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Axon regeneration mechanisms: insights from *C. elegans*

Lizhen Chen and **Andrew D. Chisholm***
Division of Biological Sciences, Section of Neurobiology, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093

Abstract

Understanding the mechanisms of axon regeneration is of great importance to develop therapeutic treatments for spinal cord injury or stroke. Axon regeneration has long been studied in various vertebrate and invertebrate models, but until recently had not been analyzed in the genetically tractable model organism *Caenorhabditis elegans*. The small size, simple neuroanatomy, and transparency of *C. elegans* allows single fluorescently labeled axons to be severed in live animals using laser microsurgery. Many neurons in *C. elegans* are capable of regenerative regrowth, and can in some cases re-establish functional connections. Large-scale genetic screens have begun to elucidate the genetic basis of axon regrowth.

Keywords

laser axotomy; MAPK cascade; DLK-1; axon fusion; microtubule dynamics; genetic screen; growth cone; calcium; cyclic AMP

Axon regeneration: *C. elegans* enters the field

In the mature mammalian Central Nervous System (CNS), axons rarely regenerate following injury, accounting for permanent functional deficits. Despite numerous efforts on mechanistic investigation of axon regeneration, the molecular basis of regrowth remains elusive. About three decades ago, it was shown that injured mature CNS axons can regrow into sciatic nerve grafts transplanted into the lesion site [1, 2], suggesting the failure of axon regeneration in mature CNS might be mainly due to the inhibitory microenvironment. Much effort has been devoted to identifying the myelin-based inhibitors, such as Nogo, Myelin Associated Glycoprotein (MAG), or Oligodendrocyte Myelin glycoprotein (OMgp), often using cultured neurons as assays [3-5]. Extrapolating these studies to whole animal models is often not straightforward, however. For example, some investigators have found improved CNS regeneration in compound mutant mice lacking MAG, Nogo and OMgp [6], whereas others have found no such improvement [7, 8]. These studies illustrate the challenge of interpreting the complex lesions involved in spinal cord injury, and the subtle effects of genetic background and functional compensation. For large-scale screening of other regeneration factors, and for systematic study of the biology of axonal regrowth, a simple genetically accessible model would be desirable.

* Corresponding Author: (chisholm@ucsd.edu) .

For decades *C. elegans* has been a tractable model for the genetic analysis of neuronal development. The nervous system of the adult hermaphrodite is composed of 302 neurons, whose genealogy, morphology. and synaptic connectivity are almost invariant in the wild type [9]. *C. elegans* axon guidance involves the same core conserved pathways (netrins, ephrins, semaphorins, Slit/Robo) as other animals. Despite the intensive analysis of *C. elegans* axon outgrowth and guidance in development, it was not until recently that regenerative regrowth of *C. elegans* axons after injury was demonstrated [10]. Several types of *C. elegans* neuron display regrowth and have been studied in depth [11-13]. Of the many pathways known to influence vertebrate axon regrowth, several play similar roles in *C. elegans* (Table 1). Conversely, pathways discovered to have critical roles in *C. elegans* regrowth, such as the Dual Leucine zipper Kinase 1 (DLK-1) cascade, play comparable roles in other organisms (Table 2). Thus, although *C. elegans* lacks myelin and other molecules specific to the vertebrate CNS, the basic biology of axon regenerative growth appears to be conserved. In this review, we highlight recent advances in axon regeneration studies using *C. elegans*, focusing on emerging insights into cellular and molecular events of regrowth.

C. elegans axon regeneration: models and techniques

The first studies of axon regrowth in *C. elegans* examined GABAergic motor neurons, which in adults comprise a series of 19 cells (Dorsal D-type motorneurons 1-6 [DD1-6], Ventral D-type motorneurons [VD1-13]) with dorsoventrally projecting commissures (Figure 1A). Motor neuron commissures are well-separated from other axons and easily severed at the lateral midline. Neither DD nor VD neurons are polarized in the ‘textbook’ sense, but extend a single process with distinct pre- and postsynaptic regions, however the commissures of DD neurons can be considered axonal, in that they extend to dorsal presynaptic termini, whereas VD neuron commissures extend to postsynaptic regions and so are more dendrite-like. Both DD and VD commissures regrow to similar extents [11], suggesting axonal or dendritic character does not strongly affect motor neuron regrowth. The peripheral mechanosensory neurons (Anterior Lateral Mechanosensory [ALM], Posterior Lateral Mechanosensory [PLM], Anterior Ventral Mechanosensory [AVM], Posterior Ventral Mechanosensory [PVM]) are also competent to regrow, and extend long, large-diameter microtubule-rich processes from posterior to anterior (Figure 1B). For simplicity, we refer to these sensory processes as ‘axons’ as they extend presynaptic branches.

Axons severed by laser microsurgery undergo a stereotyped sequence of responses. Laser surgery (under conditions used in our laboratory; [11, 14]) generates a 1-5 μm diameter break that expands to ~20 μm over the next few hours due to the retraction of the proximal and distal ends. This retraction appears distinct from the acute degeneration observed in mouse, in which the cut ends die back hundreds of microns within 30 minutes after injury [15]. The proximal stump begins to swell and extend short filopodial protrusions within 3-6 hours after injury; the exact timing of the onset of regrowth may be influenced by the method of immobilization used during axotomy. By 6 hours post axotomy, a growth cone-like structure with filopodial protrusions forms from the proximal stump and starts to extend. In mechanosensory axons, which extend along the body axis, regrowth continues for 2-3 days. Motor axon commissures extend shorter distances along the dorsoventral axis; regrowth is slower than during development [11].

In both motor and mechanosensory axons, regrowth is more accurate in early larval stages and becomes highly error-prone in later larvae and adults [11]. The overall ability of motor axons to regrow appears to decline sharply in larval development [16], whereas mechanosensory axons regrow to about the same extent in larvae and young adults [11]. Nevertheless, motor axons often re-establish functional connections [10], perhaps because of

the larger target area presented by dorsal muscle arms. It is less clear whether regrowing mechanosensory axons can restore function, although they can make synaptic branches to the appropriate target area [14].

An important consideration in regeneration studies is how to measure regrowth. In *C. elegans* regrowth has been quantitated in different ways depending on cell type. Regrowth of motor axons has been assessed either as the fraction of regrowing axons that reach the dorsal nerve cord within 24 h, the fraction that form a morphological growth cone at 24 hours, or the time taken to form a growth cone [13]. By the adult stage, about 70% of severed motor axons form growth cones, yet fewer than 10% of these reach the dorsal nerve cord. Similarly, regrowth of the ventrally directed AVM process has been measured as fraction of axons reaching the ventral cord [12]. In contrast, for ALM or PLM axons, which extend for long distances along the lateral body, it is simplest to directly measure the length of the regrown process and its branches [11]. Despite the isogenic nature of the *C. elegans* wild type, and all attempts to standardize axotomy procedures, the extent of axon regrowth varies considerably from animal to animal, with standard deviations of ~1/3 of the average regrowth. It is not clear whether this stochastic variation reflects unavoidable experimental variability in axotomy or inherent biological variation in regrowth.

Almost all *C. elegans* neurons survive axotomy, but the regrowth response depends greatly on the lesion location. Most axotomy paradigms use lesions distal from the cell body (>50 μm away in *C. elegans*), after which the severed proximal stump reforms a growth cone. When axons are severed closer (<30 μm) to the cell body, the cell responds by sprouting new processes from the soma [12]. Similar somatic or dendritic sprouting responses have been observed in many other neurons after proximal axotomy [17, 18], but it is unclear to what extent such processes resemble the regrowth from severed axon stumps.

An alternative to laser axotomy is to study spontaneous axon breakage, either in the wild type or in axon fragility mutants. The only reported example of spontaneous breakage in wild type *C. elegans* involves pruning of branches of the Posterior Ventral neuron D (PVD) [19], although this has not yet been exploited in screens for regeneration defects. Mutants with reduced β-spectrin/UNC-70 undergo repeated cycles of axon breakage and regrowth [20], and have provided a highly sensitized assay for modifiers of regrowth. Although the resulting nervous system is aberrant, this genetic background partly obviates the need for laser axotomy to induce axon breaks, and facilitates large-scale screens.

Injury triggered signals: the roles of Ca^{2+} and cAMP

Axon injury activates a number of processes in the injured cell that collectively allow transformation of the proximal axon stump into a motile growth cone (Figure 2). We first focus on the signaling cascades that appear to be directly triggered by axonal injury. Ca^{2+} and cAMP signals have long been implicated in axon regeneration in cultured neurons [21], in rat sciatic nerve [22] and in the zebrafish CNS [23]. Elevated cAMP levels can overcome the inhibitory effects of CNS myelin on dorsal root ganglion (DRG) axons in the 'conditioning lesion' paradigm [24]. As in other organisms, *C. elegans* axons respond to injury with a rapid and dramatic increase in axonal Ca^{2+} [14]. Genetic and pharmacological manipulations showed that the level of Ca^{2+} correlates with subsequent regeneration. As the Ca^{2+} response to damage is transient, it remains to be determined how such a brief elevation in Ca^{2+} is distinguished from normal neuronal activity, and how it is transduced to promote regrowth many hours later. Nevertheless, both Ca^{2+} and cAMP appear to be rate-limiting for PLM axon regrowth. Among other effects, both Ca^{2+} and cAMP can promote the reconnection of proximal and distal axon fragments by fusion, preventing degeneration of the distal fragment (Box 1). The regrowth-promoting effects of Ca^{2+} and cAMP are

mediated by protein kinase A (PKA), as treatment with the PKA inhibitor H89 reduces axon regrowth while genetic elevation of PKA activity has the opposite effect. The targets of the Ca^{2+}/cAMP/PKA pathway include the bZip transcription factor CRH-1/CREB, which is not required for overall regrowth, but appears to function in formation of synaptic branches [14].

In addition to PKA, other second messenger-regulated kinases play roles in regrowth. A chemical genetic screen of ~100 small molecules with kinase-modulating effects found that protein kinase C (PKC) promotes *C. elegans* axon regrowth [25]. The role of PKC in regrowth can vary depending on the experimental system: in some animals PKC has been correlated with regrowth [26], but in mammals PKC mediates glial inhibition of regrowth [27]. It is possible that different isoforms of PKC account for these divergent roles; *C. elegans* encodes several PKC-related proteins, and it is not yet known which are sensitive to the drugs used in the small-molecule screen [25].

Transducing injury signals: the central role of the DLK-1 pathway

In 2009, studies in *C. elegans* identified the conserved MAPKKK DLK-1 as essential for axon regeneration in motor and mechanosensory neurons [13, 28]. DLK-1 was first identified as a regulator of *C. elegans* synapse formation [29]. DLK-1 is not required for axon outgrowth in development yet is essential for axon regrowth, both after laser axotomy and in axon fragility mutants [28] [13]. DLK-1 acts cell autonomously and at the time of injury to promote early steps in growth cone formation; moreover, overexpression of DLK-1 is sufficient to enhance regenerative growth. Thus, DLK-1 is a rate-limiting switch in axon regeneration. In mechanosensory neurons, the DLK-1 MAPK cascade functions via MAK-2, a MAPKAP kinase, to promote mRNA stability and local axonal translation of the CCAAT/enhancer-binding factor homolog, CEBP-1 [28]. The targets of CEBP-1 in *C. elegans* axon regrowth remain to be identified. Why should a bZip transcription factor be locally translated in the injured axon? Does CEBP-1 function locally in the axon, or does it get retrogradely transported to the nucleus, or both? Other members of the CCAAT/enhancer-binding protein family activate transcription of α-tubulin in response to axon injury in mammalian neurons [30, 31]. Speculatively, activation of the DLK-1 pathway might have both local and transcription-mediated effects on the microtubule (MT) cytoskeleton. Fascinatingly, the DLK-1 pathway is also required for a transcriptional response to MT depolymerization in *C. elegans* neurons [32]. It will be important to determine if this MT-sensing role of the DLK pathway is related to regeneration.

The DLK family of kinases is critical for axon regeneration in insects and mammals. Wallenda (Wnd), the *Drosophila* ortholog of DLK-1, is upstream of a cell-autonomous injury signaling cascade that involves JNK and Fos [33]. Intriguingly, vesicular transport of Wnd in the injured axon is required for retrograde injury signaling and regeneration. DRG neurons derived from DLK gene trap mutant mice display reduced regrowth; this function of DLK in promoting regeneration appears to require c-Jun [34]. Thus, the DLK cascade has emerged as a key conserved pathway in axon regrowth.

Given the importance of DLK activity in axon regrowth, it is imperative to understand how DLK is regulated in response to damage. In *C. elegans* and *Drosophila*, DLK-1 or Wnd are negatively regulated by the ubiquitin E3 ligases RPM-1 and Highwire, respectively, during synapse formation [29, 35]. It has also been proposed that Phr1, the mouse ortholog of RPM-1, degrades DLK in axons during development [36]. Injury signals could downregulate RPM-1, or interrupt the interaction between RPM-1 and DLK-1, preventing DLK-1 from degradation. However, although *rpm-1* mutants display slightly improved motor neuron regeneration [13], they do not display significant differences in mechanosensory neurons [13, 28]. It is important to note that the RPM-1 ligase probably

only targets active DLK-1 [29][37], suggesting DLK-1 may be activated by other injury signals, and that its activation state may or may not be prolonged in *rpm-1* mutants depending on the cell type and site of axotomy. DLK kinases are thought to autophosphorylate, and may be activated by inhibition of other negative regulators [38]. As one of the earliest responses to damage is an elevation of intracellular Ca^{2+} it is possible that Ca^{2+} plays a role in DLK activation. Indeed, the effects of elevating Ca^{2+} or cAMP require DLK-1, consistent with Ca^{2+} acting upstream of DLK [14].

Another MAP kinase pathway involving the MAPKKK MLK-1 contributes to growth cone formation of *C. elegans* GABA motor neurons [16] (Fig. 2). Loss of function in the MLK-1 cascade results in reduced regrowth, although the regeneration block is not as severe as that of *dlk-1* mutants. The DLK and MLK pathways likely cross-activate and may share downstream targets. Overall, the DLK-1 pathway seems to play a more central role, in that overexpression of DLK-1 almost completely suppresses the effects of loss of MLK-1, whereas overexpression of MLK-1 only partly rescues the effects of loss of DLK-1. Why might two closely related MAPK pathways both be required in regrowth? Answering this question will require a more detailed examination of the dynamics of regrowth in single and double mutants and in other cell types. It is possible that coordinated activation of the two parallel cascades could be important in ensuring robustness of the regrowth response.

Local activation of factors such as DLK-1 at the site of injury must be somehow relayed to the cell body, and the role of retrogradely transported injury signals in larger neurons has long been studied [39]. In *Drosophila*, Wnd/DLK is retrogradely transported on vesicles after injury [33]. Although as yet there is no direct evidence for retrograde signals in *C. elegans*, results from a large-scale genetic screen suggest vesicle trafficking is critical in regrowth [40]. Unexpectedly, several genes required for PLM regrowth are thought to have specific roles in synaptic vesicle (SV) endocytosis, including UNC-26/Synaptojanin and UNC-57/Endophilin [40]. As many genes required for general synaptic transmission are not required for regrowth, the role of the SV recycling genes in regeneration seems independent of synaptic function. Endocytic trafficking may play a role in vesicular transport of retrograde injury signals [41]. The requirement for UNC-57/Endophilin can be bypassed by elevated DLK-1 activity [40], suggesting endocytosis genes might function in transduction of molecules such as DLK-1 itself.

The cell biology of regeneration: dynamics of the MTcytoskeleton

During axon regeneration the reformation and extension of growth cones is intimately dependent on the MT cytoskeleton. In mature axons, MTs are assumed to be stabilized in a polarized fashion, with plus ends away from the cell body (Figure 3). The effects of axotomy on the axonal MT cytoskeleton have been extensively studied in cultured neurons [17, 42, 43]. During formation of a new growth cone, axonal MTs must become highly dynamic and are likely nucleated from new sites near the area of injury [44]. Recent progress in *C. elegans* has allowed imaging of MT dynamics directly in regenerating axons [40].

For effective growth cone extension, MT dynamic instability must be precisely moderated: excessive instability (caused by MT catastrophe or MT severing) is likely as detrimental to axon regrowth as is excessive stability or insufficient severing [45]. These considerations indicate the difficulty of addressing the role of MTs in axon regrowth via genetics or pharmacology *in vivo*. Nevertheless, MT stabilization can promote axon regeneration in mammalian CNS, possibly by a combination of direct effects on axon regrowth and indirect effects on glial scarring [46, 47]. In these studies, the clinically approved anti-cancer drug Paclitaxel (Taxol) was used to stabilize microtubules. Taxol binds to polymerized β-tubulin

and at low concentrations promotes polymerization at the plus end [42]. These results suggest moderate levels of MT stabilization can promote axon regrowth.

A genetic screen for regulators of regeneration has identified potential endogenous regulators of axonal MT dynamics [40]. Several genes involved in MT dynamics, including the end-binding protein EBP-1 are required for regrowth *in vivo*. Conversely, this screen also identified a novel intrinsic inhibitor, EFA-6. EFA-6 is a member of the Exchange Factor for Arf6 (Arf6 GEF) family, yet studies in *C. elegans* embryos suggest it also functions independent of Arf6 to negatively regulate MT growth [48]. In adults, loss of function in EFA-6 enhanced regrowth whereas overexpression of EFA-6 blocked regrowth [40]. In *efa-6* mutant axons MTs are partly stabilized, whereas EFA-6 overexpression reduces the number of dynamic MTs. Notably, the effects of EFA-6 overexpression could be partially overcome by injection of Taxol, further supporting the notion that EFA-6 is a catastrophe factor in the axonal MT cytoskeleton [48]. These findings, in concert with work on mammalian MT stabilization, underscore the critical importance of MT dynamics in regrowth. It will be interesting to explore the roles of mammalian EFA6 family members in axon regrowth, and whether injury-induced signaling pathways interact directly with factors involved in MT dynamics.

Navigating a strange environment: axon guidance and the extracellular environment in regrowth

Regenerating axons must navigate an environment very different in spatial scale and molecular composition from that in which they developed. Most notably, the inability of axons to regrow in the adult mammalian CNS is partly attributable to changes in the composition of adult CNS myelin. Other extracellular matrix (ECM) molecules important for axon outgrowth can be growth-promoting or growth-inhibiting during regeneration (Table 1). In *C. elegans*,several axon guidance cues with known roles in development are either not required in regrowth or play very different roles, depending on the cell type. For instance, Slit/SLT-1 plays a repulsive role during AVM development, yet becomes inhibitory for regrowth [12, 49]. SLT-1 is attractive for PLM development, yet becomes inhibitory in PLM regrowth [40]. Moreover, UNC-40/DCC and UNC-129/TGFβ are required for AVM axon guidance during development, but dispensable for guidance of regrowing AVM axons. In contrast, CED-10/Rac1, UNC-34/Ena/VASP and MIG-10/Lamellipodin are important for AVM regrowth, but not in development [12]. In summary, there is only partial overlap between factors required in development versus those required in adult regrowth.

Axon injury in mammals results in a ‘glial scar’. The effects of the glial scar on regrowth are extremely complex: scars contain inhibitory factors such as chondroitin sulfated proteoglycans (CSPGs) [50] but may also have regrowth-promoting roles [51]. While no exact analog of the glial scar has yet been studied in *C. elegans* axon injury models, the basement membrane may have a related role. Loss of function in peroxidasin/PXN-2, a conserved extracellular matrix peroxidase involved in basement membrane biosynthesis, leads to increased axon regeneration, suggesting the *C. elegans* ECM contributes to an inhibitory environment in regrowth [52]. Interestingly, recent work in zebrafish has shown that hydrogen peroxide (H_2O_2) released from epidermal wounds promotes axon regeneration [53]. As peroxidases use H_2O_2 as substrate, it is also possible that reduced peroxidasin levels result in higher extracellular H_2O_2, leading to enhanced regrowth. A more detailed analysis of H_2O_2 and the basement membrane during regrowth is clearly warranted.

Concluding remarks

With the advent of laser axotomy, *C. elegans* has rapidly emerged as a simple model for studies of axon regeneration. Despite the many differences between the biology of mammalian and nematode neurons and the undoubted differences between laser surgery and spinal cord injury, indications are that some aspects of the *C. elegans* model can be extrapolated to more complex paradigms. Conversely, laser axotomy has now been extended to other organisms, including zebrafish [54, 55], *Drosophila* [56], and mouse [57]. Clearly, the study of *C. elegans* axon regeneration is in its infancy, and many questions remain unanswered (Box 2). We end by sketching three areas where *C. elegans* can make an impact on axon regeneration studies in the future.

First, a *raison d'etre* of studying axon regrowth in *C. elegans* must be its accessibility to large-scale screens. It has already been possible with routine methods to screen over 650 genes for roles in regrowth [40]. Axon fragility mutants [20] also facilitate large scale screening without performing axotomy. Much effort is being devoted to developing microfluidic technologies for high throughput laser surgery and imaging [25, 58]. Such technologies will have an increasing impact as they become more widespread, and raise the prospects of genome-wide screening for modulators of regrowth.

A second strength of the *C. elegans* model is the ease of analyzing combinatorial genetic interactions. A lack of inhibitory signaling alone is not sufficient to promote axonal regrowth in the mammalian CNS, suggesting combinatorial approaches will be needed to achieve maximal regrowth [59]. The analysis of such combinatorial interactions through genetics is straightforward in *C. elegans* by the ease of construction of compound mutants. Although the number of *C. elegans* protein-coding genes is close to that of humans, most gene families are represented by a smaller number of genes in the worm, thus it is possible to test a larger fraction of relevant combinations.

Finally, *C. elegans* offers excellent prospects for *in vivo* cell biology of axon regrowth. Single *C. elegans* axons are easily visualized, and MT-based transport dynamics can be studied in intact animals [60, 61]. As genetic and chemical screens identify molecules with key roles in regrowth we can expect to see increasing emphasis on protein localization and dynamics in response to injury in the intact animal.

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Box 1

Degeneration of the distal fragment and fusion of axon fragments

In many organisms the distal axon fragment created by axotomy undergoes a stereotyped and regulated degeneration process known as Wallerian degeneration [62]. Wallerian degeneration may promote regeneration by allowing development of a microenvironment that facilitates regrowth from the proximal stump [55]. In *C. elegans*, distal fragments seem to undergo degeneration as indicated by beading and disappearance of the GFP label in distal fragments [11]. It is unclear whether this distal degeneration is mechanistically related to Wallerian degeneration in other organisms, nor whether degeneration of the distal fragment is stimulatory for axon regeneration in *C. elegans*.

The mechanism of distal degeneration has yet to be fully explored in *C. elegans*. Interestingly, the DLK MAPK cascade required for regrowth of the proximal axon is also important for distal degeneration in *Drosophila* and mouse [63, 64]. In DLK/Wnd mutant flies, degeneration of olfactory neuron distal axons after injury is much slower than in wild type. However, DLK/Wnd has also been reported to suppress a Wallerian degeneration-like phenomenon in spectrin mutant flies [63]. DLK might have degeneration-promoting and degeneration-repressing effects depending on the cellular context; it will be interesting to learn if *C. elegans* DLK-1 affects distal degeneration.

Degeneration of the distal axonal fragment can be prevented by direct reconnection (fusion) of the regenerating axon with distal fragments. Reconnection by fusion is frequently observed after axotomy of *C. elegans* mechanosensory axons [14, 65]. Two modes of fusion can be distinguished: direct ‘end-to-end’ or type I fusion involves reconnection of the severed stumps, versus ‘end to side’ or type II connection of the regrowing proximal axon to the distal fragment (Figure I) [14]. Ultrastructural studies show that the membranes of the two fragments become physically continuous [14], as does their cytoplasm [66]. The end-to-side pattern of fusion is of particular interest as it suggests regrowing axons may be attracted to, and specifically recognize, their distal fragments, and that the distal fragment itself actively participates in reconnection. Experiments in which two adjacent axons of different subtypes are severed suggest fusion may exhibit cell-type specificity [66].

Fusion of severed axon fragments has been described in several organisms [67-70], although the molecular mechanisms remain little understood. With a few exceptions, such as the giant axons of molluscs [71] and the recently discovered autofusions of PVD dendrites in *C. elegans* [19], neuronal processes generally do not fuse in development, suggesting injury triggers a non-developmental program. The membrane fusogen EFF-1 is important for fusion of severed axon fragments [14] and for the autofusion of PVD dendritic branches. Expression of membrane fusogens such as EFF-1 could provide a means to promote rapid regeneration by fusion.

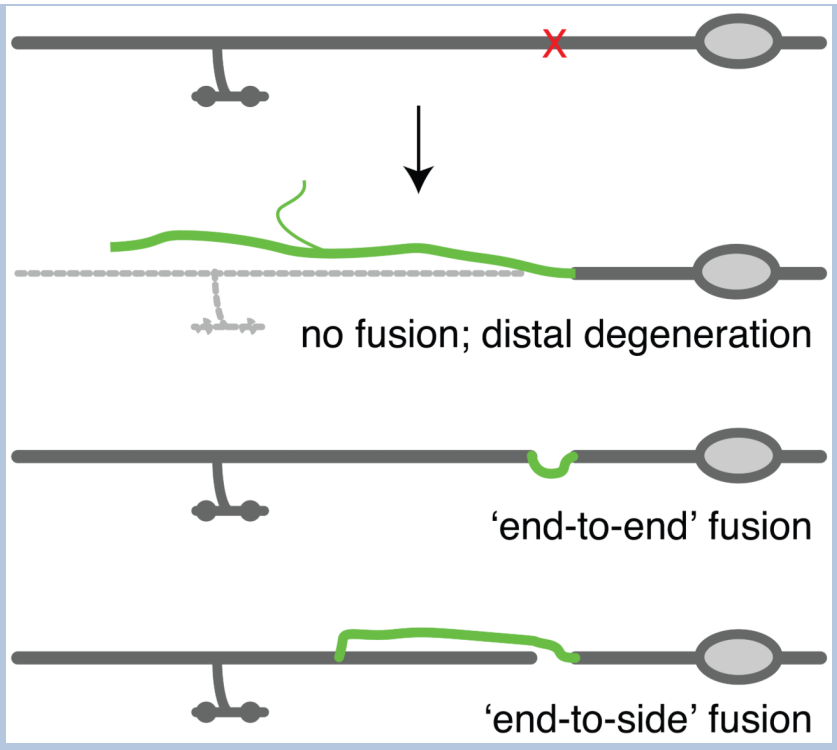

Figure I. Distal degeneration and fusion in mechanosensory axons

Mechanosensory (PLM) axons typically undergo any of three types of regrowth. Usually, the proximal axon regrows without contacting the distal fragment; the distal fragment undergoes degeneration. Less often, the regrowing axon fuses with the distal fragment, preventing distal degeneration. Fusion can be 'end to end' or 'end to side'; the frequency of reconnection by fusion is somewhat sensitive to the transgenic background and the method of axotomy [14].

Box 2

Outstanding Questions

- How are DLK kinases regulated by axonal injury? Do they respond to alterations in Ca^{2+} or in MT dynamics?
- What are the cellular targets of the DLK pathway? Does locally translated CEBP-1 play a local role in axons?
- How are new MTs nucleated after axotomy, and how are their dynamics regulated during regrowth?
- How do axonal fragments fuse? Is the regrowing axon specifically attracted to the distal fragment? Are membrane fusogens such as EFF-1 induced by injury, and can their overexpression promote reconnection?
- What factors contribute to the age-dependent decline in axon regeneration? How do they relate to the observed structural changes in *C. elegans* neurons? Can they be overcome?

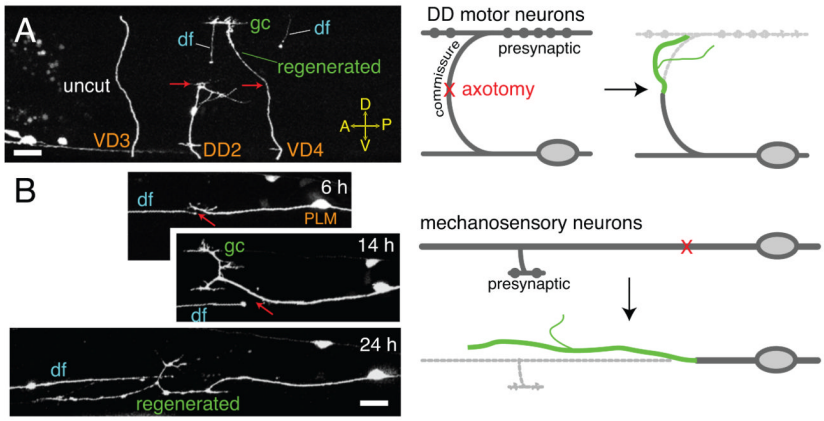

Figure 1. Types of neurons used to study regeneration

(A) Diagram (right) and images (left) of GABAergic motor neuron axotomy and regrowth at 24 h postaxotomy. df, distal fragment; gc, growth cone. Regrown process shown in green. Transgenic marker P*unc-25*-GFP(*juIs76*). (B) Diagram (right) of the PLM mechanosensory neuron showing position of laser axotomy relative to the synaptic branch and cell body. Confocal images (left) of PLM regrowth at 6 h, 14 h and 24 h postaxotomy (different animals). Transgenic marker, P*mec-7*-GFP(*muIs32*). Scales, 10 μm.

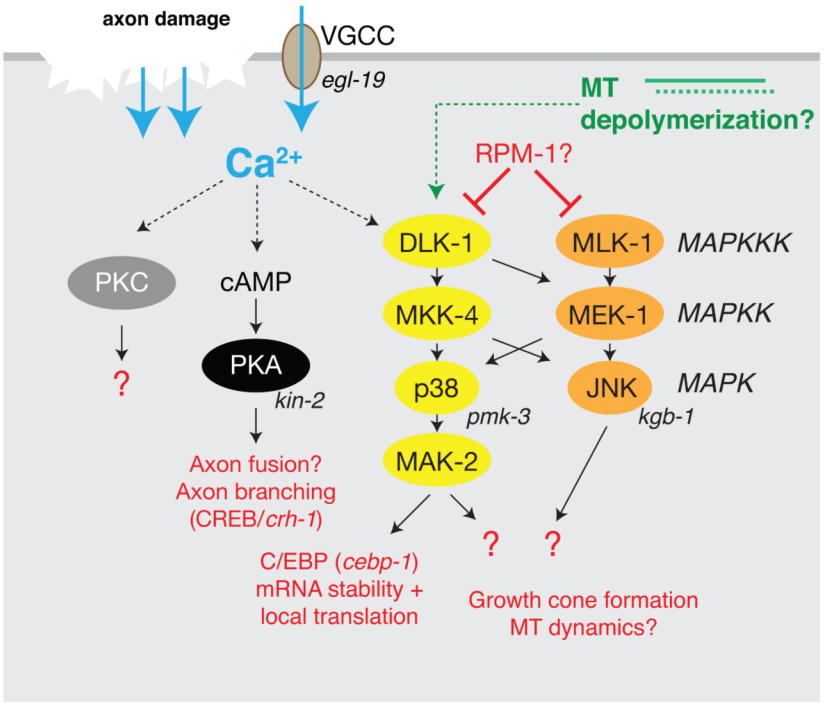

Figure 2. Injury-triggered signaling pathways

Axon injury triggers elevation of axonal Ca^{2+} by several mechanisms. Ca^{2+} elevation leads to activation of adenylyl cyclase and elevated cAMP levels, leading to PKA activation. PKC may be also activated by the injury-induced Ca^{2+} transient. Injury signals, possibly Ca^{2+} or MT depolymerization, result in activation of DLK-1 and the entire DLK-1/MKK-4/PMK-3 cascade. The MLK-1/MEK-1/KGB-1 pathway is activated in parallel by unknown signals. The DLK-1 pathway stabilizes CEBP-1 mRNA in axons, and is likely to have other targets.

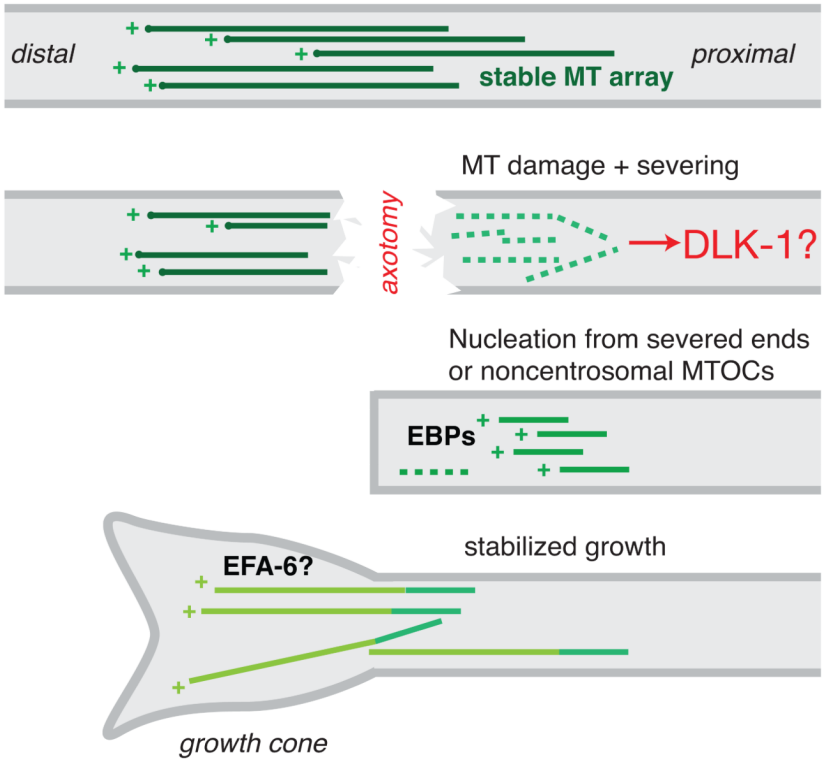

Figure 3. The MT cytoskeleton in axon regrowth

Highly simplified overview of possible changes in the MT cytoskeleton during regrowth, based on work in many organisms. Mature axons have stable polarized MT arrays with MT plus ends located distally from the soma. Axotomy disrupts MTs directly, and may trigger additional local severing of MTs into tubulin subunits, either via calpains or specific MT-severing enzymes. MT depolymerization may be sensed by the DLK-1 pathway. New axonal MTs are then nucleated, possibly from the newly formed plus ends or from as yet unknown noncentrosomal MT organizing centers (MTOCs). How polarity is re-established in the regrowing axon is not yet clear. Newly formed MTs may be highly dynamic (i.e. undergoing repeated catastrophe and regrowth) but for successful growth cone extension, MT growth must become more stable. In *C. elegans* neurons the plus end binding protein EBP-1 is required for regrowth, and the putative MT catastrophe factor EFA-6 inhibits regrowth [40].

Table 1

Roles of conserved regrowth pathways in *C. elegans*

	Gene or pathway	Function in axon regeneration (studies in other organisms)	*C. elegans* ortholog or equivalent	Function in *C. elegans* regrowth*
Second messengers	Ca^{2+}	Promoting/facilitating [72] (through PKA)	Ca^{2+}	Promoting [14]
	cAMP	Promoting through CREB [73, 74]	cAMP	Promoting [14]
Axon guidance molecules	Ephrin/Eph	Inhibitory [75-77]	EFNs/VAB-1	Guidance [11]
	Netrin/DCC	Inhibitory [78]	UNC-6/UNC-40	Inhibitory [12]
	Semaphorin/plexin	Inhibitory [76, 79]	MAB-20, SMP-1,2/PLX-1,2	Small effect [40]
	Wnts	Various; Inhibitory [76, 80]	5 Wnts	CWN-2 required [40]
	Slit/Robo	Promoting [81]	SLT-1/SAX-3	Inhibitory [12] [40]
Signal transduction	TGFβ	Inhibitory [50]	UNC-129, DBL-1, etc.	No effect [12]
	Neurotrophic factor/TrK	Promoting [82]	TRK-1	No effect [40]
GTPases, kinases and phosphatases	PKA	Promoting [26, 73]	KIN-1,2	Promoting [14]
	PKC	Promoting [26, 83] Inhibitory [27]	PKC-1-3	Promoting [25]
	PI3K	Promoting [84]	AAP-1, AGE-1	No effect [40]
	PTEN	Inhibitory [85]	DAF-18	No effect [40]
ECM, cell surface, cell adhesion	CSPGs	Inhibitory (via Rho/ROCK) [86]	Nonsulfated chondroitins	Not tested
	L1CAM	Promoting[87]	SAX-7	Required [40]
	Nogo, MAG, Omgp	Inhibitory[5]	not present	
Gene expression	KLF4	Inhibitory [88]	KLF-1	Not tested
	c-Jun	Required [89]	JUN-1	Required [14]
	CREB	Promoting [90]	CRH-1	Required for branching [14]

* results mostly refer to mechanosensory axons.

Table 2

Axon regeneration genes identified in *C. elegans* studies

	Genes and pathways	Function in *C. elegans* axon regeneration	Homologs in other species	Regenerative function in other species
Kinases	DLK-1	Essential [13, 28], function in part through regulating axonal translation [28]	Wallenda	Promoting [33]
			MAP3K12	Promoting [34]
	MLK-1	Promoting [16]	MLK-4	Unknown
	EFF-1	Involved in fusion [14]	(nematode-specific)	
ECM	PXN-2	Inhibitory [52]	Peroxidasin	Unknown
Signaling	EFA-6	Inhibitory [40]	EFA6	Unknown