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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 central nervous system do not regenerate, and even neurons in the peripheral nervous system 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 postdevelopmental role for the Notch pathway as a repressor of axon regeneration in vivo.

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 and tensin homolog (PTEN) reduces regeneration in both the mammalian central nervous system and peripheral nervous system, 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 postmitotic 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 an intrinsic inhibitor of nerve regeneration in mature *C. elegans* neurons and show that regeneration is improved 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 postinjury 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 (Figure 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, whereas 32% of axons failed to successfully regenerate (Figure 1C; see Table S1 available online 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 Experimental Procedures). 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 (Figure 1B) (Greenwald et al., 1983). After laser surgery, however, axons in Notch/*lin-12* loss-of-function animals regenerated significantly better than wild-type (Figure 1C). In Notch/*lin-12* loss-of-function animals, nearly all axons successfully regenerated, and failure of regeneration was reduced more than 2-fold, to 12%. In addition, two Notch/*lin-12*

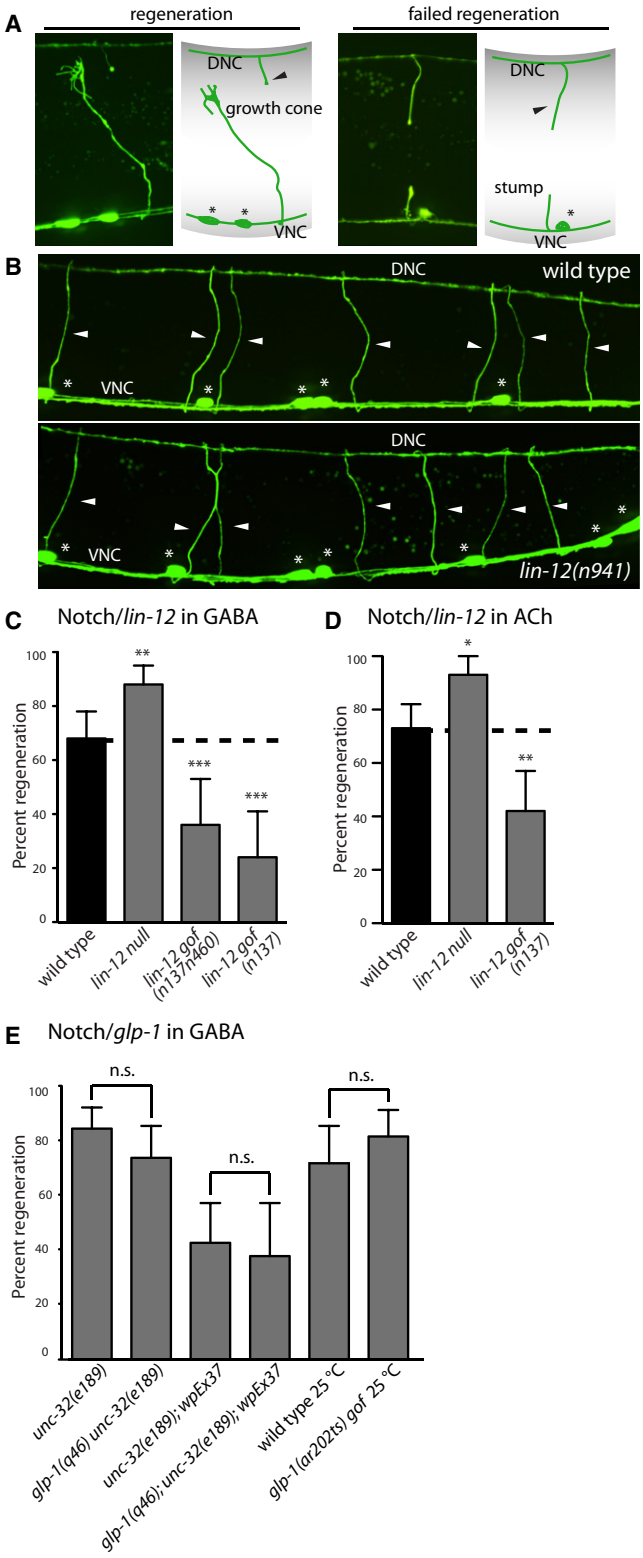

Figure 1. Notch Signaling Inhibits Nerve Regeneration

(A) Axon regeneration in *C. elegans* GABA neurons. Some injured axons regenerate (left panels), whereas 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.

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 (Figure 1D). By contrast, Notch/*glp-1* did not affect regeneration (Figure 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 min after injury (Hammarlund et al., 2009). Because 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 hr after severing their axons (Figures 2A and 2B). Consistent with previous results, in wild-type animals, only a small percentage of axons had initiated growth cones at these early time points (6 hr: 9/113 axons with growth cones, 8%). By contrast, Notch/*lin-12* mutant animals displayed a significant increase in growth cone initiation at 6 hr 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 (Figure 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

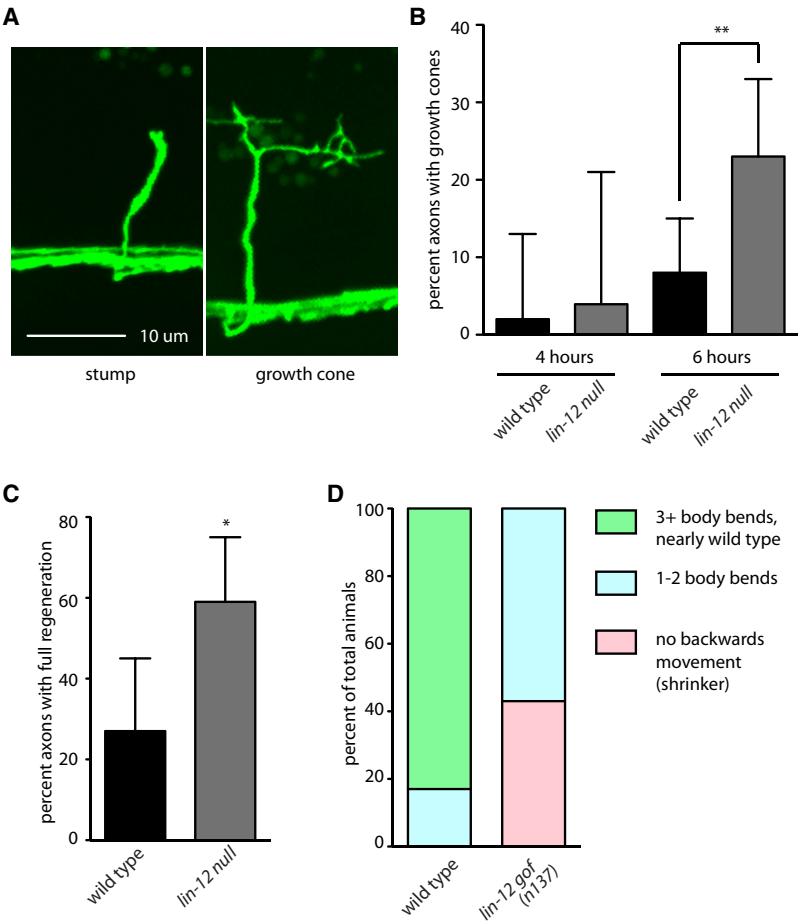

Figure 2. Notch Affects Growth Cone Formation and Behavioral Recovery

(A) Representative axons that have not formed a growth cone (left) or have formed a growth cone (right) at 6 hr 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.

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 (Figure 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 hr 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 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 intramembrane 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 (Figure 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 (Figure 3B). A loss-of-function mutant in ADAM17/*adm-4* did not affect regeneration (Figure 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. Because both single mutants already have regeneration that approaches 100%, we conducted this analysis by examining growth cone initiation at the 6 hr time point. We found that ADAM10/*sup-17* mutants, like Notch/*lin-12* mutants, have increased growth cone initiation at 6 hr relative to wild-type (Figure 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* (Figure 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.

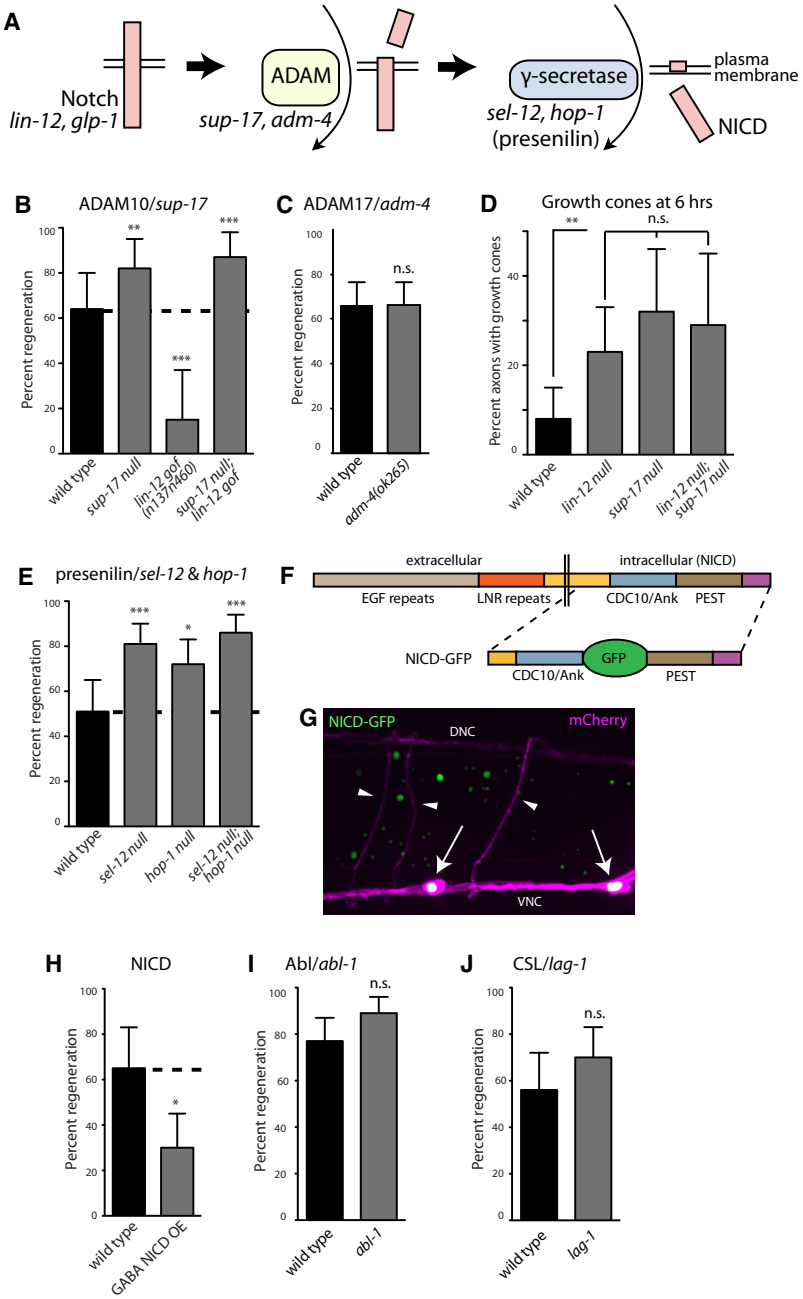

Figure 3. Notch Inhibits Regeneration via a Canonical Activation Pathway

(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, co-localization. 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 (B)–(E) and (H)–(J) show percentage of axons that initiated regeneration; error bars show 95% confidence interval (CI). *p < 0.05, **p < 0.01, ***p < 0.001 (See also Table S1.)

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 (Figure 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* and *hop-1* comprise a linear pathway that inhibits regeneration. Further, because the function of ADAM10 and gamma secretase is to liberate the 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 green fluorescent protein (GFP)-tagged version of the Notch/*lin-12* intracellular domain (NICD-GFP; Figure 3F). When this construct was expressed in wild-type animals, the NICD-GFP signal was concentrated in a subcellular distribution consistent with nuclear localization (Figure 3G). Expression of NICD-GFP resulted in significantly reduced regeneration compared to control (Figure 3H) and was similar to regeneration in gain-of-function Notch mutants (Figure 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 noncanonical mechanism is active in limiting regeneration, we examined regeneration in Abl/*abl-1*

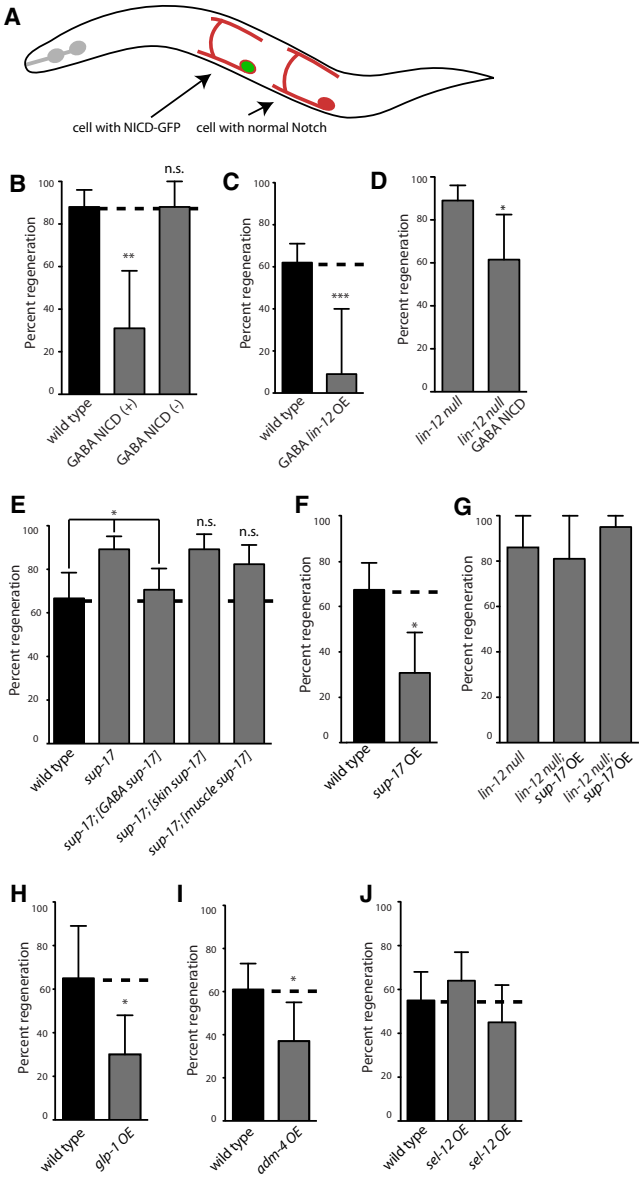

Figure 4. Notch/*lin-12* Functions Cell Intrinsically to Limit Regeneration

(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 (B)–(J) show percentage of axons that initiated regeneration; error bars show 95% CI. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (See also Table S1.)

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 (Figure 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 (Figure 3J). We conclude that Notch signaling probably 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 chondroitin sulfate proteoglycans). 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 (Figures 3F–3H). 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, whereas cells in the same animal that were 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 (Figure 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 (Figure 4B), similar to gain-of-function Notch/*lin-12* mutant axons (Figure 1C). By contrast, NICD-GFP(−) axons from the same animals had normal regeneration (Figure 4B). Third, we observed a similar overall inhibition of regeneration when we overexpressed full-length Notch/*lin-12* cDNA only in the GABA neurons (Figure 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 nontransgenic

Notch/*lin-12* null mutants: animals had protruding vulvas and were completely sterile. However, these animals had decreased regeneration in their GABA neurons (Figure 4D), compared to the increased regeneration normally found in Notch/*lin-12* null mutants (Figure 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 (Figure 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 (Figure 4E). Additionally, we found that overexpression in wild-type animals of ADAM10/*sup-17* in the GABA neurons inhibits regeneration (Figure 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 (Figure 4G). Taken together, these data demonstrate that Notch acts cell autonomously to inhibit regeneration and establish that Notch signaling is an 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 (Figure 3A) (Jarriault and Greenwald, 2005). However, only one Notch gene (Notch/*lin-12*) and one ADAM (ADAM/*sup-17*) inhibit regeneration in GABA neurons (Figures 1 and 3). Because 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 (Figure 4H), similar to overexpression of Notch/*lin-12* NICD-GFP (Figure 3H). GABA-specific overexpression of ADAM/*adm-4* also inhibited regeneration (Figure 4I), similar to overexpression of ADAM/*sup-17* (Figure 4F). By contrast, GABA-specific overexpression of presenilin/*sel-12* did not limit regeneration (Figure 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 nonpermissive temperature after surgery (Figures 5A–5C). These data demonstrate that Notch signaling is active after injury in mature neurons and that this postinjury 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 and Wang, 2007). Because 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 μM DAPT or a control solution (Figure 5D, immediate DAPT). Animals treated immediately with DAPT had increased regeneration and fewer regeneration failures than control animals (Figure 5E), similar to genetic manipulations that reduce Notch signaling (Figure 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 (Figure 3E). DAPT injection in these animals did not further increase regeneration, demonstrating that DAPT acts by inhibiting gamma secretase (Figure 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 2 hr after surgery ("DAPT + 2 hr," Figure 5D). These animals did not regenerate better than controls (Figure 5G). Thus, by 2 hr 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 may 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). Because Notch signaling inhibits regeneration, loss of the ligand that activates Notch should result in increased regeneration, similar to loss of Notch signaling itself (Figures 1

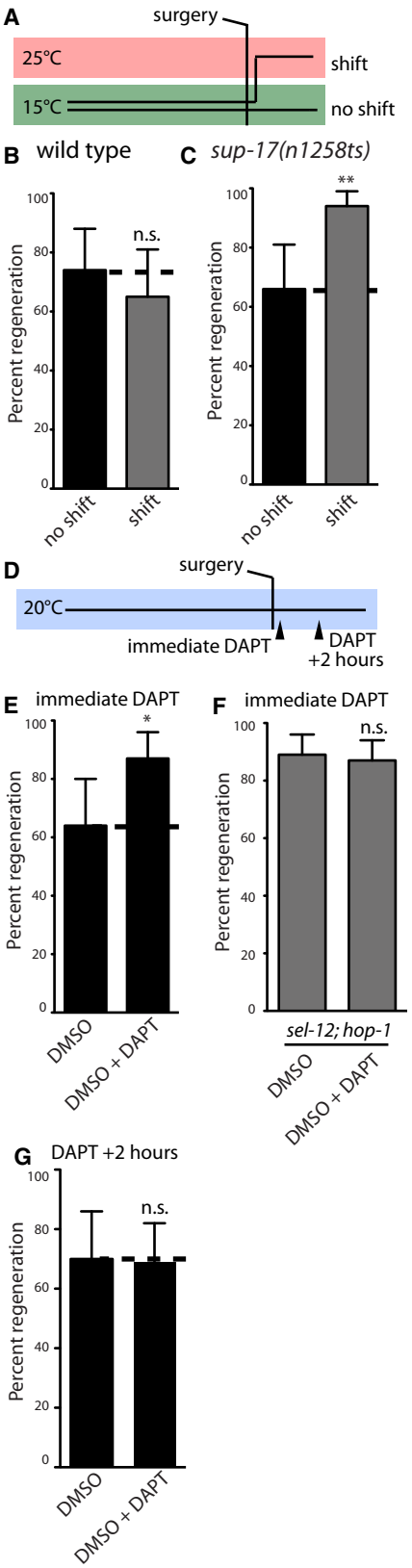

Figure 5. Notch Signaling Functions at the Time of Injury to Inhibit Regeneration, and Chemical Inhibition of Notch Improves Regeneration in Wild-Type Animals

(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 nonpermissive temperature after axotomy.

(D) DAPT in DMSO or control (DMSO) was injected immediately after axotomy or after a 2 hr 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 hr after axotomy has no effect on regeneration. Bars in (B), (C), (E), and (F) show percentage of axons that initiated regeneration; error bars show 95% CI. *p < 0.05, **p < 0.01, ***p < 0.001. (See also Table S2.)

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 (Figure 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 (Figure 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 (Figure 6C). Consistent with these data, blocking Notch signaling in aged animals does not increase regeneration compared to aged wild-type animals (Figure 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 (Figure 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.

Table 1. Individual Notch Ligands Are Not Required for Notch Function in Regeneration

Description	Genotype	Strain	Number of Animals	Number of 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		

DISCUSSION

Our results identify a postdevelopmental 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 postmitotic 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 nontranscriptional function of the NICD (Giniger, 1998; Le Gall et al., 2008), does not regulate axon regeneration (Figure 3I). Second, a GFP-tagged Notch/*lin-12* NICD localizes to the nucleus and inhibits regeneration (Figures 3F–3H), consistent with a transcriptional function. Because 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 postmitotic 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, because *lag-2* mutants have decreased regeneration (Table 1). It is possible that loss of *lag-2* triggers

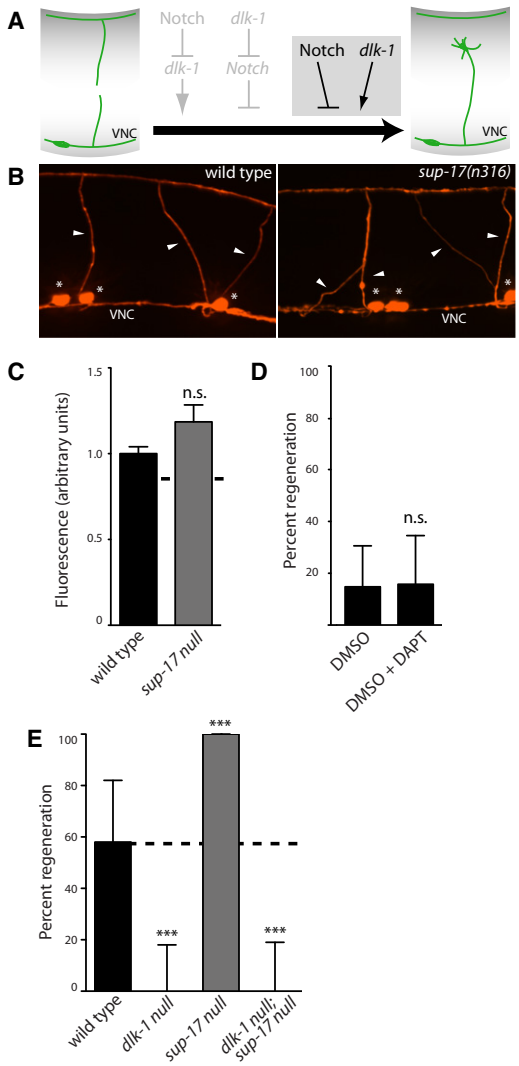

Figure 6. Notch Regulates Regeneration Independently of the *dlk-1* MAP Kinase Pathway

(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; error bars show 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; error bars show 95% CI. *p < 0.05, **p < 0.01, ***p < 0.001. (See also Table S3.)

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 (Figures 2A and 2B). 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, whereas blocking activation 2 hr after injury does not increase regeneration (Figures 5E and 5G). 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 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 (Figures 2C and 2D).

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 can act after nerve injury to suppress axon regeneration.

EXPERIMENTAL PROCEDURES

C. elegans Strains

Animals were maintained on nematode growth medium 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 that segregated 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 Tables S1–S3.

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) 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 hr later for scoring. At least 30 axons were scored for most genotypes (2–3 cut axons per animal); see Tables S1–S3. 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 hr after axotomy were scored as positive.

For growth cone initiation at 4 and 6 hr, axons with a growth cone were scored as positive. 95% confidence intervals were calculated by the Wald method, and two-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 hr after axotomy. At 1 hr after axotomy, all animals responded by shrinking and were unable to initiate backward locomotion. Animals were scored at 24 hr after axotomy into one of the following categories: (1) no backward movement (shrink); (2) one or two body bends backward; or (3) three 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 Supplemental Experimental Procedures.

Transgenics

Transgenic animals were obtained by microinjection as described (Mello et al., 1991). Transgene name, content, and concentrations are listed in Supplemental Experimental Procedures. For most strains, stable transgenic lines were selected based on GFP expression in the pharyngeal muscles from a P*myo-2*:GFP coinjection 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 hr 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. Twenty-one 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 Table S3 for numbers and statistics.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three tables and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.11.017.

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REFERENCES

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*, 770–776.

Atwal, J.K., Pinkston-Gosse, J., Syken, J., Stawicki, S., Wu, Y., Shatz, C., and Tessier-Lavigne, M. (2008). PirB is a functional receptor for myelin inhibitors of axonal regeneration. Science *322*, 967–970.

Austin, J., and Kimble, J. (1989). Transcript analysis of *glp-1* and *lin-12*, homologous genes required for cell interactions during development of *C. elegans*. Cell *58*, 565–571.

Berezovska, O., McLean, P., Knowles, R., Frosh, M., Lu, F.M., Lux, S.E., and Hyman, B.T. (1999). Notch1 inhibits neurite outgrowth in postmitotic primary neurons. Neuroscience *93*, 433–439.

Chao, M.Y., Larkins-Ford, J., Tucey, T.M., and Hart, A.C. (2005). *lin-12* Notch functions in the adult nervous system of *C. elegans*. BMC Neurosci. *6*, 45.

Christie, K.J., Webber, C.A., Martinez, J.A., Singh, B., and Zochodne, D.W. (2010). PTEN inhibition to facilitate intrinsic regenerative outgrowth of adult peripheral axons. J. Neurosci. *30*, 9306–9315.

Crowner, D., Le Gall, M., Gates, M.A., and Giniger, E. (2003). Notch steers Drosophila ISNb motor axons by regulating the Abl signaling pathway. Curr. Biol. *13*, 967–972.

Dovey, H.F., John, V., Anderson, J.P., Chen, L.Z., de Saint Andrieu, P., Fang, L.Y., Freedman, S.B., Folmer, B., Goldbach, E., Holsztynska, E.J., et al. (2001). Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. J. Neurochem. *76*, 173–181.

Filbin, M.T. (2008). PirB, a second receptor for the myelin inhibitors of axonal regeneration Nogo66, MAG, and OMgp: implications for regeneration in vivo. Neuron *60*, 740–742.

Fortini, M.E. (2009). Notch signaling: the core pathway and its posttranslational regulation. Dev. Cell *16*, 633–647.

Fournier, A.E., GrandPre, T., and Strittmatter, S.M. (2001). Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. Nature *409*, 341–346.

Franklin, J.L., Berechid, B.E., Cutting, F.B., Presente, A., Chambers, C.B., Foltz, D.R., Ferreira, A., and Nye, J.S. (1999). Autonomous and non-autonomous regulation of mammalian neurite development by Notch1 and Delta1. Curr. Biol. *9*, 1448–1457.

Geling, A., Steiner, H., Willem, M., Bally-Cuif, L., and Haass, C. (2002). A gamma-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. EMBO Rep. *3*, 688–694.

Giniger, E. (1998). A role for Abl in Notch signaling. Neuron *20*, 667–681.

Gordon, W.R., Arnett, K.L., and Blacklow, S.C. (2008). The molecular logic of Notch signaling—a structural and biochemical perspective. J. Cell Sci. *121*, 3109–3119.

Greenwald, I. (2005). LIN-12/Notch signaling in *C. elegans.* In WormBook, The *C. elegans* Research Community, ed. 10.1895/wormbook.1.10.1, http://www.wormbook.org.

Greenwald, I., and Seydoux, G. (1990). Analysis of gain-of-function mutations of the *lin-12* gene of *Caenorhabditis elegans*. Nature *346*, 197–199.

Greenwald, I.S., Sternberg, P.W., and Horvitz, H.R. (1983). The *lin-12* locus specifies cell fates in *Caenorhabditis elegans*. Cell *34*, 435–444.

Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E.M., and Bastiani, M. (2009). Axon regeneration requires a conserved MAP kinase pathway. Science *323*, 802–806.

Hassan, B.A., Bermingham, N.A., He, Y., Sun, Y., Jan, Y.N., Zoghbi, H.Y., and Bellen, H.J. (2000). *atonal* regulates neurite arborization but does not act as a proneural gene in the *Drosophila* brain. Neuron *25*, 549–561.

Hunt-Newbury, R., Viveiros, R., Johnsen, R., Mah, A., Anastas, D., Fang, L., Halfnight, E., Lee, D., Lin, J., Lorch, A., et al. (2007). High-throughput in vivo analysis of gene expression in *Caenorhabditis elegans*. PLoS Biol. *5*, e237.

Jarriault, S., and Greenwald, I. (2005). Evidence for functional redundancy between *C. elegans* ADAM proteins SUP-17/Kuzbanian and ADM-4/TACE. Dev. Biol. *287*, 1–10.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E.H., Kopan, R., and Israel, A. (1995). Signalling downstream of activated mammalian Notch. Nature *377*, 355–358.

Lambie, E.J., and Kimble, J. (1991). Two homologous regulatory genes, *lin-12* and *glp-1*, have overlapping functions. Development *112*, 231–240.

Le Gall, M., De Mattei, C., and Giniger, E. (2008). Molecular separation of two signaling pathways for the receptor, Notch. Dev. Biol. *313*, 556–567.

Levitan, D., and Greenwald, I. (1995). Facilitation of *lin-12*-mediated signalling by *sel-12*, a *Caenorhabditis elegans* S182 Alzheimer's disease gene. Nature *377*, 351–354.

Li, X., and Greenwald, I. (1997). HOP-1, a *Caenorhabditis elegans* presenilin, appears to be functionally redundant with SEL-12 presenilin and to facilitate LIN-12 and GLP-1 signaling. Proc. Natl. Acad. Sci. USA *94*, 12204–12209.

Lieber, T., Kidd, S., Alcamo, E., Corbin, V., and Young, M.W. (1993). Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. Genes Dev. *7*, 1949–1965.

Louvi, A., and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. Nat. Rev. Neurosci. *7*, 93–102.

McGee, A.W., and Strittmatter, S.M. (2003). The Nogo-66 receptor: focusing myelin inhibition of axon regeneration. Trends Neurosci. *26*, 193–198.

Mello, C.C., Kramer, J.M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. *10*, 3959–3970.

Ouellet, J., Li, S., and Roy, R. (2008). Notch signalling is required for both dauer maintenance and recovery in *C. elegans*. Development *135*, 2583–2592.

Park, K.K., Liu, K., Hu, Y., Smith, P.D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M., and He, Z. (2008). Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science *322*, 963–966.

Priess, J.R. (2005). Notch signaling in the *C. elegans* embryo. In WormBook, The *C. elegans* Research Community, ed. 10.1895/wormbook.1.4.1, http://www.wormbook.org.

Qiao, L., Lissemore, J.L., Shu, P., Smardon, A., Gelber, M.B., and Maine, E.M. (1995). Enhancers of *glp-1,* a gene required for cell-signaling in *Caenorhabditis elegans*, define a set of genes required for germline development. Genetics *141*, 551–569.

Rand, M.D., Grimm, L.M., Artavanis-Tsakonas, S., Patriub, V., Blacklow, S.C., Sklar, J., and Aster, J.C. (2000). Calcium depletion dissociates and activates heterodimeric notch receptors. Mol. Cell. Biol. *20*, 1825–1835.

Redmond, L., Oh, S.R., Hicks, C., Weinmaster, G., and Ghosh, A. (2000). Nuclear Notch1 signaling and the regulation of dendritic development. Nat. Neurosci. *3*, 30–40.

Rossi, F., Gianola, S., and Corvetti, L. (2007). Regulation of intrinsic neuronal properties for axon growth and regeneration. Prog. Neurobiol. *81*, 1–28.

Schuske, K., Beg, A.A., and Jorgensen, E.M. (2004). The GABA nervous system in *C. elegans*. Trends Neurosci. *27*, 407–414.

Sestan, N., Artavanis-Tsakonas, S., and Rakic, P. (1999). Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. Science *286*, 741–746.

Shih, Ie.M., and Wang, T.L. (2007). Notch signaling, gamma-secretase inhibitors, and cancer therapy. Cancer Res. *67*, 1879–1882.

Singh, K., Chao, M.Y., Somers, G.A., Komatsu, H., Corkins, M.E., Larkins-Ford, J., Tucey, T., Dionne, H.M., Walsh, M.B., Beaumont, E.K., et al. (2011). *C. elegans* Notch signaling regulates adult chemosensory response and larval molting quiescence. Curr. Biol. *21*, 825–834.

Smith, P.D., Sun, F., Park, K.K., Cai, B., Wang, C., Kuwako, K., Martinez-Carrasco, I., Connolly, L., and He, Z. (2009). SOCS3 deletion promotes optic nerve regeneration in vivo. Neuron *64*, 617–623.

Solomon, A., Mian, Y., Ortega-Cava, C., Liu, V.W., Gurumurthy, C.B., Naramura, M., Band, V., and Band, H. (2008). Upregulation of the *let-7* microRNA with precocious development in *lin-12*/Notch hypermorphic *Caenorhabditis elegans* mutants. Dev. Biol. *316*, 191–199.

Stiernagle, T. (2006) Maintenance of *C. elegans*. In WormBook, The C. elegans Research Community, ed. 10.1895/wormbook.1.101.1, http://www.wormbook.org.

Struhl, G., Fitzgerald, K., and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. Cell *74*, 331–345.

Tax, F.E., Thomas, J.H., Ferguson, E.L., and Horvitz, H.R. (1997). Identification and characterization of genes that interact with *lin-12* in *Caenorhabditis elegans*. Genetics *147*, 1675–1695.

Wen, C., Metzstein, M.M., and Greenwald, I. (1997). SUP-17, a *Caenorhabditis elegans* ADAM protein related to *Drosophila* KUZBANIAN, and its role in LIN-12/NOTCH signalling. Development *124*, 4759–4767.

Wittenburg, N., Eimer, S., Lakowski, B., Röhrig, S., Rudolph, C., and Baumeister, R. (2000). Presenilin is required for proper morphology and function of neurons in *C. elegans*. Nature *406*, 306–309.

Yan, D., Wu, Z., Chisholm, A.D., and Jin, Y. (2009). The DLK-1 kinase promotes mRNA stability and local translation in *C. elegans* synapses and axon regeneration. Cell *138*, 1005–1018.

Yanik, M.F., Cinar, H., Cinar, H.N., Chisholm, A.D., Jin, Y., and Ben-Yakar, A. (2004). Neurosurgery: functional regeneration after laser axotomy. Nature *432*, 822.

Yiu, G., and He, Z. (2006). Glial inhibition of CNS axon regeneration. Nat. Rev. Neurosci. *7*, 617–627.

Yochem, J., and Greenwald, I. (1989). *glp-1* and *lin-12*, genes implicated in distinct cell-cell interactions in *C. elegans*, encode similar transmembrane proteins. Cell *58*, 553–563.

Yochem, J., Weston, K., and Greenwald, I. (1988). The *Caenorhabditis elegans lin-12* gene encodes a transmembrane protein with overall similarity to *Drosophila* Notch. Nature *335*, 547–550.

Yoo, A.S., Bais, C., and Greenwald, I. (2004). Crosstalk between the EGFR and LIN-12/Notch pathways in *C. elegans* vulval development. Science *303*, 663–666.