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The Phr1 Ubiquitin Ligase Promotes Injury-Induced Axon Self-Destruction

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SUMMARY

Axon degeneration is an evolutionarily conserved process that drives the loss of damaged axons and is an early event in many neurological disorders, so it is important to identify the molecular constituents of this poorly understood mechanism. Here, we demonstrate that the Phr1 E3 ubiquitin ligase is a central component of this axon degeneration program. Loss of Phr1 results in prolonged survival of severed axons in both the peripheral and central nervous systems, as well as preservation of motor and sensory nerve terminals. Phr1 depletion increases the axonal level of the axon survival molecule nicotinamide mononucleotide adenyltransferase 2 (NMNAT2), and NMNAT2 is necessary for Phr1-dependent axon stability. The profound long-term protection of peripheral and central mammalian axons following Phr1 deletion suggests that pharmacological inhibition of Phr1 function may be an attractive therapeutic candidate for amelioration of axon loss in neurological disease.

INTRODUCTION

Axon degeneration is a hallmark of many neurological disorders including neuropathies and neurodegenerative diseases. An active and evolutionarily conserved mechanism drives axon loss (Wang and Barres, 2012), so inhibiting this process is an attractive therapeutic target for treating a wide range of neurological disorders. Acute severing of axons leads to a process commonly referred to as Wallerian degeneration, consisting of axon breakdown, glial and macrophage responses, and clearance of myelin debris (George and Griffin, 1994). Wallerian degeneration is delayed for at least a week by the ectopic expression of the Wallerian degeneration slow (Wld^S) protein, which acts via a gain-of-function mechanism requiring the axonal relocalization of its active component nicotinamide mononucleotide adenylyltransferase, NMNAT1 (Babetto et al., 2010; Sasaki et al., 2009a). Axon degeneration occurs not only in response to trauma, but also occurs in metabolic, inflammatory, and hereditary neuropathies as well as neurodegenerative

diseases (Vargas and Barres, 2007). Remarkably, expression of Wld^S or axonal NMNAT can delay axon loss in many of these disease models suggesting the existence of a general axon loss program (Wang and Barres, 2012).

While ectopic expression of NMNAT orthologs can delay axon loss, the endogenous mechanisms that promote axon degeneration are largely unknown. Others and we have recently identified neuron intrinsic pathways that promote axon death (Miller et al., 2009; Osterloh et al., 2012; Wakatsuki et al., 2011; Wishart et al., 2012) and have designed screens to identify additional components (Bhattacharya et al., 2012; Fang et al., 2012; Gerdts et al., 2011). In a hypomorphic mouse mutant of dual-leucine zipper kinase (DLK), we demonstrated axon protection for at least 52 hr after axotomy and identified JNK and SCG10 as downstream signaling effectors (Miller et al., 2009; Shin et al., 2012). However, only deletion of the sterile alpha- and armadillo-motif-containing protein (SARM) protein confers Wld^S-comparable axon protection in mice for days after peripheral nerve transection, yet its mechanism of action remains unknown (Osterloh et al., 2012). To advance our understanding of the endogenous processes orchestrating axon death, it is thus necessary to identify essential components of the degeneration program and to define the underlying mechanisms.

PHR (PAM-Highwire-Rpm-1) ligases are an evolutionarily conserved family of large E3 ubiquitin ligases that are central regulators of multiple aspects of axonal biology. Mutations of PHR orthologs from worms to mice lead to dramatic defects in the development of synapses and axons (Bloom et al., 2007; Culican et al., 2009; D'Souza et al., 2005; Lewcock et al., 2007; Schaefer et al., 2000; Wan et al., 2000; Zhen et al., 2000). Recent work in invertebrates demonstrates that PHR proteins also accelerate the regenerative response following nerve injury (Nix et al., 2011; Xiong et al., 2010) and delay synapse loss in genetic models of cytoskeletal instability (Massaro et al., 2009). The involvement of Phr1 (also known as Mycbp2) in fundamental aspects of axon biology prompted us to test the role of Phr1 in axon degeneration in mice. Here, we demonstrate that Phr1 is a central component of the axonal degeneration program. Excision of Phr1 in adult mice leads to long-term preservation of peripheral nervous system (PNS) and CNS axons, and Phr1 promotes the self-destruction of injured axons by limiting the availability of the essential axonal maintenance factor NMNAT2. This identifies Phr1 as a candidate therapeutic target for inhibiting axonal loss due to trauma and disease.

RESULTS

Phr1 Deletion Protects Axons and Nerve Terminals In Vivo

Loss of Phr1 during development in mice impairs axon guidance and leads to perinatal lethality (Bloom et al., 2007; Lewcock et al., 2007), so we disrupted Phr1 in young adults using a previously characterized conditional *Phr1* allele (Bloom et al., 2007) and a tamoxifen-inducible Cre recombinase under the control of the β-actin promoter (Hayashi and McMahon, 2002). Tamoxifen administration induced the excision of the floxed region within the *Phr1* gene (Experimental Procedures), and we confirmed in vitro that Cre-dependent excision leads to a decrease in Phr1 protein levels (Figure S1A). Phr1 adult conditional knockout (Phr1 aKO) mice appeared healthy for up to 6 weeks after tamoxifen administration.

In Phr1 aKO mice, we observed profound axon preservation following injury, comparable to that described in Wld[S] (Mack et al., 2001) and SARM KO mutants (Osterloh et al., 2012) (Figures 1A–1D). Axons degenerate about 2 days after sciatic nerve axotomy in wild-type (WT) mice (Beirowski et al., 2004), with axons of smaller caliber degenerating faster than large caliber axons (Vargas and Barres, 2007). In Phr1 aKO mutants, there is a dramatic delay in loss of both small and large axons, with ~75% of myelinated fibers structurally intact 5 days after axotomy, and ~25% of myelinated fibers persisting for at least 10 days (Figures 1A and 1B). Electron microscopy demonstrates intact axonal organelles and cytoskeleton 10 days after injury (Figure 1C), as well as persistence of unmyelinated sensory axons with their associated glia for at least 3 days (Figure 1C). Biochemical analysis confirms maintenance of cytoskeletal elements; neurofilament subunits are degraded within 3 days of injury in WT tibial nerves, but are preserved for at least 10 days in axotomized Phr1 aKO nerves (Figure 1D). Taken together, these results show that Phr1 genetic ablation confers a remarkable delay of axon degeneration in adult mice, protecting small, large, and unmyelinated sensory axons.

Many debilitating neurodegenerative conditions affect central axons, so we tested if Phr1 also regulates axon degeneration in the CNS. We axotomized retinal ganglion neurons, and assayed axon survival quantitatively in the optic nerve distal to the transection site. Five days after lesion, WT nerves display extensive axon degeneration with gliosis and axonal replacement by fibrotic tissue. In contrast, lesioned optic nerves from Phr1 aKO mice appear similar to unlesioned contralateral nerves, with preservation of the vast majority of axons (Figures 1E–1G).

We next explored whether Phr1 regulates the stability of terminal specializations such as motor and sensory endings, which are particularly vulnerable in a variety of neuropathies. We first assessed the integrity of neuromuscular junctions (NMJs). In wild-type mice, tibialis anterioris muscles are completely denervated by 3 days after sciatic nerve axotomy. In axotomized Phr1 aKO mice, motor nerve terminal degeneration is delayed, with significant numbers of NMJs remaining occupied (Figures 2A and 2B). We next examined skin sensory endings, finding an ~3-fold increase in the number of intraepidermal fibers (IEPFs) remaining in the Phr1 aKO compared to WT 3 days after injury (p < 0.05) (Figure S2). Hence, loss of

Phr1 preserves both axons and their specialized motor and sensory arbors following injury.

Phr1 Loss of Function Protects Neurites Cell-Autonomously after Disparate Insults

To investigate whether Phr1 KO promotes axon survival in a neuron-autonomous manner, we cultured embryonic dorsal root ganglia neurons (DRGs) of Phr1-floxed mice in the absence of other cell types. We confirmed loss of the neuronal Phr1 protein after lentivirus transduction of Cre recombinase (Figure S1A). Distal WT neurites degenerate within 6 hr after transection displaying features such as swelling and fragmentation (Figures 3A and 3B). Axon degeneration is delayed for at least 24 hr following axotomy in Phr1 KO neurons (Figures 3A and 3B), demonstrating that Phr1 can act autonomously in the neuron to control axonal stability. Using this culture model, we next demonstrated that axonal degeneration induced by the neurotoxin vincristine, a chemotherapeutic agent that causes peripheral neuropathy, is also delayed in Phr1 KO neurons (Figures 3A and 3B). Thus, Phr1 functions downstream of distinct insults. Finally, we decreased Phr1 levels using small hairpin RNA (shRNA) to exclude the possibility that axon preservation is an allele-specific phenotype. Three independent shRNAs for *Phr1* decrease Phr1 protein levels and delay axon degeneration following axotomy (Figures S1B and S1C). Taken together, these in vitro studies demonstrate that Phr1 can function cell autonomously within neurons to promote axon degeneration induced by both traumatic and neurotoxic insults. While this in vitro protection is clear, it is less robust than that conferred by Wld[S] in vitro even though the in vivo protection in these two mutants is similar (Babetto et al., 2010). This difference is consistent with Phr1 having additional axoprotective functions in nonneuronal cells and highlights the importance of in vivo analysis to identify the most significant axoprotective pathways.

Phr1 Regulates DLK Levels in the Mouse

To investigate the mechanism of action of Phr1, we initially focused on the MAP3K DLK, which is the best-characterized target of Phr1-family ubiquitin ligases in invertebrates (Collins et al., 2006; Nakata et al., 2005; Xiong et al., 2010). While we previously found no alteration of DLK levels in embryonic Phr1 KO whole-head lysates (Bloom et al., 2007), here we measured DLK levels in Phr1 KO DRG cultures and found a 3.9 ± 0.2-fold increase compared to controls (p < 0.005) (Figure S3A). The difference between our current findings in DRGs and prior findings in embryonic head may reflect a cell-type difference or we may have missed a change in DLK levels in embryonic CNS neurons because they were masked by expression in nonneuronal cells. The increase in DLK abundance in the DRG demonstrates that regulation of DLK levels by PHR ligases is evolutionarily conserved in invertebrates and vertebrates.

While DLK levels are increased in the Phr1 KO cells, this increase is unlikely to mediate the axoprotective Phr1 KO phenotype because loss of DLK, rather than its activation, promotes axon survival (Miller et al., 2009). To test directly whether elevated levels of DLK can protect axons, we overexpressed DLK in cultured DRG neurons. DLK overexpression does not delay neurite degeneration after axotomy (9 hr after cut, WT
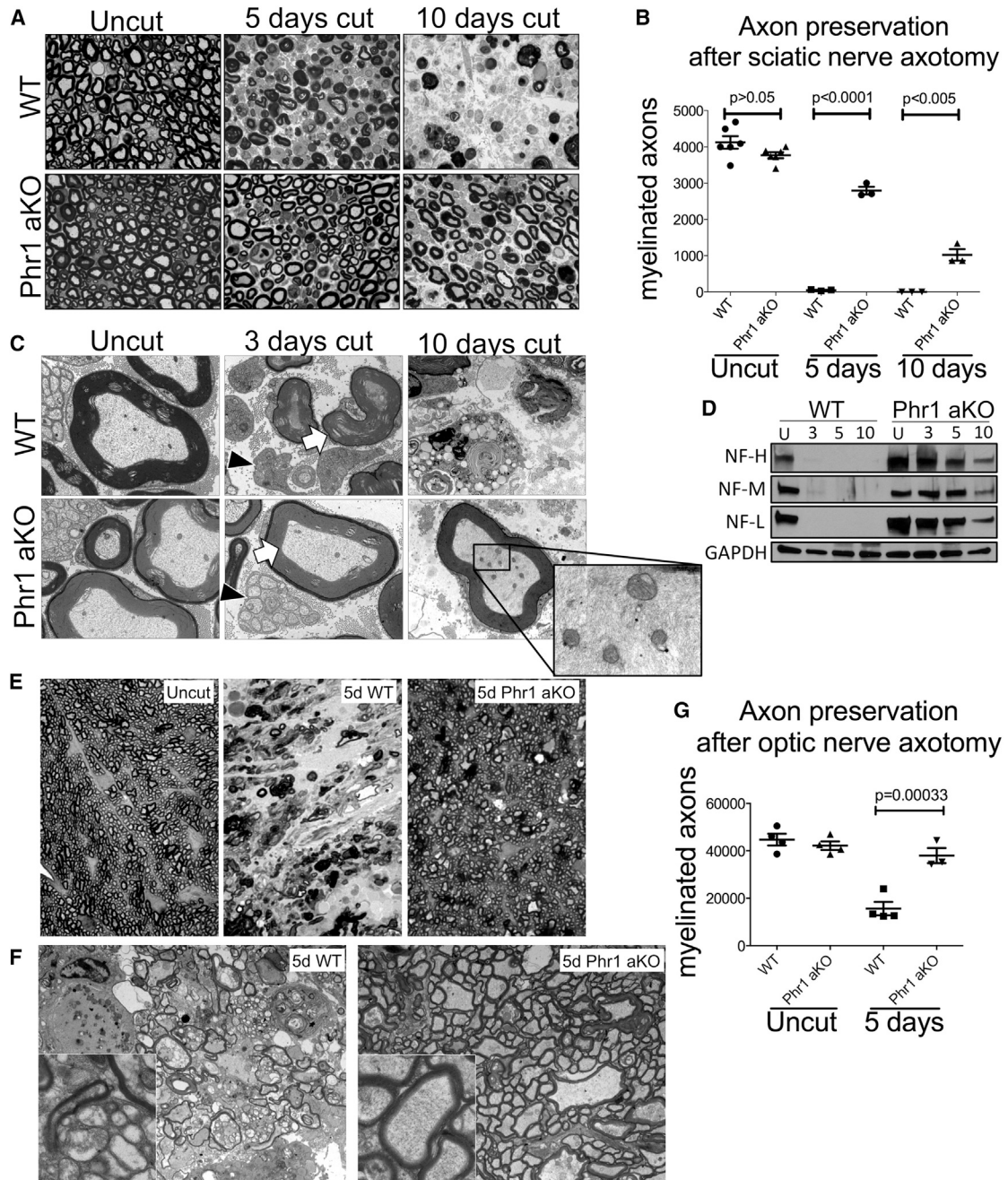
Figure 1. Phr1 Adult Conditional KO Delays Axon Degeneration in the CNS and PNS

(A) Toluidine-blue-stained cross-sections of axotomized sciatic nerve distal to the cut site, showing delayed axon degeneration in Phr1 adult conditional KO (aKO) compared to littermate controls (WT).

(B) Quantification of intact myelinated axons of cut and unlesioned contralateral sciatic nerves. Error bars indicate SE.

(C) Electron micrographs of the nerves shown in (A). Black arrowheads point to preserved small unmyelinated sensory fibers (Remak bundles) in Phr1 aKO nerves. White arrows point to the preservation of large caliber axons; whose cytoskeleton and organelles remain intact for at least 10 days (inset).

(D) Representative western blot of tibial nerves at the indicated time points after transection, showing preservation of neurofilaments (heavy, NF-H; medium, NF-M; light, NF-L chains) in Phr1 aKO mice (n = 3).

(E) Toluidine-blue-stained cross-sections of optic nerve distal to the cut site demonstrate almost complete preservation of axons in Phr1 aKO mice 5 days after eye enucleation, a time point when WT optic nerves are extensively degenerated, as quantified in (G).

(F) Electron micrographs of the same optic nerves shown in (E), demonstrating axon death in WT samples, and morphological axon preservation in Phr1 aKO samples (insets) 5 days after lesion.

(G) Quantification of intact myelinated axons of cut and unlesioned contralateral optic nerves. Error bars indicate SEM.

See also Figure S1.

A Uncut   3 days

WT

Phr1 aKO

B

not occupied
partially occupied
fully occupied

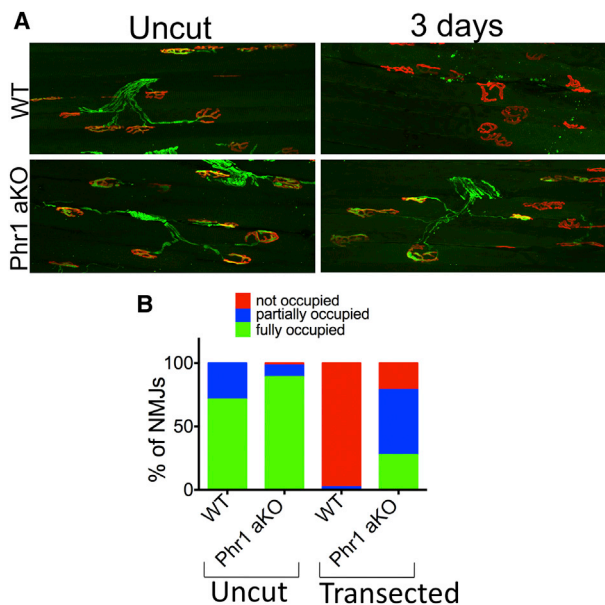% of NMJs

Uncut   Transected

Figure 2. Loss of Phr1 Slows Neuromuscular Junction Degeneration

(A) Presynaptic motoneuron terminals (antisynaptophysin and antineurofilament M, green) and postsynaptic endplates (bungarotoxin, red) from the tibialis anterioris muscle remain apposed in Phr1 aKO mice 3 days after sciatic nerve transection, whereas complete denervation is observed in WT samples, as quantified in (B). See also Figure S2.

A WT   Phr1 KO

Untreated   500 μm

Axotomy
(24h)

Vincristine
(24h)

B

Degeneration index

Hours after cut

Degeneration index

Hours after
vincristine treatment

WT
Phr1 KO

Figure 3. Phr1 Functions within the Neuron to Promote Degeneration in Response to Both Traumatic and Neurotoxic Insults

(A) Phase-contrast images of transected or vincristine-treated neurites are shown from Phr1 KO or control (WT) embryonic dorsal root ganglia (DRG) cultures at the indicated time points after injury. The time course of axonal fragmentation, quantified as degeneration index (see Experimental Procedures) is shown in (B). Values indicated are the mean ± SE and are representative of at least three repeats of each analysis (axotomy and vincristine treatment). See also Figure S1.

degeneration index = 0.51 ± 0.06 SEM; overexpressed DLK degeneration index = 0.59 ± 0.04 SEM, p > 0.1, n = 3). These results indicate that Phr1 has a different functionally relevant target in axons that mediates the change in axon stability.

Phr1 Protects Axons via Increased Levels of the Axonal Survival Factor NMNAT2

The Phr1 axon protection phenotype in vivo is similar to that afforded by Wld[S] (Mack et al., 2001), whose active component is NMNAT1. In mice and humans, there are three isoforms of NMNATs, and each promotes the survival of axons when overexpressed (Gilley and Coleman, 2010; Sasaki et al., 2009a; Yahata et al., 2009). Of these, NMNAT2 is necessary for nerve development (Hicks et al., 2012) and behaves as an endogenous axon survival factor. NMNAT2 is enriched in neurons, is axonally transported, and has a much shorter half-life than the other NMNAT isoforms. In vitro loss of NMNAT2 triggers axonal degeneration and its overexpression delays axon degeneration (Gilley and Coleman, 2010). Therefore, we tested the hypothesis that Phr1 may limit neuronal NMNAT2 abundance.

The lack of both reliable and sensitive antibodies to NMNAT2 precluded an assessment of endogenous NMNAT2 levels. Instead, we overexpressed a functional hisMyc-tagged NMNAT2 in DRG cultures, and found that NMNAT2 levels in Phr1 KO cultures are 2.0-fold higher (±0.2 SEM, p < 0.05) than in control cultures (Figure 4A). Analogous experiments with tagged NMNAT1 and NMNAT3 found no difference in the levels of these proteins in Phr1 KO neurons compared to control (Figure S3B). Hence, Phr1-dependent regulation is selective for the NMNAT isoform with a short half-life and is not a general
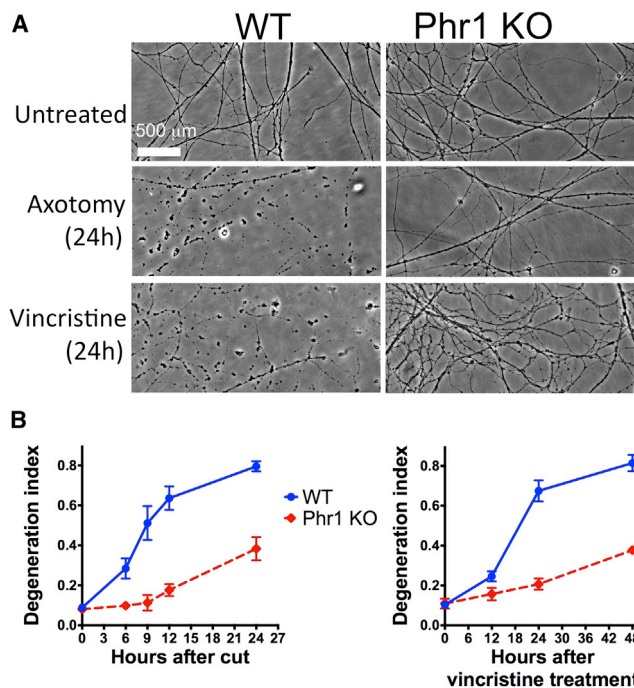
response to viral-mediated expression (Figure S3B). Moreover, endogenous NMNAT2 mRNA levels are not significantly different in Phr1 KO and control neurons (quantitative PCR, p > 0.1), consistent with posttranscriptional regulation of NMNAT2 by Phr1. To investigate the subcellular localization of this excess NMNAT2, we stained Phr1 KO and control cultures expressing hisMyc-tagged NMNAT2. NMNAT2 is increased in Phr1 KO neurites (Figures 4B and 4C) but not cell bodies (Figure S3C). Following injury NMNAT2 protein is rapidly lost from distal axons as previously described (Gilley and Coleman, 2010), reaching background levels by 2.5 hr after injury in control axons. In contrast, Phr1 KO axons maintain higher levels of NMNAT2 after injury, falling to uncut control levels 5 hr after axotomy (Figures 4B and 4C). Hence, NMNAT2 levels are elevated in Phr1 KO axons during the commitment phase of axonal degeneration, the period during which exogenous NMNAT must be present to block axonal degeneration (Sasaki and Milbrandt, 2010).

The increase in NMNAT2 protein levels in Phr1 KO neurons is consistent with the Phr1 E3 ubiquitin ligase directly targeting NMNAT2 for degradation. To test this hypothesis, we assayed for direct binding between NMNAT2 and Fbxo45, the substrate-recognition subunit of the Phr1 ubiquitin ligase complex (Wu et al., 2007; Saiga et al., 2009). When coexpressed in cultured cells, reciprocal coimmunoprecipitation experiments
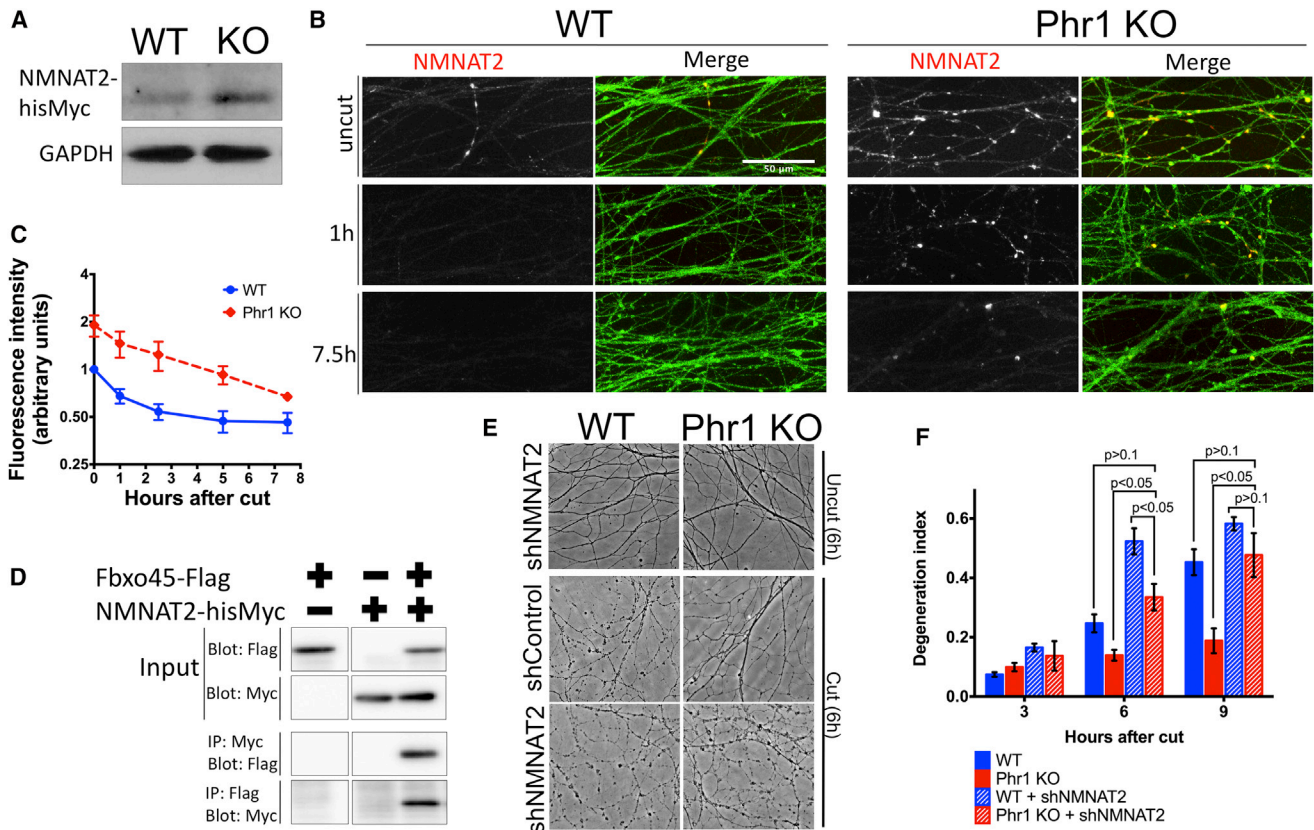
Figure 4. Increased Levels of NMNAT2 Mediates Phr1 KO Protection

(A) Western blot of embryonic DRGs transduced with NMNAT2-hisMyc, showing ∼2-fold increase of NMNAT2 protein in the Phr1 KO (n = 3).

(B) Representative anti-Myc immunostaining (NMNAT2), counterstained for axons (anti-βIII-tubulin, green), shows NMNAT2 in axons at the indicated time points after axotomy. NMNAT2 levels decrease after injury in both WT and Phr KO samples, but are consistently higher in Phr1 KO samples, as quantified in (C) (n = 3, error bars show SE).

(C) Quantification of the anti-myc immunofluorescence intensity (detecting NMNAT2-hisMyc) in neurites at the indicated time points after cut. Error bars show SE.

(D) Coimmunoprecipitation studies from cultured cells demonstrate binding between Fbxo45-Flag, the substrate recognition domain of the Phr1 ligase, and NMNAT2-hisMyc.

(E) Phase contrast images of uncut and cut DRGs (6 hr after axotomy) showing reversion of the Phr1 KO protection when NMNAT2 is knocked down via shRNA (shNMNAT2), as quantified in the time course shown in (F).

(F) Quantification of the degree of fragmentation (degeneration index) of Phr1 KO cultures and control, with or without NMNAT2 knockdown, at the indicated time points after cut. Error bars indicate SEM.

See also Figure S3.

demonstrate binding between Fbxo45 and NMNAT2 (Figure 4D). While this finding is consistent with Phr1 acting as an ubiquitin ligase to regulate NMNAT2 protein levels, the eventual loss of NMNAT2 protein from injured Phr1 mutant axons indicates that additional mechanisms must also promote NMNAT2 degradation.

Taken together, these data suggest that loss of Phr1 delays axonal degeneration by elevating the levels of the axon survival factor NMNAT2. This model predicts that reducing the levels of NMNAT2 should revert the axon protection phenotype in the Phr1 mutant. To test this model, we used lentiviral shRNA expression to reduce neuronal NMNAT2 transcript levels by 75% as measured by quantitative RT-PCR (not shown). This partial knockdown of endogenous NMNAT2 does not induce spontaneous axonal degeneration, and only slightly accelerates axotomy-induced axonal degeneration in control cultures (Fig-

ures 4E and 4F). In contrast, this reduction in NMNAT2 potently reverts the delay of axonal degeneration following axotomy of Phr1 KO axons (Figures 4E and 4F). Degeneration is comparable to control preparations 6 hr after axotomy, and no Phr1-dependent protection is evident 9 hr after axotomy (Figure 4F). These findings support the model that elevated levels of endogenous NMNAT2 mediate the axon protection in the Phr1 KO.

DISCUSSION

This study demonstrates a dramatic delay of Wallerian degeneration in Phr1 mutant mice. The axon protection is evident in several neuronal types including myelinated and unmyelinated axons, central and peripheral nerves, and sensory and motor terminals. Although there may be differences in the mechanisms of degeneration in these different neurons (Ebenezer et al., 2007;

Gillingwater and Ribchester, 2001), the broad protection afforded by the Phr1 KO strongly suggests that Phr1 is a core regulatory component of a fundamental axon degeneration mechanism in mammals. Indeed, the axoprotective phenotype of the Phr1 KO is similar to that of the recently described SARM1 mutant mice (Osterloh et al., 2012), with preservation of axotomized peripheral axons for more than a week and persistence of NMJs for at least 3 days. For Phr1, we have identified the axon survival protein NMNAT2 as a functional downstream target. Importantly, we report a regulatory factor for NMNAT2 in mammals. Modulating the levels of NMNAT2 alters the rate of Wallerian degeneration not only after axotomy in vitro, but also in mouse models of human tauopathy (Ljungberg et al., 2012). Hence, strategies to manipulate NMNAT2 are particularly attractive therapeutic candidates for treating axonopathies in a broad range of conditions.

Phr1 plays an evolutionarily conserved role in various aspects of axon biology (Po et al., 2010). Our findings now demonstrate that PHR proteins play an evolutionarily conserved role in the control of axonal degeneration. In mutants of the Phr1 ortholog *highwire* in *Drosophila*, motor nerve terminals are protected from genetic disruption of the cytoskeleton (Massaro et al., 2009). In addition, a recent study in *Drosophila* showed that Highwire promotes axon degeneration by limiting the levels of the *Drosophila* NMNAT enzyme (Xiong et al., 2012). Hence, PHR regulation of axonal degeneration via control of NMNAT isoform levels is an evolutionary conserved component of a program that likely balances axonal maintenance and degeneration. How does Phr1/Highwire regulate NMNAT2 and *Drosophila* NMNAT? Phr1 is an E3 ubiquitin ligase that can ubiquitinate and degrade targets (Han et al., 2008) and we show here that NMNAT2 binds to Fbxo45, the substrate recognition domain of the Phr1 ubiquitin ligase complex. In addition, NMNAT2 and *Drosophila* NMNAT accumulate after MG132-mediated inhibition of degradative pathways, so NMNAT2 is likely regulated via the ubiquitin proteasome system (Gilley and Coleman, 2010; Xiong et al., 2012). However, others and we have yet to detect ubiquitination of NMNAT2 (Xiong et al., 2012), raising the possibility of additional regulatory mechanisms.

The extent of Phr1 aKO protection is similar to that of the gain-of-function Wld^S mutant (Mack et al., 2001). In the Wld^s mutant the axoprotective NMNAT1 enzyme is mistargeted to axons and functionally compensates for the loss of NMNAT2 after injury. However, in contrast to the chimeric Wld^S protein, which is a gain-of-function mutation, Phr1 is an endogenous protein that limits the levels of NMNAT2 and promotes axonal degeneration. Two interesting questions arise from this finding. First, how do increased levels of NMNAT2 protect axons when the protein is still rapidly lost within hours of injury? This is an issue both in the Phr1 mutant and when NMNAT2 is overexpressed in wild-type neurons. Indeed, overexpression of NMNAT2 in wild-type neurons leads to long-term protection but the protein is undetectable within a few hours after injury (Figure 4; Gilley and Coleman, 2010). Elevated levels of NMNAT2 may have a unique function in the first hours after injury, or low levels of residual protein may still function long after the injury. In the Phr1 KO, additional targets may also contribute to axonal protection. Second, why do neurons have a mechanism to restrict the levels

of an axoprotective molecule such as NMNAT2? Short-lived axoprotective molecules are maintained in the axon by constant replenishment via anterograde axonal transport, and so their presence in the axon can serve as a signal of a healthy connection with the cell body (Gilley and Coleman, 2010; Shin et al., 2012). Axons that are not well connected can therefore be rapidly and efficiently eliminated, reducing the possibility of damaging adjacent axons, and clearing the way for regeneration. Such a mechanism would require molecules such as Phr1 to regulate the levels of axoprotective factors such as NMNAT2.

In conclusion, we have identified Phr1 as a key regulator of a core axon degeneration program that balances axon survival and loss in various neuronal cell types. The profound long-term protection of injured mammalian axons following deletion of Phr1 suggests that pharmacological inhibition of Phr1 function could be an attractive therapeutic strategy for ameliorating axon loss in neurological diseases in which axonopathy is a major component, such as peripheral neuropathies, glaucoma, multiple sclerosis, and some neurodegenerative diseases (Vargas and Barres, 2007). The great challenge will be in designing specific inhibitors of the Phr1 ubiquitin ligase, as efforts to target ubiquitin ligases are in their infancy (Zhao and Sun, 2012).

EXPERIMENTAL PROCEDURES

Mouse Dorsal Root Ganglia Cultures

Spot DRG cultures were described previously (Shin et al., 2012). E13.5 *Phr1* floxed-allele (Bloom et al., 2007) or CD1 (Charles River) DRGs were cultured with 1 μM 5-fluoro-2′-deoxyuridine and 1 μM uridine (Sigma) to block non-neuronal cell division. To avoid confounding effects from somatic death, at 2 days in vitro (DIV) DRGs were transduced with Bcl-XL lentivirus, which blocks apoptosis but not injury-induced degeneration (Vohra et al., 2010). Cre recombinase (KO) or FCIV (WT) were introduced via lentiviral transduction in *Phr1*-floxed DRGs from the same preparation at DIV 2–4. ShNMNAT2, or shControl, was applied at DIV 4 to avoid complete NMNAT2 knockdown and subsequent spontaneous axon degeneration. ShPhr1, or DLK overexpressing lentiviruses were applied to CD1 DRGs at DIV 2. See Extended Experimental Procedures for details of constructs. At DIV 8–9, after complete neurite development, cultures were lysed, axotomized, or treated with vincristine as per the specific experiment, and neurite degeneration were analyzed as described (Sasaki et al., 2009b).

Ultrastructural Studies

For assessing optic axon survival mice were perfused transcardially (ice-cold 3% glutaraldehyde [Polysciences] in 0.1 M phosphate buffer). Sciatic nerves and optic nerve segments distal to the axotomy site were harvested, and the first millimeter was discarded. See Extended Experimental Procedures for production of semithin sections and ultrathin sections. From these sections, a blinded investigator quantified intact axons 5 mm distally from the axotomy site by established methods (Beirowski et al., 2009, 2011; Marina et al., 2010).

Western Blot

Immunoprecipitated material (obtained as described in the Extended Experimental Procedures) and DRGs were lysed in Laemmli buffer. Snap-frozen tibial nerves were homogenized in RIPA buffer with protease inhibitor tablet (cOmplete Mini EDTA-free, Roche). Western blot was performed with standard protocols. Antibodies are listed in the Extended Experimental Procedures. Intensities of protein bands were normalized by comparison with the loading control (βIII-tubulin or GAPDH).

Immunocytochemistry

Immunostaining was carried out on glass slides (BD Falcon) as described (Shin et al., 2012). Neurons were stained for NMNAT2-hisMyc using anti-Myc

antibody (9B11, Cell Signaling, 1:500) and counterstained for βIII-tubulin (TUJ, Aves lab, 1:500), with the appropriate Alexa-conjugated secondary antibodies (Invitrogen). Confocal stacks were obtained with a Nikon D-Eclipse C1 confocal microscope and z-projected. The axonal NMNAT2 level was assessed with ImageJ. Images were thresholded for βIII-tubulin immunoreactivity to define the axonal area, and the average anti-Myc immunofluorescence intensity was measured within the defined area and background was subtracted.

Mouse Husbandry and Surgery

Phr1-floxed mice (Bloom et al., 2007) were bred to B6.Cg-Tg(CAG-cre/Esr1*) 5Amc/J mice (Jackson lab) that express a tamoxifen-inducible Cre-recombinase. Tamoxifen (Sigma) was injected intraperitoneally (0.1 mg/g) to all littermates, including those without the *Cre* allele (wild-type, WT), for 2 consecutive days for 3 weeks starting at 60–80 days of age. Surgical axotomy is described in the Extended Experimental Procedures. At the indicated times, mice were euthanized, or intracardially perfused, and tissue was dissected. To test for Cre recombination, a small portion of the tail tip was collected before tamoxifen injection, and DRGs were dissected at the endpoint of the experiment. PCR analysis (Bloom et al., 2007) verified the appearance of a recombination-specific band in DRGs from mice expressing Cre. Mouse handling and surgery were supervised by the Division of Comparative Medicine of the Washington University in Saint Louis.

Neuromuscular Junction Staining and Quantification

Surgery was performed 2 weeks after the first tamoxifen injection. Tibialis anterior muscles were collected from mice intracardially perfused (4% paraformaldehyde in 0.1 M PBS) and postfixed for 2 hr at room temperature. Cryopreservation, cryosectioning of 60-μm-thick sections, and staining procedures were performed as described (Babetto et al., 2010; Extended Experimental Procedures). For morphological NMJ quantification, endplate occupancy was assessed by a masked investigator. Full and partial occupancy was defined as a state when the postsynaptic bungarotoxin labeling was completely or partially opposed by the axon terminal. Fifty to 100 NMJs were quantified for each muscle preparation, from three mice per genotype.

Statistical Analysis

A Student's t test or ANOVA, with Tukey post-hoc, was used to test statistical significance. The p values > 0.05 were considered not significant. Data are presented as mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.04.013.

LICENSING INFORMATION

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