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Notch Signaling Inhibits Axon Regeneration

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Summary

Many neurons have limited capacity to regenerate their axons after injury. Neurons in the mammalian CNS do not regenerate, and even neurons in the PNS often fail to regenerate to their former targets. This failure is likely due in part to pathways that actively restrict regeneration; however, only a few factors that limit regeneration are known. Here, using single-neuron analysis of regeneration *in vivo*, we show that Notch/*lin-12* signaling inhibits the regeneration of mature *C. elegans* neurons. Notch signaling suppresses regeneration by acting autonomously in the injured cell to prevent growth cone formation. The metalloprotease and gamma-secretase cleavage events that lead to Notch activation during development are also required for its activity in regeneration. Furthermore, blocking Notch activation immediately after injury improves regeneration. Our results define a novel, post-developmental role for the Notch pathway as a repressor of axon regeneration *in vivo*.

Keywords

Notch; ADAM; gamma-secretase; regeneration; *C. elegans*

Introduction

The ability of an injured axon to regenerate varies widely between neurons, and is regulated by both negative and positive signaling pathways (Filbin, 2008; McGee and Strittmatter, 2003; Rossi et al., 2007; Yiu and He, 2006). For example, neuronal receptors that respond to myelin-derived factors—including NogoR (Fournier et al., 2001) and PirB (Atwal et al., 2008)—inhibit axon regeneration by regulating the neuronal cytoskeleton. The dual phosphatase PTEN reduces regeneration in both the mammalian CNS and PNS, at least in part by limiting mTor activity and protein synthesis (Christie et al., 2010; Park et al., 2008). SOCS3 inhibits regeneration by negatively regulating JAK-STAT signaling and affecting gene transcription (Smith et al., 2009). Such inhibitory pathways are attractive candidates for therapy after nerve damage or disease. However, only a few factors that limit regeneration *in vivo* are known.

The Notch signaling pathway is a highly-conserved signal transduction pathway that controls inductive cell fate decisions and differentiation during metazoan development (Artavanis-Tsakonas et al., 1999; Fortini, 2009; Priess, 2005), and also regulates the development of post-mitotic neurons (Berezovska et al., 1999; Franklin et al., 1999; Hassan et al., 2000; Redmond et al., 2000; Sestan et al., 1999). No function for Notch signaling in axon regeneration has been described. Here, we identify Notch signaling as a novel inhibitor of nerve regeneration in mature *C. elegans* neurons, and show that regeneration is improved

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when Notch signaling is genetically disrupted or pharmacologically inhibited after nerve injury.

Results

Notch/*lin-12* inhibits regeneration

C. elegans neurons whose axons are severed by a pulsed laser can respond by regenerating (Yanik et al., 2004). Successful axon regeneration is characterized by a post-injury morphological transition in which severed axons produce a stable growth cone and begin regenerative growth. In neurons that fail to successfully regenerate, the axon stump appears healthy but quiescent (Fig. 1a). Long-term imaging has demonstrated that these stumps do not initiate growth cones, even transitory ones (Hammarlund et al., 2009). Consistent with previous results, we found that axons in wild type animals often fail to regenerate: only 68% of axons regenerated, while 32% of axons failed to successfully regenerate (Fig. 1c; see Table S1 for full genotypes and data). The failure of many neurons to regenerate suggests that regeneration may be limited by inhibitory pathways.

To determine the function of Notch signaling in axon regeneration, we characterized regeneration in Notch mutant animals after laser axotomy (see Methods). During development, Notch functions to limit neurite extension (Berezovska et al., 1999; Franklin et al., 1999; Hassan et al., 2000; Redmond et al., 2000; Sestan et al., 1999), raising the possibility that Notch signaling may inhibit regeneration of the mature nervous system. Notch signaling in *C. elegans* is mediated by two transmembrane Notch proteins, encoded by the genes *lin-12* and *glp-1* (Austin and Kimble, 1989; Yochem and Greenwald, 1989; Yochem et al., 1988). The GABA nervous system of homozygous Notch/*lin-12(n941)* null mutants was indistinguishable from wild type animals, except in the vulval region, where Notch/*lin-12* signaling is required for normal vulval morphogenesis (Fig. 1b) (Greenwald et al., 1983). After laser surgery, however, axons in Notch/*lin-12* loss of function animals regenerated significantly better than wild type (Fig. 1c). In Notch/*lin-12* loss of function animals, nearly all axons successfully regenerated, and failure of regeneration was reduced more than two-fold, to 12%. In addition, two Notch/*lin-12* gain-of-function alleles, *lin-12(n137)* and *lin-12(n137n460)*, both of which have increased Notch/*lin-12* signaling (Greenwald and Seydoux, 1990), had reduced regeneration. Notch/*lin-12* also inhibited regeneration of cholinergic motor neurons (Fig. 1d). By contrast, Notch/*glp-1* did not affect regeneration (Fig 1e). Thus, Notch/*lin-12* is a potent inhibitor of nerve regeneration.

Notch/*lin-12* inhibits growth cone formation after nerve injury

Previously, we showed that growth cone initiation is a critical step of regeneration. Neurons that fail to regenerate do not initiate growth cones after injury, but rather remain indefinitely as quiescent stumps. Conversely, neurons that do regenerate initiate growth cones, typically between 200 and 600 minutes after injury (Hammarlund et al., 2009). Since loss of Notch increases overall regeneration, we hypothesized that Notch acts to restrict growth cone initiation after injury, and that loss of Notch would result in increased growth cone initiation. To test this idea, we examined neurons 4 and 6 hours after severing their axons (Fig. 2a and b). Consistent with previous results, in wild type animals only a small percentage of axons had initiated growth cones at these early time points (6 hours: 9/113 axons with growth cones, 8%). By contrast, Notch/*lin-12* mutant animals displayed a significant increase in growth cone initiation at 6 hours after surgery (19/82 axons with growth cones, 23%; $p = 0.004$). Thus, releasing Notch inhibition results in earlier growth cone formation, suggesting that Notch inhibits regeneration by preventing the initiation of growth cones.

Notch/*lin-12* affects functional recovery after nerve injury

Functional regeneration requires completion of the regeneration program, restoring connectivity between injured neurons and their former targets. To determine whether Notch inhibition of regeneration affects functional regeneration, we first measured the ability of injured axons to grow all the way back to their former position at the dorsal nerve cord ('full regeneration') in wild type and mutant animals. We found that Notch/*lin-12* mutant animals displayed significantly more full regeneration than wild type (Fig. 2c; wild type: 8/30 axons with full regeneration, 27%; *lin-12(n941)*: 19/32, 59%; $p = 0.01$). Thus, using a morphological assay, release of Notch inhibition allows more injured axons to reach their target. To determine whether Notch can also affect functional regeneration, we used a behavioral assay for GABA neuron function. The GABA motor neurons make inhibitory connections onto body wall muscles. These neurons are particularly important for backward movement, and animals that lack GABA neuron function cannot move backward when prodded on the nose (Schuske et al., 2004). It has been demonstrated that severing all GABA neurons results in characteristic backward movement defects, and that normal behavior is recovered as the neurons regenerate (Yanik et al., 2004). In order to assess the effect of Notch/*lin-12* activity on functional regeneration, we assessed behavioral recovery in the gain of function allele *lin-12(n137)*, which has increased Notch signaling and decreased regeneration (Fig. 1c). (Notch/*lin-12* null animals have morphogenetic defects that make it impossible to assess recovery of backward movement.) We cut all right-side GABA motor neurons in wild type and Notch gain-of-function mutants, and scored backward movement 24 hours after surgery (Figure 2d). We found that, as previously described, most wild type animals showed robust behavioral recovery. By contrast, animals with increased Notch signaling recovered poorly. These data provide the first evidence in *C. elegans* for a signaling pathway that can affect behavioral recovery after nerve injury, and demonstrate that Notch can act to limit functional as well as morphological regeneration.

Notch/*lin-12* inhibits regeneration via a canonical activation mechanism

Notch activation in *C. elegans* involves sequential cleavage of the Notch protein, first by a transmembrane ADAM metalloprotease (known as 'site 2 cleavage'), followed by intra-membrane cleavage by the intracellular gamma-secretase complex ('site 3 cleavage') (Fortini, 2009; Gordon et al., 2008). These cleavages release the Notch intracellular domain (NICD) into the cytoplasm (Fig. 3a). To determine whether Notch inhibits regeneration via its canonical activation pathway, we first tested regeneration in mutant animals that lack functional ADAM metalloproteases. In *C. elegans*, two genes encode ADAM metalloproteases that mediate Notch signaling: ADAM10/*sup-17* and ADAM17/*adm-4* (Jarriault and Greenwald, 2005; Tax et al., 1997; Wen et al., 1997). Axon regeneration in loss of function mutants in ADAM10/*sup-17(n316)* was similar to mutants that disrupt Notch/*lin-12* itself: loss of ADAM10/*sup-17* significantly improved regeneration (Fig. 3b). A loss of function mutant in ADAM17/*adm-4* did not affect regeneration (Fig. 3c). Thus, ADAM10/*sup-17* inhibits axon regeneration.

Metalloproteases have multiple cellular targets. To determine whether Notch/*lin-12* is the specific target of ADAM10/*sup-17* in axon regeneration, we analyzed double mutant animals. If ADAM10/*sup-17* has other relevant cellular targets besides Notch/*lin-12*, the double mutant should have higher regeneration than either single mutant. Since both single mutants already have regeneration that approaches 100%, we conducted this analysis by examining growth cone initiation at the 6-hour time point. We found that ADAM10/*sup-17* mutants, like Notch/*lin-12* mutants, have increased growth cone initiation at 6 hours relative to wild type (Fig. 3d). Animals that lacked both Notch/*lin-12* and ADAM10/*sup-17* did not display any additional increase in growth cone formation. Together, these data suggest that Notch/*lin-12* is the major target of ADAM10/*sup-17* in axon regeneration. Next, we

examined the converse question: whether Notch/*lin-12* can use alternate activation mechanisms that are independent of ADAM10/*sup-17*. We tested whether ADAM10/*sup-17* is required for all the inhibitory effects of gain-of function Notch/*lin-12(n137n460)* on regeneration. We found that the gain-of function Notch/*lin-12* allele failed to inhibit regeneration in double mutants that also lacked ADAM10/*sup-17* (Fig. 3b). Thus, the inhibition of regeneration by Notch/*lin-12* requires metalloprotease processing by ADAM10/*sup-17*. Together, these data demonstrate that Notch/*lin-12* and ADAM10/*sup-17* function together to inhibit regeneration.

To investigate the function of the gamma-secretase complex during axon regeneration, we tested regeneration in mutant animals that lack presenilin, the catalytic component of the gamma-secretase complex. Presenilin in *C. elegans* is encoded by two genes, *sel-12* and *hop-1* (Levitan and Greenwald, 1995; Li and Greenwald, 1997). We found that double-mutant *sel-12(ok2078); hop-1(ar179)* animals, which lack functional gamma-secretase, were similar to Notch/*lin-12* mutants: they displayed significantly increased regeneration compared to wild type animals (Fig. 3e). Thus, elimination of functional gamma-secretase has an effect similar to elimination of Notch/*lin-12*: increased regeneration. Together, these data suggest that Notch/*lin-12*, ADAM10/*sup-17*, and gamma-secretase/*sel-12* & *hop-1* comprise a linear pathway that inhibits regeneration. Further, since the function of ADAM10 and gamma-secretase is to liberate the Notch intracellular domain (NICD), they suggest that inhibition of axon regeneration is specifically mediated by this domain of Notch.

The NICD is required for all known Notch functions (Jarriault et al., 1995; Lieber et al., 1993; Struhl et al., 1993). To test whether NICD is sufficient to inhibit regeneration, we constructed a GFP-tagged version of the Notch/*lin-12* intracellular domain (NICD-GFP; Fig. 3f). When this construct was expressed in wild type animals, the NICD-GFP signal was concentrated in a subcellular distribution consistent with nuclear localization (Fig. 3g). Expression of NICD-GFP resulted in significantly reduced regeneration compared to control (Fig. 3h), and was similar to regeneration in gain-of-function Notch mutants (Fig. 1c). Thus, a canonical activation mechanism culminating in active NICD mediates inhibition of regeneration by Notch/*lin-12*.

NICD contains the CDC10/ankyrin repeats that mediate Notch transcriptional activation, and most Notch functions involve transcriptional regulation. However, a transcription-independent mechanism of Notch action has been described. In this transcription-independent mechanism, NICD does not require its CDC10/ankyrin repeats, and acts via inhibiting the receptor tyrosine kinase Abl pathway (Giniger, 1998; Le Gall et al., 2008). To determine whether this non-canonical mechanism is active in limiting regeneration, we examined regeneration in Abl/*abl-1* mutant animals: if Notch inhibits regeneration by inhibiting Abl, these mutants should have decreased regeneration. However, regeneration in Abl/*abl-1* mutant animals was not different from wild type controls (Fig. 3i), suggesting that Abl signaling does not function in regeneration and does not mediate the inhibitory effects of Notch signaling. These data suggest that Notch acts by regulating transcription. Typically, Notch signaling regulates transcription via a CSL-family transcription factor; in *C. elegans*, the single known Notch target is the CSL protein *lag-1* (Greenwald, 2005). To determine whether Notch/*lin-12* acts via CSL/*lag-1* to limit regeneration, we sought to test regeneration in CSL/*lag-1* mutant animals. However, loss of *lag-1* is lethal, and viable alleles of *lag-1* fail to block some known functions of Notch/*lin-12* signaling (Lambie and Kimble, 1991; Solomon et al., 2008). We tested regeneration in the strongest available viable allele (Qiao et al., 1995), and found that it did not affect regeneration (Fig. 3j). We conclude that Notch signaling likely acts via a transcriptional mechanism, but the identity of the transcriptional cofactor and the function of CSL/*lag-1* remain to be determined.

Notch/*lin-12* acts cell-intrinsically to inhibit regeneration

Previous studies have identified factors that inhibit regeneration by functioning in the injured neuron (such as the Nogo receptor and PTEN), and factors that inhibit regeneration due to expression in the surrounding cells (such as myelin-derived factors and CSPGs). Several results indicate that Notch acts cell-autonomously, in the injured neuron, to limit regeneration. First, overexpression of the constitutively active NICD-GFP under a GABA neuron-specific promoter inhibits regeneration in the GABA neurons (Fig. 3f-h). Second, we found that expressing the constitutively active NICD-GFP in a mosaic manner inhibits regeneration only in the individual cells that express NICD-GFP, while cells in the same animal but without the transgene were not inhibited. We expressed NICD-GFP in an unstable transgene under the GABA-specific P*unc-47* promoter. We introduced this transgene into animals that also expressed soluble mCherry in the GABA neurons (Fig. 4a). We used mCherry fluorescence to cut both NICD-GFP(+) and NICD-GFP(−) axons and quantified axon regeneration separately for each group. NICD-GFP(+) axons had significantly decreased regeneration compared to control wild-type animals (Fig. 4b), similar to gain-of-function Notch/*lin-12* mutant axons (Fig. 1d). By contrast, NICD-GFP(−) axons from the same animals had normal regeneration (Fig. 4b). Third, we observed a similar overall inhibition of regeneration when we overexpressed full-length Notch/*lin-12* cDNA only in the GABA neurons (Fig. 4c). Fourth, we found that NICD-GFP is able to cell-autonomously inhibit regeneration in animals that otherwise lack Notch/*lin-12*. We expressed NICD-GFP only in the GABA neurons of null Notch/*lin-12* mutant animals. The gross phenotype of this strain was identical to non-transgenic Notch/*lin-12* null mutants: animals had protruding vulvas and were completely sterile. However, these animals had decreased regeneration in their GABA neurons (Fig. 4d), compared to the *increased* regeneration normally found in Notch/*lin-12* null mutants (Fig. 1c). Together, these results suggest that cell-autonomous Notch signaling is sufficient to inhibit axon regeneration.

To determine whether intrinsic Notch signaling is *necessary* to inhibit regeneration, we performed tissue-specific rescue of ADAM10/*sup-17*. Regenerating GABA neurons contact only two tissues: body-wall muscles and skin. ADAM10/*sup-17* null mutants have increased regeneration (Fig. 3b). We found that expression of wild-type ADAM10/*sup-17* in muscles or skin did not affect this phenotype. Only when wild-type ADAM10/*sup-17* was expressed in GABA neurons was regeneration inhibited back to wild-type levels (Fig. 4e). Additionally, we found that overexpression in wild type animals of ADAM10/*sup-17* in the GABA neurons inhibits regeneration (Fig. 4f). Consistent with Notch/*lin-12* being the relevant target of ADAM10/*sup-17*, overexpression of ADAM10/*sup-17* in Notch/*lin-12* null mutants does not inhibit regeneration (Fig. 4g). Taken together, these data demonstrate that Notch acts cell-autonomously to inhibit regeneration, and establish that Notch signaling is a novel intrinsic inhibitor of axon regeneration.

In *C. elegans*, Notch itself and the ADAM metalloprotease that mediates Notch activation are encoded by two genes, with overlapping but different functions (Fig. 3a) (Jarriault and Greenwald, 2005). However, only one Notch gene (Notch/*lin-12*) and one ADAM (ADAM/*sup-17*) inhibit regeneration in GABA neurons (Figs. 1 and 3). Since Notch inhibition of regeneration is cell-autonomous, we tested whether the remaining Notch components could also limit regeneration when overexpressed in GABA neurons. We found that GABA-specific overexpression of Notch/*glp-1* NICD-mCh inhibited regeneration (Fig. 4h), similar to overexpression of Notch/*lin-12* NICD-GFP (Fig. 3h). GABA-specific overexpression of ADAM/*adm-4* also inhibited regeneration (Fig. 4i), similar to overexpression of ADAM/*sup-17* (Fig. 4f). By contrast, GABA-specific overexpression of presenilin/*sel-12* did not limit regeneration (Fig. 4j). Together, these data suggest that activated Notch signaling in general inhibits regeneration.

Notch signaling functions at the time of injury to inhibit regeneration

Notch signaling functions during development to regulate cell fate specification (Artavanis-Tsakonas et al., 1999; Fortini, 2009; Priess, 2005), axon guidance (Crowner et al., 2003) and neurite extension (Franklin et al., 1999). Notch signaling is also present in mature neurons: in *C. elegans*, for example, Notch acts in mature neurons to regulate dauer decisions (Ouellet et al., 2008), thermotaxis (Wittenburg et al., 2000) and locomotory behavior (Chao et al., 2005). To determine when Notch signaling acts to limit nerve regeneration, we employed a temperature-sensitive allele of ADAM10/*sup-17*, *sup-17(n1258ts)* (Tax et al., 1997). These animals have normal Notch signaling at the permissive temperature of 15 °C, but have reduced Notch signaling at the restrictive temperature of 25 °C. The temperature-sensitive ADAM10/*sup-17* animals regenerated like the wild type at the permissive temperature, but had increased regeneration and fewer regeneration failures than the wild type when shifted to the non-permissive temperature after surgery (Fig. 5a-c). These data demonstrate that Notch signaling is active after injury in mature neurons, and that this post-injury Notch signaling is necessary to limit regeneration.

Notch signaling can be blocked by pharmacological inhibition of gamma-secretase, and gamma-secretase inhibitors are under active development for treatment of cancer and Alzheimer's disease (Dovey et al., 2001; Shih Ie and Wang, 2007). Since Notch signaling after nerve injury is required for suppression of regeneration, we hypothesized that regeneration in wild-type animals might be improved by drug inhibition of Notch signaling after nerve injury. To test whether gamma-secretase inhibition can increase regeneration, we employed the small molecule N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), which is a potent inhibitor of gamma-secretase activity and Notch signaling (Geling et al., 2002). We performed axotomy on wild-type animals and then immediately microinjected their pseudocoelom with either 100 uM DAPT or a control solution (Fig. 5d, ‘immediate DAPT’). Animals treated immediately with DAPT had increased regeneration and fewer regeneration failures than control animals (Fig. 5e), similar to genetic manipulations that reduce Notch signaling (Fig. 1c). To confirm that gamma-secretase is the relevant target of DAPT, we performed DAPT injection in double-mutant *sel-12(ok2078); hop-1(ar179)* animals, which lack functional gamma-secretase and have increased regeneration (Fig. 3e). DAPT injection in these animals did not further increase regeneration, demonstrating that DAPT acts by inhibiting gamma-secretase (Fig. 5f). These data show that Notch signaling is active in mature neurons, and that Notch signaling after injury is required to inhibit regeneration. Furthermore, this experiment suggests that direct microinjection after laser axotomy in *C. elegans* could be used to test potential agents aimed at improving regeneration.

DAPT acts by inhibiting gamma-secretase and blocking Notch activation. DAPT injection immediately after injury prevents Notch signaling from inhibiting regeneration. To determine the temporal requirements for Notch activation after injury, we injected animals with DAPT two hours after surgery (‘DAPT + 2 hours’, Fig. 5d). These animals did not regenerate better than controls (Fig. 5g). Thus, by 2 hours after surgery, Notch is already sufficiently activated to inhibit regeneration. Together, our data demonstrate that Notch signaling is unable to inhibit regeneration unless Notch is activated immediately following injury. It is possible that this temporal requirement is because injury itself activates Notch. Alternatively, activated Notch signals could need to interact with other cellular events triggered by injury in order to limit regeneration.

Individual Notch ligands are not required for inhibition of regeneration

Notch signaling is activated by DSL-family ligands. To identify the ligand that activates Notch inhibition of regeneration, we assayed regeneration in all available DSL-family ligand

mutants (Table 1). Since Notch signaling inhibits regeneration, loss of the ligand that activates Notch should result in increased regeneration, similar to loss of Notch signaling itself (Figs. 1 and 3). Surprisingly, however, no ligand mutant displayed increased regeneration. Rather, all ligand mutants regenerated at wild type levels, with the single exception of DSL/*lag-2*, which displayed *decreased* regeneration. We conclude that no single ligand is necessary to activate Notch for inhibiting regeneration (see Discussion).

Notch/*lin-12* signaling and the DLK-1 MAP kinase pathway

The MAP kinase pathway defined by the MAP3K *dlk-1* promotes regeneration by functioning in injured neurons at the time of injury (Hammarlund et al., 2009; Yan et al., 2009). Thus, both Notch signaling and the *dlk-1* pathway act in the same cell at the same time to regulate axon regeneration. However, two lines of evidence suggest these two pathways may regulate axon regeneration independently of one another (Fig. 6a). First, we determined that constitutive absence of Notch signaling does not increase activity of the *dlk-1* pathway. We monitored *dlk-1* pathway activity in Notch pathway mutants by assessing expression of a *cebp-1* fluorescent reporter gene (Fig. 6b). Expression of this reporter is increased about 6 fold in mutants that increase *dlk-1* pathway activity (Yan et al., 2009). However, reporter expression was not increased in ADAM10/*sup-17* mutants (which lack Notch signaling), suggesting that Notch does not suppress regeneration by constitutively inhibiting the *dlk-1* pathway (Fig. 6c). Consistent with these data, blocking Notch signaling in aged animals does not increase regeneration compared to aged wild-type animals (Fig. 6d). By contrast, *dlk-1* overexpression can restore regeneration in aged animals (Hammarlund et al., 2009). Next, we determined that the DLK-1 pathway does not regulate regeneration via Notch. We found that absence of Notch signaling—which increases regeneration—is unable to bypass the requirement for *dlk-1*. We examined regeneration in *dlk-1; sup-17* double mutants, which lack both Notch signaling and *dlk-1* signaling. These animals regenerated as poorly as *dlk-1* single mutants, suggesting that inhibition of Notch is not the major effect of the *dlk-1* pathway (Fig. 6e). Together, these experiments suggest that Notch and *dlk-1* signaling may act independently to regulate regeneration. Alternatively, Notch may act at the time of injury to acutely limit activity of the *dlk-1 pathway*.

Discussion

Our results identify a novel, post-developmental role for Notch signaling: inhibition of axon regeneration. Notch signaling inhibits regeneration via a canonical activation pathway, involving Notch/*lin-12*, the metalloprotease ADAM10/*sup-17*, and the gamma-secretase complex. These factors release the NICD of Notch/*lin-12* into the cytoplasm. The NICD localizes to the nucleus and is sufficient to inhibit regeneration, suggesting that a nuclear function of the NICD mediates Notch inhibition of regeneration. In the GABA neurons studied in this work, not all Notch pathway components affect regeneration. Specifically, the other *C. elegans* Notch, Notch/*glp-1*, and the other metalloprotease that mediates Notch signaling, ADAM17/*adm-4*, do not affect regeneration of the GABA neurons. However, both the NICD of Notch/*glp-1* and ADAM17/*adm-4* inhibit regeneration when overexpressed in GABA neurons. These data suggest that the different effects of the endogenous Notch components on axon regeneration are not due to different target specificities or intracellular activation mechanisms. Rather, lack of expression of Notch/*glp-1* and ADAM17/*adm-4* in the GABA neurons could account for the lack of endogenous inhibitory activity of these genes. Consistent with this idea, Notch/*glp-1* is expressed in some post-mitotic neurons, but not in GABA neurons (Ouellet et al., 2008), and ADAM/*adm-4* is not expressed in adult neurons (Hunt-Newbury et al., 2007). Thus, Notch signaling can function generally to restrict regeneration, at least in GABA neurons.

Notch signaling usually acts by regulating gene transcription via a CSL-family transcription factor. Although we were unable to demonstrate a role in inhibition of regeneration for the single *C. elegans* CSL factor, CSL/*lag-1*, two lines of evidence suggest that regulation of gene transcription may account for Notch's ability to inhibit regeneration. First, the Abl signaling pathway, which mediates non-transcriptional function of the NICD (Giniger, 1998; Le Gall et al., 2008), does not regulate axon regeneration (Fig. 3i). Second, a GFP-tagged Notch/*lin-12* NICD localizes to the nucleus and inhibits regeneration (Fig. 3f-h), consistent with a transcriptional function. Since Notch signaling usually *activates* gene transcription (Greenwald, 2005), the targets of Notch signaling in regeneration are likely to be factors that themselves limit regeneration. Although no direct Notch targets in mature *C. elegans* neurons are currently known, some candidate genes have been identified (Singh et al., 2011; Yoo et al., 2004). Identification of the relevant targets would provide insight into the mechanism of Notch inhibition of regeneration, and could also shed light on how Notch generally inhibits the growth of post-mitotic neurons (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999).

How is Notch activated to inhibit regeneration? Our data indicate that no single Notch ligand is required for this activation (Table 1). However, it is possible that two or more ligands function redundantly to mediate Notch activation. Alternatively, Notch activation could occur via a ligand-independent mechanism. In normal cellular contexts, DSL ligands activate Notch by changing Notch's relationship to the plasma membrane, allowing ADAM cleavage to occur. It is possible that nerve injury and consequent relaxation of plasma membrane tension alter the conformation of Notch relative to the membrane, and allow ADAM cleavage of Notch even without ligand binding. Interestingly, the DSL ligand DSL/*lag-2* promotes regeneration, rather than inhibiting it, since *lag-2* mutants have decreased regeneration (Table 1). It is possible that loss of *lag-2* triggers compensatory mechanisms that result in decreased regeneration. These mechanisms could involve increased Notch signaling, either via activation by a different ligand or by a ligand-independent mechanism; alternatively, loss of *lag-2* could trigger Notch-independent inhibition of regeneration.

Our data demonstrate that Notch signaling regulates a very early stage of regeneration: growth cone initiation (Fig. 2a and b). To limit growth cone initiation, Notch must act soon after injury. Consistent with this result, blocking Notch activation at the time of injury is sufficient to prevent Notch from inhibiting regeneration, while blocking activation two hours after injury does not increase regeneration (Fig. 5e and g). It is possible that Notch is active in GABA neurons even before injury, but that continued activation is necessary because the downstream targets of Notch are short-lived. Alternatively, Notch could be activated by injury, either by acute ligand upregulation, changes in local calcium (Rand et al., 2000), or a ligand-independent mechanism. In either case, Notch signaling affects not only growth cone initiation after injury but also has profound effects on the eventual success of regeneration, limiting both morphological and functional recovery after nerve injury (Fig. 2c and d).

Notch has multiple functions in neuronal development. During early development, Notch signaling maintains neuronal progenitors and inhibits neuronal differentiation (Louvi and Artavanis-Tsakonas, 2006). After differentiation, Notch signaling inhibits neurite extension in cultured vertebrate neurons and in the neonatal mouse cortex (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999) and modulates axon guidance in *Drosophila* (Crowner et al., 2003). Our results demonstrate that Notch's function in regulating the growth potential of neurons is not limited to development. Rather, Notch signaling can function long after development is complete, and acts after nerve injury to suppress axon regeneration.

Experimental Procedures

C. elegans strains

Animals were maintained on NGM agar plates with *E. coli* OP50 as a source of food (Stiernagle, 2006). Temperature was controlled at 20 °C unless otherwise stated. Null mutations in *lin-12* result in sterility, so we characterized homozygous mutant progeny segregating from a balanced heterozygous strain. Maternal contributions of wild-type Notch/*lin-12* allow these mutants to survive and develop into viable adults. Many of these adults rupture from their vulva; we used only normally sized, healthy animals in these experiments. Strain names, genotypes and complete data with P values can be found in Supplementary Tables 1-4.

Axotomy

All experiments were performed in parallel with a matched control. L4-stage hermaphrodites were mounted in a slurry of 0.1 μm diameter polystyrene beads (Polysciences Inc) or in 50 mM of the GABA agonist, muscimol, (Sigma M1523) to immobilize the animals. No difference in regeneration rates was observed between beads and muscimol: Wild type animals regenerated at a similar rate under both conditions, and Notch signaling mutants had increased regeneration under both conditions (data not shown). Commissures in the tail region of the animal posterior to the vulva were severed (GABA neurons: VD and DD; acetylcholine neurons: AS and DB). Commissures were visualized with a Nikon Eclipse 80i microscope using a 100× Plan ApoVC lens (1.4 NA) and a Hamamatsu Orca camera. Selected axons were cut using a Micropoint laser from Photonic Instruments (10 pulses, 20 Hz). Axotomized animals were recovered to agar plates and remounted 18-24 hours later for scoring. At least 30 axons were scored for most genotypes (2-3 cut axons per animal); see Sup. Tables 1-4. Only axons with a distal stump as evidence of a complete cut were scored. Axons with a visible growth cone that had progressed past the cut site, and axons that had regenerated to the dorsal nerve cord, were scored as positive. Axons with no growth or with only filopodial extensions and no progression past the cut site were counted as negative.

When scoring full regeneration, only axons that showed visual evidence of reconnection to the dorsal cord 24 hours after axotomy were scored as positive. For growth cone initiation at 4 and 6 hours, axons with a growth cone were scored as positive. 95% confidence intervals were calculated by the Wald method, and 2-tailed P values were calculated using Fisher's exact test (http://www.graphpad.com/quickcalcs/).

Functional recovery

All visible GABA commissures (~16/ animal) were severed in healthy wild type and *lin-12(n137)* gain of function L4-stage animals. Axotomized animals were recovered onto fresh plates with food and probed on the nose 1 hour after axotomy. At 1 hour after axotomy all animals responded by shrinking and were unable to initiate backward locomotion. Animals were scored at 24 hours after axotomy into one of the following categories: 1) no backwards movement (shrink); 2) 1 or 2 body bends backwards; 3) 3 or more body bends and efficient backing up but not wild type. No axotomized animals recovered completely wild type locomotion after axotomy.

Molecular biology

Plasmids were assembled using Gateway recombination (Invitrogen). Entry clones were generated using Phusion DNA polymerase (Finnzymes). Primers, templates and Plasmid names are listed in Supplementary Experimental Procedures.

Transgenics

Transgenic animals were obtained by microinjection as described (Mello et al., 1991). Transgene name, content and concentrations are listed in Supplementary Experimental Procedures. For most strains, stable transgenic lines were selected based on GFP expression in the pharyngeal muscles from a P*myo-2*::GFP co-injection marker. For XE1291 *wpEx107 lin-12(n941)(III)/hT2(I;III)*, transgenics were selected based on mCherry expression in GABA neurons. For XE1271 *wpEx102*, transgenics were selected based on mCherry expression in the cholinergic motor neurons. For XE1139 and XE1208, *unc-32* rescued animals were picked based on wild-type movement.

DAPT injections

N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was obtained from Tocris Bioscience (Cat. No. 2634) and prepared in DMSO. This stock was diluted in M9 medium to a final concentration of 100 μM DAPT and 1% DMSO. The control solution contained 1% DMSO in M9. Wild type EG1285 *oxIs12* or *sel-12(ok2078); hop-1(ar179)* (derived from XE1207 balanced strain) hermaphrodites were axotomized at the L4-stage (or 5 days post-L4 for the experiment in aged animals). Small numbers of animals (~10) were axotomized at one time to minimize timing errors. The animals were promptly recovered to agar plates with food. Animals were then mounted for injections either immediately or after a 2 hour delay. Injections were performed into the pseudocoelom using standard microinjection techniques. Injected animals were recovered to new agar plates and scored for regeneration as previously described.

Fluorescence quantification

Expression of the mCherry *cebp-1* reporter (*juEx1735)* (Yan et al., 2009) was analyzed in uninjured animals using an UltraVIEW VoX (PerkinElmer) spinning disc confocal and a 40× CFI Plan Apo, NA 1.0 oil objective. Cell body fluorescence was quantified using Volocity (Improvision) and the average fluorescence per cell body was used to calculate the mean. 21 wild type (*juEx1735*) and 19 *sup-17(n316); juEx1735* animals were analyzed, and the average fluorescence intensity per animal was determined for each genotype. See Supplementary Table 4 for numbers and statistics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GABA

OE

CSPG

NICD

ADAM

DAPT

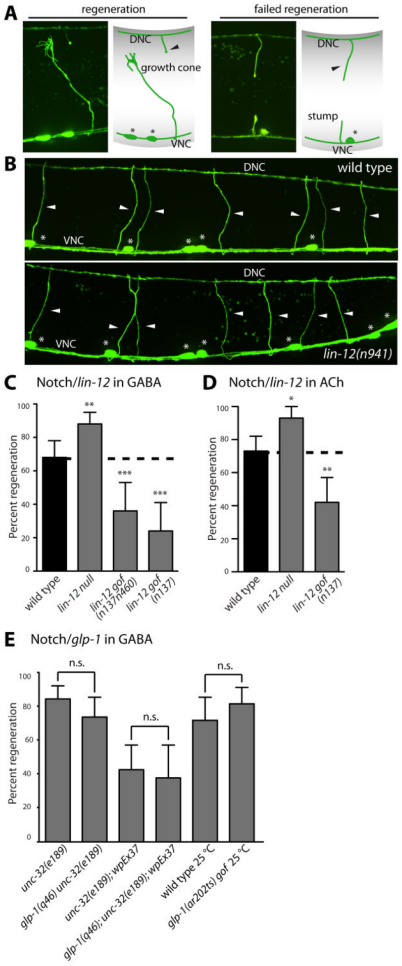

Fig. 1.

Notch signaling inhibits nerve regeneration. (**a**) Axon regeneration in *C. elegans* GABA neurons. Some injured axons regenerate (left panels), while some do not (right panels). Arrowheads in diagrams indicate distal axon fragments; stars indicate cell bodies. (**b**) GABA neurons in wild type and in Notch/*lin-12* null mutants. Arrowheads indicate commissures; stars indicate cell bodies. DNC, dorsal nerve cord; VNC, ventral nerve cord. (**c**) Notch/*lin-12* inhibits regeneration of GABA neurons. (**d**) Notch/*lin-12* inhibits regeneration of acetylcholine neurons. (**e**) Notch/*glp-1* does not inhibit regeneration of GABA neurons.

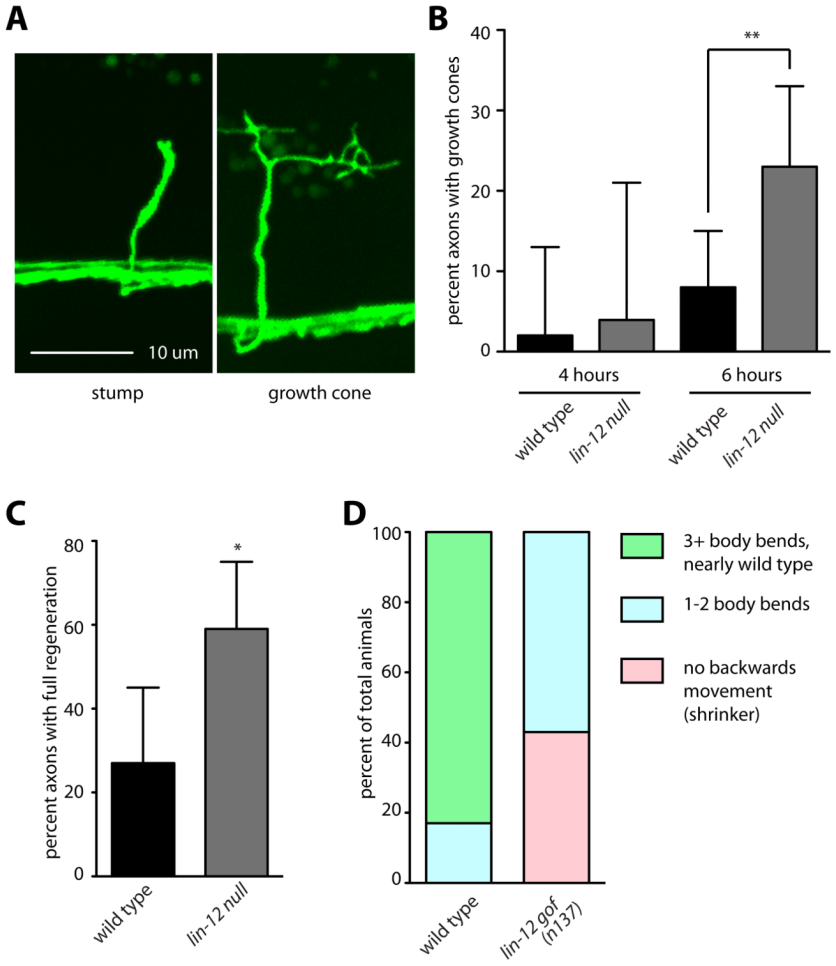

Fig. 2.

Notch affects growth cone formation and behavioral recovery. (**a**) Representative axons that have not formed a growth cone (left) or formed a growth cone (right) at 6 hours after injury. (**b**) Growth cone formation after injury in wild type and Notch/*lin-12* null mutants. (**c**) Notch/*lin-12* inhibits complete morphological regeneration. (**d**) Notch/*lin-12* inhibits behavioral recovery after nerve injury.

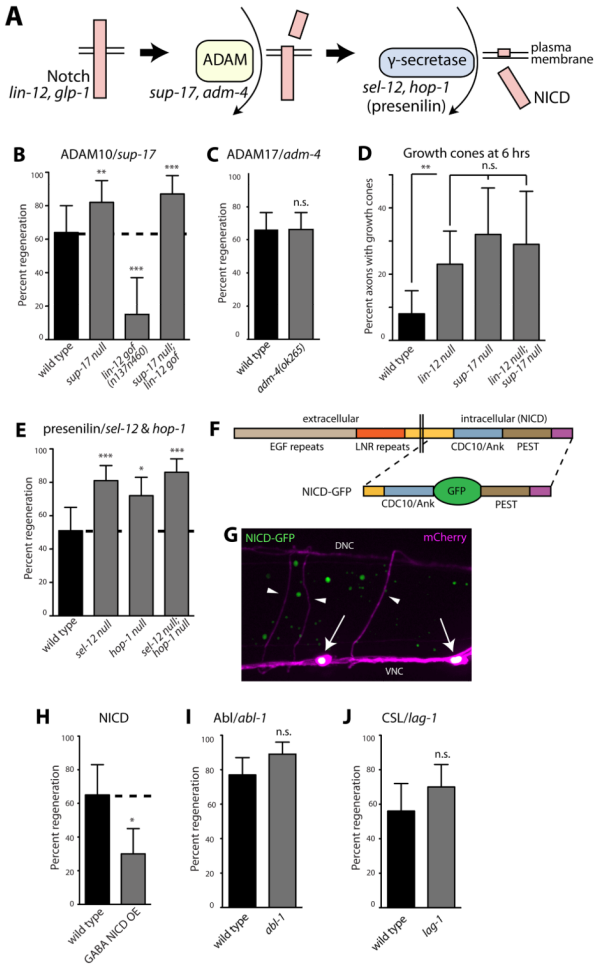

Fig. 3.

Notch inhibits regeneration via a canonical activation pathway. (**a**) Notch signaling in *C. elegans*.(**b**) ADAM10/*sup-17* inhibits regeneration, and is required for Notch/*lin-12* to inhibit regeneration. (**c**) ADAM17/*adm-4* does not inhibit regeneration in GABA neurons. (**d**) Notch/*lin-12* and ADAM10/*sup-17* function together to inhibit regeneration. (**e**) Presenilin/*sel-12* and *hop-1* inhibit regeneration. (**f**) Notch protein domains and design of the GFP-tagged NICD construct (NCID-GFP). (**g**) NICD-GFP is localized to nuclei in GABA neurons. Green, NICD-GFP; purple, soluble mCherry; white, colocalization. Arrows indicate cell bodies; arrowheads indicate commissures. (**h**) Expression of NICD-GFP in wild-type animals inhibits regeneration. (**i**) Abl/*abl-1* does not affect regeneration in GABA neurons. (**j**) A weak allele of CSL/*lag-1* does not affect regeneration in GABA neurons. Bars in panels b-e and h-j show percentage of axons that initiated regeneration and 95% confidence interval (CI). *P < 0.05, **P < 0.01, ***P < 0.001 (see also Table S1).

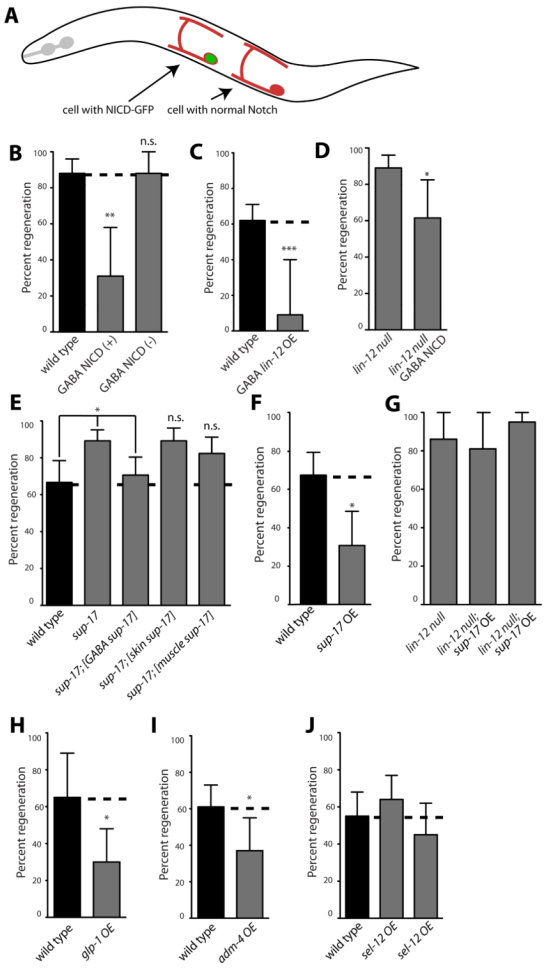

Fig. 4.

Notch/*lin-12* functions cell-intrinsically to limit regeneration. (**a**) Mosaic animals allow identification of NICD-GFP-expressing individual neurons. (**b**) Notch/*lin-12* NICD-GFP overexpression inhibits regeneration cell-intrinsically. (**c**) Notch/*lin-12* overexpression in GABA neurons inhibits GABA neuron regeneration. (**d**) NICD expression in Notch/*lin-12* mutants in GABA neurons inhibits GABA neuron regeneration. (**e**) Mosaic expression of ADAM10/*sup-17* in GABA neurons, but not in muscle or skin, inhibits GABA neuron regeneration. (**f**) ADAM10/*sup-17* overexpression in GABA neurons inhibits GABA neuron regeneration. (**g**) ADAM10/*sup-17* overexpression in GABA neurons in Notch/*lin-12* null mutants does not inhibit GABA neuron regeneration. (**h**) Notch/*glp-1* NICD-mCh overexpression inhibits regeneration in GABA neurons. (**i**) ADAM/*adm-4* overexpression inhibits regeneration in GABA neurons. (**j**) Presenilin/*sel-12* overexpression does not inhibit regeneration in GABA neurons. Bars in panels b-j show percentage of axons that initiated regeneration and 95% confidence interval (CI). *P < 0.05, **P < 0.01, ***P < 0.001. (See also Table S2)

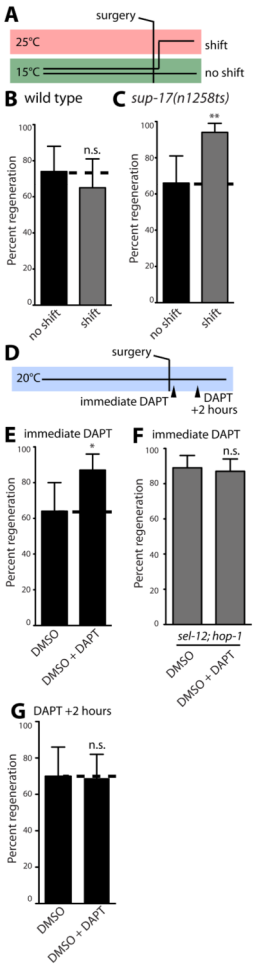

Fig. 5.

Notch signaling functions at the time of injury to inhibit regeneration, and chemical inhibition of Notch improves regeneration in wild type animals. (**a**) Temperature shift or control ('no shift') was performed immediately after axotomy. (**b**) Temperature manipulations do not affect regeneration in wild type animals. (**c**) Regeneration is increased when temperature-sensitive ADAM10/*sup-17* animals are shifted to the non-permissive temperature after axotomy. (**d**) DAPT in DMSO or control ('DMSO') was injected immediately after axotomy, or after a 2-hour delay. (**e**) Injecting DAPT immediately after axotomy increases regeneration. (**f**) Injecting DAPT immediately after axotomy into *sel-12; hop-1* mutant animals does not increase regeneration. (**g**) Injecting DAPT 2 hours after axotomy has no effect on regeneration. Bars in panels b, c, and e-f show percentage of axons that initiated regeneration and 95% confidence interval (CI). *P < 0.05, **P < 0.01, ***P < 0.001. (See also Table S3).

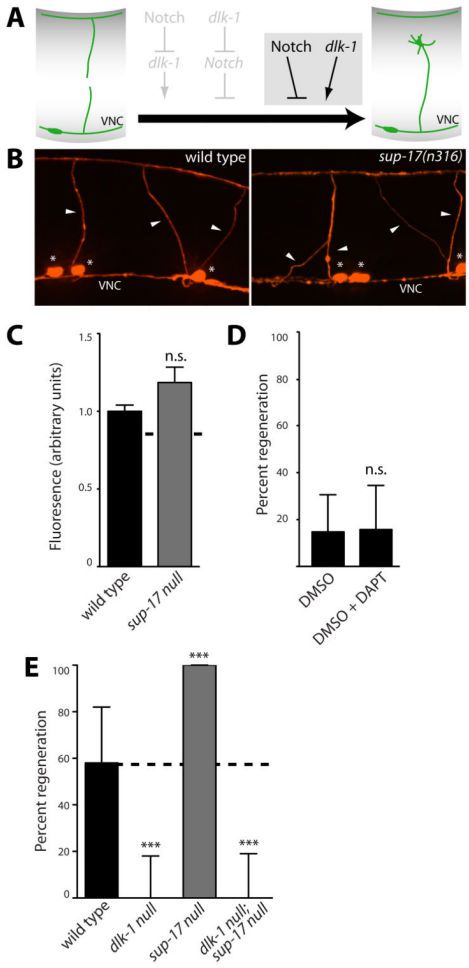

Fig. 6.

Notch regulates regeneration independently of the *dlk-1* MAP kinase pathway. (**a**) Three models describing the relationship of Notch and *dlk-1* signaling during axon regeneration. (**b**) Expression of a *cebp-1* reporter in GABA neurons in the wild type and in ADAM10/*sup-17* mutants. Arrowheads indicate commissures; stars indicate cell bodies. (**c**) Removing Notch signaling does not increase *cebp-1* fluorescence in GABA neurons. Bars show mean fluorescence and SEM. (**d**) Blocking Notch activation immediately after injury does not improve regeneration in aged animals. (**e**) *dlk-1* does not promote regeneration by inhibiting Notch. Bars show percentage of axons that initiated regeneration and 95% confidence interval (CI). *P < 0.05, **P < 0.01, ***P < 0.001. (See also Table S4).

Table 1

Individual Notch ligands are not required for Notch function in regeneration.

Description	Genotype	Strain	# animals	# axons	% regeneration	P value
wild type	*oxIs12*	EG1285	111	323	69	
wild type	*juIs76*	OH4121	33	113	73	
lag-2	*lag-2(q420)ts; oxIs12*	XE1199	22	53	30	<0.0001
osm-11	*osm-11(rt142); juIs76*	XE1276	16	55	71	0.85
osm-7	*osm-7(n1515); oxIs12*	XE1418	18	47	66	0.7
osm-7	*osm-7(tm2256); oxIs12*	XE1419	21	46	72	0.73
apx-1 allele 1	*apx-1(or22); oxIs12*	Derived from XE1408	9	26	77	0.5
apx-1 allele 2	*apx-1(or15); oxIs12*	Derived from XE1407	9	25	76	0.5
dsl-1	*dsl-1(ok810); oxIs12*	XE1411	18	44	77	0.29
dsl-3	*dsl-3(ok3411); oxIs12*	XE1412	19	52	60	0.2
dsl-4	*dsl-4(ok1020); juIs76*	XE1413	13	32	84	0.2
dsl-5	*dsl-5(ok588); oxIs12*	XE1409	15	38	68	1.0
dsl-6	*dsl-6(ok2265); oxIs12*	XE1410	11	30	73	0.68
dos-1	*dos-1(ok2398); oxIs12*	XE1414	21	53	74	0.14
dos-2	*dos-2(tm4515); oxIs12*	XE1415	12	31	84	0.1
dos-3	*dos-3(tm4899); oxIs12*	XE1416	19	51	65	0.63
arg-1	*arg-1(ok3127); juIs76*	XE1417	19	49	84	0.162
apx-1 balanced strain 1	*apx-1(or22)/nT1; oxIs12*	XE1408		N/A		
apx-1 balanced strain 2	*apx-1(or15)/nT1; oxIs12*	XE1407		N/A		