, J. W., Sullivan, P. G., and Freeman, M. R. (2012). WldS prevents axon degeneration through increased mitochondrial flux and enhanced mitochondrial Ca2+ buffering. Curr Biol *22*, 596-600.

Avery, M. A., Sheehan, A. E., Kerr, K. S., Wang, J., and Freeman, M. R. (2009). Wld S requires Nmnat1 enzymatic activity and N16-VCP interactions to suppress Wallerian degeneration. J Cell Biol *184*, 501-513.

Babetto, E., Beirowski, B., Janeckova, L., Brown, R., Gilley, J., Thomson, D., Ribchester, R. R., and Coleman, M. P. (2010). Targeting NMNAT1 to axons and synapses transforms its neuroprotective potency in vivo. J Neurosci *30*, 13291-13304.

Barrientos, S. A., Martinez, N. W., Yoo, S., Jara, J. S., Zamorano, S., Hetz, C., Twiss, J. L., Alvarez, J., and Court, F. A. (2011). Axonal degeneration is mediated by the mitochondrial permeability transition pore. J Neurosci *31*, 966-978.

Baumann, F., Henderson, R. D., Ridall, P. G., Pettitt, A. N., and McCombe, P. A. (2012). Use of Bayesian MUNE to show differing rate of loss of motor units in subgroups of ALS. Clin Neurophysiol *123*, 2446-2453.

Bedi, S. S., and Glanzman, D. L. (2001). Axonal rejoining inhibits injury-induced long-term changes in Aplysia sensory neurons in vitro. J Neurosci *21*, 9667-9677.

Beirowski, B., Adalbert, R., Wagner, D., Grumme, D. S., Addicks, K., Ribchester, R. R., and Coleman, M. P. (2005). The progressive nature of Wallerian degeneration in wild-type and slow Wallerian degeneration (WldS) nerves. BMC Neurosci *6*, 6.

Beirowski, B., Babetto, E., Gilley, J., Mazzola, F., Conforti, L., Janeckova, L., Magni, G., Ribchester, R. R., and Coleman, M. P. (2009). Non-nuclear Wld(S) determines its neuroprotective efficacy for axons and synapses in vivo. J Neurosci *29*, 653-668.

Beirowski, B., Berek, L., Adalbert, R., Wagner, D., Grumme, D. S., Addicks, K., Ribchester, R. R., and Coleman, M. P. (2004). Quantitative and qualitative analysis of Wallerian degeneration using restricted axonal labelling in YFP-H mice. J Neurosci Methods *134*, 23-35.

Beirowski, B., Morreale, G., Conforti, L., Mazzola, F., Di Stefano, M., Wilbrey, A., Babetto, E., Janeckova, L., Magni, G., and Coleman, M. P. (2010). WldS can delay Wallerian degeneration in mice when interaction with valosin-containing protein is weakened. Neuroscience *166*, 201-211.

Belinda, L. W., Wei, W. X., Hanh, B. T., Lei, L. X., Bow, H., and Ling, D. J. (2008). SARM: a novel Toll-like receptor adaptor, is functionally conserved from arthropod to human. Mol Immunol *45*, 1732-1742.

Berger, F., Lau, C., Dahlmann, M., and Ziegler, M. (2005). Subcellular compartmentation and differential catalytic properties of the three human nicotinamide mononucleotide adenylyltransferase isoforms. J Biol Chem *280*, 36334-36341.

Bernier, B., Castejon, S., Culver, D. G., and Glass, J. D. (1999). Axonal neurofilaments are resistant to calpain-mediated degradation in the WLD(S) mouse. Neuroreport *10*, 1423-1426.

Blum, E. S., Abraham, M. C., Yoshimura, S., Lu, Y., and Shaham, S. (2012). Control of nonapoptotic developmental cell death in Caenorhabditis elegans by a polyglutamine-repeat protein. Science *335*, 970-973.

Bosco, D. A., Morfini, G., Karabacak, N. M., Song, Y., Gros-Louis, F., Pasinelli, P., Goolsby, H., Fontaine, B. A., Lemay, N., McKenna-Yasek, D., Frosch, M. P., Agar, J.

N., Julien, J. P., Brady, S. T., and Brown, R. H. J. (2010). Wild-type and mutant SOD1 share an aberrant conformation and a common pathogenic pathway in ALS. Nat Neurosci *13*, 1396-1403.

Brand, A. H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.
Brikos, C., and O'Neill, L. A. (2008). Signalling of toll-like receptors. Handb Exp Pharmacol 21-50.

Broadus, J., Fuerstenberg, S., and Doe, C. Q. (1998). Staufen-dependent localization of prospero mRNA contributes to neuroblast daughter-cell fate. Nature *391*, 792-795.

Broadus, J., Skeath, J. B., Spana, E. P., Bossing, T., Technau, G., and Doe, C. Q. (1995). New neuroblast markers and the origin of the aCC/pCC neurons in the Drosophila central nervous system. Mech Dev *53*, 393-402.

Brown, M. C., Lunn, E. R., and Perry, V. H. (1992). Consequences of slow Wallerian degeneration for regenerating motor and sensory axons. J Neurobiol *23*, 521-536.

Buckmaster, E. A., Perry, V. H., and Brown, M. C. (1995). The rate of Wallerian degeneration in cultured neurons from wild type and C57BL/WldS mice depends on time in culture and may be extended in the presence of elevated K+ levels. Eur J Neurosci *7*, 1596-1602.

Bull, N. D., Chidlow, G., Wood, J. P., Martin, K. R., and Casson, R. J. (2012). The mechanism of axonal degeneration after perikaryal excitotoxic injury to the retina. Exp Neurol *236*, 34-45.

Buss, R. R., Sun, W., and Oppenheim, R. W. (2006). Adaptive roles of programmed cell death during nervous system development. Annu Rev Neurosci *29*, 1-35.

Cahoy, J. D., Emery, B., Kaushal, A., Foo, L. C., Zamanian, J. L., Christopherson, K. S., Xing, Y., Lubischer, J. L., Krieg, P. A., Krupenko, S. A., Thompson, W. J., and Barres, B. A. (2008). A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J Neurosci *28*, 264-278.

Caraganis, A., Benn, S., Cudkowicz, M., and Brown, R. H. J. (2008). Thrombopoietin is ineffective in a mouse model of motor neuron disease. Amyotroph Lateral Scler *9*, 354-358.

Carty, M., Goodbody, R., Schroder, M., Stack, J., Moynagh, P. N., and Bowie, A. G. (2006). The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. Nat Immunol *7*, 1074-1081.

Cavanagh, J. B. (1964). The significance of the "dying back" process in experimental and human neurological disease. Int Rev Exp Pathol *3*, 219-267.

Chang, C., Hsieh, Y. W., Lesch, B. J., Bargmann, C. I., and Chuang, C. F. (2011). Microtubule-based localization of a synaptic calcium-signaling complex is required for left-right neuronal asymmetry in C. elegans. Development *138*, 3509-3518.

Chen, C. Y., Lin, C. W., Chang, C. Y., Jiang, S. T., and Hsueh, Y. P. (2011). Sarm1, a negative regulator of innate immunity, interacts with syndecan-2 and regulates neuronal morphology. J Cell Biol *193*, 769-784.

Chuang, C. F., and Bargmann, C. I. (2005). A Toll-interleukin 1 repeat protein at the synapse specifies asymmetric odorant receptor expression via ASK1 MAPKKK signaling. Genes Dev *19*, 270-281.

Cleveland, D. W., and Rothstein, J. D. (2001). From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. Nat Rev Neurosci *2*, 806-819.

Clyne, P. J., Warr, C. G., Freeman, M. R., Lessing, D., Kim, J., and Carlson, J. R. (1999). A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in Drosophila. Neuron *22*, 327-338.

Coleman, M. (2005). Axon degeneration mechanisms: commonality amid diversity. Nat Rev Neurosci *6*, 889-898.

Coleman, M. (2011). Molecular signaling how do axons die? Adv Genet *73*, 185-217.
Coleman, M. P., Conforti, L., Buckmaster, E. A., Tarlton, A., Ewing, R. M., Brown, M. C., Lyon, M. F., and Perry, V. H. (1998). An 85-kb tandem triplication in the slow Wallerian degeneration (Wlds) mouse. Proc Natl Acad Sci U S A *95*, 9985-9990.

Coleman, M. P., and Freeman, M. R. (2010). Wallerian degeneration, wld(s), and nmnat. Annu Rev Neurosci *33*, 245-267.

Coleman, M. P., and Perry, V. H. (2002). Axon pathology in neurological disease: a neglected therapeutic target. Trends Neurosci *25*, 532-537.

Conforti, L., Fang, G., Beirowski, B., Wang, M. S., Sorci, L., Asress, S., Adalbert, R., Silva, A., Bridge, K., Huang, X. P., Magni, G., Glass, J. D., and Coleman, M. P. (2007). NAD(+) and axon degeneration revisited: Nmnat1 cannot substitute for Wld(S) to delay Wallerian degeneration. Cell Death Differ *14*, 116-127.

Conforti, L., Tarlton, A., Mack, T. G., Mi, W., Buckmaster, E. A., Wagner, D., Perry, V. H., and Coleman, M. P. (2000). A Ufd2/D4Cole1e chimeric protein and overexpression

of Rbp7 in the slow Wallerian degeneration (WldS) mouse. Proc Natl Acad Sci U S A *97*, 11377-11382.

Conforti, L., Wilbrey, A., Morreale, G., Janeckova, L., Beirowski, B., Adalbert, R., Mazzola, F., Di Stefano, M., Hartley, R., Babetto, E., Smith, T., Gilley, J., Billington, R. A., Genazzani, A. A., Ribchester, R. R., Magni, G., and Coleman, M. (2009). Wld S protein requires Nmnat activity and a short N-terminal sequence to protect axons in mice. J Cell Biol *184*, 491-500.

Couillault, C., Pujol, N., Reboul, J., Sabatier, L., Guichou, J. F., Kohara, Y., and Ewbank, J. J. (2004). TLR-independent control of innate immunity in Caenorhabditis elegans by the TIR domain adaptor protein TIR-1, an ortholog of human SARM. Nat Immunol *5*, 488-494.

Court, F. A., Hendriks, W. T., MacGillavry, H. D., Alvarez, J., and van Minnen, J. (2008). Schwann cell to axon transfer of ribosomes: toward a novel understanding of the role of glia in the nervous system. J Neurosci *28*, 11024-11029.

Couto, A., Alenius, M., and Dickson, B. J. (2005). Molecular, anatomical, and functional organization of the Drosophila olfactory system. Curr Biol *15*, 1535-1547.
Dalod, M. (2007). Studies of SARM1 uncover similarities between immune and neuronal responses to danger. Sci STKE *2007*, pe73.

De Vos, K. J., Grierson, A. J., Ackerley, S., and Miller, C. C. (2008). Role of axonal transport in neurodegenerative diseases. Annu Rev Neurosci *31*, 151-173.

Deckwerth, T. L., and Johnson, E. M. J. (1994). Neurites can remain viable after destruction of the neuronal soma by programmed cell death (apoptosis). Dev Biol *165*, 63-72.

Deriemer, S. A., Elliott, E. J., Macagno, E. R., and Muller, K. J. (1983). Morphological evidence that regenerating axons can fuse with severed axon segments. Brain Res *272*, 157-161.

Ding, W. X., and Nam Ong, C. (2003). Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. FEMS Microbiol Lett *220*, 1-7.

Dobritsa, A. A., van der Goes van Naters, W., Warr, C. G., Steinbrecht, R. A., and Carlson, J. R. (2003). Integrating the molecular and cellular basis of odor coding in the Drosophila antenna. Neuron *37*, 827-841.

Doherty, J., Logan, M. A., Tasdemir, O. E., and Freeman, M. R. (2009). Ensheathing glia function as phagocytes in the adult Drosophila brain. J Neurosci *29*, 4768-4781.
Eddleman, C. S., Ballinger, M. L., Smyers, M. E., Fishman, H. M., and Bittner, G. D. (1998). Endocytotic formation of vesicles and other membranous structures induced by Ca2+ and axolemmal injury. J Neurosci *18*, 4029-4041.

Fang, C., Bernardes-Silva, M., Coleman, M. P., and Perry, V. H. (2005). The cellular distribution of the Wld s chimeric protein and its constituent proteins in the CNS. Neuroscience *135*, 1107-1118.

Fekonja, O., Avbelj, M., and Jerala, R. (2012). Suppression of TLR signaling by targeting TIR domain-containing proteins. Curr Protein Pept Sci *13*, 776-788.

Feng, Y., Yan, T., Zheng, J., Ge, X., Mu, Y., Zhang, Y., Wu, D., Du, J. L., and Zhai, Q. (2010). Overexpression of Wld(S) or Nmnat2 in mauthner cells by single-cell electroporation delays axon degeneration in live zebrafish. J Neurosci Res *88*, 3319-3327.

Ferri, A., Sanes, J. R., Coleman, M. P., Cunningham, J. M., and Kato, A. C. (2003). Inhibiting axon degeneration and synapse loss attenuates apoptosis and disease progression in a mouse model of motoneuron disease. Curr Biol *13*, 669-673.

Finn, J. T., Weil, M., Archer, F., Siman, R., Srinivasan, A., and Raff, M. C. (2000). Evidence that Wallerian degeneration and localized axon degeneration induced by local neurotrophin deprivation do not involve caspases. J Neurosci *20*, 1333-1341.

Fischer, J. A., Giniger, E., Maniatis, T., and Ptashne, M. (1988). GAL4 activates transcription in Drosophila. Nature *332*, 853-856.

Fischer, L. R., Culver, D. G., Davis, A. A., Tennant, P., Wang, M., Coleman, M., Asress, S., Adalbert, R., Alexander, G. M., and Glass, J. D. (2005). The WldS gene modestly prolongs survival in the SOD1G93A fALS mouse. Neurobiol Dis *19*, 293-300.

Forsberg, K., Jonsson, P. A., Andersen, P. M., Bergemalm, D., Graffmo, K. S., Hultdin, M., Jacobsson, J., Rosquist, R., Marklund, S. L., and Brannstrom, T. (2010). Novel antibodies reveal inclusions containing non-native SOD1 in sporadic ALS patients. PLoS One *5*, e11552.

Fruhbeis, C., Frohlich, D., and Kramer-Albers, E. M. (2012). Emerging roles of exosomes in neuron-glia communication. Front Physiol *3*, 119.

George, E. B., Glass, J. D., and Griffin, J. W. (1995). Axotomy-induced axonal degeneration is mediated by calcium influx through ion-specific channels. J Neurosci *15*, 6445-6452.
Gerdts, J., Sasaki, Y., Vohra, B., Marasa, J., and Milbrandt, J. (2011). Image-based screening identifies novel roles for IkappaB kinase and glycogen synthase kinase 3 in axonal degeneration. J Biol Chem *286*, 28011-28018.

Ghosh-Roy, A., and Chisholm, A. D. (2010). Caenorhabditis elegans: a new model organism for studies of axon regeneration. Dev Dyn *239*, 1460-1464.

Gilley, J., Adalbert, R., and Coleman, M. P. (2011). Modelling early responses to neurodegenerative mutations in mice. Biochem Soc Trans *39*, 933-938.

Gilley, J., and Coleman, M. P. (2010). Endogenous Nmnat2 is an essential survival factor for maintenance of healthy axons. PLoS Biol *8*, e1000300.

Gillingwater, T. H., Ingham, C. A., Coleman, M. P., and Ribchester, R. R. (2003). Ultrastructural correlates of synapse withdrawal at axotomized neuromuscular junctions in mutant and transgenic mice expressing the Wld gene. J Anat *203*, 265-276.

Gillingwater, T. H., Ingham, C. A., Parry, K. E., Wright, A. K., Haley, J. E., Wishart, T. M., Arbuthnott, G. W., and Ribchester, R. R. (2006a). Delayed synaptic degeneration in the CNS of Wlds mice after cortical lesion. Brain *129*, 1546-1556.

Gillingwater, T. H., Thomson, D., Mack, T. G., Soffin, E. M., Mattison, R. J., Coleman, M. P., and Ribchester, R. R. (2002). Age-dependent synapse withdrawal at axotomised neuromuscular junctions in Wld(s) mutant and Ube4b/Nmnat transgenic mice. J Physiol *543*, 739-755.

Gillingwater, T. H., Wishart, T. M., Chen, P. E., Haley, J. E., Robertson, K., MacDonald, S. H., Middleton, S., Wawrowski, K., Shipston, M. J., Melmed, S., Wyllie, D. J., Skehel, P. A., Coleman, M. P., and Ribchester, R. R. (2006b). The neuroprotective WldS gene regulates expression of PTTG1 and erythroid differentiation regulator 1-like gene in mice and human cells. Hum Mol Genet *15*, 625-635.

Glass, J. D., Brushart, T. M., George, E. B., and Griffin, J. W. (1993). Prolonged survival of transected nerve fibres in C57BL/Ola mice is an intrinsic characteristic of the axon. J Neurocytol *22*, 311-321.

Glass, J. D., Schryer, B. L., and Griffin, J. W. (1994). Calcium-mediated degeneration of the axonal cytoskeleton in the Ola mouse. J Neurochem *62*, 2472-2475.

Golic, K. G., and Lindquist, S. (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. Cell *59*, 499-509.

Gonzalez, M. A., Van, B., Derek, Hulme, W., Ulloa, R. H., Lebrigio, R. F. A., Osterloh, J., Logan, M., Freeman, M., and Zuchner, S. (2012). Whole Genome Sequencing and a New Bioinformatics Platform Allow for Rapid Gene Identification in D. melanogaster EMS Screens. Biology *1*, 766-777.

Gould, T. W., Buss, R. R., Vinsant, S., Prevette, D., Sun, W., Knudson, C. M., Milligan, C. E., and Oppenheim, R. W. (2006). Complete dissociation of motor neuron death from motor dysfunction by Bax deletion in a mouse model of ALS. J Neurosci *26*, 8774-8786.

Grether, M. E., Abrams, J. M., Agapite, J., White, K., and Steller, H. (1995). The head involution defective gene of Drosophila melanogaster functions in programmed cell death. Genes Dev *9*, 1694-1708.

Grueber, W. B., Ye, B., Yang, C. H., Younger, S., Borden, K., Jan, L. Y., and Jan, Y. N. (2007). Projections of Drosophila multidendritic neurons in the central nervous system: links with peripheral dendrite morphology. Development *134*, 55-64.

Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C., Polchow, C. Y., Alexander, D. D., Caliendo, J., Hentati, A., Kwon, Y. W., Deng, H. X., and et, a. (1994). Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science *264*, 1772-1775.

Guruharsha, K. G., Rual, J. F., Zhai, B., Mintseris, J., Vaidya, P., Vaidya, N., Beekman, C., Wong, C., Rhee, D. Y., Cenaj, O., McKillip, E., Shah, S., Stapleton, M., Wan, K. H., Yu, C., Parsa, B., Carlson, J. W., Chen, X., Kapadia, B., VijayRaghavan, K., Gygi, S. P., Celniker, S. E., Obar, R. A., and Artavanis-Tsakonas, S. (2011). A protein complex network of Drosophila melanogaster. Cell *147*, 690-703.

Hattori, K., Naguro, I., Runchel, C., and Ichijo, H. (2009). The roles of ASK family proteins in stress responses and diseases. Cell Commun Signal *7*, 9.

Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., Frenguelli, B. G., and Hardie, D. G. (2005). Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab *2*, 9-19.

Hayakawa, T., Kato, K., Hayakawa, R., Hisamoto, N., Matsumoto, K., Takeda, K., and Ichijo, H. (2011). Regulation of anoxic death in Caenorhabditis elegans by mammalian apoptosis signal-regulating kinase (ASK) family proteins. Genetics *187*, 785-792.

Hoopfer, E. D., McLaughlin, T., Watts, R. J., Schuldiner, O., O'Leary, D. D., and Luo, L. (2006). Wlds protection distinguishes axon degeneration following injury from naturally occurring developmental pruning. Neuron *50*, 883-895.

Hsieh, Y. W., Chang, C., and Chuang, C. F. (2012). The microRNA mir-71 inhibits calcium signaling by targeting the TIR-1/Sarm1 adaptor protein to control stochastic L/R neuronal asymmetry in C. elegans. PLoS Genet *8*, e1002864.

Hummel, T., Vasconcelos, M. L., Clemens, J. C., Fishilevich, Y., Vosshall, L. B., and Zipursky, S. L. (2003). Axonal targeting of olfactory receptor neurons in Drosophila is controlled by Dscam. Neuron *37*, 221-231.

Ikegami, K., and Koike, T. (2003). Non-apoptotic neurite degeneration in apoptotic neuronal death: pivotal role of mitochondrial function in neurites. Neuroscience *122*, 617-626.

Ilieva, H., Polymenidou, M., and Cleveland, D. W. (2009). Non-cell autonomous toxicity
in neurodegenerative disorders: ALS and beyond. J Cell Biol *187*, 761-772.

Inoue, A., Sawatari, E., Hisamoto, N., Kitazono, T., Teramoto, T., Fujiwara, M., Matsumoto, K., and Ishihara, T. (2013). Forgetting in C. elegans Is Accelerated by Neuronal Communication via the TIR-1/JNK-1 Pathway. Cell Rep *3*, 808-819.

Jenkins, K. A., and Mansell, A. (2010). TIR-containing adaptors in Toll-like receptor signalling. Cytokine *49*, 237-244.

Jia, H., Yan, T., Feng, Y., Zeng, C., Shi, X., and Zhai, Q. (2007). Identification of a critical site in Wld(s): essential for Nmnat enzyme activity and axon-protective function. Neurosci Lett *413*, 46-51.

Johnson, A. C., McNabb, A. R., and Rossiter, R. J. (1950). Chemistry of wallerian degeneration; a review of recent studies. Arch Neurol Psychiatry *64*, 105-121.

Kaneko, M., Park, J. H., Cheng, Y., Hardin, P. E., and Hall, J. C. (2000). Disruption of synaptic transmission or clock-gene-product oscillations in circadian pacemaker cells of Drosophila cause abnormal behavioral rhythms. J Neurobiol *43*, 207-233.

Kaneko, S., Wang, J., Kaneko, M., Yiu, G., Hurrell, J. M., Chitnis, T., Khoury, S. J., and He, Z. (2006). Protecting axonal degeneration by increasing nicotinamide adenine dinucleotide levels in experimental autoimmune encephalomyelitis models. J Neurosci *26*, 9794-9804.

Kanning, K. C., Kaplan, A., and Henderson, C. E. (2010). Motor neuron diversity in development and disease. Annu Rev Neurosci *33*, 409-440.

Kerschensteiner, M., Schwab, M. E., Lichtman, J. W., and Misgeld, T. (2005). In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. Nat Med *11*, 572-577.

Kim, Y., Zhou, P., Qian, L., Chuang, J. Z., Lee, J., Li, C., Iadecola, C., Nathan, C., and Ding, A. (2007). MyD88-5 links mitochondria, microtubules, and JNK3 in neurons and regulates neuronal survival. J Exp Med *204*, 2063-2074.

Klein, T. (2008). Immunolabeling of imaginal discs. Methods Mol Biol *420*, 253-263.
Knoferle, J., Koch, J. C., Ostendorf, T., Michel, U., Planchamp, V., Vutova, P., Tonges, L., Stadelmann, C., Bruck, W., Bahr, M., and Lingor, P. (2010). Mechanisms of acute axonal degeneration in the optic nerve in vivo. Proc Natl Acad Sci U S A *107*, 6064-6069.

Koch, J. C., Knoferle, J., Tonges, L., Ostendorf, T., Bahr, M., and Lingor, P. (2010). Acute axonal degeneration in vivo is attenuated by inhibition of autophagy in a calcium-dependent manner. Autophagy *6*, 658-659.

Kurz, C. L., Shapira, M., Chen, K., Baillie, D. L., and Tan, M. W. (2007). Caenorhabditis elegans pgp-5 is involved in resistance to bacterial infection and heavy metal and its regulation requires TIR-1 and a p38 map kinase cascade. Biochem Biophys Res Commun *363*, 438-443.

Lasek, R. J., Gainer, H., and Barker, J. L. (1977). Cell-to-cell transfer of glial proteins to the squid giant axon. The glia-neuron protein trnasfer hypothesis. J Cell Biol *74*, 501-523.

Laser, H., Conforti, L., Morreale, G., Mack, T. G., Heyer, M., Haley, J. E., Wishart, T. M., Beirowski, B., Walker, S. A., Haase, G., Celik, A., Adalbert, R., Wagner, D., Grumme, D., Ribchester, R. R., Plomann, M., and Coleman, M. P. (2006). The slow Wallerian degeneration protein, WldS, binds directly to VCP/p97 and partially redistributes it within the nucleus. Mol Biol Cell *17*, 1075-1084.

Lau, C., Dolle, C., Gossmann, T. I., Agledal, L., Niere, M., and Ziegler, M. (2010). Isoform-specific targeting and interaction domains in human nicotinamide mononucleotide adenylyltransferases. J Biol Chem *285*, 18868-18876.

Lee, J. J., and Swain, S. M. (2006). Peripheral neuropathy induced by microtubule-stabilizing agents. J Clin Oncol *24*, 1633-1642.

Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of
gene function in neuronal morphogenesis. Neuron *22*, 451-461.

Lee, T., and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. Trends Neurosci *24*, 251-254.
Liberati, N. T., Fitzgerald, K. A., Kim, D. H., Feinbaum, R., Golenbock, D. T., and Ausubel, F. M. (2004). Requirement for a conserved Toll/interleukin-1 resistance domain protein in the Caenorhabditis elegans immune response. Proc Natl Acad Sci U S A *101*, 6593-6598.

Liu, L., Gonzalez, P. K., Barrett, C. F., and Rittenhouse, A. R. (2003). The calcium channel ligand FPL 64176 enhances L-type but inhibits N-type neuronal calcium currents. Neuropharmacology *45*, 281-292.

Lubinska, L. (1977). Early course of Wallerian degeneration in myelinated fibres of the rat phrenic nerve. Brain Res *130*, 47-63.

Lunn, E. R., Perry, V. H., Brown, M. C., Rosen, H., and Gordon, S. (1989). Absence of Wallerian Degeneration does not Hinder Regeneration in Peripheral Nerve. Eur J Neurosci *1*, 27-33.

Luo, L., and O'Leary, D. D. (2005). Axon retraction and degeneration in development and disease. Annu Rev Neurosci *28*, 127-156.

Lyon, M. F., Ogunkolade, B. W., Brown, M. C., Atherton, D. J., and Perry, V. H. (1993). A gene affecting Wallerian nerve degeneration maps distally on mouse chromosome 4. Proc Natl Acad Sci U S A *90*, 9717-9720.

Ma, M., Ferguson, T. A., Schoch, K. M., Li, J., Qian, Y., Shofer, F. S., Saatman, K. E., and Neumar, R. W. (2013). Calpains mediate axonal cytoskeleton disintegration during Wallerian degeneration. Neurobiol Dis

MacDonald, J. M., Beach, M. G., Porpiglia, E., Sheehan, A. E., Watts, R. J., and Freeman, M. R. (2006). The Drosophila cell corpse engulfment receptor Draper mediates glial clearance of severed axons. Neuron *50*, 869-881.

MacInnis, B. L., and Campenot, R. B. (2005). Regulation of Wallerian degeneration and nerve growth factor withdrawal-induced pruning of axons of sympathetic neurons by the proteasome and the MEK/Erk pathway. Mol Cell Neurosci *28*, 430-439.

Mack, T. G., Reiner, M., Beirowski, B., Mi, W., Emanuelli, M., Wagner, D., Thomson, D., Gillingwater, T., Court, F., Conforti, L., Fernando, F. S., Tarlton, A., Andressen, C.,

Addicks, K., Magni, G., Ribchester, R. R., Perry, V. H., and Coleman, M. P. (2001). Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. Nat Neurosci *4*, 1199-1206.

Martin, N., Jaubert, J., Gounon, P., Salido, E., Haase, G., Szatanik, M., and Guenet, J. L. (2002). A missense mutation in Tbce causes progressive motor neuronopathy in mice. Nat Genet *32*, 443-447.

Martin, S. M., O'Brien, G. S., Portera-Cailliau, C., and Sagasti, A. (2010). Wallerian degeneration of zebrafish trigeminal axons in the skin is required for regeneration and developmental pruning. Development *137*, 3985-3994.

Massoll, C., Mando, W., and Chintala, S. (2013). Excitotoxicity Up-regulates SARM1 Protein Expression and Promotes Wallerian-like Degeneration of Retinal Ganglion Cells and their Axons. Invest Ophthalmol Vis Sci

McGoldrick, P., Joyce, P. I., Fisher, E. M., and Greensmith, L. (2013). Rodent models of amyotrophic lateral sclerosis. Biochim Biophys Acta

Medana, I. M., and Esiri, M. M. (2003). Axonal damage: a key predictor of outcome in human CNS diseases. Brain *126*, 515.

Meyer zu Horste, G., Miesbach, T. A., Muller, J. I., Fledrich, R., Stassart, R. M., Kieseier, B. C., Coleman, M. P., and Sereda, M. W. (2011). The Wlds transgene reduces axon loss in a Charcot-Marie-Tooth disease 1A rat model and nicotinamide delays post-traumatic axonal degeneration. Neurobiol Dis *42*, 1-8.

Mi, W., Beirowski, B., Gillingwater, T. H., Adalbert, R., Wagner, D., Grumme, D., Osaka, H., Conforti, L., Arnhold, S., Addicks, K., Wada, K., Ribchester, R. R., and Coleman, M. P. (2005). The slow Wallerian degeneration gene, WldS, inhibits axonal spheroid pathology in gracile axonal dystrophy mice. Brain *128*, 405-416.

Miguel-Aliaga, I., Thor, S., and Gould, A. P. (2008). Postmitotic specification of Drosophila insulinergic neurons from pioneer neurons. PLoS Biol *6*, e58.

Miller, B. R., Press, C., Daniels, R. W., Sasaki, Y., Milbrandt, J., and DiAntonio, A. (2009). A dual leucine kinase-dependent axon self-destruction program promotes Wallerian degeneration. Nat Neurosci *12*, 387-389.

Mink, M., Fogelgren, B., Olszewski, K., Maroy, P., and Csiszar, K. (2001). A novel human gene (SARM) at chromosome 17q11 encodes a protein with a SAM motif and structural similarity to Armadillo/beta-catenin that is conserved in mouse, Drosophila, and Caenorhabditis elegans. Genomics *74*, 234-244.

Moldovan, M., Alvarez, S., and Krarup, C. (2009). Motor axon excitability during Wallerian degeneration. Brain *132*, 511-523.

Mukherjee, P., Woods, T. A., Moore, R. A., and Peterson, K. E. (2013). Activation of the Innate Signaling Molecule MAVS by Bunyavirus Infection Upregulates the Adaptor Protein SARM1, Leading to Neuronal Death. Immunity

Nave, K. A., and Trapp, B. D. (2008). Axon-glial signaling and the glial support of axon function. Annu Rev Neurosci *31*, 535-561.

Neumann, B., Nguyen, K. C., Hall, D. H., Ben-Yakar, A., and Hilliard, M. A. (2011). Axonal regeneration proceeds through specific axonal fusion in transected C. elegans neurons. Dev Dyn *240*, 1365-1372.

Newsome, T. P., Asling, B., and Dickson, B. J. (2000). Analysis of Drosophila photoreceptor axon guidance in eye-specific mosaics. Development *127*, 851-860.

Ng, C. H., Guan, M. S., Koh, C., Ouyang, X., Yu, F., Tan, E. K., O'Neill, S. P., Zhang, X., Chung, J., and Lim, K. L. (2012). AMP kinase activation mitigates dopaminergic dysfunction and mitochondrial abnormalities in Drosophila models of Parkinson's disease. J Neurosci *32*, 14311-14317.

Nikolaev, A., McLaughlin, T., O'Leary, D. D., and Tessier-Lavigne, M. (2009). APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. Nature *457*, 981-989.

O'Neill, L. A. (2006). DisSARMing Toll-like receptor signaling. Nat Immunol *7*, 1023-1025.

O'Neill, L. A., Fitzgerald, K. A., and Bowie, A. G. (2003). The Toll-IL-1 receptor adaptor family grows to five members. Trends Immunol *24*, 286-290.

Osterloh, J. M., Yang, J., Rooney, T. M., Fox, A. N., Adalbert, R., Powell, E. H., Sheehan, A. E., Avery, M. A., Hackett, R., Logan, M. A., MacDonald, J. M., Ziegenfuss, J. S., Milde, S., Hou, Y. J., Nathan, C., Ding, A., Brown, R. H. J., Conforti, L., Coleman, M., Tessier-Lavigne, M., Zuchner, S., and Freeman, M. R. (2012). dSarm/Sarm1 is required for activation of an injury-induced axon death pathway. Science *337*, 481-484.

Panneerselvam, P., Singh, L. P., Ho, B., Chen, J., and Ding, J. L. (2012). Targeting of pro-apoptotic TLR adaptor SARM to mitochondria: definition of the critical region and residues in the signal sequence. Biochem J *442*, 263-271.

Panneerselvam, P., Singh, L. P., Selvarajan, V., Chng, W. J., Ng, S. B., Tan, N. S., Ho, B., Chen, J., and Ding, J. L. (2013). T-cell death following immune activation is mediated by mitochondria-localized SARM. Cell Death Differ *20*, 478-489.
Park, J. Y., Jang, S. Y., Shin, Y. K., Koh, H., Suh, D. J., Shinji, T., Araki, T., and Park, H. T. (2013). Mitochondrial swelling and microtubule depolymerization are associated with energy depletion in axon degeneration. Neuroscience

Parnas, I., Dudel, J., and Atwood, H. L. (1991). Synaptic transmission in decentralized axons of rock lobster. J Neurosci *11*, 1309-1315.

Pasinelli, P., and Brown, R. H. (2006). Molecular biology of amyotrophic lateral sclerosis: insights from genetics. Nat Rev Neurosci *7*, 710-723.

Peng, J., Yuan, Q., Lin, B., Panneerselvam, P., Wang, X., Luan, X. L., Lim, S. K., Leung, B. P., Ho, B., and Ding, J. L. (2010). SARM inhibits both TRIF- and MyD88-mediated AP-1 activation. Eur J Immunol *40*, 1738-1747.

Perry, V. H., Brown, M. C., Lunn, E. R., Tree, P., and Gordon, S. (1990a). Evidence that Very Slow Wallerian Degeneration in C57BL/Ola Mice is an Intrinsic Property of the Peripheral Nerve. Eur J Neurosci *2*, 802-808.

Perry, V. H., Lunn, E. R., Brown, M. C., Cahusac, S., and Gordon, S. (1990b). Evidence that the Rate of Wallerian Degeneration is Controlled by a Single Autosomal Dominant Gene. Eur J Neurosci *2*, 408-413.

Polilov, A. A. (2012). The smallest insects evolve anucleate neurons. Arthropod Struct Dev *41*, 29-34.

Press, C., and Milbrandt, J. (2008). Nmnat delays axonal degeneration caused by mitochondrial and oxidative stress. J Neurosci *28*, 4861-4871.

Press, C., and Milbrandt, J. (2009). The purine nucleosides adenosine and guanosine delay axonal degeneration in vitro. J Neurochem *109*, 595-602.

Pudla, M., Limposuwan, K., and Utaisincharoen, P. (2011). Burkholderia pseudomallei-induced expression of a negative regulator, sterile-alpha and Armadillo motif-containing protein, in mouse macrophages: a possible mechanism for suppression of the MyD88-independent pathway. Infect Immun *79*, 2921-2927.

Racioppi, L., and Means, A. R. (2012). Calcium/calmodulin-dependent protein kinase kinase 2: roles in signaling and pathophysiology. J Biol Chem *287*, 31658-31665.

Raff, M. C., Whitmore, A. V., and Finn, J. T. (2002). Axonal self-destruction and neurodegeneration. Science *296*, 868-871.

Rallis, A., Lu, B., and Ng, J. (2013). Molecular chaperones protect against JNK- and Nmnat-regulated axon degeneration in Drosophila. J Cell Sci *126*, 838-849.

Reyes, N. A., Fisher, J. K., Austgen, K., VandenBerg, S., Huang, E. J., and Oakes, S. A. (2010). Blocking the mitochondrial apoptotic pathway preserves motor neuron viability and function in a mouse model of amyotrophic lateral sclerosis. J Clin Invest *120*, 3673-3679.

Rothstein, J. D. (2009). Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. Ann Neurol *65 Suppl 1*, S3-9.

Saggu, S. K., Chotaliya, H. P., Blumbergs, P. C., and Casson, R. J. (2010). Wallerian-like axonal degeneration in the optic nerve after excitotoxic retinal insult: an ultrastructural study. BMC Neurosci *11*, 97.

Sajadi, A., Schneider, B. L., and Aebischer, P. (2004). Wlds-mediated protection of dopaminergic fibers in an animal model of Parkinson disease. Curr Biol *14*, 326-330.

Salzberg, A., Prokopenko, S. N., He, Y., Tsai, P., Pal, M., Maroy, P., Glover, D. M., Deak, P., and Bellen, H. J. (1997). P-element insertion alleles of essential genes on the third chromosome of Drosophila melanogaster: mutations affecting embryonic PNS development. Genetics *147*, 1723-1741.

Samsam, M., Mi, W., Wessig, C., Zielasek, J., Toyka, K. V., Coleman, M. P., and Martini, R. (2003). The Wlds mutation delays robust loss of motor and sensory axons in a genetic model for myelin-related axonopathy. J Neurosci *23*, 2833-2839.

Sasaki, Y., Araki, T., and Milbrandt, J. (2006). Stimulation of nicotinamide adenine dinucleotide biosynthetic pathways delays axonal degeneration after axotomy. J Neurosci *26*, 8484-8491.

Sasaki, Y., and Milbrandt, J. (2010). Axonal degeneration is blocked by nicotinamide mononucleotide adenylyltransferase (Nmnat) protein transduction into transected axons. J Biol Chem *285*, 41211-41215.

Sasaki, Y., Vohra, B. P., Baloh, R. H., and Milbrandt, J. (2009). Transgenic mice expressing the Nmnat1 protein manifest robust delay in axonal degeneration in vivo. J Neurosci *29*, 6526-6534.

Saxena, S., and Caroni, P. (2007). Mechanisms of axon degeneration: from development to disease. Prog Neurobiol *83*, 174-191.

Schlaepfer, W. W., and Bunge, R. P. (1973). Effects of calcium ion concentration on the degeneration of amputated axons in tissue culture. J Cell Biol *59*, 456-470.

Schoenmann, Z., Assa-Kunik, E., Tiomny, S., Minis, A., Haklai-Topper, L., Arama, E., and Yaron, A. (2010). Axonal degeneration is regulated by the apoptotic machinery or a NAD+-sensitive pathway in insects and mammals. J Neurosci *30*, 6375-6386.

Shefner, J. M., Cudkowicz, M., and Brown, R. H. J. (2006). Motor unit number estimation predicts disease onset and survival in a transgenic mouse model of amyotrophic lateral sclerosis. Muscle Nerve *34*, 603-607.

Shefner, J. M., Cudkowicz, M. E., and Brown, R. H. J. (2002). Comparison of incremental with multipoint MUNE methods in transgenic ALS mice. Muscle Nerve *25*, 39-42.

Sheller, R. A., Tytell, M., Smyers, M., and Bittner, G. D. (1995). Glia-to-axon communication: enrichment of glial proteins transferred to the squid giant axon. J Neurosci Res *41*, 324-334.

Shiizaki, S., Naguro, I., and Ichijo, H. (2013). Activation mechanisms of ASK1 in response to various stresses and its significance in intracellular signaling. Adv Biol Regul *53*, 135-144.

Sievers, C., Platt, N., Perry, V. H., Coleman, M. P., and Conforti, L. (2003). Neurites undergoing Wallerian degeneration show an apoptotic-like process with Annexin V positive staining and loss of mitochondrial membrane potential. Neuroscience research *46*, 161-169.

Simmen, T. (2011). Hax-1: a regulator of calcium signaling and apoptosis progression with multiple roles in human disease. Expert Opin Ther Targets *15*, 741-751.

Simonin, Y., Ferrer-Alcon, M., Ferri, A., and Kato, A. C. (2007). The neuroprotective effects of the WldS gene are correlated with proteasome expression rather than apoptosis. Eur J Neurosci *25*, 2269-2274.

Smith, R. S., and Bisby, M. A. (1993). Persistence of axonal transport in isolated axons of the mouse. Eur J Neurosci *5*, 1127-1135.

Song, Y. H. (2013). A Memory Molecule, Ca(2+)/Calmodulin-Dependent Protein Kinase II and Redox Stress; Key Factors for Arrhythmias in a Diseased Heart. Korean Circ J *43*, 145-151.

Spira, M. E., Oren, R., Dormann, A., and Gitler, D. (2003). Critical calpain-dependent ultrastructural alterations underlie the transformation of an axonal segment into a growth cone after axotomy of cultured Aplysia neurons. J Comp Neurol *457*, 293-312.

St Johnston, D. (2002). The art and design of genetic screens: Drosophila melanogaster. Nat Rev Genet *3*, 176-188.

Stengl, M. (1995). Pigment-dispersing hormone-immunoreactive fibers persist in crickets which remain rhythmic after bilateral transection of the optic stalks. J Comp Physiol A *176*, 217-228.

Szretter, K. J., Samuel, M. A., Gilfillan, S., Fuchs, A., Colonna, M., and Diamond, M. S. (2009). The immune adaptor molecule SARM modulates tumor necrosis factor alpha production and microglia activation in the brainstem and restricts West Nile Virus pathogenesis. J Virol *83*, 9329-9338.

Tamsett, T. J., Picchione, K. E., and Bhattacharjee, A. (2009). NAD+ activates KNa channels in dorsal root ganglion neurons. J Neurosci *29*, 5127-5134.

Tang-Schomer, M. D., Johnson, V. E., Baas, P. W., Stewart, W., and Smith, D. H. (2012). Partial interruption of axonal transport due to microtubule breakage accounts for the formation of periodic varicosities after traumatic axonal injury. Exp Neurol *233*, 364-372.

Theodosiou, N. A., and Xu, T. (1998). Use of FLP/FRT system to study Drosophila development. Methods *14*, 355-365.

Van Den Bosch, L. (2011). Genetic rodent models of amyotrophic lateral sclerosis. J Biomed Biotechnol *2011*, 348765.

Vande Velde, C., Garcia, M. L., Yin, X., Trapp, B. D., and Cleveland, D. W. (2004). The neuroprotective factor Wlds does not attenuate mutant SOD1-mediated motor neuron disease. Neuromolecular Med *5*, 193-203.

Vohra, B. P., Sasaki, Y., Miller, B. R., Chang, J., DiAntonio, A., and Milbrandt, J. (2010).
Amyloid precursor protein cleavage-dependent and -independent axonal degeneration programs share a common nicotinamide mononucleotide adenylyltransferase 1-sensitive pathway. J Neurosci *30*, 13729-13738.

Vosshall, L. B., Amrein, H., Morozov, P. S., Rzhetsky, A., and Axel, R. (1999). A spatial map of olfactory receptor expression in the Drosophila antenna. Cell *96*, 725-736.

Vukosavic, S., Dubois-Dauphin, M., Romero, N., and Przedborski, S. (1999). Bax and Bcl-2 interaction in a transgenic mouse model of familial amyotrophic lateral sclerosis. J Neurochem *73*, 2460-2468.

Waller, A. (1850). Experiments on the section of the glossopharyngeal and hypoglossal nerves of the frog, and observation of the alterations produced thereby in the structure of their primitive fibres. Philos Trans R Soc Lond B Biol Sci *140*, 423-429.

Wang, H., and Macagno, E. R. (1998). A detached branch stops being recognized as self by other branches of a neuron. J Neurobiol *35*, 53-64.

Wang, J., and He, Z. (2009). NAD and axon degeneration: from the Wlds gene to neurochemistry. Cell Adh Migr *3*, 77-87.

Wang, J., Zhai, Q., Chen, Y., Lin, E., Gu, W., McBurney, M. W., and He, Z. (2005). A local mechanism mediates NAD-dependent protection of axon degeneration. J Cell Biol *170*, 349-355.

Wang, J. T., and Barres, B. A. (2012). Axon degeneration: where the Wlds things are. Curr Biol *22*, R221-3.

Wang, M., Wu, Y., Culver, D. G., and Glass, J. D. (2001). The gene for slow Wallerian degeneration (Wld(s)) is also protective against vincristine neuropathy. Neurobiol Dis *8*, 155-161.

Wang, M. S., Davis, A. A., Culver, D. G., and Glass, J. D. (2002). WldS mice are resistant to paclitaxel (taxol) neuropathy. Ann Neurol *52*, 442-447.

Wang, P. H., Gu, Z. H., Wan, D. H., Zhu, W. B., Qiu, W., Weng, S. P., Yu, X. Q., and He, J. G. (2013). Litopenaeus vannamei sterile-alpha and armadillo motif containing protein (LvSARM) is involved in regulation of Penaeidins and antilipopolysaccharide factors. PLoS One *8*, e52088.

Watanabe, M., Tsukiyama, T., and Hatakeyama, S. (2007). Protection of vincristine-induced neuropathy by WldS expression and the independence of the activity of Nmnat1. Neurosci Lett *411*, 228-232.

Whitmore, A. V., Lindsten, T., Raff, M. C., and Thompson, C. B. (2003). The proapoptotic proteins Bax and Bak are not involved in Wallerian degeneration. Cell Death Differ *10*, 260-261.

Wilbrey, A. L., Haley, J. E., Wishart, T. M., Conforti, L., Morreale, G., Beirowski, B., Babetto, E., Adalbert, R., Gillingwater, T. H., Smith, T., Wyllie, D. J., Ribchester, R. R., and Coleman, M. P. (2008). VCP binding influences intracellular distribution of the slow Wallerian degeneration protein, Wld(S). Mol Cell Neurosci *38*, 325-340.

Williams, T., Courchet, J., Viollet, B., Brenman, J. E., and Polleux, F. (2011). AMP-activated protein kinase (AMPK) activity is not required for neuronal development but regulates axogenesis during metabolic stress. Proc Natl Acad Sci U S A *108*, 5849-5854.

Wishart, T. M., Paterson, J. M., Short, D. M., Meredith, S., Robertson, K. A., Sutherland, C., Cousin, M. A., Dutia, M. B., and Gillingwater, T. H. (2007). Differential proteomics analysis of synaptic proteins identifies potential cellular targets and protein mediators of synaptic neuroprotection conferred by the slow Wallerian degeneration (Wlds) gene. Mol
Cell Proteomics *6*, 1318-1330.

Wishart, T. M., Rooney, T. M., Lamont, D. J., Wright, A. K., Morton, A. J., Jackson, M., Freeman, M. R., and Gillingwater, T. H. (2012). Combining comparative proteomics and molecular genetics uncovers regulators of synaptic and axonal stability and degeneration in vivo. PLoS Genet *8*, e1002936.

Wright, A. K., Wishart, T. M., Ingham, C. A., and Gillingwater, T. H. (2010). Synaptic protection in the brain of WldS mice occurs independently of age but is sensitive to gene-dose. PLoS One *5*, e15108.

Xia, R. H., Yosef, N., and Ubogu, E. E. (2010). Dorsal caudal tail and sciatic motor nerve conduction studies in adult mice: technical aspects and normative data. Muscle Nerve *41*, 850-856.

Xie, Y., Moussaif, M., Choi, S., Xu, L., and Sze, J. Y. (2013). RFX Transcription Factor DAF-19 Regulates 5-HT and Innate Immune Responses to Pathogenic Bacteria in Caenorhabditis elegans. PLoS Genet *9*, e1003324.

Xiong, X., Hao, Y., Sun, K., Li, J., Li, X., Mishra, B., Soppina, P., Wu, C., Hume, R. I., and Collins, C. A. (2012). The Highwire ubiquitin ligase promotes axonal degeneration by tuning levels of Nmnat protein. PLoS Biol *10*, e1001440.

Xue, Y., Liu, Z., Cao, J., Ma, Q., Gao, X., Wang, Q., Jin, C., Zhou, Y., Wen, L., and Ren, J. (2011). GPS 2.1: enhanced prediction of kinase-specific phosphorylation sites with an algorithm of motif length selection. Protein Eng Des Sel *24*, 255-260.

Yahata, N., Yuasa, S., and Araki, T. (2009). Nicotinamide mononucleotide adenylyltransferase expression in mitochondrial matrix delays Wallerian degeneration. J Neurosci *29*, 6276-6284.

Yamamoto, M., and Takeda, K. (2010). Current views of toll-like receptor signaling pathways. Gastroenterol Res Pract *2010*, 240365.

Yamanaka, K., Chun, S. J., Boillee, S., Fujimori-Tonou, N., Yamashita, H., Gutmann, D. H., Takahashi, R., Misawa, H., and Cleveland, D. W. (2008). Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. Nat Neurosci *11*, 251-253.

Yuan, S., Wu, K., Yang, M., Xu, L., Huang, L., Liu, H., Tao, X., Huang, S., and Xu, A. (2010). Amphioxus SARM involved in neural development may function as a suppressor of TLR signaling. J Immunol *184*, 6874-6881.

Zhai, Q., Wang, J., Kim, A., Liu, Q., Watts, R., Hoopfer, E., Mitchison, T., Luo, L., and He, Z. (2003). Involvement of the ubiquitin-proteasome system in the early stages of wallerian degeneration. Neuron *39*, 217-225.

Zhang, B., Carroll, J., Trojanowski, J. Q., Yao, Y., Iba, M., Potuzak, J. S., Hogan, A. M., Xie, S. X., Ballatore, C., Smith, A. B. r., Lee, V. M., and Brunden, K. R. (2012). The microtubule-stabilizing agent, epothilone D, reduces axonal dysfunction, neurotoxicity, cognitive deficits, and Alzheimer-like pathology in an interventional study with aged tau transgenic mice. J Neurosci *32*, 3601-3611.

Ziegenfuss, J. S., Doherty, J., and Freeman, M. R. (2012). Distinct molecular pathways mediate glial activation and engulfment of axonal debris after axotomy. Nat Neurosci *15*, 979-987.

Ziv, N. E., and Spira, M. E. (1993). Spatiotemporal distribution of Ca2+ following axotomy and throughout the recovery process of cultured Aplysia neurons. Eur J Neurosci *5*, 657-668.