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# Fragile Axons Forge the Path to Gene Discovery: A MAP Kinase Pathway Regulates Axon Regeneration

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**The nematode *Caenorhabditis elegans* is emerging as a promising model for studying the molecular control of axon regeneration. A forward genetic screen identified the DLK-1 (dual leucine zipper-bearing kinase 1) MAP (mitogen-activated protein) kinase pathway as a positive regulator of growth cone formation during axon regeneration. Although DLK-1 pathway mutant animals display a dramatic defect in regeneration, their axons have no apparent defects in initial outgrowth. The DLK-1 pathway also plays a role in synaptogenesis, but this role appears to be separate from its function in regeneration. Understanding how the DLK-1 pathway acts in development, plasticity, and regeneration may shed light on the evolution of mechanisms regulating axon regeneration.**

## A Genetic Approach to Identify Genes Controlling Axon Regeneration

An axon's ability to regenerate after damage is influenced by a balance of extrinsic factors that inhibit or promote axon outgrowth and guidance, as well as intrinsic processes that regulate axon growth potential. In the vertebrate central nervous system (CNS), inhibitory pathways predominate, effectively preventing functional recovery from axon injury. Studies of axon regeneration in the mammalian CNS have focused primarily on a few extrinsic signals that limit axon regeneration, most prominently, the myelin-associated inhibitors that signal through the Nogo receptor, and chondroitin sulfate proteoglycans in glial scars (*1*). However, knocking down genes in these pathways so far has allowed only partial improvements in regeneration (*2*, *3*). It may be necessary to manipulate additional extrinsic or intrinsic pathways to robustly improve axon regeneration.

Unbiased forward genetic screening could be a powerful approach for identifying genes that regulate axon regeneration, but such large-scale genetic screening is technically difficult in rodent models. The nematode *Caenorhabditis elegans* has emerged as a promising model for studying axon regeneration. The development of precise laser axotomy methods has made it possible to monitor the regeneration of single axons in live animals (*4*). Studies in *C. elegans* combining laser axotomy with candidate gene analysis have provided insight into the process of axon guidance during regeneration (*5*, *6*). However, even in *C. elegans*, laser axotomy would likely be too laborious to use as an assay in a comprehensive genetic screen. Hammarlund *et al*. devised a clever scheme to solve this problem: make the worms break their own axons (*7*) (Fig. 1A). To achieve this, they took advantage of a loss-of-function mutation in the *unc-70* gene, the *C. elegans* homolog of β-spectrin, a component of the cortical cytoskeleton (*8*). In β-spectrin mutants, the nervous system develops normally, but axons are fragile and break as animals move (*9*). This chronic damage to the nervous system induces continuous, error-prone regeneration. Regrowing axons can be directly visualized with a fluorescent reporter for γ-aminobutyric acid (GABA)–releasing motor neurons, which extend their axons from the ventral to dorsal nerve cord and control the sinusoidal movement of the worm. A large-scale screen for *unc-70* mutant animals with diminished regenerative growth of GABAergic motor neurons identified a requirement for the MAP kinase kinase kinase (MAPKKK) DLK-1 (dual leucine zipper-bearing kinase 1) in axon regeneration (*7*). Laser axotomy in *dlk-1* loss-of-function mutants confirmed that DLK-1 was required for axon regeneration, regardless of the mechanism of injury.

The inability of severed axons in *dlk-1* mutants to regenerate appeared to result from an inability to generate new growth cones (*7*). Time-lapse movies revealed that although filopodia extended from severed axons in *dlk-1* mutants, motile growth cones did not form. This defect was seen even when axotomy was performed during active outgrowth, suggesting that DLK-1 is required for growth cone initiation after injury at any stage, rather than just in mature axons. GABAergic neurons overexpressing DLK-1 in a wild-type background had a greater capacity for regeneration after axotomy: Growth cones initiated more quickly, and a higher percentage of axons grew to the dorsal cord compared with wild-type. *dlk-1* mutants displayed no apparent defects in initial motor neuron outgrowth, indicating that DLK-1 is required for axon regeneration but not for development.

## MAP Kinase Signaling in *C. elegans* Regeneration and Synaptogenesis

In *C. elegans*, members of all three major classes of MAP kinases—extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), and p38—play roles in development and neuronal physiology (*10*). DLK-1 is a member of a conserved MAPK cascade that includes the MAP kinase kinase MKK-4 and the p38 MAP kinase PMK-3 (Fig. 1B). Loss-of-function mutations in either the *mkk-4* or *pmk-3* genes caused the same axon regeneration defect as *dlk-1* mutants, indicating that these genes act together in a pathway to promote growth cone formation in injured axons (*7*). Loss-of-function mutations in members of a distinct p38 pathway (either the MAPKKK *mlk-1* or the MAPKK *mek-1*) also caused defects in axon regeneration, whereas a loss-of-function mutation in the JNK signaling pathway (*jnk-1*) improved regeneration (*7*). In mammals, axon damage induces ERK and JNK activity, which is thought to carry retrograde signals from the site of axotomy to the cell body to mediate responses to injury, such as the induction of gene transcription (*11*). p38 is implicated in the formation of growth cones in vitro, perhaps by controlling local translation in the axon (*12*). Characterizing the downstream effects of each of the *C. elegans* MAP kinase pathways that influence axon regeneration may clarify whether they affect the same or different steps of regeneration.

Both JNK and p38 signaling in *C. elegans* also control presynaptic structures (*13*, *14*),

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raising the possibility that synapse development and axon regeneration are linked processes. One potential model is that synapse formation decreases growth-related molecules, including DLK-1, and consequently diminishes the capacity for regeneration. In *C. elegans,* the ubiquitin ligase RPM-1, a member of the PHR (Pam/Highwire/RPM-1) family of proteins that also regulates synaptogenesis in flies and mice (*15*–*17*), affects synapse development in part by targeting DLK-1 for degradation (*14*). Hammarlund *et al*. verified the relationship between these genes in regeneration by performing axotomies in *rpm-1* loss-of-function mutants (*7*), which exhibited improved axon regeneration relative to wild type, similar to GABAergic neurons overexpressing DLK-1. Conversely, overexpression of RPM-1 caused defects in axon regeneration, similar to those seen in *dlk-1* loss-of-function mutants. Axon injury could therefore activate DLK-1 directly, or indirectly by inhibiting its regulator RPM-1. RPM-1 also functions in a parallel pathway to activate *glo-1* and *glo-4*, genes that encode a Rab and Rab guanine exchange factor (GEF), respectively, that regulate vesicular trafficking to presynaptic terminals (*18*). Hammarlund *et al*. performed axotomies in *glo-1* and *glo-4* loss-of-function mutants and found that the capacity for regeneration was not strongly affected (*7*). Thus, RPM-1 affects regeneration primarily through its regulation of DLK-1, rather than through its other effects on synapse formation.

It is possible that the roles of the RPM-1 and DLK-1 proteins in regeneration are independent of their roles in synaptogenesis and relates instead to a separate function in axon outgrowth. Motor axons in *rpm-1* mutants often grow aberrantly beyond their target in the dorsal cord (*19*). Thus, although DLK-1 is not required for axon development, its regulator, RPM-1, limits axon growth. One potential explanation for this observation is that DLK-1 acts redundantly with another gene (perhaps MLK-1) to promote growth cone initiation during development. The activity of this other pathway could weaken after initial axon outgrowth, revealing DLK-1's role in growth cone initiation. Consistent with the model that DLK-1 plays a role in axon outgrowth, a DLK-1 homolog in mice is required for axon development and cell migration in the brain (*20*).

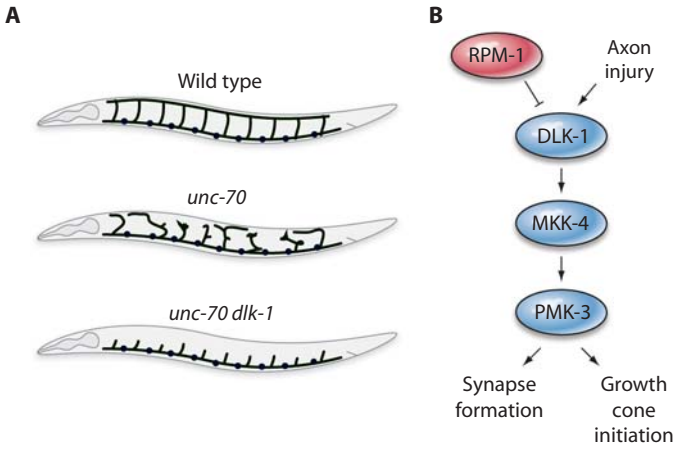


**Fig. 1.** A MAP kinase pathway is required for axon regeneration in *C. elegans*. (**A**) In wild-type animals, ventral GABA motor neurons extend to the dorsal nerve cord (top). In *unc-70* β-spectrin mutants, axons continuously break and regenerate growth cones that extend dorsally, albeit in an error-prone manner (middle). A screen for GABA motor neuron regeneration in an *unc-70* mutant background identified DLK-1 as a positive regulator of regeneration: When the function of the *dlk-1* gene was disrupted by RNA interference (RNAi) or mutation, growth cone initiation was blocked (bottom). (**B**) The DLK-1 pathway consists of the MAPKKK DLK-1, the MAPKK MKK-4, and the p38 MAPK PMK-3. This pathway, which is inhibited by the ubiquitin ligase RPM-1, is required for both synapse development and axon regeneration.

## Development, Regeneration, and Plasticity

Axon regeneration recapitulates many of the steps of axon development: A growth cone must be generated, the axon must navigate through a complex environment, and synapses must form on the appropriate targets. However, during regeneration, axons grow through a substantially different environment than the one they initially encountered. At later stages, guidance cues may no longer be expressed by target tissues and there may be molecular and physical barriers to growth that were not present during development. In both vertebrates and *C. elegans*, a distinct set of guidance molecules influence axon extension during development versus during regeneration (*5*, *6*, *21*). In *C. elegans*, spontaneous regeneration occurs after injury, but successful target reinnervation is rare. Hammarlund *et al*. reported that only 5% of regenerating GABAergic motor axons reached the dorsal cord (*7*), which suggests that even in a relatively permissive environment for regeneration, growth is often aberrant due to a lack of guidance cues or a decrease in guidance receptors on the axon.

The molecular mechanisms controlling axon regeneration might overlap not just with pathways that function during initial axon outgrowth but also with pathways controlling adult structural plasticity. Structural and synaptic changes in mature axons occur as the nervous system responds to its environment, allowing animals to learn and store memories. A striking example of the connection between regeneration and plasticity is the observation that several of the molecules limiting regenerative axon outgrowth in the vertebrate CNS are also required for limiting the critical period for ocular dominance plasticity in the cortex (*22*–*25*). This correlation indicates that axons face the same barriers to growth whether regenerating after injury or undergoing plasticity. These barriers arise at the closure of critical periods, so that an axon regenerating after injury faces distinct challenges that were not present during development.

The observation that mutants in the DLK-1 pathway have no axon outgrowth phenotype during development, but a dramatic one during regeneration, raises questions concerning the evolution of mechanisms controlling regeneration. It is possible that the need to repair axon damage exerts a selective pressure sufficient to have favored the evolution of a mechanism dedicated specifically to promoting axon regeneration. However, this model seems unlikely, given that wild-type *C. elegans* do not normally break their axons, at least not in a laboratory setting. Another possibility is that DLK-1's function in axon regeneration is a secondary consequence of a role in synaptogenesis or a cryptic role in developmental axon outgrowth, as discussed above. A third, perhaps more intriguing, alternative is that evolution selected mech-

anisms controlling adult plasticity, which consequently had effects on axon regenerative capacity. In this model, inhibitors of axon regeneration in mammals may have evolved primarily to block plasticity and thus stabilize neural circuits. Similarly, positive regulators of regeneration, such as the DLK-1 pathway, may have evolved to promote plasticity in appropriate contexts. Although neuronal plasticity is less well characterized in *C. elegans* than in vertebrates, mature axons and synaptic connections do remodel as part of normal developmental programs (*26*, *27*) and in response to changes in activity (*28–31*). It will be exciting to learn whether the DLK-1 pathway is required for any of these remodeling processes and whether its roles in either regeneration or synapse formation can contribute to regulating neuronal plasticity. Further studies of DLK-1 function may thus shed light not only on how axon regeneration is regulated but also on why those regulatory mechanisms evolved.

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