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The Core Apoptotic Executioner Proteins CED-3 and CED-4 Promote Initiation of Neuronal Regeneration in Caenorhabditis elegans

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Abstract

A critical accomplishment in the rapidly developing field of regenerative medicine will be the ability to foster repair of neurons severed by injury, disease, or microsurgery. In C. elegans, individual visualized axons can be laser-cut in vivo and neuronal responses to damage can be monitored to decipher genetic requirements for regeneration. With an initial interest in how local environments manage cellular debris, we performed femtosecond laser axotomies in genetic backgrounds lacking cell death gene activities. Unexpectedly, we found that the CED-3 caspase, well known as the core apoptotic cell death executioner, acts in early responses to neuronal injury to promote rapid regeneration of dissociated axons. In ced-3 mutants, initial regenerative outgrowth dynamics are impaired and axon repair through reconnection of the two dissociated ends is delayed. The CED-3 activator, CED-4/Apaf-1, similarly promotes regeneration, but the upstream regulators of apoptosis CED-9/Bcl2 and BH3-domain proteins EGL-1 and CED-13 are not essential. Thus, a novel regulatory mechanism must be utilized to activate core apoptotic proteins for neuronal repair. Since calcium plays a conserved modulatory role in regeneration, we hypothesized calcium might play a critical regulatory role in the CED-3/CED-4 repair pathway. We used the calcium reporter cameleon to track in vivo calcium fluxes in the axotomized neuron. We show that when the endoplasmic reticulum calcium-storing chaperone calreticulin, CRT-1, is deleted, both calcium dynamics and initial regenerative outgrowth are impaired. Genetic data suggest that CED-3, CED-4, and CRT-1 act in the same pathway to promote early events in regeneration and that CED-3 might act downstream of CRT-1, but upstream of the conserved DLK-1 kinase implicated in regeneration across species. This study documents reconstructive roles for proteins known to orchestrate apoptotic death and links previously unconnected observations in the vertebrate literature to suggest a similar pathway may be conserved in higher organisms.

Introduction

In the injured vertebrate central nervous system (CNS), neurons often survive and sprout but encounter extrinsic and intrinsic barriers to functional regeneration [1], with devastating consequences for victims. The successful repair of neurons severed by accident or surgery is an obvious goal of modern regenerative medicine. A more detailed understanding of the fundamental molecular mechanisms of neuronal regeneration within a physiological context will be required for design of novel and effective therapies that could shift treatment goals from palliative care to restoration of function.

Considerable understanding of regeneration responses consequent to neuronal injury has been generated via study of vertebrate models in vivo and in vitro. More recently, laser technology advanced the precision of in vivo investigation to the single axon level by enabling the axotomy of individual processes in genetic model organisms [2,3]. Moreover, the opportunity to...
Author Summary

Clinical success in reconnecting neurons damaged by injury will require detailed molecular understanding of how mature axons respond to being severed. To decipher intrinsic molecular pathways that stimulate axon regeneration, we use the simple transparent model, Caenorhabditis elegans, in which individual labeled axons can be laser-severed without damage to neighboring tissue, and regrowing axons can be observed directly in the living animal. We find that the apoptotic protein CED-3, well known for its developmental roles in cell death, also unexpectedly acts in a beneficial role to promote regeneration of severed axons. Initial post-surgery outgrowth is impaired in a ced-3 mutant, suggesting that CED-3 is involved in the early steps of axonal regeneration. The activation of CED-3 caspase in this context occurs independently of major cell death regulatory pathways, but efficient regeneration does require the caspase activator CED-4/Apaf-1, the conserved regeneration kinase DLK-1, and calreticulin-dependent calcium fluxes within the severed neuron. Our data suggest a novel conserved pathway for neuronal reconstruction, and call into question the practice of blocking caspases to treat neuronal injury in the clinic.

Results

CED-3 Caspase Activity Is Needed for Efficient Axonal Regeneration

With an initial interest in whether neurons might activate death pathways in soma or dissociated fragments in response to severe physical injury such as axon severing, we performed femtosecond laser microsurgeries on individual GFP-visualized C. elegans neurons. We find that ALM mechanosensory neurons and D-type motor neurons rarely die after laser axotomy in adult C. elegans. Moreover, the severed dissociated processes generally persist for several days post-surgery (Figure S1) and can remain functional, as axotomized animals were touch-sensitive 6 h after surgery and remained so up to at least 1 wk post-surgery (see data note in Materials and Methods). As observed previously [2,3,9,12,13], severed processes display substantial regeneration from the soma-proximal side, with the severed stump regenerating a structure that first extends multiple spike-like filopodia and then directs further axonal extension (see Movie S1 for a typical depiction of wild type (WT) regeneration). At 24 h, roughly one third of axotomized ALM axons grew back to track along the severed distal process (see below), and the remaining axons displayed dramatic outgrowth with long and branched processes (Figure 1ai–iii, v). We also noted limited regrowth responses from the end of the severed soma-distal side (see below and Movies S1 and S2).

As one approach toward quantitation of the regeneration response, we measured total new outgrowth length of the proximal fragment 24 h following laser surgery for those neurons that did not regrow back into the original severed process (Figure laii, v; those processes that did track back to the old distal process could not be measured as the new process could not be distinguished from the old persisting process). Somewhat unexpectedly, four independent mutants of ced-3 caspase, the central apoptosis executor protease required for all C. elegans programmed cell deaths [11], showed markedly reduced regenerative ALM outgrowth in this timeframe (Figure laiv, vi, 1b), a phenotype also exhibited by ced-3 D-type motor neurons (Figure 1c). ced-3 regenerative defects in severed ALM neurons diminished with time and were no longer apparent at 3 d post-surgery (Figure 1d). Most severe ALM deficits in ced-3 mutants occur in L4 larvae, although significant regeneration differences are apparent in young adults (Figure 1e). Notably, mutant phenotypes in the ced-3(n2433) active-site point mutant, which is deficient in vitro protease activity [14], indicate that caspase activity itself is necessary for efficient axonal regeneration.

Axonal regeneration involves a complex interplay of biochemical activities within the injured neuron and interactions of the neuron with signals and structures in its environment. Thus, caspases might act directly in injured neurons, in the synaptic partners that provide guidance cues, or in the surrounding tissue (hypodermis for touch neurons) to set up conditions permissive for regeneration. To address whether CED-3 caspase activity is required within the severed neuron to facilitate regeneration, we expressed ced-3 in the mechanosensory neurons of the ced-3(n2433) mutant. Although like others [15] we found that expression of caspase transgenes is most often associated with cell toxicity, making the generation of transgenic lines extremely challenging, we identified one low copy number transgenic line with only moderate touch neuron loss (Figure S2). We found that the regeneration defect induced by ced-3(n2433) was rescued by specific expression of ced-3 in the mechanosensory neurons (Figure 1f), supporting that CED-3 acts in the damaged neuron for regeneration. Of note, moderate overexpression of ced-3 in
Figure 1. CED-3 caspase activity is needed for efficient axonal regeneration. (a) Representative images of a pmec-4GFP-labeled ALM neuron were taken before (i), immediately after (ii, red arrow indicates cut point at 20 μm from the cell body), and 24 h after laser axotomy in WT (iii) and in the ced-3(n2433) active site mutant (iv). Images are projected z-stacks. The green traces indicate the observed regenerative outgrowth for WT (v) and ced-3(n2433) (vi); scale bar: 10 μm. Regenerative outgrowth was measured 24 h after surgery in (b) ALM neurons (four independent ced-3 alleles including deletion allele n2452 and active site mutant allele n2433) and (c) D-motor neurons for young adult animals. (d) Regenerative outgrowth was measured 3 d after surgery in ALM neurons in WT and ced-3(n2433) (no statistical difference between the two by t-test). (e) Comparison of WT (grey) and ced-3(n2433) (red) 24 h regenerative outgrowth in ALM neurons for different age animals (L3 and L4 larvae, young adults, and 2-d-old adult). (f) Cell autonomy test for ced-3 rescue of regeneration outgrowth phenotype. The length of ALM regenerative outgrowth was measured 24 h after...
surgery in young adult animals for the control transgenic strains, bearing the unc-119(+)
marker of transformation, Is[unc-119(+)] and ced-3(n2433); Is[unc-119(+)] as well as
transgenic strains expressing ced-3 in the touch neurons Is[punc-4::ced-3] and
ced-3(n2433); Is[punc-4::ced-3]. See notes on strain construction and ced-3
transgene expression toxicity in touch neurons in Figure S2. The unc-119
tagged copy (Is[unc-119]) did not affect the ced-3(n2433) defect in regeneration, and expression of
punc-4::ced-3 (Is[punc-4:ced-3]) in the mechanosensory neurons rescue the ced-3(n2433) defect,
despite some neurotoxicity. punc-4::ced-3 expression in wild type does not induce excessive
regeneration (panel f, third bar), and thus does not appear sufficient to promote
regeneration, although we cannot rule that toxicity of elevated caspase activation could
mask a potential beneficial outcome.

Number of animals assayed is indicated in (or above) each bar for this and all other figures.

**p<0.05, **p<0.005 in all cases. Number of animals assayed is indicated in (or above) each bar for this and all other figures.

CED-3 Acts at Early Steps of Axonal Regeneration

To evaluate ced-3 impact on regeneration in greater detail, we acquired time-lapse images of regrowing neurons for the first 5 h following laser axotomy. We accomplished this using neurelode
immobilization techniques that are stable over long time periods without the use of harsh anesthetics (see Materials and Methods and Figure S3) [16,17]. We found that both the rate and extent of new outgrowth were dramatically reduced in ced-3 mutants during
the initial 5 h following laser axotomy, with total outgrowth reduced by ~45% and the average outgrowth rate reduced by 55% (Figure 2a). Higher resolution analysis of initial regenerative
dynamics in WT and ced-3 mutants revealed three striking phenotypes in regenerating
ced-3 neurons that impact the sprouting of short, often transient, exploratory filopodia-like
processes that dominate during this early stage of outgrowth: (1) there is a significant delay in outgrowth onset after axotomy, with first signs of re-growth appearing after 91±13 min on average in
ced-3 mutant axons compared to 43±8 min characteristic of WT
axons (Figure 2b); (2) the number of sprouts initiated in ced-3 mutants is greatly diminished 0–5 h post-surgery, with the greatest
effect observed during the initial 0–45 min (Figure 2c); and (3) ced-3
extensions often appear defective or stunted, resulting in short,
wide, persistent bleb-like outgrowths that are distinctly different from the transient, dynamically active filopodia-like extensions of
WT neurons (Figure 2d,e, Movies S2 and S3). These dramatic
defects in the initiation of regrowth responses to axotomy in ced-3
contrast with overall outgrowth scores 3 d post-surgery, which no
longer show differences from WT (Figure 1d). We conclude that the
CED-3 apoptosis caspase impacts very early events in post-
axotomy filopodia extension but is not essential to regrowth per se,
suggesting that, like in other C. elegans regeneration studies [18],
additional gene activities may act in parallel to promote
regeneration.

ced-3 mutant neurons are not generally defective in develop-
mental growth cone formation or guidance. In ced-3 mutants, we
observed that developmental growth cones of migrating VD
motor neurons in L1 larvae exhibit wild-type behaviors when they
contact a new surrounding tissue: rounded in the hypodermis,
and anvil-shaped when contacting the lateral nerve cord or body wall
muscle cells (Figure S4) [19]. In addition, when we examined the
AVM touch neuron projection to the ventral nerve cord (VNC) (a
model for in vivo regenerative axon guidance [2]) by laser
dissecting the AVM process half way to the VNC, we found that the
ced-3(n2433) mutant shows the same ability to reach the ventral
nerve cord 24 h post-surgery as the wild type (WT: 63%±9.3%
reach the VNC, N=27 [2]; ced-3(n2433): 64.3%±9.1%, N=28,
no statistical difference by t test). Together, these observations
suggest that ced-3 defects in early filopodia extension dynamics and outgrowth might be limited to injury responses, although detailed
eventual quantification of developmental outgrowth and guidance needs to
be accomplished before relative roles in development versus injury
can be definitively assigned.

Interestingly, our high-resolution time lapse studies also revealed that the distal part of the axotomized axon, disconnected from
the cell body, exhibited regrowth attempts by blebbing and extending exploratory processes initially similar in appearance to
those in the proximal end (Figure 2d purple arrow, Movies S2 and
S3). However, in the ced-3 mutant 0–5 h post-surgery, growth from the distal side of the laser cut was both delayed in onset (58±13 min in WT versus 111±22 min in ced-3 distal termini,
p<0.05) and diminished in extent (1.8±0.2 vs WT explorationary processes in
WT versus 1.3±0.2 in ced-3, p<0.05) (Figure S5). These initial regenerative responses of axon segments separated from the cell
body must therefore be driven by ced-3 proteins or transcripts
already present in the injured axon [20]. Thus, it appears that a
nucleus-independent mechanism of CED-3 caspase activation lies
in wait in healthy processes prior to injury.

CED-3 Is Needed for Rapid Reconnection Following

Axotomy

In C. elegans, injured neurons can reconnect to reestablish the
cytoplasmic connection of the proximal axon with the dissociated
distal region of the axon [8,13]. To generate a more complete
picture of the consequences of ced-3 deficiency, we assayed
regenerative capacities of those WT and ced-3 mutant neurons
that tracked back to the dissociated process (i.e., those not
counted for overall outgrowth due to coincidence of old and new
processes) using a cytoplasmic reconnection assay. To score for
reconnection, we adapted a fluorescence transfer protocol for use
with GFP (see Materials and Methods for details) [21]. In our
assay, we isolated a segment of the previously severed fragment
by introducing a second cut more distal to the initial injury/
potential reconnection site; we then selectively photo-bleached
GFP within this distal segment (Figure 3a). Rapid recovery of
GFP fluorescence within this segment revealed free diffusion
of GFP from the non-photo-bleached regenerating proximal axon
into the formerly severed fragment, and thus a re-established
cytoplasmic connection.

To assay regeneration phenotypes of those neurons that regrew
to come in proximity to the dissociated process, we compared WT and
ced-3 mutant neurons for restored cytoplasmic continuity. We
found that ced-3 mutant neurons were somewhat diminished in
their capacity to rapidly track back to the dissociated fragment
(Figure 3b), but, of the neurons that grew back to, and appear to be
in contact with, the dissociated distal process at 12 h, 92±2% of
WT versus 20%±18% of ced-3 processes successfully reconnect
(p<0.05 Fisher’s exact test) (Figure 3c). When we sum data for all
axotomies at 12 h post-surgery, 34%±2% of total WT ALM
axons severed were reconnected at this time point, as compared to
4%±4% of ced-3 mutant axons (Figure 3d). As is true for the
outgrowth phenotype, reconnection can approach WT levels after a significant time lag (Figure 3e). We conclude that a consequence of ced-3 caspase inactivation is delayed reconnection. Although the reconnection defect might be an indirect consequence of slow initial outgrowth, it is clear that CED-3 caspase deficiency impairs both initiation of axonal regeneration and reparative timing. In cultured Aplysia neurons, the time to reconnection can influence long-term function of the neuron [22], so the speed to reconnection might hold physiological relevance in invertebrate physiology.

CED-4/Apaf-1, But No Other C. elegans Apoptosis Regulator, Is Required for Efficient Regeneration

A pressing question raised by the discovery of the role of CED-3 caspase in post-axotomy neuronal responses is whether other apoptotic pathway components modulate neuronal regeneration. During C. elegans developmental apoptosis, the expression of EGL-1 (BH3 domain only protein) inhibits CED-9 (Bcl-2 family member), releasing CED-4 (apoptosis protease activating factor-1 Apaf-1 homolog), which in turn activates CED-3 caspase [23]; CED-8 modulates the timing of developmental apoptosis [24]. Physiological germline apoptosis requires ced-9 transcription directed by the lin-35 Rb ortholog [25], and under conditions of radiation stress, both the C. elegans BH3-only domain proteins EGL-1 and CED-13 are needed for CED-3-dependent apoptosis [26]. To address how CED-3 caspase might be activated by axotomy, we tested roles of known apoptosis regulators in regeneration using the amount of 24 h outgrowth as a measure. We found that ced-4(n1162) and ced-4(n1416) mutants displayed diminished regeneration similar to ced-3(n2433), establishing that CED-4 functions in axonal regeneration as well as in apoptosis (Figure 4a). The double mutant ced-4(n1162); ced-3(n2433) is impacted to the same degree as either single mutant, suggesting that ced-3 and ced-4 work in the same pathway to influence

Figure 2. CED-3 caspase contributes to early dynamics of axonal regeneration. (a) Time-lapse regenerative outgrowth measurements during the 0–5 h time period following laser surgery for WT (grey) and ced-3(n2433) (red). Data points indicate outgrowth of individual neurons, and lines indicate average outgrowth (shaded region areas ± s.e.m.). The insert shows total outgrowth rates over the 0–5 h time period (calculated using a regression fit of the displayed outgrowth data, restricted to pass through the origin). (b) Mean time of initial outgrowth after laser surgery for WT (grey) and ced-3(n2433) (red) mutant worms as determined from time lapse measurements. (c) Mean number of individual exploratory processes generated during the 0–45 min and 0–5 h time periods following laser surgery. (d) Representative images showing numerous exploratory outgrowths, sprouting of small often short-lived processes, in the WT background, compared to (e) relatively few such protrusions in the ced-3(n2433) mutant background. Green arrows mark new exploratory processes, green arrowheads mark stunted or stalled processes, purple arrow marks an exploratory process from the disconnected distal axon segment, and time is indicated in minutes post-laser surgery. For bar graphs, data are expressed as mean ± s.e.m. *p<0.05, **p<0.005 versus wild type by Student’s t test.
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Figure 3. CED-3 caspase contributes to reconnection to the dissociated distal fragment. (a) Photo-bleaching test for successful reconnection (see Materials and Methods for details). (i, ii) Compressed z-stack images 12 h post-laser surgery of neurons displaying apparent reconnection. Red arrow indicates the original cut point. (iii, iv) Magnified image of distal segment (single z-frame). (v, vi) A second laser cut (yellow arrow) is followed by selective photo-bleaching between the two cut points. (vii) Recovery of GFP fluorescence in the original distal segment within 15 min indicates the existence of a fusion between the regenerating proximal axon segment and the distal segment, and (viii) a lack of fluorescence recovery indicates no such reconnection (with a cutoff of <7.7% fluorescence score, see Materials and Methods). White brackets indicate the portion of process analyzed for fluorescence recovery; numbers indicate percent recovery of fluorescence. (b) Percent of re-growing axons that track to the place of the dissociated distal fragment at 12 h (i.e., appear to be in physical contact). Note that although ced-3 mutant axons tend to track less often to the dissociated distal fragment, the differences are not statistically significant (p = 0.217). (c) Percent of neurons, of those that track to the dissociated distal fragment, that are also scored to have reconnected at 12 h. Specific reconnection events (in addition to poor tracking) appear delayed in ced-3 mutant axons. (d) Percentage of total neurons at 12 h post-surgery, for which the regenerating proximal axon successfully reconnected with the disconnected distal axon segment. (e) Percentage of total neurons at 72 h post-surgery, for which the regenerating proximal axon successfully reconnected with the disconnected distal axon segment. Note that reconnected axons do not show filopodial extensions, suggesting this trait might be suppressed in reconnected neurons as well as in intact neurons. All comparisons are by Fisher’s exact test, with *p<0.05.

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regenerative outgrowth (Figure 4a). We also found that expression of our one minimally toxic ced-3 transgene in the touch neurons partially rescued the ced-4(n1162) defect, consistent with ced-3 acting downstream of ced-4 in axonal regeneration (the same as the order of CED-4 and CED-3 action in apoptosis) (Figure 4a). As with ced-3, regenerative defects of ced-4 mutant animals were no longer apparent after 3 d (Figure 4b). We conclude that ced-4 is needed for efficient regeneration and acts upstream in the same pathway as ced-3.

Other known upstream regulators of apoptosis, including loss-of-function (lf) allele ced-9(n950), gain-of-function (gf) allele ced-9(n950), egf-1 if mutants egf-1(n1084n3082) and egf-1(n886), the egf-1; ced-13 double mutant lacking both C. elegans BH3-only domain proteins, and lin-35(n745), did not affect regeneration proficiency, revealing an alternative regulatory mechanism for CED-4 and CED-3 activation in the response to axotomy (Figure 4c). Likewise, because the ced-9(n1950) mutation did not impact regeneration, we conclude that the delayed regeneration response in ced-3 mutants is unlikely to be the consequence of timing-regulator ced-9 action in axonal regeneration. Overall, our data reveal an unexpected reconstructive role for the core apoptotic proteins CED-3 and CED-4 that is mobilized via a novel regulatory mechanism distinct from known apoptosis regulatory pathways.

DLK-1 Kinase and CED-3 Appear to Act in the Same Pathway to Promote Regeneration

The DLK-1 p38-like MAPK pathway has been shown to play a critical role in C. elegans neuronal regeneration [8–10]. Our detailed phenotypic analysis of ced-3 suggests action early in axonal regeneration, influencing initial exploratory sprouting (Figure 2), and similarly, the dlk-1 mutant has a drastic reduction in primary growth cone formation consequent to axotomy [9]. We therefore addressed whether DLK-1 might act together with CED-3 and CED-4 in the same molecular pathway, or alternatively, might act in parallel. Using our femtosecond laser and immobilization protocol, we find that the single mutant dlk-1(ju476) displays ALM regenerative outgrowth similar to that of ced-3 mutants, with a ~50% reduction as measured at the 24-h time-point but wild-type regeneration proficiency at 3 d (Figure 5a,b). In the dlk-1(ju476) mutant background, weak regeneration of touch neurons severed in the adult contrasts with total block of regeneration of D-type motoneurons severed at the L4 larval stage, as we measured no regeneration outgrowth following axotomy in 22/22 D-type motoneurons (unpublished data) [8–10], underscoring that different molecular mechanisms might control regeneration in different cell types or developmental stages and, more specifically, that multiple redundant pathways may influence regeneration in adult ALM neurons. Interestingly, the double mutant dlk-1(ju476); ced-3(n2433) exhibited ALM regeneration impairment similar to that of single mutants, both at 24 h and at 3 d post-surgery (Figure 5a,b), suggesting action in the same pathway. Additionally, the double mutant dlk-1(ju476); ced-4(n1162) showed the same regeneration defect as the single mutants at 24 h (Figure 5a), further genetic evidence in support of action in the same pathway. Finally, expression of ced-3 in the touch neurons did not ameliorate regeneration deficiencies in dlk-1 mutants (Figure 5a), suggesting that ced-3 may act upstream of dlk-1 to promote early events in regeneration of ALM touch neurons in adult C. elegans.

Kinase KGB-1 of the (JNK) MAPK pathway has recently been shown to operate in parallel to DLK-1 to promote axon regeneration [18]. We found that although mutant kgb-1(um3) is defective in ALM regeneration, the double mutant kgb-1(um3)/ced-3(n2433) is significantly more impaired in overall regrowth scores

Figure 4. The ced-4 core apoptotic gene, but not known C. elegans upstream regulators of developmental, germline, or radiation-induced apoptosis, are needed for efficient axonal regeneration. (a) We measured mean regenerative outgrowth in ALM neurons 24 h after laser surgery for WT and mutant strains affecting ced-3 and ced-4 (two independent alleles) core apoptotic genes, compound mutants, and in ced-4; lin-35(unc-7)ced-3. Because some studies [9,10,18] documented mutant neurons that show virtually no post-axotomy regeneration and we find that the kgb-1 ced-3 double mutant exhibits lower regeneration than the ced-3 single mutant (Figure 56), the partial phenotype can become more severe in compound mutants. (b) Regenerative outgrowth was measured 3 d after surgery in ALM neurons in WT and ced-4(n1162) (no statistical difference by t test). (c) Regenerative outgrowth was measured 24 h after surgery in ALM neurons for mutants in upstream apoptosis regulators as well as compound mutants (which show no statistical difference by one-way ANOVA test). Bar graphs depict mean ± s.e.m. For (a), the Student’s t test, with a Dunn-Sidak adjustment for multiple comparisons, was used to determine the statistical significance of differences versus WT, with brackets indicating direct Student’s t test between two specific values. *p<0.05, **p<0.005. doi:10.1371/journal.pbio.1001331.g004

Death Executors Promote Neuronal Regeneration
than either of the kgb-1 or ced-3 single mutants (Figure S6). Our
data suggest that, similar to dlk-1, ced-3 acts in a separate
regeneration pathway from kgb-1. Together, these studies define
two parallel processes, one involving ced-4, ced-3, and dlk-1, and
the other involving kgb-1, that act in ALM axon regeneration.

**CED-3 Caspase Promotes Axonal Regeneration in a
Calreticulin- Calcium-Dependent Pathway**

Calcium signaling is known to play a fundamental role in the
neuronal responses to damage and subsequent recovery, with
acute cellular insult inducing large intracellular calcium transients
important for regrowth [7,8]. To address whether calcium
signaling could play a role in the CED-3/CED-4 molecular
pathway during regeneration, we performed in vivo measurements
of cytoplasmic calcium levels in the touch neuron cell soma during
dlaxotomy using two versions of the genetically encoded
fluorophore cameleon (see Materials and Methods). Laser
axotomy of WT neurons initiates an immediate (within <3 s)
and dramatic increase of cellular calcium levels reported by
cameleon-based FRET (Figure 6a). In two independent crt-1
mutants, which lack the ER calcium-binding chaperone calretici-

**Figure 5. The MAPKKK dlk-1 might act together with ced-3 to
promote regeneration.** (a) Mean regenerative outgrowth in ALM
neurons was measured 24 h after laser surgery for WT and mutant
strains for ced-3, ced-4, dlk-1, double mutants dlk-1; ced-3 and dlk-1; ced-
4, as well as dlk-1; blp4 mec-4 ced-3). Note that double mutant kgb-1 ced-3
exhibits lower regeneration than ced-3 alone (Figure S6), as would be
predicted for action in a parallel regeneration pathway, so it is
experimentally possible for a double mutant to exhibit lower
regeneration. (b) Regenerative outgrowth in ALM neurons was
measured 3 d after laser surgery for WT and mutant strains dlk-1 and
dlk-1; ced-3. Bar graphs depict mean ± s.e.m. For (a), the Student’s t

dlak-1; ced-3 transgene in the touch neurons partially

minimally toxic ced-3 transgene in the touch neurons partially

rescued the crt-1(bz29) defect (Figure 6b), suggesting that CRT-1/ calcium
elevation might act upstream of CED-3 activation during
axonal regeneration. This is in agreement with the calcium
imaging data in Figure 6a showing a defect in calcium signaling in
the crt-1 mutants but not in the ced-3 or ced-4 mutants. Finally, the
double mutant dlk-1; crt-1 showed similar defects to the single
mutants (Figure 6b), consistent with the action of 

dlk-1 in the same pathway as 
ced-4/ced-3. Taken together, our data are consistent with a model in which 
ced-1 could act to influence 
intracellular calcium signals needed for 
ced-4-dependent localized 
caspase CED-3 activity and efficient 
regeneration initiation 
promoted in part via kinase DLK-1.

Discussion

Here we document novel roles of core apoptosis executors in 
the initiation of process regrowth in axotomized neurons. CED-3 
caspase activity within the injured neuron promotes rapid 
remodeling and outgrowth, often resulting in efficient reconnection. 
C. elegans apoptosis executor CED-3 contributes to early 
regenerative events via a process genetically implicated to include 
ced-4 and calreticulin. The DLK-1 kinase might act downstream in 
the ced-1/ced-4/ced-3 pathway. The definition of reconstructive 
roles for the core apoptosis executor CED-3 holds implications for 
regenerative medicine strategies.

Caspase CED-3 Is Needed for Early Regrowth and 
Appears Ready for Rapid Activation

High resolution video microscopy time course studies during the 
first 5 h post-axotomy revealed that in wild type proximal 
processes, distinctive filopodia-like extensions can appear within 
minutes, leading to active growth cones and extensive outgrowth. In 
ced-3, these responses are slowed and often appear markedly defective—ced-3 processes take longer to initiate outgrowth, there 
are fewer filopodia generated, and there is less overall outgrowth. 
One particularly striking phenotype is that severed processes in 
ced-3 mutants can appear to produce extensions that do not 
mature into filopodia—instead, ends persist as rounded blebs that 
lack structure and do not extend (Movie S3, Figure 2e). Previous in 
vitro screens have identified C. elegans cytoskeletal proteins, such as 
actin, tubulin, and myosin chains, as potential CED-3 targets [27]; 
and caspases can cleave mammalian cytoskeletal proteins and their 
regulators [28,29]. Thus, although critical targets in the regrowth 
mechanism remain to be identified, one possibility is that CED-3 
activity might induce structural rearrangements needed for 
efficient filopodia production by cleaving cytoskeletal proteins.

Eventually, both total regenerative outgrowth and reconnection to the 
severed distal fragment reached WT levels at longer time points in 
ced-3 mutants, 3 d post-surgery (Figure 1d and Figure 3e). This outcome is consistent with a model in which the CED-3 caspase plays a role in the kinetics of a single regeneration pathway; 
alternatively, other pathways may run in parallel to promote 
regeneration and these other pathways may eventually compensate for ced-3 defects. Given the complex processes that influence 
regeneration in C. elegans [6,18] and mammalian systems [30], and 
our genetic data that suggest kinase KGB-1 acts in parallel to 
caspase CED-3, the contribution of multiple pathways to regeneration 
seems like a probable scenario. The dramatic deficits in the 
initiation and early outgrowth dynamics suggest that CED-3 plays a 
prominent role during this critical stage of regeneration. Because 
dlk-1 is needed for early growth cone formation [9], it exhibits 
similar outgrowth defects to ced-3, the dlk-1 ced-3 double mutant shows similar regenerative outgrowth defects as single mutants, and 
elevated expression of ced-3 does not ameliorate the dlk-1 mutant 
deficit, we suggest that conserved kinase DLK-1 may be an integral 
downstream component of this early-acting mechanism.

A Caspase Ready for Repair

Interestingly, our studies reveal that both the proximal process 
(remaining in contact with the nucleus) and the dissociated distal 
process (devoid of a nucleus) exhibit early regrowth efforts, 
generating dynamic filopodial extensions. Changes in the 
dissociated end have been noted in another C. elegans regeneration study [10]. As growth cones have been observed to extend from isolated 
processes in injured cultured vertebrate neurons [31–33], this phenomenon might represent another conserved element of the 
injury response. We find that in C. elegans, the regenerative 
response in the dissociated end is significantly diminished when 
ced-3 is lacking, and thus ced-3-dependent responses can occur 
independently of a nucleus and new transcription. These observations suggest that CED-3 protein might persist at low 
levels in an inactive form in healthy axons, evidence for which has 
been previously noted in touch neurons [15] and suggested for 
other non-apoptotic caspase paradigms [31]. Low basal level caspase activity can modulate motility in some cell types [35,36] 
and might contribute to regeneration in this case. Alternatively, 
ced-3 transcript distributed throughout healthy processes might 
be translated at the injury site upon transsection, as rapid local 
translation of other messages has been documented at injury sites 
in in vitro mammalian culture models [20] and in C. elegans [10] (including dlk-1). Regardless of activation strategy, it appears that 
C. elegans neurons can rapidly employ CED-3 activity when 
regenerative repair growth is needed.

CED-3 Is Needed for Rapid Reconnection

Wild type regenerating C. elegans neurons are capable of rapidly 
locating and re-fusing with the dissociated distal process (34.4% 
successfully reconnected at 12 h; >50% reconnected by 72 h). We 
observed that ced-3 mutant processes are diminished in reconnec-
tion at the 12-h time point—fewer neurons overall reconnect 
(3.8% for ced-3 versus 34.4% for WT), and of neurons that do 
successfully track to the distal severed process, fewer ced-3 ends 
successfully reconnect (20.0% for ced-3 versus 91.7% for WT). 
Eventually, severed neurons do grow and reconnect in the ced-3 
mutant background. Thus, ced-3 is not essential for reconnection, 
but rather plays a role in promoting rapid reconnection. The 
phenomenon of reconnection raises the question as to whether 
process breaking is a natural in vivo challenge in the development 
and/or function of neurons such that a protective mechanism of 
repair has evolved. Interestingly, severed Aplysia neurons also 
reconnect in culture, with failure to reconnect associated with 
electrophysiological dysfunction of the proximal neuron [22]. 
Rapid reconnection might thus be physiologically important for 
restoring or maintaining the function of the injured neuron.

Defined Pathways That Regulate Apoptosis Are Not 
Operative in Regeneration

We tested multiple regulators of C. elegans somatic or germline 
apoptosis for an effect on neuronal regeneration but find that 
regrowth is not influenced by CED-9/Bcl-2, BH3 domain proteins 
EGL-1 and CED-13, or the LIN-35 germline apoptosis regulator. 
Likewise, regeneration responses are not altered in a 
ced-8 mutant in which the progression through apoptosis is slowed. These data 
support that CED-3 caspase must be regulated by a novel 
mechanism that transpires independently of known apoptosis 
regulatory pathways.

The one other apoptosis protein needed for efficient regener-
ation is Apaf-1/CED-4, which our genetic analysis suggests acts 
upstream in the same pathway as ced-3. In apoptosis, CED-4 
oligomerizes to form the apoptosome structure that facilitates 
procaspase cleavage [37]. It is possible that a similar reaction 
occurs in the regenerative response, although this process would 
likely need to be tightly regulated to prevent apoptosis (see below). 
ced-4 has been documented to execute some functions indepen-
Figure 6. CED-3 caspase acts in a calreticulin-, calcium-dependent pathway for efficient axonal regeneration. (a) Intracellular calcium dynamics in the ALM neurons during laser axotomy. Two different variants of the FRET-based calcium-sensitive fluorophore cameleon were used: Left panel, YC2.12; right panel, YC3.60. Differences in the wild type response (amplitude and shape) are due in part to the lower calcium affinity and larger dynamic range of the YC3.60 fluorophore compared to that of YC2.12 [58]. All laser axotomies were performed 20 μm from the cell soma at time = 0 s (red arrow). Traces represent average response at the cell soma (≠9 trials per trace), and shaded regions indicate s.e.m. (b) Mean regenerative outgrowth in ALM neurons measured 24 h after laser surgery for the indicated mutant strains and compound mutant strains defective in the ER calcium-binding chaperone calreticulin, including in the context of ced-3 expression in touch neurons (indicated as ced-1; Is[p_mec-4, ced-3]). (c) Regenerative outgrowth was measured 3 d after surgery in ALM neurons in ced-1(bz29) (no statistical difference was found by Student’s t test).
Death Executors Promote Neuronal Regeneration

Nerve Injury
CED-3 Repairs Axons: Implications for the Treatment of Nerve Injury

D. Sedlock, K. Soto, M. Gurney


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One page of a document, as well as some raw textual content that was previously extracted for it. The content is about the role of CED-3 and CED-4 in neuronal regeneration and the implications for the treatment of nerve injury.
Death Executors Promote Neuronal Regeneration

Potential reconnection events from outgrowth scores, as in those cases we could not distinguish new growth from old process (the old process persists and does not lose GFP signal). Wild-type data were generated in six distinct groups, taken months apart, in strains ZB154 (cdc39/+) and KWN177 (myi014/+ mCherry unc-119(+)) (each group consisting of experiments run on the same or adjacent days using the same reagents). We found no significant difference between 24 h regenerative outgrowth of different WT groups and different transgenic markers (by one-way ANOVA). Wild-type data from all groups were therefore pooled together to give the wild-type measurement reported in the figures.

As measured in the time-lapse analysis (see below), the effect of the ced-3 mutation in the first 5 h is quite striking, featuring a deficit in exploratory processes, stunted sprout morphology not seen in WT regenerating neurons, and a general delay in response. Although conducting 5 h scoring in all genetic studies might have maximized phenotypic differences, the 24 h time point was used to evaluate relative regeneration in most genetic comparisons (most of these studies were conducted prior to the 5 h measurements that revealed early action of ced-3). Because statistically significant differences were still apparent at 24 h, and 5 h high resolution video microscopy required laborious analysis of individual movies, it would have been impractical to redo all genetic analyses for the 0–5 h time points.

Light touch to the anterior part of C. elegans body is sensed through a pair of ALM neurons and the AVM neuron. Interestingly, when both ALM axons were cut 20 μm from the cell body (n = 10) and AVM was ablated, we found that anterior touch was not significantly reduced, suggesting that the severed distal processes, in contact with post-synaptic interneurons to mediate the escape behavior, maintain the capacity for touch transmission. When we cut both ALM axons >200 μm from the cell body (just posterior to the nerve ring, where critical contacts to interneuron targets are concentrated), touch sensitivity was diminished. Although some axons from this axotomy distance seemed to have a directed regeneration, we did not find evidence of restored touch sensitivity even several days post-axotomy, consistent with previous reports [12].

Of our scores of extent of regeneration defects differ quantitatively from some published studies. Differences may be attributed to a number of factors, including different anesthetic techniques, neuron type studied, age, and laser surgery technique [2,8,9,12]. A few teams previously reported a delay of ~10 h in the formation of the growth cone [2,12]. However, using nematode immobilization techniques that do not require harsh anesthetics (microfluidic devices (Figure S3), as well as a 10% agarose pad), we observed no such delay: neurons often displayed initial growth within minutes of the laser damage with average initial growth time <1 h (Figure 2b). We observed more robust regeneration in ALM neurons compared to that of D-motor neurons, which may explain some of our differing results with published dtk-1 mutant strains (we consistently find reduction to ~50% 24 h regrowth rather than no regrowth; minimal but non-zero regeneration in dtk-1 mutant strains has been reported in the PLM neurons in other studies [8,10]). Our laser ablation technique utilizing a 1 kHz femtosecond pulse train at ~800 nm is specifically designed to deliver precise ablation with minimal collateral damage to the animal and target neuron. Other techniques using MHz femtosecond pulse trains and conventional UV lasers produce larger regions of ablation and therefore more significant damage to the targeted neuron. Although some studies have indicated that postsurgical neuronal regeneration is unaffected by laser ablation technique [12], under certain conditions this may not hold true, leading to possible discrepancies in details among experiments. Despite these technical differences in the field and quantitative differences in extent of regeneration reported, basic conclusions have held across the field.

Reconnection Test

A significant proportion of axotomized neurons grew back to the dissociated fragment and could not be monitored for total outgrowth. To determine how ced-3 caspase disruption altered regeneration outcomes in this fraction of axotomized neurons, we scored for reconnection. ALM axons were severed 20 μm from the cell body in young adults using an zdk39/+ mCherry unc-119(+) marker to visualize processes. 12 h, 24 h, or 72 h post-surgery, neurons were inspected by eye. Neurons for which the regenerative outgrowth of the proximal axon segment appeared to track to (i.e., be in close contact with) the dissociated distal segment (Figure 3a) were further assayed for reconnection using the following photo-bleaching experiment: (a) An initial image of the neuron was recorded (frame 1, see Figure 3a, panels iii and iv). (b) Using the laser, a second cut (yellow arrow in Figure 3a, panels v and vi) was made along the distal segment ~40 μm from the initial cut point (red arrow) and ~20 μm from any potential reconnection points. This effectively isolated the distal segment, where there is potential reconnection, from the rest of the process. This was important to prevent GFP refilling from the distal side. (c) The relevant segment (i.e., between the two cut points) was selectively bleached using standard high intensity UV illumination and a restricted illumination field. A second image was acquired immediately after bleaching (frame 2, see Figure 3a, panels v and vi). (d) After 15 min a third picture was acquired (frame 3, see Figure 3a, panels vii and viii).

GFP fluorescence level in each frame was measured as the average intensity along ~15 μm of the nerve process starting at the second cut point (white brackets in Figure 3a, panels vii and viii), minus the background fluorescence measured adjacent to the nerve process (note, the same portion of the process was analyzed in each successive frame). Percent recovery was calculated as intensity increase between frames 1 and 2, relative to the intensity decrease between frames 1 and 2; percentage recovery = (frame 3-frame 2)/(frame 1-frame 2) (where f1 is the fluorescence intensity measured in frame 1, etc.). Over the short recovery time, recovery of GFP intensity indicates diffusion of non-bleached GFP into the isolated segment through a new connection point with the regenerating neuron (Figure 3a, panel viii). If there is no reconnection, the segment is truly isolated and GFP fluorescence does not recover (Figure 3a, panel vii). Control experiments, performed by severing the axon of an intact neuron twice and immediately photo-bleaching the isolated disconnected segment, gave an average “recovery” background after 15 min of 3.05%±0.55%. We therefore set a cutoff for successful reconnection at >7.57% recovery (2 sigma from the control average). The percent of reconnection at 12 h as well as additional measurements are given in Figure 3b–d.

Time-Lapse Imaging

Time-lapse movies following laser surgery were acquired using two methods of worm immobilization: (1) microfluidic devices, the design, fabrication, and use of which followed previously described methods [17], and (2) a preparation of stiff 10% agarose pads and polystyrene microspheres as described earlier [16]. Laser surgery was performed by manual alignment, but subsequent imaging was computer-automated to allow simultaneous time-lapse imaging of up to 10 regenerating neurons in separate C. elegans. Initially movies were generated using microfluidic devices at lower resolution (30 min/frame, ×40 magnification). These data were eventually pooled with that from higher resolution movies (see
Because of apparent close linkage between the \( ced-3 \), \( mec-4 \), \( bzIs17 \) and \( ced-4 \), \( lin-15(+) \) alleles at the \( ced-3 \) and \( mec-4 \) promoters, respectively. Regression fits to the data displayed in Figure 2a (by the least squared error method, KaleidaGraph, Synergy Software, and restricted to pass through the origin) were used to generate the outgrowth rates displayed in the insert. These rates therefore measure the average total outgrowth of the neurons, which at this stage is largely dominated by the creation and retraction of numerous filopodial extensions rather than the elongation of an individual branch.

To generate an accurate account of the initial regenerative dynamics (the number and timing of exploratory processes displayed in Figure 2b–e, Figure 6d,e and Movies S2–S3), higher resolution movies (10 min/frame or 15 min/frame, \( \times 60 \) magnification) were generated in two ways. Microfluidic devices were used as described above, with the addition of 0.05% tetramisole in the surrounding buffer. The tetramisole worked to partially paralyze the worms [19] in order to keep them still enough for automated re-imaging under high magnification for long time periods. Worms were also immobilized for imaging without anesthetics, using stiff 10% agarose pads and polystyrene microspheres [16]. Data were collected for 5 h post-surgery and images were analyzed by eye (counting number and timing of exploratory processes). Figure 2b,c and Figure 6d,e show the results of data pooled together from the two preparations, as no statistical difference was found between results from the micro-fluidic devices and stiff agarose protocols.

**Fluorescence Calcium Imaging**

We quantified calcium dynamics as changes in ratiometric fluorescence emission between the cyan and yellow fluorescent protein components of cameleon, in the same manner as described previously [55,56]. Two versions of cameleon were employed: YC2.12 [57] and YC3.60 [56,58]. For measurements within the \( ced-3 \) mutants we used the \( bzz17[bpec-4 YC2.12+lin-15(+)] \) allele expressing cameleon YC2.12 from the \( mec-4 \) promoter [57]. Because of apparent close linkage between the \( ced-3 \) and the \( bzz17[bpec-4 YC2.12+lin-15(+)] \) allele, we used a second allele expressing cameleon YC3.60 under the \( mec-4 \) promoter, \( bzz158 [bpec-4 YC3.60] \), for measurements in the \( ced-3 \) and in the \( ced-4 \) mutant background. Images were taken every 3 s with a 300 ms exposure time. The response of an individual neuron was measured as an integration of the fluorescence signal across the entire cell soma. For the YC2.12 measurements, animals were immobilized on a 2% agar pad containing 0.05% tetramisole. For the YC3.60 measurements, the 10% agarose preparation, described above, was used. Differences in the wild type calcium response between YC2.12- and YC3.60-expressing strains could be due to a number of factors including the larger dynamic range and lower calcium affinity of YC3.60, and the different worm immobilization techniques. For these reasons we compared calcium measurements only across genetic backgrounds expressing the same cameleon variant. Likewise, our measured intracellular calcium signals differ with that of others [8] due to a number of possibilities including differing neuron type, calcium reporter, position of cut relative to the cell body, and the portion of cell analyzed. Strains expressing cameleon YC2.12 displayed a deficit in regeneration compared to non-cameleon strains at the 24 h time point (Figure S7). Although we observed a general reduction in overall regenerative outgrowth for all strains expressing the calcium-binding cameleon YC2.12, the \( \sim 50\% \) relative reduction in outgrowth compared to WT control is maintained in the \( ced-3 \) mutant in the presence or absence of cameleon YC2.12, so basic conclusions on the requirement for cameleon YC2.12 are not compromised by the use of the cameleon YC2.12 reporter (Figure S7). The WT strain expressing cameleon YC3.60 showed no significant defect in regenerative outgrowth at the 5 h time point.

**Statistical Analysis**

Details of statistical analysis are stated in the figure legends. In general, for comparisons between two measurements a two-tailed Student’s \( t \) test was used to show statistical significance (direct \( t \) tests are indicated by brackets where they are not otherwise obvious). For group comparisons involving multiple strains (i.e., all strains within one figure panel unless otherwise indicated) the Dunn-Sidak group comparison method was used. Statistical tests were implemented using MATLAB (The MathWorks, Inc.). Outgrowth rates in Figure 2a insert were calculated by regression fits to the data as described above.

**C. elegans Strains and Media**

Strains were grown at 20°C on NGM agar seeded with *Escherichia coli* OP50 as a food source [59]. The wild type strain was *C. elegans* N2 Bristol. Standard genetic techniques were used to generate compound mutant strains. The active site point mutation allele \( ced-3(n2433) \) was used in all compound mutant strains (see Table 1).

**Note on Molecular Lesions of Alleles Studied**

The true \( ced-3 \) null allele has not been formally defined, although many loss-of-function mutants have been described in detail [60]. All four \( ced-3 \) alleles studied are strong loss-of-function. The \( n2433 \) allele encodes a point mutation that alters the caspase active site and shows weak semi-dominance regarding apoptosis; the encoded substitution generates a mutant CED-3 that has no detectable protease activity in vitro [14]. We also studied regeneration in \( ced-3(n2452) \) (a 17 Kb deletion also disrupting four other putative genes: C48D1.1, F58D2.2, F58D2.4, and F58D2.1), \( ced-3(n717) \) (mutation of the conserved acceptor site of intron #7), and \( ced-3(n2885) \) (early stop codon).

The \( ced-3(ok948) \) deletion mutant deletes all but the first 21 amino acids, including the stop codon. \( ced-3(b29) \) encodes a stop codon at position 28 and lacks immunoreactivity [61]. These \( ced-3 \) alleles have been suggested to be functional null alleles.

The \( dkl-1(m476) \) allele is a 5 bp insertion at G631 [62]; this allele has been cited to act as a null allele for axonal regeneration [9].

**Plasmid Construction and Generation of *ced-3* Transgenic Animals**

Plasmids were constructed using standard genetic techniques. The \( ppec-4 mCherry \) vector was constructed by amplification of mCherry sequence improved for expression in *C. elegans* [63] using the following primers: 5'-GGGATTCATGCGTCAAGATAGAAGGA-3' and 5'-GAATTCATGGTCTCAAAGGGT-GAAGA-3'. The PCR fragment generated was cloned into \( ppec-4 \) GFP [64], replacing GFP using BamHI and EcoRI sites.

For the construction of \( ppec-4 ced-3 \), \( ced-3 \) cDNA was amplified from a pool of *C. elegans* cDNA using primers 5'-GGATCCCATGCGTCAAGATAGAAGGA-3' and 5'-CAATTTGGTA-GACGGAGAGATTTCTGTGCG-3' and cloned into pCR2.1 using TOPO TA cloning kit (Invitrogen). For further cloning purposes, the HindIII site of *ced-3* cDNA was inactivated while introducing the silent mutation A to G at position 609 on the cDNA giving...
HindIII. The GFP fragment of pmec-4GFP [64] was replaced with
ced-3(A609G) from pCR3(A609G) using BamHI and MfeI sites. A
fragment containing the mec-4 promoter fused to ced-3(A609G)
cDNA from the previous vector was introduced using Apol and
HindIII sites into pDP#MM016b bearing unc-119(+) [65] and
giving the pmec-4.ced-3 vector construction.

The pmec-4.ced-3 vector and the pDP#MM016b [65] vector
bearing unc-119 gene were used for bombarding unc-119(ed3)
animals as described [66]. Generated transgenic lines were

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Mutations are loss-of-function unless otherwise indicated. We confirmed lesions in all ced-3, ced-4, and dhl-1 constructs by DNA sequence analysis.

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Table 1. List of strains used in this study.
and array transgenes were lost at a very high frequency, precluding our ability to test dkl-1 overexpression in ced-3 mutants.

Supporting Information

Figure S1 The severed distal fragment generated consequent to ALM axotomy often persists for days. (a) Pictures of a regenerating ALM neuron expressing the zdk-5(punc-4gfp) transgene that does not obviously regrow to the site of the dissociated fragment. Note that the severed distal end (green arrow), disconnected from the cell body, remains visible for at least 3 d post-axotomy in young adults. Red arrow indicates laser cut point. (b) To quantitate process persistence in non-reconnected neurons, we classified degeneration of the distal fragment into three types: (i) no or very minimal degeneration (apart from the formation of an end bulb at the cut point); (ii) significant degeneration consisting of apparent thinning of the axon, significant loss of GFP fluorescence, and/or beading; and (iii) fragmentation and complete degeneration (this was not observed). (c) Degeneration from ALM axotomies classified in this way in wild type (N=39) and ced-3(n2433) mutant (N=37) animals showed no significant difference. (TIF)

Figure S2 CED-3 caspase expression affects neuronal health, but one minimally toxic low copy number line can be used for rescue in touch receptor neurons. To test whether ced-3-specific expression in touch neurons could rescue the ced-3 mutation defect in regeneration, we constructed transgenic lines using biolistic transformation, which generates low copy number integrated transgenes. We first engineered a control transgenic line harboring an integrated unc-119 gene (the selectable marker used for biolistic transformation, which is also a critical gene for neuronal development, see details in Materials and Methods), indicated as Is [unc-119(+)]. Is [unc-119(+)] was crossed to the ced-3(n2433) mutant to generate ced-3(n2433); Is [unc-119(+)]. The punc-3 cDNA was expressed in touch neurons in the wild type and ced-3(n2433) backgrounds along with co-transformation marker unc-119. These strains are indicated as I[punc-3, ced-3] and ced-3(n2433); I[punc-3, ced-3]. To test for toxicity associated with ced-3 neuronal expression from I[punc-3, ced-3], we compared surviving fluorescent touch neurons visualized by the zdk-5[punc-4gfp]transgene in wild type, ced-3(n2433), the control transgenic strains Is [unc-119(+)] and ced-3(n2433); Is [unc-119(+)], as well as transgenic strains expressing ced-3 in the touch neurons I[punc-3, ced-3] and ced-3(n2433); I[punc-3, ced-3]. Mean ± s.e.m. are shown. Student’s t test, with a Dunn-Sidak adjustment for multiple comparisons, was used to determine the statistical significance: *p<0.05 versus wild type, #p<0.005 versus ced-3(n2433). Note that ced-3 mutant displays 1.1±0.8 (mean ± standard deviation) extra surviving fluorescent neurons as compared to wild type, including in transgenic backgrounds, consistent with a previous report suggesting survival of a lineage sister that does not undergo programmed cell death in this background [67]. Many transgene lines had higher levels of touch neuron death associated with ced-3 overexpression (not shown) and thus could not be used for rescue assays in our study. (TIF)

Figure S3 Immobilized C. elegans in microfluidics channels. C. elegans were physically immobilized in microfluidic devices consisting of a parallel array of 128 tapered channels or worm “clamps.” Constant suction through the device sufficiently restrained the animals for laser surgery and subsequent time-lapse imaging. This figure is related to time lapse imaging quantitated in Figure 2a. (TIF)

Figure S4 Growth cones of ced-3 mutant neurons exhibit wild type behavior during development. Migrating VN neurons exhibit stereotyped behaviors when they contact a new substratum, as visualized by the ced-3(unc-47::GFP) in the wild type and in ced-3(n2433) mutant. Rounded growth cones migrate across the epidermis (left panels). Growth cones form anvils at the lateral nerve cord (middle panels). Anvil-shaped growth cones paused at the dorsal body wall muscle extend fingers toward the dorsal nerve cord (right panels). Five larvae were observed for each genotype. Pictures are projections of z-stacks. The scale bar represents 5 μm. We conclude that ced-3 mutants do not have major systemic defects in developmental growth cones. (TIF)

Figure S5 Post-axotomy regenerative dynamics in the dissociated distal axon segment reveal that CED-3 activities can be induced in a cellular fragment devoid of a nucleus. (a) Mean time of initial outgrowth from the severed end of the distal fragment after laser surgery for WT (grey) and ced-3(n2433) (red) mutant (see Figure 2). (b) Mean number of individual exploratory processes generated from the dissociated end of the distal axon segment, during the 0–5 h time period following laser surgery. Student’s t test was used to determine the statistical significance of differences for ced-3 versus WT in each panel; *p<0.05. See also Movies S1, S2, and S3 for views of changes in dissociated distal ends. (TIF)

Figure S6 Regeneration efficiency is lower in kgb-1 ced-3 than in ced-3 and kgb-1 strains, suggesting that kgb-1 and ced-3 act in different regeneration pathways. A recent study suggested parallel kinase pathways promote C. elegans regeneration, and that kgb-1 was one kinase that might act in parallel to dkl-1 [18]. Because our genetic data suggested that dkl-1 acts in the ced-3 pathway, we elected to construct a double mutant with kgb-1 to provide proof-of-principle that double mutants impacting parallel pathways would have enhanced regeneration defects. We measured regenerative outgrowth of the axotomized ALM neuron visualized using the zdks-3(punc-4gfp) transgene and monitored 24 h post-surgery in ced-3(n2433) and kgb-1(umi3) single mutants and in the kgb-1(umi3) ced-3(n2433) double mutant. Student’s t test, with a Dunn-Sidak adjustment for multiple comparisons, was used to determine the statistical significance: **p<0.05 versus WT, #p<0.005 versus kgb-1(umi3) ced-3(n2433). (TIF)

Figure S7 ced-1 mutant axons exhibit reduced regenerative outgrowth with calcium sensor cameleon YC2.12 in the background. Since calcium-binding cameleons might sequester calcium to change regeneration events when expressed in touch neurons, we scored our cameleon strains for regenerative outgrowth. Both WT and ced-1 strains that harbor cameleon YC2.12 transgenes exhibit diminished regenerative outgrowth as compared to non-cameleon strains (WT shown, compare ced-1 data with Figure 6b). However, even with cameleon transgene expression, ced-1 mutants remain ~50% reduced in 24 h regenerative outgrowth such that conclusions on calcium signaling remain valid (see Figure 6a). The wild type strain expressing improved cameleon variant YC3.60 showed no significant defect in regenerative outgrowth at the 5 h time point but was not investigated further since a ced-1 dependence for efficient regeneration was apparent even with YC2.12. Shown is mean regenerative outgrowth 24 h after laser surgery for strains expressing the cameleon YC2.12 + bzi17[punc-4 YC2.12+lin-15(+)] (indicated as WT YC2.12), bzi17[punc-4 YC2.12+lin-15(+)]; ced-1(bz29) (indicated as ced-1(bz29) YC2.12), and bzi17[punc-4 YC2.12+lin-15(+)]; ced-1(ok948) (indicated as ced-1(ok948) YC2.12).
Brackets represent Student’s t test between the two indicated measurements, with *p<0.05, **p<0.005.

**Movie S1**  
Regeneration of an ALM neuron after femtosecond laser axotomy. The ALM dendrite was targeted 13 μm from the cell body in an adult wild type *C. elegans* (arrow). We can visualize new growth cones that direct axon extension. Note that the posterior process also initiates limited outgrowth. Frames were taken every 15 min as indicated with laser axotomy occurring at T = 0 min. Duration, 9 h 30 min. Scale bar, 10 μm.

**Movie S2**  
Time-lapse regenerative dynamics in WT. Representative time-lapse movies of initial stages (0–5 h) of neuronal regeneration. ALM neurons display numerous transient, dynamically active exploratory processes. Animals were held in microfluidic devices for laser surgery and time-lapse imaging (see Materials and Methods and Figure S3). Frames were taken every 10 min as indicated with laser axotomy occurring at T = 0 min. Scale bar, 10 μm. Select frames are displayed in Figure 2d.

**Movie S3**  
Time-lapse regenerative dynamics in *ced-3* mutant. Representative time-lapse movies of initial stages (0–5 h) of neuronal regeneration in *ced-3(n2433)* mutant background. *ced-3* mutants display significantly fewer of these extensions, and initial outgrowth is substantially delayed as compared to WT. Animals were held in microfluidic devices for laser surgery and time-lapse imaging (see Materials and Methods and Figure S3). Frames were taken every 10 min as indicated with laser axotomy occurring at T = 0 min. Scale bar, 10 μm. Select frames are displayed in Figure 2e.

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**Author Contributions**  
The author(s) have made the following declarations about their contributions. Conceived and designed the experiments: BPL CVG CPR SHE SES GMW AS MD. Performed the experiments: BPL CVG CPR SHE SES RDS JX YQ SW KR LS. Analyzed the data: BPL CVG AS MD. Wrote the paper: BPL CVG AS MD. Final approval of the version to be published: BPL CVG AS MD.

**References**  
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