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Review Article

Axon regeneration in *C. elegans*: Worming our way to mechanisms of axon regeneration

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ABSTRACT

How axons repair themselves after injury is a fundamental question in neurobiology. With its conserved genome, relatively simple nervous system, and transparent body, *C. elegans* has recently emerged as a productive model to uncover the cellular mechanisms that regulate and execute axon regeneration. In this review, we discuss the strengths and weaknesses of the *C. elegans* model of regeneration. We explore the technical advances that enable the use of *C. elegans* for in vivo regeneration studies, review findings in *C. elegans* that have contributed to our understanding of the regeneration response across species, discuss the potential of *C. elegans* research to provide insight into mechanisms that function in the injured mammalian nervous system, and present potential future directions of axon regeneration research using *C. elegans*.

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Contents

1. General features of the *C. elegans* axon regeneration model . . . 0
2. Techniques for axon injury: lasers and genetics . . . 0
3. Genetic screens identify regulators of axon regeneration . . . 0
4. Current areas of investigation . . . 0
5. Dual leucine zipper kinase-1 . . . 0
6. Cytoskeletal dynamics . . . 0
7. Age vs. axon regeneration . . . 0
8. Axon fusion . . . 0
9. Environment . . . 0
10. Future areas of investigation . . . 0
References . . . 0

1. General features of the *C. elegans* axon regeneration model

The adult *C. elegans* hermaphrodite is a transparent cylinder approximately 1 mm long that contains 302 neurons (Fig. 1). The worm's nervous system includes motor, sensory, interneuron, and polymodal neurons and can be divided into 118 distinct classes of neurons, including GABAergic, cholinergic, chemosensory, mechanosensory, oxygen sensing, osmoceptors and proprioceptors (White et al., 1986). The development and positions of individual neurons are invariant from worm to worm. Together with the transparent cuticle, the invariant development of the nervous system makes the worm a tractable model of axon regeneration. The transparent cuticle provides the ability to sever individual fluorescently labeled axons with a laser and monitor severed axons for regenerative ability in vivo. The lack of inter-worm variability allows the effects of genetic and chemical manipulations on axon regeneration of specific, individual neurons to be compared between worms.

Most neurons are contained in ganglia located in the head and tail of the worm. In the head, the nerve ring is considered 'the brain' of the worm and consists of a neuropil of predominantly sensory neurons but also motor neurons and interneurons. Additional motor and mechanosensory neurons are located along the body of the worm. Among the various types of neurons, regenerative ability has been

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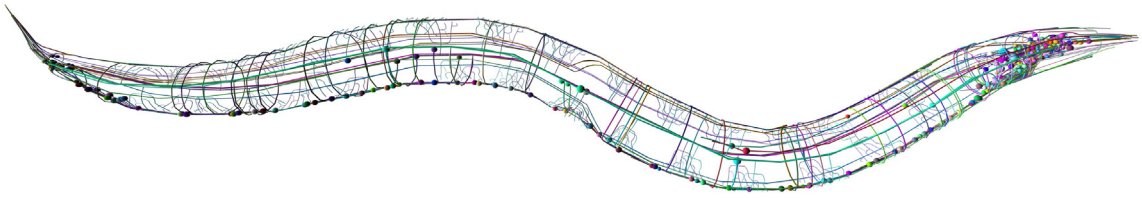

Fig. 1. *C. elegans* nervous system. The *C. elegans* nervous system consists of 302 neurons. Most cell bodies lie in the head (right) and tail (left) of the worm. GABA motor neuron cell bodies are located along the ventral nerve cord of the worm and extend commissures circumferentially to the dorsal nerve cord. Mechanosensory neurons extend axons along the lateral aspect of the worm. Image courtesy of OpenWorm.org and VirtualWorm.

characterized for the GABA motor neurons, the mechanosensory neurons, and a few other types of neurons. The regenerative ability of most neuron types has not yet been studied.

The field of *C. elegans* axon regeneration began with the discovery that GFP-labeled GABA motor neurons regenerate after being severed with a femtosecond laser (Yanik et al., 2004). The GABA neuron cell bodies are positioned along the ventral nerve cord and extend commissures circumferentially towards the dorsal nerve cord (White et al., 1986; McIntire et al., 1993) (Fig. 2a). They receive synaptic input from excitatory cholinergic neurons and make inhibitory en passant synaptic connections with body wall muscles. The commissural axons are accessible for laser surgery and imaging and have been used extensively for regeneration studies (Fig. 2b). When a GABA motor axon commissure is cut at the midline, the two severed ends retract and the axon segment proximal to the cell body forms a growth cone 6–12 h post-injury, then extends at 2–3 μm/h towards the dorsal cord (Wu et al., 2007). Axon regeneration in mature animals is imprecise, as the axons that regenerate are often branched and occasionally grow around the distal stump rather than follow the same developmental path (Yanik et al., 2004; Wu et al., 2007). Not only do the axons regenerate, the regenerated axons are able to restore functional connections with the musculature (Yanik et al., 2004; El Bejjani and Hammarlund, 2012).

The mechanosensory or 'touch' neurons extend long axons along the length of the worm and regulate response to touch (Chalfie and Sulston, 1981) (Fig. 2a). Like the GABA neuron commissures, the axons of the touch neurons are relatively easy to sever and study. Further, because these axons extend along the anterior-posterior axis, regenerative growth can be monitored over a much longer distance than the GABA neurons. Severed mechanosensory neurons regenerate more quickly, at a rate of approximately 7 μm/h. However, in these neurons the majority of growth occurs between 24 and 48 h (Wu et al., 2007). Unlike the GABA neurons, behavioral output dependent on regeneration of the touch neurons has not been described.

Because the nervous system is invariant between animals, the same single neuron can be studied across multiple animals. As such, *C. elegans* is an excellent model to analyze variability in regenerative ability in individual neurons. For example, although the GABA neurons can regenerate when severed with a femtosecond or pulsed UV laser, only 70% of injured axons form growth cones, while the remaining 30% do not (Wu et al., 2007; Hammarlund et al., 2009; Byrne et al., 2011). Variable regeneration is also observed in other neuron types, including the touch neurons (Yanik et al., 2006; Wu et al., 2007). One key determinant of regeneration success at the level of individual neurons is the rise in calcium observed after injury (Ghosh-Roy et al., 2010; Pinan-Lucarre et al., 2012; Yan and Jin, 2012). Axotomy of the PLM mechanosensory neurons causes an increase in localized calcium at the site of injury and the amount of calcium or cAMP determines regeneration success (discussed in more detail below) (Ghosh-Roy et al., 2010). It is likely that other cellular mechanisms besides calcium also contribute to the variability in regeneration.

The *C. elegans* nervous system also contains 56 glia. As in other invertebrate and early vertebrate systems, the glia wrap around nerve-nerve and nerve-muscle synapses, presumably to insulate signals from the surrounding environment (Ward et al., 1975; White et al., 1986). They also wrap around extending dendrites and axons to enhance extension and provide guidance. The role of glia in *C. elegans* axon regeneration has not been well characterized. In contrast to vertebrate glia, *C. elegans* glia do not produce myelin. The absence of myelin inhibition means the neuronal mechanisms that regulate axon regeneration can be studied in the absence of this glial inhibitory pathway in *C. elegans*. However, interactions between regenerating neurons and their supporting cells (including glia) are undoubtedly important for regeneration. Further studies are necessary to address this important question.

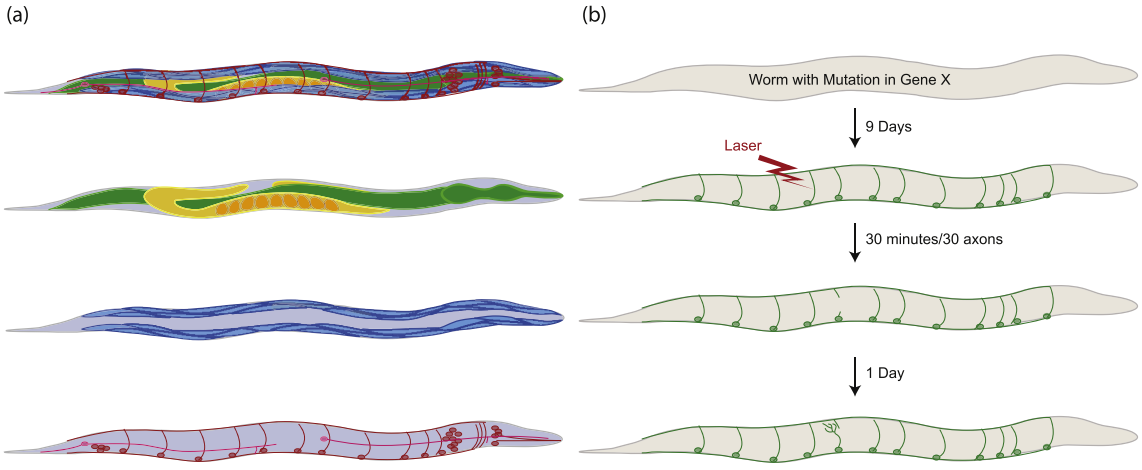

Fig. 2. Axon Regeneration in *C. elegans*. (a) Worms contain an alimentary system (green), reproductive system (yellow), muscle (blue), and nervous system (red and pink). Axon regeneration is mostly studied in the GABA motor neurons (red) and the mechanosensory neurons (pink). (b) Worms carrying a mutation in a gene of interest and expressing GFP in a specific type of neuron can be built and used to study axon regeneration in approximately 9 days. The diagram depicts a GABA axon severed with a laser and regenerating 24 h later. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Finally, *C. elegans* is a well-developed model organism, with a rapid life cycle and easy cultivation. The well-characterized genome facilitates the identification and investigation of the genetic and molecular determinants of axon regeneration (Box 1). Regulators of nervous system development and function are largely conserved between *C. elegans* and mammals. Approximately 40% of the worm's genome is conserved with the mammalian genome (Shaye and Greenwald, 2011). In addition, the worm's small size allows it to be easily cultured in large numbers on agar-filled petri plates containing non-pathogenic *E. coli*. The worm has a three-day life cycle and ten-day lifespan. The relatively short life cycle and lifespan facilitate quick construction of mutant strains and rapid analysis of the mechanisms that regulate regeneration at various ages, respectively. A strain containing a mutation in a gene of interest and a fluorescent marker expressed in a specific population of neurons can be built in approximately two weeks. Since regeneration is typically assessed within 24–48 h after injury, the role of a given gene in axon regeneration can be quickly investigated.

2. Techniques for axon injury: lasers and genetics

Traditional mechanical techniques to injure axons, such as crushing nerves with forceps or severing nerves with a scalpel, have not been applied in *C. elegans*, due to the small size of the worm. Instead, optical or genetic techniques are used to sever axons. The first investigations of axon regeneration in *C. elegans* were carried out on axons that had been severed with amplified Ti-sapphire lasers that produce femtosecond pulses of near infrared light (780–800 nm) (Yanik et al., 2004). These lasers supply 10–40 nJ of energy with 200 fs pulses at 1 kHz and vaporize 1–2 μm of axon. Subsequently, unamplified femtosecond lasers that supply an order of magnitude less energy (1–3 nJ) with approximately 150 femtosecond pulses at a higher pulse rate (80–90 MHz) have been used to sever axons (Wu et al., 2007). The amount of energy supplied is the main determinant of the size of the injury; lower amounts of energy create a smaller area of damage (Bourgeois and Ben-Yakar, 2008). However, pulse rate also influences the specificity of injury; pulse rates in the kHz range create less damage and more specific injuries than pulse rates in the MHz range (Wu et al., 2007). Contrary to expectations, high trains of low energy pulses increase regeneration frequency compared to low trains of pulses of the same energy, indicating the total amount of energy used to injure the axon is not the sole determinant of regeneration success (Bourgeois and Ben-Yakar, 2008). Together, these experiments indicate femtosecond laser ablation using high trains of low energy pulses delivered at kHz pulse rates severs axons with minimal physical or thermal damage to the surrounding tissue.

Besides Ti-sapphire lasers, many axon regeneration studies use other types of pulsed lasers, such as nitrogen-pumped dye lasers (historically used for cell ablations) or solid-state lasers (Rao et al., 2008; Williams et al., 2011). These lasers are a fraction of the price of Ti-sapphire lasers. However, since they produce longer pulses, they may create a larger area of damage than Ti-sapphire lasers. In addition, these lasers may have difficulty severing axons deep within the animal. Therefore, they are excellent for cutting widely-spaced axons located just under the skin, such as the GABA and touch neurons.

In general, laser axotomy is a widely applicable technique, with the significant experimental advantage that the time and location of injury can be precisely controlled and limited to individual axons of interest. Although laser axotomy proceeds one neuron at a time, dedicated application of laser techniques can result in the study of a large number of genes (Chen et al., 2011; Nix et al., 2014).

For experimental questions that require even higher numbers of injured axons, a genetic mutation in the gene encoding β-spectrin (*unc-70*) has been used. Lack of β-spectrin causes axons to break when the animal moves (Hammarlund et al., 2007), likely due to defects in the spectrin-based membrane cytoskeleton (Xu et al., 2013; He et al., 2016). Loss of *unc-70* function has no obvious effect on the development of motor axons. However, after the axons develop, the resulting fragile axons break as the worm moves. In this genetic background, axons successively break and regenerate without the use of laser axotomy. Thus,

Box 1

C. elegans is an efficient genetic model with a highly conserved genome.

C. elegans is a conserved, tractable, and efficient genetic system. The worm genome consists of approximately 21,000 genes on six chromosomes. Nearly half of the worm genome and most major signaling pathways, including Notch, Wnt, Insulin, and TGF-B, are conserved with mammals (Consortium, 1998; Shaye and Greenwald, 2011). Manipulating the genome and characterizing resulting phenotypes is relatively straightforward in *C. elegans*. Therefore, molecular mechanisms that control many processes, such as development, reproduction, and aging, have been investigated using genetic approaches in *C. elegans.* Knowledge gained from these studies can be applied to axon regeneration.

The conservation of function between *C. elegans* and mammalian genes predicts genes found to regulate axon regeneration in *C. elegans* may have a similar function in mammalian axon regeneration. The genetic conservation also suggests the function of genes found to regulate axon regeneration in mammals may be further characterized in *C. elegans*. Indeed, multiple recently identified regulators of axon regeneration, including PTEN and DLK, have conserved function in *C. elegans* and in mammals (Park et al., 2008; Hammarlund et al., 2009; Itoh et al., 2009; Yan et al., 2009; Shin et al., 2012; Watkins et al., 2013; Byrne et al., 2014).

C. elegans is particularly amenable to genetic and genomic approaches, both classic and modern. Worms exist as hermaphrodites capable of self-fertilization and as males. Therefore, an isogenic strain of hermaphroditic worms can be maintained without organized mating and genotyping. Males are used to introduce a desired genetic mutation or fluorescent reporter into a hermaphroditic strain. In most regeneration experiments, a mating is used to create progeny who express GFP in a specific set of neurons and have a specific genetic mutation. The worm's three-day reproductive cycle makes creating such progeny relatively quick: a neuron type-specific GFP reporter can be crossed into a strain containing a genetic mutation to produce a homozygous, double mutant (*reporter*; *mutation*), isogenic line in as few as nine days. Recently, modern CRISPR/Cas9 technology has added greatly to the ability to disrupt, express and characterize *C. elegans* genes in a spatial and temporal manner (Friedland et al., 2013). As with classic genetic approaches, the short *C. elegans* reproductive cycle makes modern genetic manipulations extremely fast compared to mammalian genetic manipulations.

The efficiency of *C. elegans* as a genetic model extends beyond time saving. In addition to a short life cycle, the worm has a relatively large brood size. A wild type hermaphroditic worm gives birth to approximately 250 isogenic progeny. As such, many identical animals are readily available for axotomy and analysis from a single parental worm. Worms also survive long-term freezing at extreme temperatures. The ability to freeze worms allows new and valuable worm strains to be maintained indefinitely with minimal effort or cost. Moreover, candidate gene analysis is aided by the availability of a large number of mutants generated by the worm community and *C. elegans* Gene Knockout Consortium, which are kept as frozen stocks at the 'Caenorhabditis Genetics Center' and at the 'National Bioresource Project for the Experimental Animal *C. elegans*'.

Together, the conservation, tractability, and efficiency of *C. elegans* genetics facilitate the relatively quick and relevant investigation of conserved biological processes such as axon regeneration.

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with this mutant, very large numbers of axons can be injured (at the expense of temporal and spatial precision), facilitating genetic and genomic screening (Hammarlund et al., 2009; Rohde et al., 2009; Nix et al., 2014). An alternative approach is to automate laser surgery, potentially increasing the number of axons that can be injured and analyzed (Guo et al., 2008; Gokce et al., 2014). The availability of diverse axon injury models in *C. elegans* enables researchers to select the optimal approach for a given experiment.

3. Genetic screens identify regulators of axon regeneration

A prominent benefit of the *C. elegans* regeneration model is the ability to conduct forward screens to identify genes involved in axon regeneration. To date, multiple screens of various types and scales have been undertaken, including RNAi screens, mutant screens, automated chemical screens, and candidate screens. These screens have made significant contributions to our understanding of axon regeneration, and have generated large amounts of data that awaits detailed analysis.

A large screen for regulators of axon regeneration in *C. elegans* took advantage of spontaneously broken axons caused by mutation of the *unc-70* (β-spectrin) gene (Hammarlund et al., 2007; Hammarlund et al., 2009; Nix et al., 2014). Mutant *unc-70* worms with GFP-labeled GABA motor neurons were screened against an RNAi library that targeted 5076 homologs of human genes (Nix et al., 2014). To induce RNAi in *C. elegans*, worms are fed bacteria that express double-stranded RNA corresponding to a particular gene (Fire et al., 1998; Timmons and Fire, 1998). Multiple independent investigations have found approximately 70% of GABA motor neurons regenerate in wild type animals (Yanik et al., 2004; Wu et al., 2007; Hammarlund et al., 2009). In contrast, when fed bacteria expressing a double stranded RNA with homology to a gene required for axon regeneration, few axons regenerate. In the first round of the screen, RNAi was used to characterize all genes in the library; in the second round, positive hits and other candidates were retested using laser axotomy in the corresponding genetic mutant background. In total, the screen identified more than 50 conserved genes that function in GABA neuron axon regeneration. Of these 50 genes, detailed analysis has been performed on the DLK-1 MAP Kinase pathway and its parallel MLK-1 MAP Kinase pathway, along with syndecan and the tRNA splicing ligase RTCB-1 have been characterized as regulators of axon regeneration (Hammarlund et al., 2009; Nix et al., 2011; Li et al., 2012; Edwards and Hammarlund, 2014; Kosmaczewski et al., 2015). The screen also led to the finding that Notch signaling inhibits axon regeneration (El Bejjani and Hammarlund, 2012). The remainder of these genes await further characterization.

Despite the positive results from this screen, it is not expected to identify all genes involved in axon regeneration. For example, not all genes were screened, and genes with functions essential to survival could not be tested for a role in axon regeneration. Further, RNAi does not always eliminate gene function in the *C. elegans* nervous system, even in a sensitized background (Kamath et al., 2001; Timmons et al., 2001; Sieburth et al., 2005; Wang et al., 2005). Therefore, other regulators of regeneration remain to be uncovered in the *C. elegans* genome.

A different type of screen was performed using laser axotomy. In this screen, 654 strains of worms with mutations in conserved genes were tested for their role in axon regeneration of PLM mechanosensory neurons (Chen et al., 2011). The screen identified 149 genes as regulators of axon regeneration. Most of the identified genes are required for axon regeneration and only 16 inhibit regeneration. The identified genes have diverse functions including, but not restricted to, synaptic vesicle endocytosis, neurotransmission, formation of the extracellular matrix, and axon guidance. Interestingly, the functions of many of the identified genes are specific to axon regeneration and not development, as the PLM axons have mostly wild type morphology pre-injury. One of the characterized hits is the conserved ARF guanine nucleotide exchange factor *efa-6*, which inhibits axon regeneration by regulating microtubule dynamics (Chen et al., 2015). Interestingly, with some notable exceptions (e.g. the DLK pathway, syndecan), there is relatively little overlap between regeneration genes identified by these two screens, suggesting that axon regeneration in these two different neurons types (one motor, one sensory) is mediated in part by different cellular mechanisms.

In vivo, whole animal, chemical screens hold significant promise to identify regulators of axon regeneration. A semi-automated chemical screen of approximately 100 small molecules was carried out using microfluidics and a femtosecond laser (Samara et al., 2010). Chemicals that targeted the cytoskeleton and protein kinases affected regeneration while chemicals that targeted HDACs and vesicle trafficking did not. In particular, the screen identified three PKC (protein kinase C) inhibitors that inhibited regeneration and a PKC activator that enhanced axon regeneration. Expanded chemical screening has the potential to identify and characterize additional regulators of axon regeneration and reveal chemical approaches that may be translated to treat injury in humans.

4. Current areas of investigation

Current investigations focus on the regulation and role of injury sensing, signal transduction, cytoskeletal dynamics, aging, and axon fusion in axon regeneration in *C. elegans*. In many cases, the investigations are motivated by the identification and characterization of genes in the screens described above. The emerging understanding of axon regeneration in *C. elegans* is that, as in the mammalian system, axon regeneration is a complex, orchestrated process regulated by the concerted function of multiple aspects of neuronal cell biology (Fig. 3, Fig. 4, and Table 1).

5. Dual leucine zipper kinase-1

The MAP kinase kinase kinase *dlk-1* (dual leucine zipper kinase) is the best characterized intrinsic regulator of axon regeneration in *C. elegans* (Nakata et al., 2005; Hammarlund et al., 2009; Yan et al., 2009). In *dlk-1* loss of function mutants, axon regeneration is critically compromised, and in animals that overexpress *dlk-1*, axon regeneration is improved compared to axon regeneration in wild type animals. *dlk-1*'s function in axon growth is specific to injury; *dlk-1* is not required for developmental axon outgrowth. In addition to its function in the *C. elegans* neuronal injury response, *dlk-1* mediates regeneration in *Drosophila* and mice (Itoh et al., 2009; Xiong et al., 2010; Shin et al., 2012; Watkins et al., 2013) and also mediates other types of neuronal injury responses including Wallerian degeneration and cell death (Miller et al., 2009; Ghosh et al., 2011; Xiong and Collins, 2012; Xiong et al., 2012; Huntwork-Rodriguez et al., 2013; Pozniak et al., 2013).

In *C. elegans*, DLK-1 functions in injured axons at the time of injury to mediate regeneration (Hammarlund et al., 2009; Yan et al., 2009). DLK-1 is activated in response to an increase in localized calcium (Yan and Jin, 2012). Injury triggers an influx of calcium via a voltage-gated calcium channel (*egl-19*) (Ghosh-Roy et al., 2010) and triggers a release of calcium from stores in the endoplasmic reticulum in response to activated ryanodine receptor channels (Pinan-Lucarre et al., 2012; Sun et al., 2014). Calcium signaling triggers dissociation of an inhibitory DLK-1 isoform to activate DLK-1 function (Yan and Jin, 2012). In addition to calcium signaling, DLK-1 is activated by colchicine-induced microtubule disassembly via RHGF-1 (PDZ-RhoGEF) (Bounoutas et al., 2011; Chen et al., 2014), and DLK-1 function requires palmitoylation (Holland et al., 2016).

DLK-1 activation initiates a mitogen-activated protein kinase (MAPK) signaling cascade that includes the downstream map kinases *mkk-4* (MAPKK) and *pmk-3* (MAPK), along with *mak-2*, a MAPKAP kinase (Nakata et al., 2005; Hammarlund et al., 2009; Yan et al., 2009). The signaling cascade stabilizes and increases translation of the mRNA encoding the CCAAT/enhancer binding protein *cebp-1* (Yan et al., 2009). *cebp-1* regulates axon regeneration by regulating transcription of at least one component of the regeneration response, the receptor

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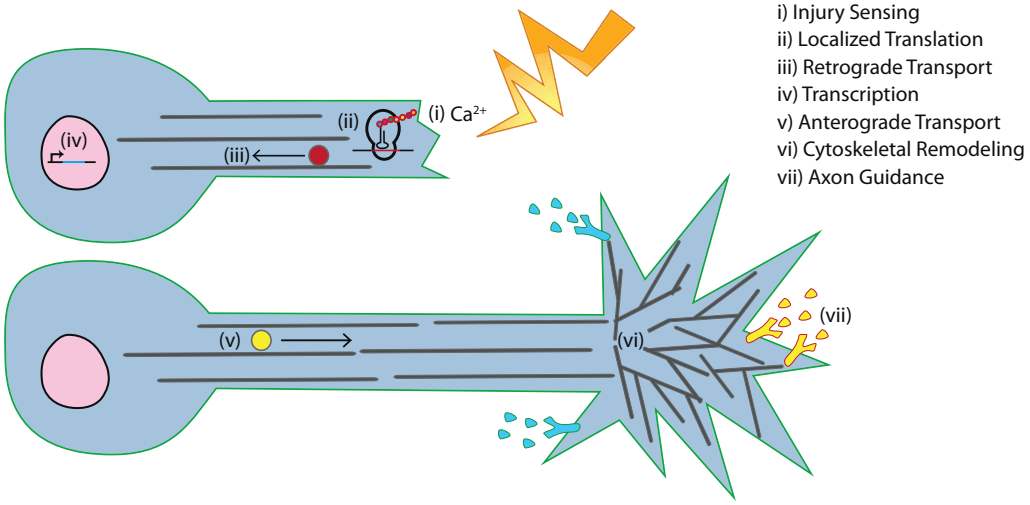

Fig. 3. Current investigations focus on multiple components of axon regeneration in *C. elegans*. A severed *C. elegans* axon (i) senses and transduces an injury signal mediated by calcium signaling, (ii) upregulates localized translation of genes, (iii) retrogradely transports vesicles along microtubules to the nucleus, (iv) upregulates transcription, (v) anterogradely transports vesicles along microtubules to the growth cone, (vi) remodels the cytoskeleton to drive new growth, (vi) and responds to guidance cues to steer itself towards its target. The precise order of these events is not understood as they likely overlap with one another.

tyrosine kinase *svh-2* (Li et al., 2012; Li et al., 2015). Other relevant targets of the DLK pathway downstream of *cebp-1* have not been identified, but presumably have an essential function in axon regeneration.

Loss of DLK function not only blocks regeneration in otherwise normal animals, it also blocks regeneration in some backgrounds with improved regeneration. For example, manipulating Notch and Insulin signaling increases regeneration, but does not restore regeneration to *dlk-1* mutants. By contrast, manipulations that affect axonal microtubules are able to produce regeneration even in mutants that lack DLK signaling (Ghosh-Roy et al., 2012; Chen et al., 2015). These data suggest a major output of DLK signaling is cytoskeletal remodeling (the role of the microtubule cytoskeleton in *C. elegans* axon regeneration is discussed below). A second form of DLK-independent regeneration is observed in axons of sensory neurons, involving reduced sensory activity (Chung et al., 2016). Thus, although DLK is a critical regeneration factor, some forms of enhanced regeneration can act without DLK. Further, at least three *C. elegans* neurons, ALM, ASJ, and ASH, can regenerate even in the absence of DLK signals (Pinan-Lucarre et al., 2012; Chung et al., 2016).

One mechanism that could account for DLK-independent regeneration, such as observed in the ALM neuron, is activation of an alternative MAP kinase pathway. The MLK-1/JNK MAP kinase pathway has been identified as regulating axon regeneration, and can exhibit cross talk with the DLK pathway (Raivich et al., 2004; Hammarlund et al., 2009; Itoh et al., 2009; Nix et al., 2011). The parallel pathway consists of JNK (c-Jun. N-terminal kinase), MLK-1 (MLK-type MAPKKK), MEK-1 (MKK7-type MAPKK), and KGB-1 (JNK-type MAPK) (Nix et al., 2011) (Fig. 4).

Interestingly, in *Drosophila melanogaster*, the DLK and JNK pathways have converged: the fruit fly's *dlk-1* homolog, Wallenda, regulates axon regeneration via c-Jun N-terminal kinase (JNK) signaling (Xiong et al., 2010). In both *C. elegans* and *D. melanogaster*, the downstream JNK target Fos, but not Jun., regulates axon regeneration (Xiong et al., 2010; Nix et al., 2014). As in worms, mammals have parallel p38 and JNK pathways. In the murine model, DLK is required for retrograde transport of p-STAT3 and p-cJun (Shin et al., 2012). These data indicate although the DLK and MLK pathways have diverged slightly throughout evolution, they have maintained a shared function in regeneration.

The *C. elegans* MAP Kinase phosphatase VHP-1, identified in the large RNAi screen described above (Nix et al., 2011; Nix et al., 2014), negatively regulates both the DLK-1/p38 and the MLK-1/JNK MAP kinase pathways. Ten downstream regulators of VHP-1 were subsequently identified by their ability to suppress lethality caused by loss of *vhp-1* gene function (Li et al., 2012). At least five suppressors of *vhp-1* (svh) genes regulate regeneration via regulation of the MLK-1 pathway (Li et al., 2012; Pastuhov et al., 2012; Li et al., 2015). The SVH-1 growth factor is homologous to the hepatocyte growth factor (HGF), macrophage stimulating protein (MSP), and plasminogen family (Li et al., 2012). SVH-1's receptor tyrosine kinase, SVH-2, is homologous to the hepatocyte growth factor receptor Met and the macrophage stimulating protein receptor Ron (Li et al., 2012). Both HGF and MSP have conserved roles in axon regeneration in rat optic nerves (Tonges et al., 2011; Yin et al., 2003). Other characterized suppressors of VHP-1 include SVH-3 and SVH-5. SVH-3 encodes a fatty acid amide hydrolase required for regeneration (Pastuhov et al., 2012). SVH-5 encodes the *ets-1* transcription factor, whose paralog *ets-4*, is required for axon regeneration (Pastuhov et al., 2012). These data suggest that expanding regeneration studies to additional neuron types will help identify key regeneration mechanisms that function together with or in parallel to DLK.

6. Cytoskeletal dynamics

In addition to affecting calcium signaling, MAP kinase signaling, and gene transcription, injury also affects cytoskeletal dynamics. A primary component of the cytoskeleton is the microtubule, which regulates cell shape, axonal transport, and axon growth. Microtubules are relatively dynamic in the growing or regenerating axon and stable in the mature axon. Upon injury, neurons must destabilize their microtubules in order to regenerate. In injured *C. elegans* mechanosensory neurons, DLK-1 signaling regulates microtubule dynamics via a microtubule depolymerase KLP-7 (kinesin-13) and via a cytosolic carboxypeptidase CCPP-6 (Ghosh-Roy et al., 2012). KLP-7 inhibits growth of uninjured axons by stabilizing microtubules and CCPP-6 promotes axon growth by modifying alpha-tubulin post-translationally. Upon injury, KLP-7 is downregulated, which results in an increased number of growing microtubules. CCPP-6 then promotes microtubule growth. Thus, regulating microtubule dynamics in a timely manner upon injury allows the axon to regenerate.

The Arf6 guanine exchange factor (GEF) EFA-6 inhibits axon regeneration by binding to the doublecortin-like kinase ZYG-8 and the transforming acidic coiled coil protein TAC-1, which are required for axon regeneration (Chen et al., 2011; Chen et al., 2015). Interestingly, EFA-6 and TAC-1 colocalize at the minus ends of microtubules with PTRN-1, which also functions in axon regeneration (Chuang et al., 2014; Chen et al., 2015). Manipulation of *efa-6* and *ptrn-1* can enhance regeneration, even in the absence of DLK signaling (Chen et al., 2011;

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Table 1
Regulators of Axon Regeneration in *C. elegans*. Regulators of *C. elegans* axon regeneration are listed, along with their role in axon regeneration. For simplicity, the table does not include all identified regulators.

	Gene	Description	Neuron	Role in regeneration	Reference
Calcium and cAMP signaling	*egl-19*	Voltage-gated calcium channel alpha1 subunit	PLM	Required	Ghosh-Roy et al., 2010
	acy-1	Adenylyl cyclase	PLM	Required	Ghosh-Roy et al., 2010
	cAMP	Cyclic AMP	PLM	Required	Ghosh-Roy et al., 2010
	kin-2	PKA subunit	PLM	Inhibitor	Ghosh-Roy et al., 2010; Chen et al., 2011
	itr-1	Inositol triphosphate receptor	PLM	Required	Ghosh-Roy et al., 2010
	pde-4	cAMP phosphodiesterase	PLM	Inhibitor	Ghosh-Roy et al., 2010
	gsa-1	Heterotrimeric G protein alpha subunit Gs	PLM	Required	Ghosh-Roy et al., 2010
	jun-1	bZIP transcription factor	PLM	Required	Ghosh-Roy et al., 2010
	crt-1	Calreticulin	ALM	Required	Pinan-Lucarre et al., 2012
	unc-68	Ryanodine receptor	ALM	Required	Sun et al., 2014
MLK signaling	*vhp-1*	MAPK phosphatase	GABA	Inhibitor	Nix et al., 2011
	svh-1	HGF, MSP, and plasminogen family	GABA	Required	Li et al., 2012
	svh-2	Receptor tyrosine kinase	GABA	Required	Li et al., 2012
	faah-1	Fatty acid amide hydrolase	GABA	Required	Pastuhov et al., 2012
	ets-4	ETS transcription factor	GABA	Required	Li et al., 2015
	mlk-1	MAPKKK	GABA	Required	Hammarlund et al., 2009; Nix et al., 2011
	mek-1	MAPKK	GABA	Required	Hammarlund et al., 2009; Nix et al., 2011
	kgb-1	MAPK	GABA	Required	Nix et al., 2011
	fos-1	bZIP transcription factor	GABA	Required	Nix et al., 2014
	goa-1	Heterotrimeric G protein alpha subunit Go	GABA	Inhibitor	Pastuhov et al., 2012
	egl-30	Heterotrimeric G protein alpha subunit Gq	GABA	Required	Pastuhov et al., 2012
	egl-8	Phospholipase C beta	GABA	Required	Pastuhov et al., 2012
	tpa-1	Protein kinase C	GABA	Required	Pastuhov et al., 2012
DLK signaling	*rpm-1*	E3 ubiquitin ligase	GABA	Inhibitor	Hammarlund et al., 2009; Yan et al., 2009
	dlk-1	MAPKKK	GABA, PLM	Required	Hammarlund et al., 2009; Yan et al., 2009
	mkk-4	MAPKK	GABA, PLM	Required	Hammarlund et al., 2009; Yan et al., 2009
	pmk-3	MAPK	GABA, PLM	Required	Hammarlund et al., 2009; Yan et al., 2009
	mak-2	MAPKAP kinase	PLM	Required	Yan et al., 2009
	cebp-1	bZIP CCAAT/enhancer-binding protein	PLM	Required	Yan et al., 2009
Cytoskeletal dynamics	*mek-7*	β-tubulin	ALM, PLM	Required	Kirszenblat et al., 2013
	efa-6	Arf guanine nucleotide exchange factor	ALM, PLM		Chen et al., 2011; Chen et al., 2015
	klp-7	Kinesin-13 family member	PLM	Inhibitor	Ghosh-Roy et al., 2012
	ccpp-6	Cytosolic carboxypeptidase	PLM	Required	Ghosh-Roy et al., 2012
	ttll-5	Putative tubulin polyglutamylase	PLM	Inhibitor	Ghosh-Roy et al., 2012
	zyg-8	Doublecortin-like kinase	ALM, PLM	Required	Chen et al., 2015
	tac-1	Transforming acidic coiled coil protein	ALM, PLM	Required	Chen et al., 2015
	ptrn-1	Microtubule-binding protein	PLM	Required	Chuang et al., 2014
	ebp-1	Microtubule end-binding protein	PLM		Chen et al., 2011
Axon guidance	*sax-3*	Robo receptor	PLM	Inhibitor	Chen et al., 2011
	slt-1	Slit ligand	AVM, PLM	Inhibitor	Gabel et al., 2008, Chen et al., 2011
	vab-1	Eph receptor tyrosine kinase	PLM	Inhibitor	Wu et al., 2007
	unc-6	Netrin	AVM	Inhibitor	Gabel et al., 2008
	cwn-2	Wnt ligand	PLM	Required	Chen et al., 2011
Actin dynamics	*unc-34*	EVH1 domain-containing protein	AVM	Required	Gabel et al., 2008
	mig-10	Lamellopodin homolog	AVM	Inhibitor	Gabel et al., 2008
	ced-10	Rac	AVM	Required	Gabel et al., 2008
Aging	*daf-2*	Insulin receptor	GABA	Inhibitor	Byrne et al., 2014
	daf-16	FOXO transcription factor	GABA	Required	Byrne et al., 2014
PTEN signaling	*daf-18*	Phosphatase and tensin homolog	GABA	Inhibitor	Byrne et al., 2014
Developmental switch	*let-7*	microRNA	AVM	Inhibitor	Zou et al., 2013
	lin-41	Ring finger-B box-coiled coil protein	AVM	Required	Zou et al., 2013
	lin-29	Zinc finger transcription factor	AVM	Inhibitor	Zou et al., 2013
	alg-1	Argonaute	AVM	Inhibitor	Zou et al., 2013
Notch signaling	*lin-12*	Notch	GABA	Inhibitor	El Bejjani and Hammarlund, 2012
	sup-17	ADAM protein	GABA	Inhibitor	El Bejjani and Hammarlund, 2012
	sel-12	Presenilin	GABA	Inhibitor	El Bejjani and Hammarlund, 2012
	hop-1	Presenilin	GABA	Inhibitor	El Bejjani and Hammarlund, 2012
Extracellular matrix	*sdn-1*	Syndecan	GABA	Required	Edwards and Hammarlund, 2011
	pxn-2	Peroxidasin	PLM, ALM	Inhibitor	Gotenstein et al., 2010
Caspases	*ced-3*	Caspase	ALM	Required	Pinan-Lucarre et al., 2012
	ced-4	Caspase activator	ALM	Required	Pinan-Lucarre et al., 2012
RNA ligase	*rtcb-1*	RNA ligase	GABA	Inhibitor	Kosmaczewski et al., 2015
S6 kinase signaling	*rsks-1*	S6 kinase	PLM, ALM	Inhibitor	Hubert et al., 2014
	aak-2	AMP kinase	PLM	Required	Hubert et al., 2014
Serotonin signaling	*tph-1*	Tryptophan hydroxylase	PLM, GABA	Required	Alam et al., 2016
	hif-1	Hypoxia-induced factor	PLM, GABA	Required	Alam et al., 2016
	ser-7	5-HT receptor	GABA	Required	Alam et al., 2016
	gpa-12	G protein alpha subunit	GABA	Required	Alam et al., 2016
	rhgf-1	RhoGEF	GABA	Required	Alam et al., 2016
	rho-1	RhoGTPase	GABA	Required	Alam et al., 2016
	rga-5	RhoGAP	GABA	Inhibitor	Alam et al., 2016
DLK-independent regeneration	*egl-19*	Voltage-gated calcium channel subunit	ASJ	Inhibitor	Chung et al., 2016
	tax-4	Cyclic nucleotide-gated channel alpha subunit	ASJ	Inhibitor	Chung et al., 2016
	tax-2	Cyclic nucleotide-gated channel beta subunit	ASJ	Inhibitor	Chung et al., 2016

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Table 1 (*continued*)

Gene	Description	Neuron	Role in regeneration	Reference
osm-6	Intraflagellar transport particle component	ASJ	Inhibitor	Chung et al., 2016
unc-36	Voltage-gated calcium channel alpha2/delta subunit	ASJ	Inhibitor	Chung et al., 2016
daf-11	Transmembrane guanylate cyclase	ASJ	Inhibitor	Chung et al., 2016
sax-1	Ndr kinase	ASJ	Inhibitor	Chung et al., 2016
unc-43	Type II calcium/calmodulin-dependent protein kinase	ASJ, ALM	Inhibitor	Chung et al., 2016

Chuang et al., 2014). Therefore, as in the mammalian system, microtubule dynamics are an important component of the regeneration response and represent one way the injured axon reorganizes its subcellular resources to enable regrowth. The role of microtubules in axon regeneration has been recently reviewed (Tang and Chisholm, 2016).

7. Age vs. axon regeneration

As in the maturing mammalian central and peripheral nervous systems, *C. elegans* axons lose their ability to regenerate as they age (Pestronk et al., 1980; Tanaka et al., 1992; Verdu et al., 1995; Verdu et al., 2000; Wu et al., 2007; Gabel et al., 2008; Hammarlund et al., 2009; Nix et al., 2011; Zou et al., 2013; Byrne et al., 2014). In *C. elegans*, the loss of regenerative ability occurs in largely two phases: the first phase of decline occurs as the worm develops to adulthood (Wu et al., 2007; Gabel et al., 2008) and the second phase of decline occurs as adults age (Hammarlund et al., 2009; Nix et al., 2011; Byrne et al., 2014). Decline in regenerative ability is differentially regulated in the two phases.

The developmental decline in the number of severed mechanosensory neurons that reach their targets is partly attributable to errors in axon guidance (Wu et al., 2007; Gabel et al., 2008; Zou et al., 2013). However, the heterochronic genes *let-7* (encodes a microRNA), *lin-41* (encodes a tripartite motif protein) and *alg-1* (encodes an argonaute protein) also contribute to the developmental decline in regenerative ability of mechanosensory neurons (Zou et al., 2013). The *let-7* and *lin-41* genes interact reciprocally: in young neurons, *let-41*, which promotes axon regeneration, inhibits *let-7* expression via *alg-1*. However, *let-7* microRNA expression is upregulated throughout development and eventually inhibits *lin-41* via its 3'UTR. The conservation of *let-7* microRNA in the mammalian genome suggests that inhibiting *let-7* may be a strategy to enhance regeneration of injured mature neurons.

Axon regeneration also declines throughout adult age in worms and in mammals (Pestronk et al., 1980; Tanaka et al., 1992; Verdu et al., 1995; Verdu et al., 2000; Hammarlund et al., 2009; Nix et al., 2011;

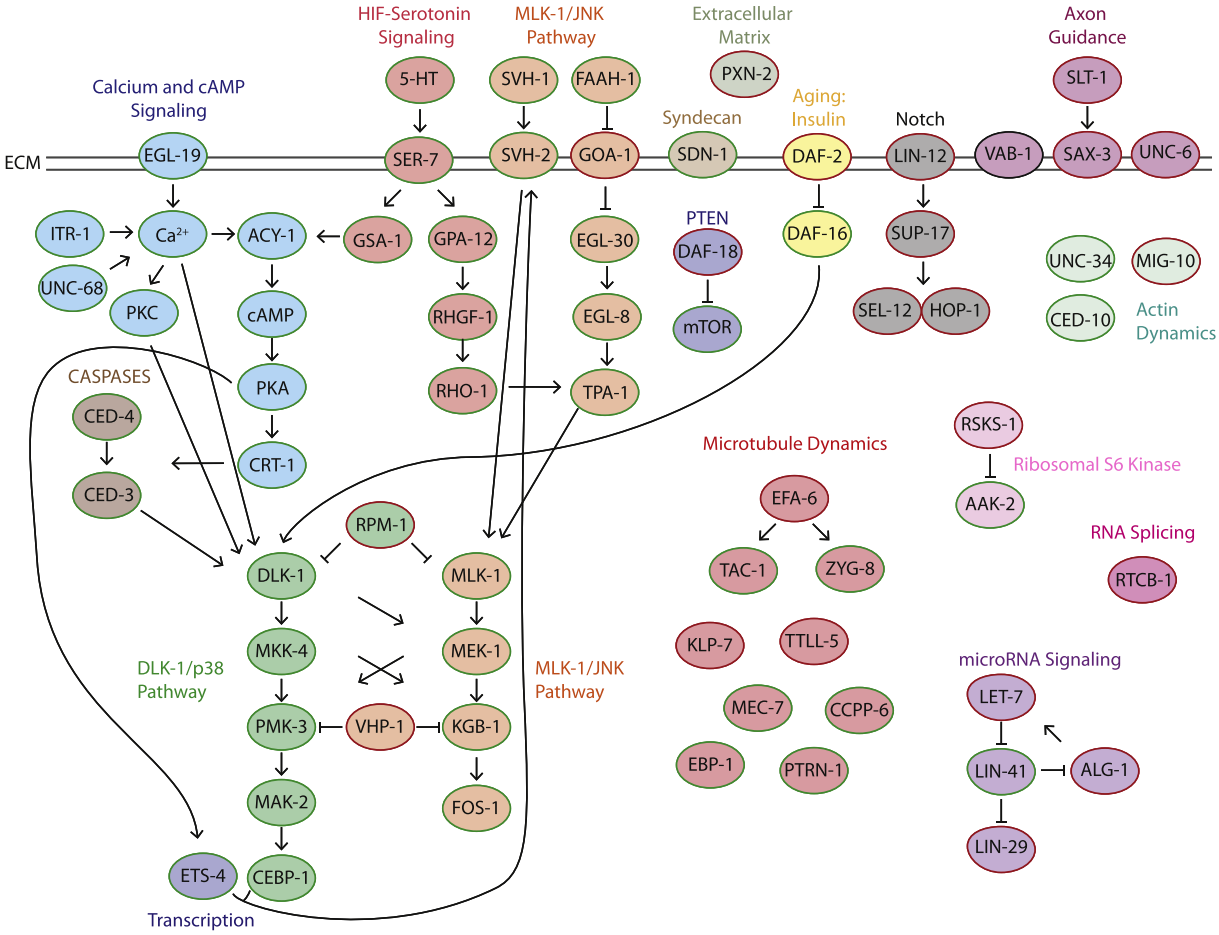

Fig. 4. Regulators of Axon Regeneration in *C. elegans*. Characterized components of pathways found to regulate axon regeneration in *C. elegans* are depicted in this model. Inhibitors of axon regeneration are outlined in red, while required components of axon regeneration are outlined in green. Note, many regulators of axon regeneration have been identified in *C. elegans*. Due to the resulting complexity of interactions between them, the model does not include all identified regulators. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Byrne et al., 2014). The loss of intrinsic regenerative ability in aging adult neurons is not merely a secondary consequence of a decrepit animal, but is a regulated process occurring specifically in neurons (Byrne et al., 2014). Thus, adult decline of axon regeneration is an early effect of neuronal aging, occurring before other effects such as aberrant axon branching, defects in synaptic transmission, and decreased kinesin function (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012; Liu et al., 2013; Li et al., 2016).

In aged GABA neurons, the loss of regenerative ability is regulated by the insulin signaling pathway (Byrne et al., 2014). This pathway is best known for its conserved role in regulating lifespan (Antebi, 2007; Kenyon, 2010). Briefly, the insulin receptor DAF-2/INSR/IGF1R promotes aging by inhibiting translocation of the transcription factor DAF-16/FOXO to the nucleus. In *daf-2* loss of function mutants, DAF-16 translocates to the nucleus and regulates transcription of downstream genes, resulting in two-fold lifespan extension (Kenyon et al., 1993). In aged *daf-2* loss of function mutants, axon regeneration is improved, but the regeneration effect can be uncoupled from lifespan extension and depends specifically on DAF-16 activity in neurons. Neuron-specific ChIP-seq analysis indicates that DAF-16 regulates a number of unique genes in neurons, including the critical regeneration factor DLK-1 (Byrne et al., 2014). These results were recently verified in a study using RNAseq (Kaletsky et al., 2015).

Further evidence supporting independent regulation of aging and regeneration is the finding that loss of function mutations in DAF-18/PTEN, a member of the canonical insulin pathway, increase axon regeneration despite shortening lifespan (Byrne et al., 2014). This result is surprising given DAF-18 functions antagonistically to DAF-2 in regulating lifespan. However, DAF-18/PTEN regulates axon regeneration via TOR (target of rapamycin), similar to its role in mice, and this function is independent of age and insulin signaling (Park et al., 2008; Byrne et al., 2014).

The above findings establish multiple molecular programs that regulate an aging neuron's response to injury. In so doing, they demonstrate that *C. elegans* is an excellent system to identify and characterize novel regulators of adult axon regeneration. The conservation of *let-7, daf-2, daf-16, dlk-1, daf-18, and* TOR pathways with the mammalian genome suggest the knowledge gained from these and from future studies may inform strategies to induce regeneration of mammalian axons that do not regenerate because of their age.

8. Axon fusion

An active area of *C. elegans* regeneration focuses on a fusion event that joins a regenerating axon to the distal segment of the severed axon. When mechanosensory axons are severed, the regenerating axon can fuse with the severed distal axon segment (Gabel et al., 2008; Neumann et al., 2011; Neumann et al., 2015). Reconnecting the severed axon segment to the cell body prevents degeneration of the severed axon (Yanik et al., 2006; Ghosh-Roy et al., 2010; Neumann et al., 2011). After injury, phosphatidylserine (PS) is externalized on the separated axon fragments, where it interacts with its receptor PSR-1 and with the transthyretin protein TTR-52, components of parallel conserved cell-corpse engulfment pathways. These signaling events are proposed to act as a 'save me' signal that mediates recognition of separated axonal fragments (Neumann et al., 2015). The fusogen EFF-1 then critically regulates fusion of the separated ends (Ghosh-Roy et al., 2010; Neumann et al., 2011; Neumann et al., 2015).

Mutation of *ced-3* caspase, the core apoptotic executioner, or its activator, *ced-4*, delays fusion of severed ALM mechanosensory axons, likely as a result of inefficient axon regeneration (Pinan-Lucarre et al., 2012). CED-4 regulates regeneration independently of its canonical upstream regulators. Rather, CED-4 functions downstream of injury-induced calcium signaling and upstream of DLK-1. It is not yet known how activation of CED-3 by CED-4 results in regeneration rather than apoptosis.

Fusion may involve some form of self-recognition, as severed neurons fuse preferentially with the severed segment to which they were originally attached (Neumann et al., 2011). Fusion is an exciting method of neuronal repair as it eliminates the need for severed axons to regenerate over long-distances and form nascent connections with target cells. Although fusion of severed axons occurs in multiple organisms, currently fusion has not been identified in vertebrates. The involvement of conserved apoptotic molecules in *C. elegans* axonal fusion suggests that ectopic induction of these mechanisms in the mammalian system may be an approach to treat injury in the future.

9. Environment

Although most research in *C. elegans* to date has focused on intrinsic mechanisms that mediate regeneration, axon regeneration in the worm is also affected by extracellular cues. For example, as in the mammalian nervous system, axon guidance pathways including netrin, ephrin, WNT, and SLT/ROBO play an important role in the ability of severed axons to regenerate towards their target (Benson et al., 2005; Fabes et al., 2007; Wu et al., 2007; Gabel et al., 2008; Liu et al., 2008; Low et al., 2008; Giger et al., 2010; Zhang et al., 2010; Chen et al., 2011). *C. elegans* lacks some environmental features of mammalian axon regeneration, such as myelin, chondroitin sulfate proteoglycans, and macrophages. The simpler environment in *C. elegans* may facilitate the investigation of conserved extrinsic mechanisms that regulate axon regeneration in addition to myelin.

10. Future areas of investigation

Research in the next decade is likely to yield great advances in understanding axon regeneration, as synergistic findings are emerging from multiple diverse approaches. With its strong knowledge base and robust research community, as well as its particular experimental advantages discussed above, *C. elegans* is positioned to make important contributions. Conservation of function between molecules that regulate axon regeneration in *C. elegans* and those that regulate axon regeneration in mammals, including DLK and PTEN, along with the conserved roles and regulators of calcium signaling, microtubule dynamics, axon guidance, and neuronal aging in axon regeneration, suggest findings in *C. elegans* will inform our understanding of mammalian axon regeneration (Neumann et al., 2002; Filbin, 2003; Raivich et al., 2004; Spencer and Filbin, 2004; Benson et al., 2005; Erturk et al., 2007; Fabes et al., 2007; Wu et al., 2007; Gabel et al., 2008; Liu et al., 2008; Low et al., 2008; Hammarlund et al., 2009; Itoh et al., 2009; Montolio et al., 2009; Yan et al., 2009; Ghosh-Roy et al., 2010; Giger et al., 2010; Zhang et al., 2010; Chen et al., 2011; Nix et al., 2011; Ghosh-Roy et al., 2012; Shin et al., 2012; Watkins et al., 2013; Byrne et al., 2014; Tang and Chisholm, 2016).

A particular strength of *C. elegans* is the ability to study cell biology in vivo. Currently, we are far from having a complete cellular understanding of axon regeneration. To date, only a few key *C. elegans* regeneration mechanisms, such as the DLK pathway and regulation of microtubules, have been subjected to detailed cell-biological analysis. By contrast, genetic approaches have resulted in identification of hundreds of genes that affect regeneration. A major challenge and opportunity for the next decade is to connect this rich genetic information to enable a detailed understanding of the cell biology of axon regeneration.

A complete understanding of the cell biology of axon regeneration will include identifying dedicated cellular mechanisms that specifically mediate the neuronal response to injury, as well as understanding how general neuronal properties are reconfigured and reused during the injury response. For example, in *C. elegans* loss of the DLK pathway strongly affects axon regeneration and the injury response, without significant disruptions of other neuronal functions. On the other hand, important regeneration factors such as Notch, syndecan, insulin signaling, and PTEN clearly have other neuronal functions as well.

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In addition, certain specific questions emerge as particularly urgent. What are the downstream effectors of DLK signaling? What factors control successful circuit reconnection, including the building of new synapses? How does fusion after axotomy work, and how is specificity achieved in this process? How do local calcium and microtubule dynamics shape the response to injury? Can *C. elegans* be used as a drug discovery platform for compounds that improve regeneration in the mammalian CNS? As new researchers enter the field of axon regeneration, *C. elegans* will contribute to answering these and other questions.

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