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A Model of Toxic Neuropathy in *Drosophila* Reveals a Role for MORN4 in Promoting Axonal Degeneration

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Axonal degeneration is a molecular self-destruction cascade initiated following traumatic, toxic, and metabolic insults. Its mechanism underlies a number of disorders including hereditary and diabetic neuropathies and the neurotoxic side effects of chemotherapy drugs. Molecules that promote axonal degeneration could represent potential targets for therapy. To identify such molecules, we designed a screening platform based on intoxication of *Drosophila* larvae with paclitaxel (taxol), a chemotherapeutic agent that causes neuropathy in cancer patients. In *Drosophila*, taxol treatment causes swelling, fragmentation, and loss of axons in larval peripheral nerves. This axonal loss is not due to apoptosis of neurons. Taxol-induced axonal degeneration in *Drosophila* shares molecular execution mechanisms with vertebrates, including inhibition by both NMNAT (nicotinamide mononucleotide adenylyltransferase) expression and loss of *wallenda*/DLK (dual leucine zipper kinase). In a pilot RNAi-based screen we found that knockdown of *retinophilin* (*rtp*), which encodes a MORN (membrane occupation and recognition nexus) repeat-containing protein, protects axons from degeneration in the presence of taxol. Loss-of-function mutants of *rtp* replicate this axonal protection. Knockdown of *rtp* also delays axonal degeneration in severed olfactory axons. We demonstrate that the mouse ortholog of *rtp*, MORN4, promotes axonal degeneration in mouse sensory axons following axotomy, illustrating conservation of function. Hence, this new model can identify evolutionarily conserved genes that promote axonal degeneration, and so could identify candidate therapeutic targets for a wide-range of axonopathies.

Introduction

Injury to axons occurs in a wide variety of pathological contexts, including diabetic neuropathy, exposure to chemotherapeutic agents during cancer treatment, and hereditary neuropathies such as Charcot-Marie-Tooth disease (Coleman, 2005). In recent years, axonal injury has been appreciated as an early event in CNS degenerative diseases such as Alzheimer's disease (Stokin et al., 2005) as well as traumatic brain injuries (Xu et al., 2007). These diverse mechanical, toxic, and metabolic insults initiate a stereotyped set of cellular changes beginning with axonal swellings that progress into breaches of membrane integrity and axonal fragmentation (Coleman and Freeman, 2010).

Axon degeneration occurs through a molecular pathway that is evolutionarily conserved. Evidence for this molecular cascade came initially from the remarkable protective effects of expression of nicotinamide mononucleotide adenylyltransferase (NMNAT), the critical component of the Wallerian degeneration slow (Wld^s) fusion protein (Mack et al., 2001; Araki et al., 2004). Using a mechanism that is still mysterious, NMNAT gain-of-function in the axon blocks axonal degeneration in *Drosophila* and mouse from a wide range of insults (Mack et al., 2001; Hoopfer et al., 2006; MacDonald et al., 2006; Press and Milbrandt, 2008; Zhai et al., 2008; Sasaki et al., 2009a). Endogenous pathways also promote axonal degeneration following injury. The dual leucine zipper kinase DLK, or its *Drosophila* ortholog *wallenda*, is required for efficient axonal degeneration in response to axotomy or chemotherapy treatment *in vitro*, and *in vivo* following sciatic nerve injury (Miller et al., 2009). That the roles of NMNAT and DLK are functionally conserved across species suggests that an ancient cascade executes the removal of irreparably damaged axons. Identification of important players in this cascade may enable strategies to block axon degeneration and could slow or prevent progression of many neurological diseases.

In human patients, the administration of chemotherapeutic agents such as vincristine and paclitaxel (taxol) causes a dose-limiting peripheral neuropathy, such that very aggressive cancers cannot be adequately controlled by these drugs (Malik and Stillman, 2008; Park et al., 2008). Since the timing of chemotherapy treatment is known, strategies to protect axons before and/or during treatment have great potential to minimize this toxic side effect (Windebank, 1999). The identification of genes that promote axon degeneration after injury is a first step toward realizing this therapeutic potential.

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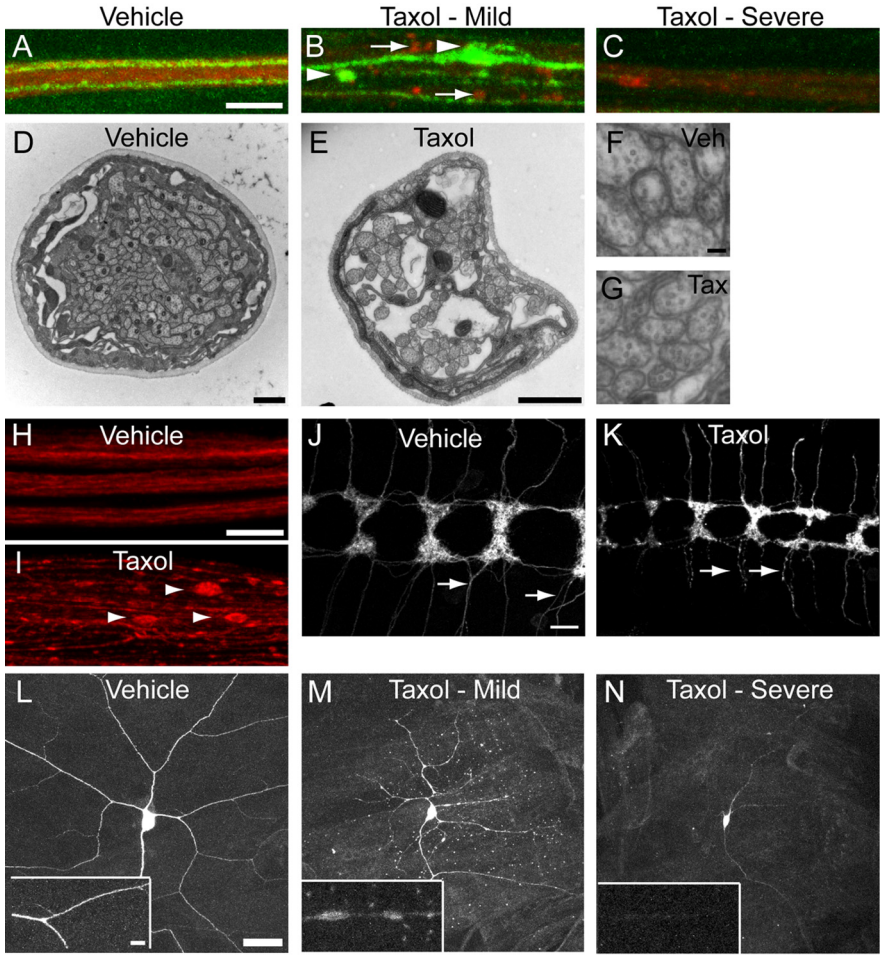

Figure 1. *Drosophila* peripheral axons are damaged or lost following taxol treatment. ***A–C***, The segmental nerve between segment A3 and A4 is visualized by Cy3-HRP (labels neuronal membranes, red). *ppkEGFP* labels a subset of sensory neurons. ***A***, Vehicle (DMSO) treated. ***B***, ***C***, 30 μM taxol treated. Axon degeneration was assessed 4 d after treatment initiation. In ***B***, an intermediate phenotype of swellings and fragmentation is visible with both EGFP (arrowheads) and with HRP (arrows). In ***C***, a more severe phenotype of EGFP and HRP loss is shown. Scale bar: (in ***A***) ***A***–D, 5 μm. ***D***, ***E***, EM cross-section images of nerves from wild-type (*Canton S*) vehicle- and taxol-treated animals. Scale bars: ***D***, ***E***, 1 μm. ***F***, ***G***, Zoomed-in images of axons from ***D*** and ***E***. Scale bar (in ***F***) ***F***, ***G***, 200 nm. ***H***, ***I***, Peripheral nerves stained with antibody to Futsch (22C10). Three nerves are shown in the vehicle; one defasciculated nerve is shown in the taxol-treated animal. Arrowheads point to prominent swellings in individual axons. Scale bar (in ***H***), ***H***, ***I***, 10 μm. ***J***, ***K***, Class IV sensory axons (*ppkgal4/+; UAS-CD8:GFP/+*) in the ventral nerve cord in vehicle (left) and taxol treated (right). Arrows point to intact axons (***J***) or swollen and fragmenting axons (***K***). Scale bar: (in ***J***) ***J***, ***K***, 10 μm. ***L–N***, Dendrites and cell body of ddaC sensory neurons in vehicle-treated (***L***) or 30 μM taxol-treated (***M***, ***N***) animals. Scale bar: (in ***L***) ***L–N***, 30 μm. Insets show portions of a dendrite from the larger images. Scale bar, 6 μm.

To identify molecules underlying axonal degeneration, we devised a novel paradigm for injuring axons in the fruit fly *Drosophila melanogaster* using the chemotherapy drug paclitaxel (taxol). In this model, axons swell, fragment, and are lost, and this loss is not caused by apoptosis. Axons injured in this paradigm can be protected by both NMNAT overexpression and loss of *wallenda/DLK*, demonstrating that the conserved axonal degeneration program mediates this taxol-induced neuropathy. To test the utility of this new model as a gene discovery platform, we performed a pilot RNAi-based screen and identified the MORN (membrane occupation and recognition nexus) family member *retinophilin* as a gene promoting degeneration. Importantly, the mouse ortholog of *retinophilin*, Morn4, also promotes mammalian axonal degeneration. Our work introduces a novel and clinically relevant axonal injury paradigm in *Drosophila* and has identified a role for MORN4 as a conserved component of the axonal degeneration program.

Materials and Methods

Fly strains. The following strains were used in this study: *Canton S* (wild-type), *elav-GAL4* (Yao and White, 1994), *pickpocket*^{5}*-EGFP* (referred to as *ppkEGFP*; Grueber et al., 2003), *pickpocket-CD8:3xGFP* (referred to as *ppkCD8GFP*; Shimono et al., 2009), *ppk-GAL4* (Grueber et al., 2007), *wnd*^{2} and *wnd*^{3} mutant alleles (Collins et al., 2006), *UAS-dmNMNAT* (MacDonald et al., 2006), *retin*^{1} (Venkatachalam et al., 2010), and *rtp*^{1} (Mecklenburg et al., 2010). All other strains are from the Bloomington Stock Center. All RNAi lines used are from the Vienna *Drosophila* RNAi Consortium (Retinophilin RNAi, 109000).

The *UAS-cytNMNAT1* expression construct was made using mouse *cytNMNAT1*, a mutant version of NMNAT1 with the nuclear localization disrupted (Sasaki et al., 2006). The full-length cytNMNAT1 was inserted into the pUAST expression vector and injected into *w*− fly embryos to create transgenic lines. A viable second chromosome insertion of this construct was used for experiments.

Genetics. For screening, females with homozygous *UAS-Dicer2, elavGAL4*, and *ppkEGFP* were crossed to homozygous *UAS-RNAi* males to generate progeny of either sex for analysis. For olfactory axotomies, females of a strain containing GAL4 driven by the promoter region of olfactory receptor 82a (*Or82a-GAL4*; Fishilevich and Vosshall, 2005), *UAS-CD8:GFP*, and *UAS-Dicer2* were crossed to *UAS-RNAi* males, and male progeny were analyzed. Other genotypes are as noted in figure legends.

Taxol treatment. Embryos from crosses were collected on grape plates, and first instar larvae of both sexes were washed off and resuspended in water with 30 μM paclitaxel (Tocris Bioscience) or an equivalent amount of DMSO (vehicle; Tissue Culture Support Center, Washington University School of Medicine). This liquid was then used to make up instant *Drosophila* media ("blue food;" Carolina Biological Supply). For large-scale screening, 12 well plates were used and plugged with Flugs (Genesee Scientific). Animals on taxol-treated food were incubated at 25°C for 4 d before dissection and analysis.

Immunocytochemistry. GFP signal was enhanced with Alexa Fluor 488-rabbit-anti-GFP (1:1000, Invitrogen). Other antibodies used were Cy3-HRP (1:1000, Jackson ImmunoResearch) and Rabbit anti-cleaved-Caspase 3 (1:200, Cell Signaling Technology). Secondary antibodies were obtained from Jackson ImmunoResearch and used at 1:1000 dilution.

Mouse DRG cultures. Embryonic day 12.5 mouse DRG neurons were cultured as described previously (Sasaki et al., 2009b) using embryos of both sexes. Lentiviral particles containing Morn4-targeting (5′-TGACCTTCTCAGATGGCTCAA-3′) or control shRNA sequences were packaged as described previously (Araki et al., 2004) and added to DRG cultures at 2 DIV. *In vitro* axotomy was performed at 7–8 DIV. Quantitative reverse transcriptase-PCR analysis of neuronal mRNA was performed using an ABI Prism 7900HT sequence detection instrument (Applied Biosystems) as described previously (Gerdts et al., 2011). Following insult, axonal degeneration was quantified from four phase-contrast images per well by ImageJ software that measures fragmentation of axon segments (Sasaki et al., 2009b). Briefly, axon images are bina-

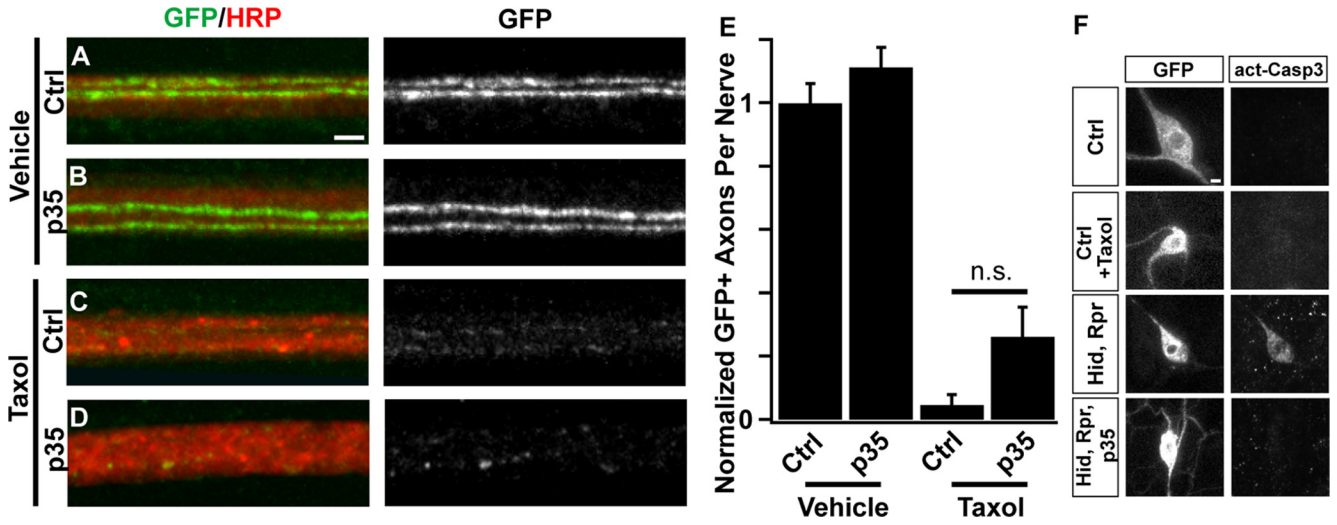

Figure 2. Axonal degeneration following taxol treatment is independent of apoptosis. ***A–D***, The viral anti-apoptotic protein p35 is unable to rescue sensory axon degeneration. Confocal images of single representative nerves labeled with *CD8:GFP* (green) and HRP (red). Genotype is *ppkgal4/+;UAS-CD8:GFP/+* (Ctrl, ***A*** and ***C***) or *ppkgal4/UAS-p35;UAS-CD8:GFP/+* (p35, ***B*** and ***D***). Animals were fed food containing vehicle (DMSO) (***A***, ***B***) or taxol (***C***, ***D***) for 4 d. Scale bar (***A***), 2 μm. ***E***, Quantification of GFP-labeled axons per nerve remaining after treatment from the genotypes shown in ***A–D***. $N = 21, 20, 18$, and 16 for the genotypes quantified. NS, Not significant ($p > 0.1$). ***F***, GFP (left column) and activated caspase 3 (right column) in control larvae (*ppkGAL4/+;UAS-CD8:GFP/+*) that are untreated (top) or treated with 30 μm taxol (second from top). Bottom two sets of images are controls showing caspase 3 activation following *ppkGAL4*-driven expression of *UAS-hid* and *UAS-reaper* (second from bottom), and loss of caspase 3 activation in flies expressing *UAS-hid*, *UAS-reaper*, and *UAS-p35* (bottom). Scale bar (***F***), 3 μm.

rized, and the ratio of fragmented to total axonal area axonal area is computed by using circularity as an indicator of fragmentation.

Microscopy. Images of *Drosophila* larval axons and adult brains were taken on a Nikon Eclipse E600 confocal microscope and are maximal *Z*-projections. Phase contrast images of DRG axons (20× objective) were taken using an inverted microscope (Eclipse TE 300; Nikon).

To analyze *Drosophila* axons, we took images of nerves between segments A3 and A4 of vehicle-treated third instar larvae or equivalently timed taxol-treated larvae. We counted the number of intact GFP-positive axons per nerve from each image (~100 μm length of nerve per picture). The average number of GFP+ axons/nerve in vehicle-treated control (wild-type) animals labeled with *ppkEGFP* was 1.97 ± 0.06 (SEM) ($n = 71$ nerves) and was not significantly different in animals labeled with *ppkCD8GFP*, or between wild-type and *rtp* mutant strains. For presentation, we normalized data to the average of the wild-type vehicle control of the relevant experiment. All analysis of peripheral sensory axons and olfactory axons was done blind to genotype. One-way ANOVA was used for statistical analysis unless otherwise specified. All error bars are SEM.

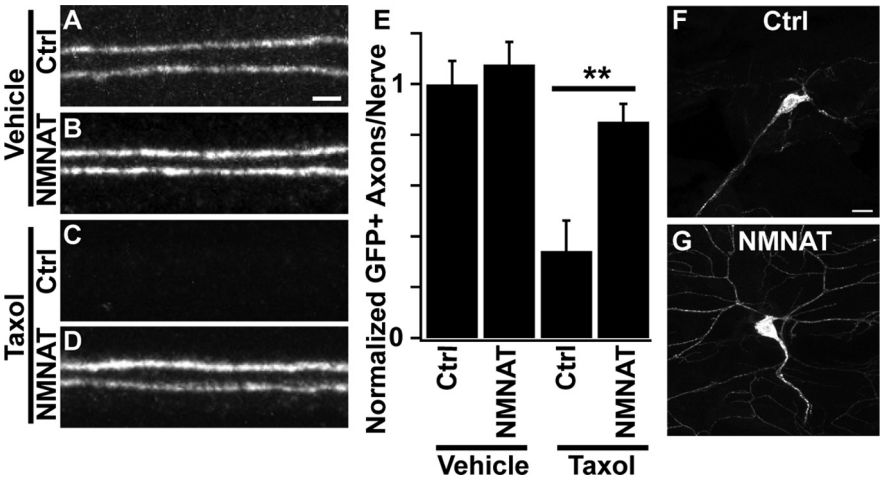

Figure 3. Overexpression of mouse NMNAT protects axons and dendrites from taxol-induced degeneration. ***A–D***, Confocal images of single representative nerves labeled with *ppkEGFP*. Genotypes are *elavgal4, ppkEGFP/+* (Ctrl, ***A*** and ***C***), or *UAS-cytNMNAT1/+;elavgal4, ppkEGFP/+* (NMNAT, ***B*** and ***D***). Animals were treated with DMSO (***A***, ***B***) or taxol (***C***, ***D***) for 4 d and then analyzed. Scale bar (***A***), 2 μm. ***E***, Quantification of GFP-labeled axons per nerve following taxol treatment in the genotypes shown in ***A–D***. $N = 10, 17, 17$, and 22 for the genotypes quantified. $^{**}p < 0.01$. ***F***, ***G***, Dendrites and cell bodies of ddaC neurons treated with taxol from control (same genotype as ***A*** and ***C***) and *UAS-cytNMNAT1*-expressing (same genotype as ***B*** and ***D***) animals. Scale bar (***F***), 10 μm.

For ultrastructural analysis of the *Drosophila* peripheral nerves, samples were fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc.) in 100 mM phosphate buffer, pH 7.2 overnight at 4°C. Samples were washed in phosphate buffer and postfixed in 0.5% osmium tetroxide (Polysciences Inc.)/0.08% potassium ferricyanide (Electron Microscopy Sciences)/100 mM phosphate buffer for 1 h, and subsequently in 1% tannic acid (Electron Microscopy Sciences, Fort Washington, PA)/100 mM phosphate buffer for 1 h. Samples were then rinsed extensively in dH_2O before *en bloc* staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 h. Following several rinses in dH_2O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 100 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate. Electron micrographs were taken on a transmission electron microscope (H-7500; Hitachi). Quantification of axon number was performed on 10 nonconsecutive sections of nerves from at least three independent larvae per treatment condition.

Results

Paclitaxel causes axonal injury and loss in *Drosophila*

Because only a few genes have been identified that regulate axonal degeneration, we sought to develop a model of axonal degeneration in *Drosophila* amenable to screens for genes that promote axonal demise. Loss-of-function of a protein participating in the axonal degeneration cascade would be predicted to cause axonal

protection following an insult and would be a putative therapeutic target. To maximize throughput, we wanted to devise an axonal injury paradigm that did not depend on mechanically injuring each animal individually. In addition, we wanted to use a clinically relevant insult. *Drosophila* has been used as a model of neurodegeneration following cisplatin toxicity (Podratz et al., 2011), and so may respond to chemotherapeutics in a manner analogous to mammals. We therefore attempted to mimic the neuropathy caused in human patients by paclitaxel (taxol) administration. To do this, we fed larval-stage animals food laced with taxol. By beginning treatment after animals have reached the larval stage, we avoid disrupting neurogenesis or axon pathfinding, events that are complete at the time of hatching (for review, see Prokop, 1999). Following treatment, we examined larval peripheral axons using a genetically encoded GFP that labels class IV sensory neurons and their projections (*ppkEGFP*) (Grueber et al., 2003). Because this marker highlights only a few neurons per hemisegment, it allows single axon resolution within the segmental nerves leading to the ventral nerve cord.

We find that treatment with taxol causes axonal destruction and loss (Fig. 1). Axonal injury following taxol treatment was apparent after 3 d, with maximal injury after 4 d. In the majority of nerves, GFP is severely fragmented or lost following taxol treatment (Fig. 1*C*). Interestingly, in nerves where taxol has not caused complete GFP-positive axon loss, we see axonal swellings and debris, hallmarks of mammalian axonal degeneration (Coleman, 2005) (Fig. 1*B*,*I*). HRP labeling occasionally shows axonal swellings (Fig. 1*B*) or overall staining intensity decreases (Fig. 1*C*), likely due to the degeneration of non-GFP-labeled nerves. Examination of nerves using electron microscopy reveals an approximately twofold decrease in axon number (vehicle, 80.9 ± 3.9 axons per nerve; taxol, 42.7 ± 5.1 axons per nerve; $p < 0.001$, Student's *t* test) (Fig. 1*D*,*E*), suggesting that the GFP loss often corresponds to the frank loss of axons. Nerves in taxol-treated animals have large holes (Fig. 1*E*), and free organelles are occasionally seen in these areas. However, remaining axons appear relatively unaffected and contain microtubule profiles (Fig. 1*F*,*G*). In addition to the phenotypes in GFP-labeled axons, we also observe peripheral nerve defasciculation. In these defasciculated nerves, swellings of single axons that accumulate the microtubule-associated protein Futsch are apparent (Fig. 1*H*,*I*). Sensory axons are still present within the neuropil of the ventral nerve cord following taxol treatment, however the ladder pattern of sensory endings is often disrupted and individual axons are fragmented (Fig. 1*J*,*K*). Using the same *ppkEGFP* label, we also examined the effects of taxol on the dendrites of multidendritic sensory neurons in the larval cuticle. Similar to its effects on axons, taxol causes sensory dendrite swelling and loss (Fig. 1*L*–*N*). To assess whether taxol treatment caused "dying back"-like retraction, we examined larval neuromuscular synapses. Unlike genetic models in *Drosophila* that disrupt the cytoskeleton (Massaro et al., 2009), we observe normal apposition of presynaptic and postsynaptic markers following taxol treatment, indicating that our model of taxol-induced degeneration does not cause motoneuron synaptic instability (data not shown). Together, these results highlight the range of degenerative phenotypes resulting from paclitaxel treatment in flies and reveal morphological similarities to degenerating mammalian axons.

Taxol-Induced Axonal Degeneration is Not Secondary to Apoptosis

In patients, neuronal cell death is not a major contributor to paclitaxel-induced neuropathy (Lee and Swain, 2006; Windebank and Grisold, 2008). To test whether this is also true in our *Drosophila* model, we overexpressed the baculoviral anti-apoptotic protein p35 in sensory neurons (Hay et al., 1994). Expression of p35 with the *ppkGAL4* driver (Grueber et al., 2007) effectively blocks activation of Caspase 3 following induction of apoptosis in sensory neurons via the forced expression of the proapoptotic genes *hid* and *reaper* (Fig. 2*F*). However, when p35-expressing animals are treated with taxol, their peripheral sensory axons are not preserved (Fig. 2*A*–*E*), indicating that axonal loss in this model is not secondary to apoptosis in the sensory cell bodies. In addition, cell bodies of taxol-treated animals fail to label with anti-activated Caspase-3 antibodies (Fig. 2*F*). These results are consistent with studies that have found a role for caspases in dendrite pruning but not in injury-induced degeneration (Schoenmann et al., 2010; Tao and Rolls, 2011). These data highlight the independence of the mechanism of taxol-induced axonal degeneration from that of apoptosis.

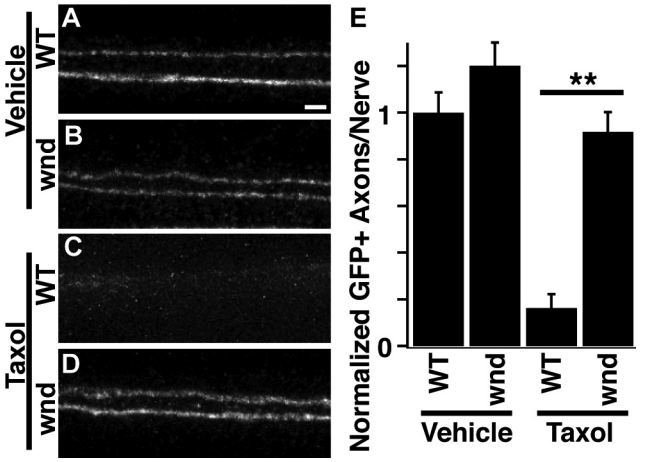

Figure 4. Wallenda is required for taxol-induced axonal degeneration in *Drosophila*. ***A–D***, Confocal images of single representative nerves labeled with *ppkEGFP*. Animals were treated with DMSO (***A***, ***C***) or taxol (***B***, ***D***) for 4 d and then analyzed. WT genotype is heterozygous $wnd^3/+$; *wnd* genotype is wnd^3/wnd^2. ***A***, Wild-type vehicle; ***B***, *wnd* mutant vehicle; ***C***, wild-type taxol; ***D***, *wnd* mutant taxol. Scale bar (***A***), 2 μm. ***E***, Quantification of axonal preservation in the genotypes shown in ***A–D***. $N = 54, 54, 58$, and 77 for genotypes quantified. $^{**}p < 0.01$.

NMNAT overexpression can block paclitaxel-induced axonal degeneration

To determine whether paclitaxel-induced axonal degeneration in flies shares molecular mechanisms with axonal degeneration in mammalian sensory neurons, we asked whether molecules known to block axonal degeneration in mice also protect axons in our *Drosophila* model. First, we tested whether overexpression of NMNAT could protect fly sensory axons from degeneration. NMNAT is protective for both sensory axons and dendrites in other injury paradigms in the fly (Tao and Rolls, 2011; Wen et al., 2011). We made transgenic flies that allow for the targeted expression of cytoplasmically targeted mouse NMNAT1 (cyt-mNMNAT1) (Sasaki et al., 2009a). Following taxol treatment, axons from neurons overexpressing cyt-mNMNAT1 were preserved, whereas control axons were lost (Fig. 3). cyt-mNMNAT1 expression also blocked dendrite degeneration following taxol treatment (Fig. 3*F*,*G*). These results illustrate conservation of the protective effects of NMNAT for taxol-induced neuropathy between invertebrates and vertebrates (Wang et al., 2002).

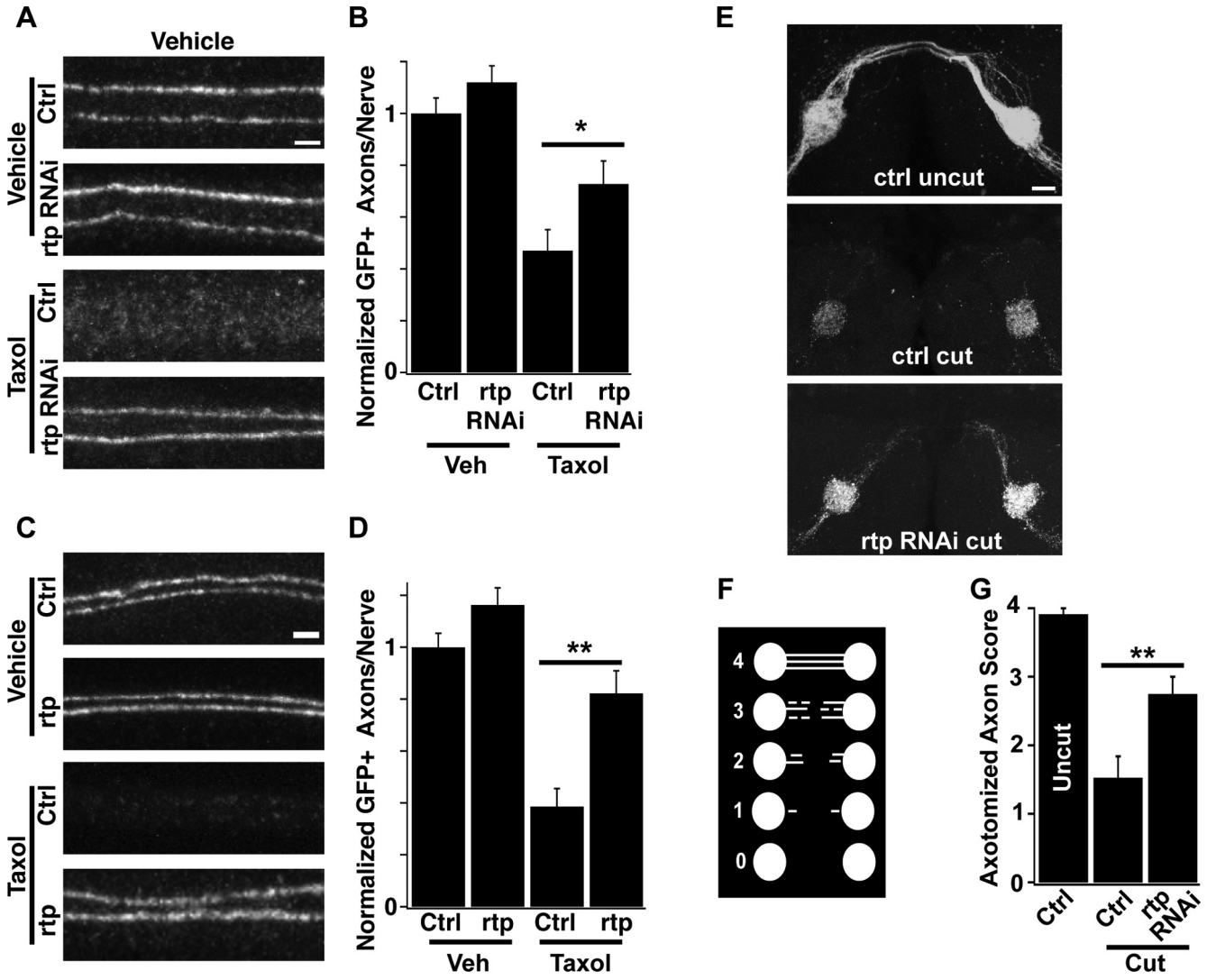

Figure 5. Retinophilin promotes axonal degeneration. ***A***, Comparison of GFP-labeled axonal maintenance with knockdown of *retinophilin* in vehicle-treated (top two images) or taxol-treated animals (bottom two images). Genotypes are *UAS-DCR/+;elavGAL4, ppkEGFP/+* (Ctrl) and *UAS-DCR/+;UAS-rtpRNAi/+;elavGAL4, ppkEGFP/+* (rtp RNAi). Scale bar, 2 μm. ***B***, Quantification of *rtp RNAi* and control axons as shown in ***A***. For the graph, $N = 23$, 26, 38, and 33 nerves for each genotype and treatment. $^*p < 0.05$ between taxol-treated groups, Student's *t* test. ***C***, Images of GFP-labeled axons in vehicle-treated (top two images) or taxol-treated animals (bottom two images) that are of the genotypes *ppkCD8GFP/+* (Ctrl) or *ppkCD8GFP/+;rtp^1/retin^1* (*rtp*). ***D***, Quantification of *rtp* mutant and control axons as shown in ***C***. For the graph, $N = 58$, 37, 51, and 40 nerves for each genotype and treatment. $^{**}p < 0.01$. ***E***, Representative images of olfactory axons. All are labeled with *Or82a-gal4,UAS-CD8:GFP*. Top, Uncut control (no RNAi); middle, cut control; bottom, *rtp* RNAi expressed by *Or82a-gal4*. Scale bar, 15 μm. ***F***, Schematic of quantification scheme for olfactory axotomies. A score of 0 indicates the lack of any detectable axons outside of glomeruli; a score of 4 indicates that axons reach and cross the central commissure. Scores of 1–3 rank axons by number and length of remaining axons or axonal fragments. ***G***, Axotomy scores of the genotypes shown in ***C***. $N = 18$, 12, and 10 for genotypes quantified. $^{**}p < 0.01$.

Wallenda/DLK is required for taxol-induced axonal degeneration in *Drosophila*

To examine whether endogenous genes required for axonal degeneration in mammals also participate in taxol-induced degeneration in flies, we examined the effect of loss of the MAP3K Wallenda (DLK in mammals). Wallenda participates in the degeneration cascade following *Drosophila* olfactory axotomy or mouse axonal injury (Miller et al., 2009) and is required for injury signaling following *Drosophila* nerve crush (Xiong et al., 2010). Following taxol treatment, *wallenda* mutants displayed robust protection of peripheral axons (Fig. 4). Similar protection is observed when transgenic RNAi is used to knockdown *wallenda* exclusively in neurons (data not shown). These data demonstrate that known pathways used in mammalian axonal degeneration are conserved members of the taxol-induced degeneration cascade in flies. Furthermore, it illustrates that loss-of-function analysis in taxol-treated animals could be useful to identify novel players in axonal degeneration that may be relevant to mammalian axonal degeneration.

RNAi interference identifies a role For *retinophilin* in axonal degeneration

We undertook a pilot RNAi screen looking for genes that, similar to *wallenda*, would block axonal degeneration when knocked down. We used a collection of inducible, transgenic RNAi strains available from an international consortium (Dietzl et al., 2007). These RNAi lines were expressed under the control of UAS elements using the pan-neuronal driver *elav-GAL4* to express both the RNAi and Dicer2, which enhances efficacy of RNAi knockdown. Under these conditions, ~80% of genes with known phenotypes in the nervous system give the expected phenotype when knocked down (Dietzl et al., 2007).

We limited our analysis to *Drosophila* genes with clear mammalian orthologs as determined by Homologene NCBI software.

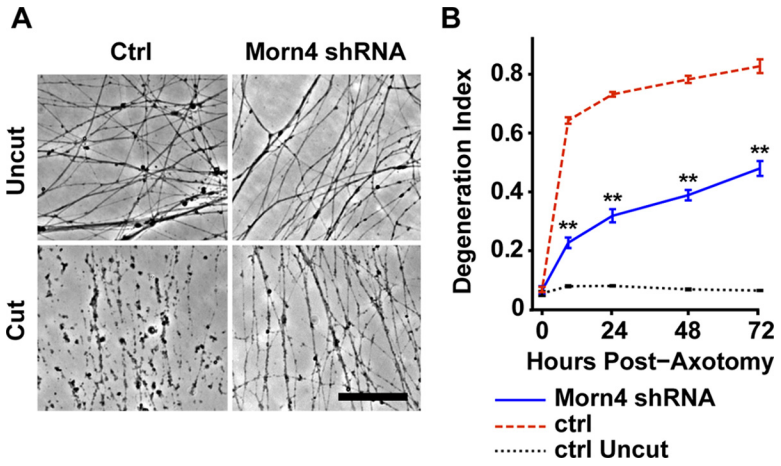

Figure 6. Mouse Morn4 participates in axonal degeneration. ***A***, Representative images of axons from cultured mouse DRG neurons infected with shRNAs targeting luciferase (ctrl) or Morn4. Axons are uncut (top images) or 24 h post-axotomy (bottom images). Scale bar, 100 μm. ***B***, Quantification of degeneration index of control shRNA (red trace)- or Morn4 shRNA (blue trace)-infected axons at 0, 9, 24, 48, and 72 h post-axotomy, and uncut control shRNA-infected axons (black trace). Double asterisks indicate $p < 0.001$ between cut control shRNA and Morn4 shRNA.

A subset of 490 RNAi strains was chosen for our pilot screen that was enriched for proteins with enzymatic functions and for genes known to function in the nervous system; this selection was done using Flybase QueryBuilder (www.flybase.org). These strains were screened for degeneration or preservation of *ppkEGFP*-labeled axons following taxol intoxication. From this collection we identified the MORN family member *retinophilin* [*rtp*, also called *undertaker* (*uta*)]. Retinophilin participates in termination of phototransduction in the fly retina (Mecklenburg et al., 2010; Venkatachalam et al., 2010) and in store-operated calcium release and phagocytosis in fly embryonic macrophages (Cuttell et al., 2008). It contains four MORN repeats, regions that can bind to phospholipid membranes (Ma et al., 2006). Knockdown of *retinophilin* protected axons from chemotherapy-induced peripheral neuropathy (Fig. 5*A*). The RNAi protection is quantitatively modest, but consistent (Fig. 5*B*). To test whether loss of Retinophilin is responsible for this phenotype, we generated a transheterozygote between two previously characterized loss-of-function mutant alleles of *rtp* (Mecklenburg et al., 2010; Venkatachalam et al., 2010). Axons in this *rtp* mutant are preserved following taxol treatment (Fig. 5*C*,*D*), confirming that removal of retinophilin is indeed axoprotective.

Retinophilin could be specifically involved in chemotherapy-induced neuropathy in the peripheral nervous system, or could play a broader role in injury-induced axonal degeneration. To test whether *retinophilin* participates in other types of degeneration, we turned to the well established model of olfactory axotomy in *Drosophila* (MacDonald et al., 2006; Doherty et al., 2009). The cell bodies of olfactory neurons are located in the antenna, and their projections extend into the brain, cross a central commissure, and synapse onto bilateral glomeruli (Fig. 5*E*, top). By removing the third antennal segment, olfactory axons are severed (MacDonald et al., 2006). We labeled a subset of olfactory axons via expression of GFP driven by the *Or82a-GAL4* line (Fishilevich and Vosshall, 2005). Twenty-four hours after axotomy, wild-type axons have degenerated almost completely, with only a few axonal fragments remaining outside the glomeruli (Fig. 5*E*, middle). In contrast, axons in which *retinophilin* has been knocked down exclusively within the labeled neurons show a delay in this degeneration, with many axons still extending near the commissure (Fig. 5*E*, bottom). Using a scoring system to rank the number and length of remaining axons, we find that axon loss is inhibited following *rtp* knockdown, but much less so than via expression of cyt-mNMNAT1 (NMNAT score = 3.8 ± 0.1) (Fig. 5*F*,*G*). Together, these results suggest that Retinophilin promotes the normal axonal degeneration program initiated by both traumatic and toxic axonal injury, and that it functions in a cell-autonomous manner.

The mammalian *rtp* ortholog Morn4 is required for axonal degeneration

The closest mammalian ortholog to *Drosophila retinophilin* is Morn4 (50% identity at the amino acid level) (Mecklenburg, 2007). Morn4 is an uncharacterized protein in mouse and human that also contains four MORN repeats. To test whether Morn4 functions like Retinophilin in promoting axonal degeneration, we knocked down Morn4 expression in cultured mouse embryonic DRG neurons and then cut their axons. Control axons swell and then degenerate within the first 12 h, a process that can be quantified using image analysis software that detects axon fragments as circular particles (Sasaki et al., 2009b; Gerdts et al., 2011). Knockdown of Morn4 was very efficient, with only ~1% of mRNA remaining by quantitative PCR analysis (1.3 ± 0.7%). Knockdown of Morn4 significantly reduced axonal degeneration following axotomy (Fig. 6*A*,*B*). Protection was evident for at least 72 h, with knockdown cultures displaying signs of early degeneration (swellings) but no fragmentation. Thus Morn4 may participate in an intermediate step of axonal degeneration, following axonal swelling but preceding fragmentation. The involvement of mouse Morn4 in axonal degeneration highlights the ability of this new *Drosophila* chemotherapy-induced neuropathy model to identify genes that participate in axonal degeneration in mammals.

Discussion

Here we describe a novel *Drosophila* model of chemotherapy-induced peripheral neuropathy and demonstrate that the process shares molecular components with the mammalian axonal degeneration program. We then use this platform to identify the Retinophilin/Morn4 protein as a component of an evolutionarily conserved axonal self-destruction cascade.

A Drosophila model of chemotherapy-induced peripheral neuropathy

Chemotherapy drugs such as paclitaxel (taxol) and vincristine cause a dose-limiting peripheral neuropathy in humans. Taxol causes sensory neuropathy which manifests as pain and loss of sensation in extremities, while vincristine causes both sensory and motor dysfunction. These drugs are used widely in both pediatric and adult patients to treat aggressive metastatic cancer (Sparano, 2000; Bradley and Hussain, 2008; Marsh, 2009; Moore and Pinkerton, 2009; Grossi et al., 2010). Because we know so little about the mechanisms causing this severe side effect, we currently have no way to prevent axonal degeneration in these patients.

Axonal degeneration is not only a component of chemotherapy-induced neuropathy but is also a hallmark of many neurological

diseases including hereditary neuropathies, neurodegenerative diseases such as Alzheimer's Disease, and trauma including spinal cord injury. Hence, the need is great to identify therapeutic strategies that could slow or block axon loss and thereby prevent progression of neurological symptoms in these and other disorders. To identify such targets, we have developed a model of toxic neuropathy in *Drosophila*. Treating larval-stage *Drosophila* with paclitaxel causes axonal degeneration that is anatomically and molecularly similar to that occurring in rodents and, potentially, in human patients undergoing chemotherapy treatment. In *Drosophila*, as in humans, paclitaxel preferentially damages sensory rather than motor axons (Park et al., 2011). This conservation provides a platform to identify molecules that could serve as future therapeutic targets.

Retinophilin/Morn4 participates in axonal degeneration

We identified Rtp/Morn4 as an evolutionarily conserved component of the axonal degeneration cascade. Furthermore, we observe that Rtp/Morn4 must be present for efficient degeneration following both neurotoxicity (i.e., chemotherapy administration) and traumatic injury (i.e., axotomy). Hence, while the screen uses a neurotoxin as the axonal damaging agent, it can identify components that function downstream of multiple stimuli. Importantly, our screen was able to identify a subtle effect of *rtp* on the timing of axonal demise. When using RNA interference to implicate a gene in a process, the ability to detect small changes is useful since gene expression is not completely eliminated. Importantly, we were able to confirm this finding using a true loss-of-function mutant in flies. While identification of a *Drosophila* gene that promotes axonal degeneration is important, we are most interested in genes with an evolutionarily conserved role in the process. Lentiviral-mediated gene knockdown in mouse sensory neurons (Gerdts et al., 2011) is a powerful secondary screen allowing us to identify such genes. Using these methods in combination, we demonstrate that the role of Rtp/Morn4 in the progression of axonal demise is evolutionarily conserved.

While we do not know the mechanism of action of Morn4 in axonal degeneration, Retinophilin has been implicated in both localization of signaling molecules required for termination of phototransduction (Mecklenburg et al., 2010; Venkatachalam et al., 2010) and regulating cytoplasmic calcium levels preceding phagocytosis (Cuttell et al., 2008). Morn4 contains four membrane occupation and recognition nexus (MORN) repeats that can target proteins to lipid membranes (Ma et al., 2006). Potentially, these domains could bring Morn4 to a membrane surface to modulate lipid activity or second messenger generation. Interestingly, Morn4 has recently been implicated in the neuroprotective process of preconditioning following oxygen-glucose deprivation (Dai et al., 2010). Further work to clarify the molecular role of Morn4 in these two seemingly opposite cascades will be necessary.

Axonal degeneration mechanisms and implications for neurological disease

While we know a great deal about the molecular control of the apoptotic process and how it can contribute to disease, we know comparatively little about axonal degeneration despite its importance in a wide variety of pathological states. Many activating insults ultimately use a common pathway (Vohra et al., 2010) that includes the mitochondrial permeability transition pore (Forte et al., 2007; Barrientos et al., 2011) and can be blocked by axonal expression of NMNAT isoforms (Araki et al., 2004; Conforti et al., 2009; Sasaki et al., 2009a; Yahata et al., 2009; Babetto et al., 2010; Sasaki and Milbrandt, 2010). In addition, kinases including DLK, JNK, IKK, and GSK3 promote axonal degeneration (Miller et al., 2009; Gerdts et al., 2011). The relationships among these proteins, as well as the identity of upstream and downstream components of the axonal degeneration program, are still mysterious.

Screens in both *Drosophila* and mouse (Gerdts et al., 2011) have the potential to reveal molecular targets for therapeutic intervention to block axonal degeneration in the early stages of neurodegenerative diseases, hereditary neuropathies, or before chemotherapeutic treatment. Here we have used a clinically relevant insult in *Drosophila* to identify a role for Retinophilin/Morn4 in promoting axonal degeneration. Future screening should yield additional candidates whose inhibition could disable this degenerative process.

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