# Axon regeneration requires coordinate activation of p38 and JNK MAPK pathways

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**Signaling pathways essential for axon regeneration, but not for neuron development or function, are particularly well suited targets for therapeutic intervention. We find that the parallel PMK-3(p38) and KGB-1(JNK) MAPK pathways must be coordinately activated to promote axon regeneration. Axon regeneration fails if the activity of either pathway is absent. These two MAPKs are coregulated by the E3 ubiquitin ligase RPM-1(Phr1) via targeted degradation of the MAPKKKs DLK-1 and MLK-1 and by the MAPK phosphatase VHP-1(MKP7), which negatively regulates both PMK-3 (p38) and KGB-1(JNK).**

*Caenorhabditis elegans* | MAPK signaling | laser axotomy

Neuronal regeneration has been studied in humans and other animal model systems for over 100 years, yet we still do not have a comprehensive molecular model or an effective treatment for axotomy due to injury or disease (1–9). In vivo and in vitro model systems have been developed to focus on identifying the molecular differences between vertebrate neurons that can and cannot regenerate (e.g., PNS versus CNS and embryonic versus adult), or between animals that exhibit major differences in their regenerative capacities (e.g., salamander versus mouse). Many exciting discoveries have been made that illustrate the importance of both the extracellular molecular environment and the intrinsic cellular "state" of the neuron (10–13). Classic experiments showing that adult CNS neurons can indeed regenerate if given the right environment, and the identification of many inhibitory molecules associated with CNS myelin and the glial scar stand out as turning points reigniting interest in potential therapies for spinal injury (4, 14). Genetic and cellular studies identifying genes regulating neuron development, motility, and pathfinding have added to the excitement and provided many new environmental and cell intrinsic molecular signaling pathways as potential regulators of neural regeneration (2).

In 2004, laser axotomy was introduced to *Caenorhabditis elegans* by Yanik et al. (15), who showed for the first time that axon regeneration in this model system is robust. Subsequent studies by several laboratories have described the similar features of axon regeneration in *C. elegans* compared with mammals, and provided some insights into the genetic basis of axon regeneration (16–21). The development of microfluidic chambers to automate the immobilization of worms, combined with laser axotomy, raises the hope for high throughput axon regeneration screening assays (22–25). We made an observation that makes it possible to screen for genes that affect axon regeneration in *C. elegans* without laser axotomy (26). We discovered that embryonic neurons lacking β-spectrin develop normally but, after hatching, undergo a spontaneous movement-induced axotomy followed by regeneration. Most commissural axons in each animal break and regenerate before the animal reaches adulthood.

In a previous study, we identified the MAPKKK DLK-1 in our unc-70(β-spectrin) based RNAi screen for genes required for axon regeneration in *C. elegans* (18). MAPKKKs are components of the mitogen-activated protein kinase (MAPK) signaling pathways that respond to extracellular stimuli and generate a diverse array of physiological responses within the cell (27). MAPKs are regulators of cell proliferation, differentiation, migration, regeneration, stress response, and apoptosis. In neurons, MAPK signaling has been implicated in axonal regeneration and degeneration, where loss of these molecules slows distal degeneration and inhibits proximal neurite outgrowth (28–31). DLK-1 and its downstream targets, the MAPKK MKK-4, and the p38 MAPK PMK-3, are dispensable for nervous system development but are essential for regeneration. Loss of function mutations in any of these genes causes regeneration to fail completely, whereas increased activity of the pathway improves regeneration. We identified additional MAPK components in the RNAi screen and here we describe a second MAPK pathway essential for axon regeneration.

## Results

**MLK-1/MEK-1/KGB-1 MAPK Pathway Is Required for Axon Regeneration.** We identified the MAPKKK MLK-1 in an RNAi screen for genes required for axon regeneration (18). MLK-1 and the downstream MAPKK, MEK-1, had been shown to be important for heavy metal stress response by activating the JNK-like MAPK KGB-1 (32). We used laser axotomy to assay the requirement for each of these genes in regeneration.

Wild-type regeneration in GABA motor neurons follows a steep, age-dependent decline (Fig. 1*A* and see Tables S1 and S2 for raw data of all axotomy experiments). Axons in very young animals (L1/L2 stage) respond to injury nearly 100% of the time with regeneration back to their original targets in the dorsal cord. At the L4 stage, most injured axons still respond by initiating and extending a growth cone. However, growth cone migration is rarely successful; growth cone motility is impaired and eventually the growth cone stalls before reaching the dorsal nerve cord (Fig. 2 and Movie S1). By adulthood, even growth cone initiation is compromised. Scoring regeneration at the L4 stage provides a suitable window for observing both increased and decreased regeneration. In wild-type L4 animals, 70% of severed GABA neurons initiate and extend a growth cone within 24 h. In contrast, only 23% of *mlk-1* and 27% of *mek-1* mutant axons are capable of initiating a growth cone, whereas nearly all of the *kgb-1* neurons failed to regenerate (Fig. 1*B*). Thus, KGB-1 activity is essential for axon regeneration.

Overexpression of wild-type MLK-1 in the GABA neurons led to improved regeneration (Figs. 1*B* and 3 and Movie S2). Both the frequency of growth cone initiation and the successful migration of growth cones to target sites were improved relative to the wild type (Fig. 1 *B* and *C*). These data indicate that MLK-1 functions cell-autonomously to promote regeneration. Overexpressing MLK-1 also restored some of the lost regenerative ability of older animals.

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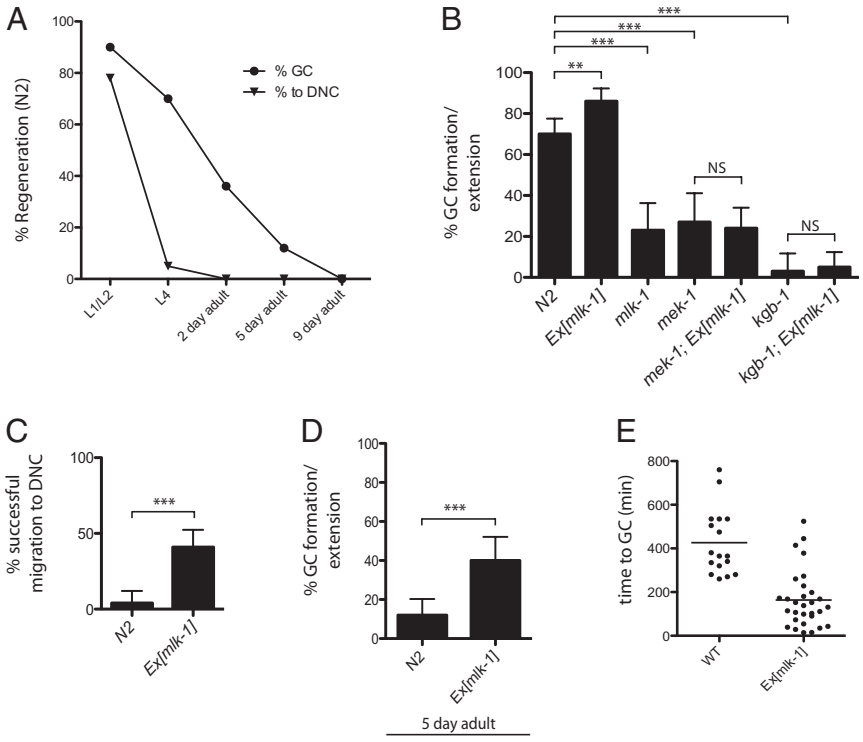


**Fig. 1.** MLK-1/MEK-1/KGB-1 MAPK pathway is required for axon regeneration. (*A*) Age-dependent decline of axon regeneration in the wild type. Regeneration to the dorsal nerve cord (DNC) fails first, followed by failure of axotomized axons to initiate growth cones (GC). %GC is the percentage of severed axons that form a growth cone (GC). % to DNC is the percentage of growth cone-forming axons that regrow to the dorsal nerve cord. (*B*) Regeneration is blocked in mutants of the MLK-1/MEK-1/KGB-1 MAPK module, whereas MLK-1 overexpression improves regeneration. (*C*–*E*) Improved regeneration in animals overexpressing MLK-1 as scored by: (*C*) the percentage of regenerating axons that successfully migrate back to targets in the dorsal nerve cord (DNC), (*D*) growth cone (GC) formation/extension in older animals, and (*E*) timing of first growth cone (GC) appearance. All axotomy performed at L4 stage unless indicated. Ex[*mlk-1*], *mlk-1* transgene expression from an extrachromosomal array. *P* determined by Fisher's exact test. $^{**}P < 0.01$, $^{***}P < 0.001$, NS not significant. Error bars indicate 95% confidence interval.

The age-related decline in regeneration renders most 5-d old adult axons incapable of regenerating (12% growth cone initiation). Overexpressing MLK-1 significantly improved this to 40% (Fig. 1*D*). Finally, the timing of growth cone initiation was improved in neurons overexpressing MLK-1 (Fig. 1*E*). In wild-type injured axons there is a delay of about 7 h before the appearance of a growth cone, whereas neurons overexpressing MLK-1 initiate a growth cone, on average, at 3 h (many within the first hour).

We constructed strains overexpressing MLK-1 in combination with mutants in the downstream genes *mek-1* and *kgb-1*. Both *mek-1* and *kgb-1* mutants suppressed the effects of overexpressing MLK-1 (Fig. 1*B*). Following laser axotomy, *mek-1*; Ex[*mlk-1*] initiated a growth cone 24% of the time, whereas *kgb-1*; Ex[*mlk-1*] initiated a growth cone 5% of the time. Both of these phenotypes are indistinguishable from the single mutants alone, confirming that *mek-1* and *kgb-1* act in a linear pathway downstream of *mlk-1* (Fig. 4*A*).

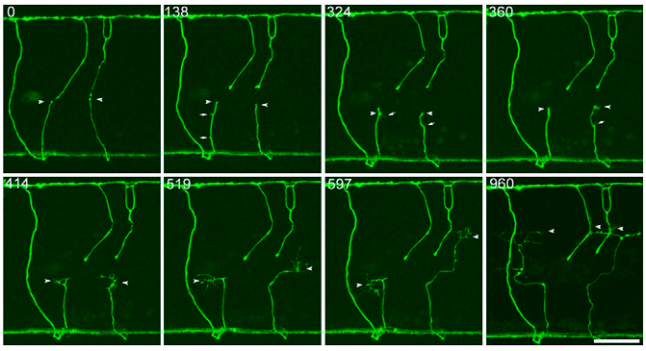


**Fig. 2.** Time-lapse recording illustrates characteristic features of axon regeneration by wild-type L4 GABA motor neurons. Dorsal nerve cord is at the top and ventral nerve cord is at the bottom of each frame. 0 min shows site of laser axotomy and beginning formation of retraction bulbs. 138 and 324 min show microspike activity and remodeling of the axon stump. At 360 min, the right stump initiates a growth cone. By 414 min, both growth cones are beginning to migrate. 519 and 597 min show dystrophic growth cones that make poor progress toward the dorsal nerve cord. At 960 min the dystrophic processes are stalled and it is apparent that regeneration has failed. See Movie S1. Arrowheads point to axon stump and growth cones. Arrows point to microspikes. (Scale bar, 20 μm.)

**Crosstalk Between PMK-3(p38) and KGB-1(Jnk) MAPK Pathways.** The phenotypes of *mlk-1* and *mek-1* mutants are not as severe as *kgb-1* mutants. Although *mlk-1* and *mek-1* function upstream of *kgb-1*, we tested the possibility of crosstalk between the DLK-1 and MLK-1 pathways. First, overexpression of DLK-1 suppresses the loss of *mlk-1* (Fig. 4*B*). Second, some regeneration occurs in *mkk-4* mutants overexpressing DLK-1 compared with the single mutants that fail to regenerate (Fig. 4*C*). When both *mkk-4* and *mek-1* MAPKKs are eliminated in animals overexpressing either DLK-1 or MLK-1, regeneration is blocked (Fig. 4*C*). Taken together, these data demonstrate crosstalk between the two pathways and suggest that MEK-1 is a substrate for DLK-1 (Fig. 4*A*). Overexpression of MLK-1 similarly suppresses the loss of *dlk-1* (Fig. 4*B*). However, *mek-1* mutants themselves are not suppressed by MLK-1 overexpression (Fig. 1*B*), and regeneration is blocked in *dlk-1; mek-1* double mutants overexpressing MLK-1 (Fig. 4*B*). These observations suggest that MKK-4 is unlikely to be a substrate for MLK-1 and that the remaining regeneration in *mlk-1* and *mek-1* single mutants (23% and 27%, respectively) is likely due to the activity of the DLK-1 pathway. When a *mek-1* mutant overexpressing MLK-1 is paired with a mutant from the DLK-1 pathway regeneration is blocked (Fig. 4 *B* and *C* and Table S1).

We also observed evidence of crosstalk at the MAPK level. Overexpression of DLK-1 suppresses the loss of *mek-1* such that regeneration is 73%, whereas overexpression of DLK-1 in *kgb-1* mutants resulted in only 34% regeneration (Fig. 4*D*). We interpret this difference as evidence that KGB-1 may also be activated by MKK-4 (Fig. 4*A*). Similarly, overexpression of MLK-1 increased regeneration in *mkk-4* mutants to 52%, whereas regeneration in *pmk-3* was increased only to 10% (Fig. 4*D*). This difference could be due to activation of PMK-3 by MEK-1 (Fig. 4*A*).

**VHP-1 Phosphatase Acts on Both KGB-1 and PMK-3 to Inhibit Regeneration.** The dual-specificity MAPK phosphatases (MKPs) remove phosphate groups from activated MAPKs and thus, are

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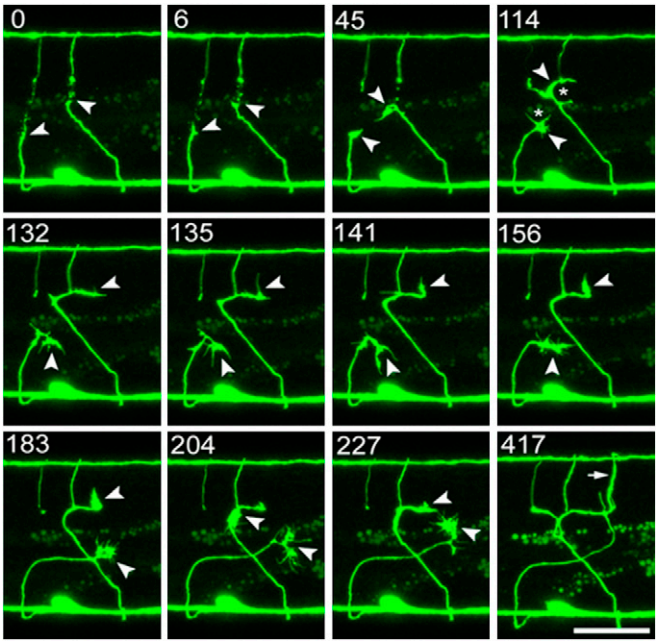


**Fig. 3.** Axon regeneration by GABA neurons expressing higher levels of MLK-1. Dorsal nerve cord is at the top and ventral nerve cord is at the bottom of every frame. 0 min shows retraction bulb formation shortly after laser axotomy. The first sign of stump remodeling is seen at 6 min. At 45 min growth cones are initiated from both stumps. 114 min shows the growth cones extending around their respective sites of laser damage (asterisks). Notice as the growth cone migrates it successfully remodels to reconstitute an embryonic like growth cone (156, 183, and 227 min). By 417 min, both axons have successfully reached the dorsal nerve cord. See Movie S2. Arrowheads point to proximal axon stump and growth cones. (Scale bar, 20 μm.)

important regulators of MAPK signaling (33). In *C. elegans*, the *vhp-1* gene was identified based on its sequence homology to MKPs (MKP7 specifically) and *vhp-1* null mutants arrest as young larvae (at the L2-L3 stage) (32). Loss-of-function mutations in *mlk-1*, *mek-1*, or *kgb-1* can suppress this early larval arrest phenotype (32). These observations indicate that VHP-1 negatively regulates the KGB-1 MAPK pathway and chronically increased activity of the pathway in *vhp-1* mutants leads to a growth arrest phenotype. We tested whether *vhp-1* may also play a role in regeneration. Although most *vhp-1* null mutants arrest as early larvae, rare escapers may be isolated that develop to L4 or adult stages. We assayed regeneration in a small number of these escapers and found that regeneration at L4 is improved to 92% (Fig. 5*A*). Furthermore, 46% of regenerated axons successfully reached the dorsal nerve cord (Fig. 5*B*). A second *vhp-1* allele, *sa366*, encodes an early stop in the sequence, but animals are fully viable. *vhp-1(sa366)* mutants also show improved regeneration of 90%, with 45% successful migration to the dorsal cord (Fig. 5 *A* and *B*). Next, we determined whether increased KGB-1 activity in *vhp-1* mutants could compensate for the loss of PMK-3 signaling. Indeed, a small, although significant number of axons (15%) regenerated in *pmk-3*; *vhp-1* double mutants compared with no regeneration in *pmk-3* single mutants (Fig. 5*A*). *vhp-1* suppressed the *kgb-1* regeneration phenotype more strongly. Forty-eight percent of axons regenerated in *kgb-1; vhp-1* double mutants, compared with 3% in *kgb-1* single mutants (Fig. 5*A*). We observed no regeneration in *kgb-1 pmk-3; vhp-1* triple mutants. Taken together, these data suggest that VHP-1 acts on both KGB-1 and PMK-3 to negatively regulate MAPK signaling and inhibit regeneration (Fig. 7*D*). We confirmed the interaction between VHP-1 and PMK-3 by immunoprecipitation. When VHP-1 was coexpressed with PMK-3, VHP-1 immunoprecipitated complexes also contained PMK-3 (Fig. 5*D*).

Because *vhp-1* acts on both MAPKs to inhibit regeneration, we tested whether mutations in the DLK-1/MKK-4/PMK-3 pathway could also suppress the larval arrest phenotype of *vhp-1* null mutants. *dlk-1* and *pmk-3* mutants fully suppressed *vhp-1* larval arrest, whereas *mkk-4* mutants did not (Fig. 5*C*). It is possible that MEK-1 activity in the absence of MKK-4 could allow for minimal

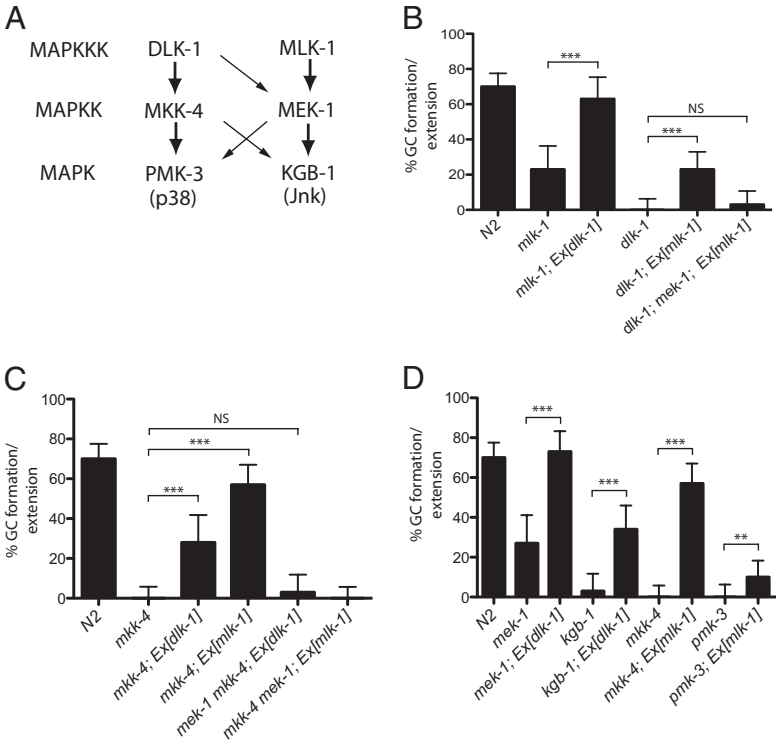


**Fig. 4.** Crosstalk between p38 and JNK MAPK pathways. (*A*) Diagram of two MAPK pathways essential for axon regeneration with arrows indicating crosstalk between kinases. (*B* and *C*) Crosstalk by MAPKKK. DLK-1 can activate both MKK-4 and MEK-1, whereas MLK-1 activates only MEK-1. (*B*) DLK-1 overexpression suppresses *mlk-1* loss of function and MLK-1 overexpression suppresses *dlk-1* loss of function. (*C*) *mkk-4* mutants are rescued by overexpressing either DLK-1 or MLK-1. *mkk-4* rescue by MAPKKK overexpression requires functional MEK-1. (*D*) Crosstalk by MAPKK. MKK-4 and MEK-1 each activate PMK-3 and KGB-1. Ex[*dlk-1*] and Ex[*mlk-1*], *dlk-1* and *mlk-1* transgene expression from an extrachromosomal array, respectively. P determined by Fisher's exact test. $^{**}P < 0.01$, $^{***}P < 0.001$, NS not significant. Error bars indicate 95% confidence interval. GC, growth cone.

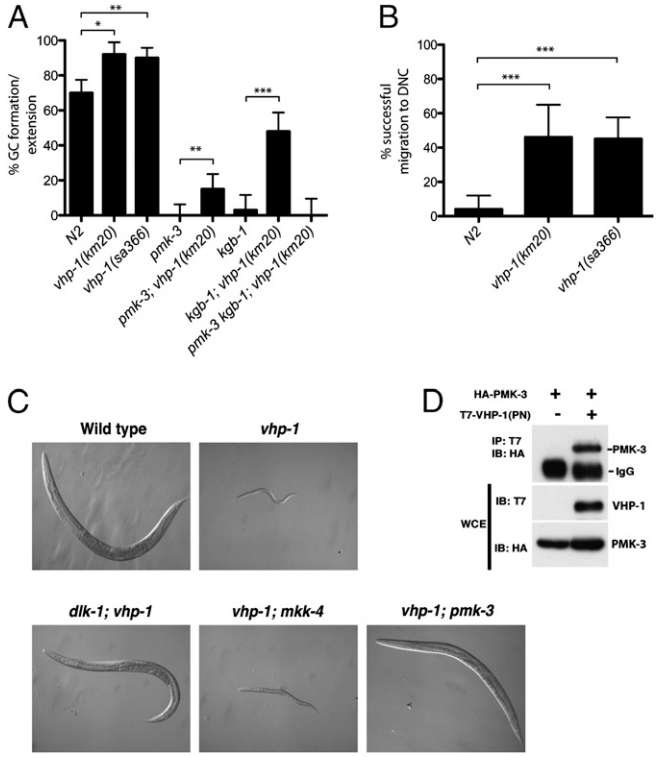


**Fig. 5.** VHP-1 acts on both PMK-3 and KGB-1 MAPK. (*A*) *vhp-1* mutants show improved regeneration of growth cones (GC) and suppress the loss of either *pmk-3* or *kgb-1*. (*B*) Successful migration back to dorsal nerve cord (DNC) is improved in *vhp-1* mutants. (*C*) *dlk-1* or *pmk-3* mutants suppress *vhp-1* larval arrest. Loss of *mkk-4* does not suppress *vhp-1* larval arrest. Scale bar is 100 μm. (*D*) Complexes immunoprecipitated (IP) with anti-T7 (targeting T7-VHP-1) and immunoblotted (IB) with anti-HA (targeting HA-PMK-3). Whole cell extracts (WCE) were also probed with anti-T7 and anti-HA antibodies. *P* determined by Fisher's exact test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Error bars indicate 95% confidence interval.

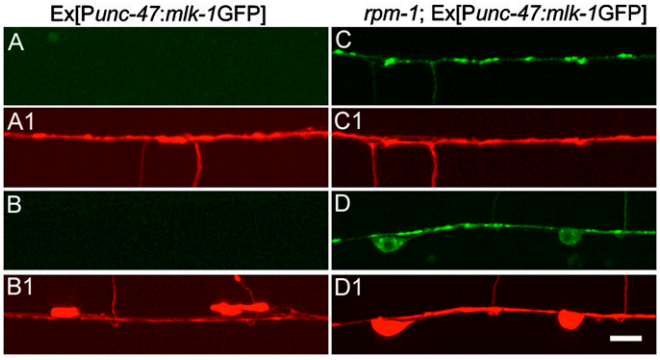


**Fig. 6.** MLK-1 is targeted for degradation by the E3 ubiquitin ligase RPM-1. The red (*A1–D1*) is cytoplasmic mCherry to show the GABA neuron morphology. The green (*A–D*) is MLK-1:GFP. There is no visible MLK-1:GFP in the wild-type background (dorsal nerve cord in *A* and ventral nerve cord in *B*). (*C*) The punctate localization of MLK-1 to synaptic zones in the dorsal nerve cord and diffuse labeling in axons. (*D*) Punctate localization in ventral nerve cord synaptic zones and diffuse labeling in the cell cytoplasm. Notice that MLK-1:GFP is excluded from the nucleus. (Scale bar, 5 μm.)

activation of PMK-3 and thus, failure to rescue *vhp-1* larval arrest. This activity may not be sufficient to support regeneration because regeneration is blocked in *mkk-4* mutants. Alternatively, an additional MAPKK may function with MKK-4 to activate PMK-3 during development.

**RPM-1 E3 Ubiquitin Ligase Targets MLK-1 for Degradation.** DLK-1 is localized to synapses and directly targeted for degradation by the E3 ubiquitin ligase RPM-1 (34). DLK-1:GFP expression was elevated in *rpm-1* mutants compared with wild type. We tested whether MLK-1 might also be a target of RPM-1 by constructing a strain expressing *mlk-1*:GFP in the GABA neurons. We observed occasional faint cell body GFP expression in an otherwise wild-type background, indicating that MLK-1 is normally kept below detectable levels (Fig. 6 *A* and *B*). When *mlk-1*:GFP was expressed in an *rpm-1* mutant background we observed increased GFP expression in a punctate pattern along the dorsal and ventral cords and faintly in the commissures (Fig. 6 *C* and *D*). These data suggest that, like DLK-1, MLK-1 is targeted for degradation by RPM-1. Consistent with these results, we find that *rpm-1* mutants enhance regeneration, but the enhancement requires *kgb-1* (Fig. 7*A*). *rpm-1* single mutants improved regeneration to 86%, whereas *rpm-1; kgb-1* double mutants regenerated only 10%. Thus, loss of *rpm-1* results in the activation of both PMK-3 and KGB-1 MAPK pathways (Fig. 7*D*).

Given the role of *rpm-1* in regulating the activity of both DLK-1 and MLK-1 signaling, we tested whether RPM-1 may also be important for establishing the critical period of regeneration. In previous work, we showed that DLK-1 is required at the time of injury to promote regeneration (18). *dlk-1* expressed either hours before or hours after injury resulted in less or no regeneration. We concluded that an important step following axotomy is the transport and accumulation of DLK-1 at the injured stump. Is the narrow temporal window for DLK-1 activity determined in part by coaccumulation of RPM-1 that targets DLK-1 and MLK-1 for degradation? We expressed DLK-1 at fixed time points using the heat-inducible promoter *Phsp-16.2* and compared regeneration in *dlk-1* single mutants with *dlk-1; rpm-1* double mutants. In *dlk-1* mutants, 40% of axons initiated a growth cone when heat shock was applied 8 h after axotomy, and 13% of axons initiated a growth cone when heat shock was applied 24 h after axotomy (Fig. 7*B*). Regeneration was not significantly improved in *dlk-1; rpm-1* double mutants under the same heat-shock conditions. Thus, the absence of *rpm-1* expression did not extend the critical period for DLK-1 expression. These data suggest that RPM-1 does not play a rate-limiting role in the critical period for axon regeneration and therefore is unlikely to be an effective drug target for improving regenerative outcomes after injury.

Downstream targets of the DLK-1 signaling pathway were identified as suppressors of *rpm-1* synaptic defects (35). These include the MAP kinase-activated protein kinase (MAPKAPK) MAK-2 and the C/EBP family transcription factor CEBP-1. Yan et al. (35) showed that axotomy-induced signaling via DLK-1 to MAK-2 promoted local translation and stabilization of *cebp-1* transcript. This accumulation of *cebp-1* transcript was required for regeneration. Our results have shown a cooperative relationship between the p38 MAPK PMK-3 and the JNK-like MAPK KGB-1. Both pathways are essential for regeneration, and cross-activation occurs between the pathways. We tested whether the KGB-1 pathway might also signal via the same downstream molecules, MAK-2 and CEBP-1.

A *mak-2* loss-of-function mutation resulted in a significant, but mild, defect in the ability of GABA neurons to regenerate (Fig. 7*C*). This is consistent with observations in the PLM mechanosensory neuron (35). Although Yan et al. demonstrated that the elevated levels of *cebp-1* transcript observed in *rpm-1* mutants require *mak-2*, our data suggest that additional elements must also affect *cebp-1* levels in the absence of *mak-2* during regeneration. Furthermore, the improved regeneration we observed in *vhp-1* mutants was not affected by loss of *mak-2*. *vhp-1* single mutants regenerated 90%, whereas *vhp-1*; *mak-2* regenerated 80% (Fig. 7*C*). This finding suggests that although PMK-3 may activate MAK-2, KGB-1 likely signals through a different intermediate.

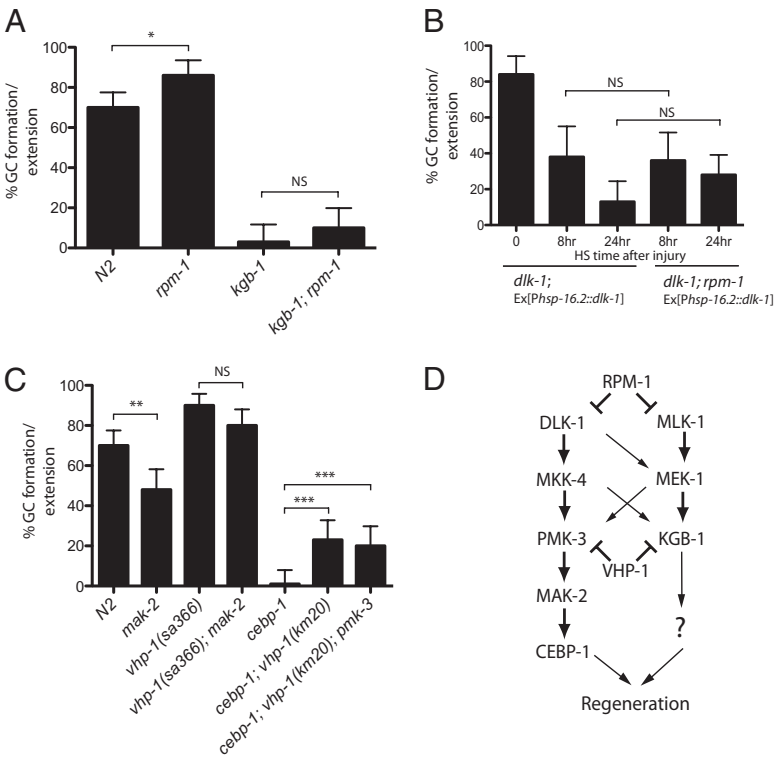


**Fig. 7.** Upstream regulation and downstream targets of MAPK signaling in regeneration. (*A*) *rpm-1* mutants show improved regeneration of growth cones (GC) which requires functional KGB-1. (*B*) RPM-1 does not influence the critical period for regeneration. (*C*) PMK-3 and KGB-1 promote regeneration via different downstream effectors. Improved regeneration in *vhp-1* mutants was not affected by loss of *mak-2*. *cebp-1* mutants fail to regenerate, but can be partially suppressed by *vhp-1* loss-of-function. The *cebp-1; vhp-1; pmk-3* triple mutant reveals that suppression occurs through KGB-1 activity. (*D*) Summary of signaling interactions highlighting the coordinated regulation of both PMK-3(p38) and KGB-1(JNK) MAPK pathways by the E3 ubiquitin ligase RPM-1(Phr1), the MAPKKKs DLK-1 and MLK-1, and the MKP VHP-1(MKP7). P determined by Fisher's exact test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, NS not significant. Error bars indicate 95% confidence interval. GC, growth cone. HS, heat shock. Ex[P*hsp-16.2:dlk-1*], *dlk-1* transgene under the control of a heat shock promoter and expressed as an extrachromosomal array.

*cebp-1* mutants had a much stronger phenotype and failed to regenerate (Fig. 7*C*). However, we found that *vhp-1* mutants partially suppress the loss of *cebp-1*. Both *vhp-1*; *cebp-1* double mutants and *pmk-3; vhp-1; cebp-1* triple mutants regenerated about 20% of the time. These data suggest that although *cebp-1* represents a major target of PMK-3, other factors promoting regeneration may be influenced by KGB-1 activity (Fig. 7*D*).

## Discussion

In this study, we extended our characterization of MAPK signaling during *C. elegans* axonal regeneration and identify an additional MAPK pathway, MLK-1/MEK-1/KGB-1(JNK), that together with DLK-1/MKK-4/PMK-3(p38), is essential for regeneration. Both kinase signals are tightly regulated by similar mechanisms, including targeted degradation of activating MAPKKKs by the E3 ubiquitin ligase RPM-1 and dephosphorylation of MAPKs by the MAPK phosphatase VHP-1.

Overexpression of MLK-1 has a similar beneficial effect on axonal regeneration as seen when DLK-1 is overexpressed (18), consistent with the cross-activation of PMK-3 and KGB-1 by both MAPKKKs. Both DLK-1 and MLK-1 cause an increase in the frequency and decrease in the latency of growth cone initiation after axotomy. They also both improve the performance of regenerating growth cones as assayed by successful migration back to the target. This correlates with the transformation of the regenerating growth cone into a more embryonic-like morphology and behavior. However, there is a limit to the effect of MLK-1 and DLK-1 signaling. Although overexpression of either MAPKKK extends the period of regeneration in older animals, an age-related decline is still apparent and there must be other molecular components limiting regeneration. One possibility was that the E3 ubiquitin ligase RPM-1 (Phr1) might limit DLK-1 and MLK-1 activity during regeneration. However, we found that although RPM-1 regulates the basal level of DLK-1 and MLK-1 in the uninjured cell, and therefore affects axon regeneration, RPM-1 does not influence regeneration after axotomy or play a rate-limiting role in the critical period.

In most model systems the downstream target of DLK or MLK is typically JNK (36, 37), not p38. Mammalian JNK activation and its phosphorylated target, the transcription factor c-jun, are also consistent markers associated with axotomy and regeneration (28, 38). When our initial work demonstrated the requirement for DLK-1/MKK-4/PMK-3(p38) in regeneration, the distinction could have been attributed to species differences (39). However, experiments in cultured cells indicate that DLK and MLK may also activate the p38 pathway (40, 41). We now show that in *C. elegans*, DLK-1 and MLK-1 activate *both* p38 and JNK, and dual activation of both MAPKs is essential for regeneration. It seems likely that similar coactivation of MAPK pathways occurs in other systems as well. Recent experiments in mouse DRG neurons support a role for DLK in mammalian regeneration (42). Neurite regeneration was impaired in DLK-deficient mice, and the axotomized neurons showed a decrease in phosphorylated c-Jun compared with wild-type mice. p38 has similarly been implicated in regeneration. Application of a p38 inhibitor immediately before axotomy inhibited growth cone formation in rat DRG neurons (43).

The parallel DLK-1 and MLK-1 pathways activating PMK-3(p38) and KGB-1(JNK) may also be relevant to the studies demonstrating a role for DLK in regulating axon degeneration in *Drosophilia* (30) and microtubule regulated gene expression in *C. elegans* (44). The model proposed by Bounoutas et al. suggests that an unknown MAPKK may activate PMK-3 in parallel to MKK-4 (44). Our data would implicate MEK-1 for this role and further suggest that MLK-1 and KGB-1 may also be involved in the response. Miller et al. found that DLK activated JNK (but not p38) plays an active role in axon degeneration in *Drosophila* (30). MLK/Slpr may also be involved in the activation of this pathway because phosphorylated JNK is still present in axons and neuropil of DLK/Wnd mutants suggesting other activation pathways do exist (45). It is interesting to note that the critical period for JNK inhibition (around the time of axotomy) to protect against Wallerian degeneration is similar to that seen for DLK activity to promote axon regeneration.

Crosstalk between MAPKs is an important regulatory mechanism during various cellular responses (46). Multiple stimuli often simultaneously activate both p38 and JNK pathways resulting in synergistic effects on downstream targets. This could increase the intensity and duration of the signal necessary to promote local cytoskeletal changes, as well as retrograde transport of signals to the nucleus. We find that a single MKP, VHP-1 (MKP7), targets both pathways to effectively inhibit axon regeneration. Our findings highlight that potential therapies aimed toward improving recovery after traumatic axotomy will likely need to target both cognate p38 and JNK MAPK pathways.

## Materials and Methods

**Strains.** Animals were maintained on HB101 *Escherichia coli* seeded NGM plates according to standard methods. See Tables S1 and S3 for a complete list of strains and genotypes used in this study.

**Molecular Biology and Transgenics.** Plasmids generated for this study were constructed using multisite Gateway cloning (Invitrogen) as follows:

pPN60 (*Punc-47:mlk-1*): a [1-2] Gateway entry clone (pPN59) was generated by amplifying an 8-kb N2 genomic DNA fragment with the following primers:

PN156 GGGGACAAGTTTGTACAAAAAAGCAGGCTGGgaacaggcttcggtcccgtc

PN157 GGGGACCACTTTGTACAAGAAAGCTGGGTGatagatctcggttccttcgac

PCR was completed with Phusion DNA polymerase (Finnzymes). The final construct was obtained in a multisite Gateway reaction combining pMH522 (P*unc-47* [4-1]), pPN59 (*mlk-1* [1-2]), pMH473 (*unc-54* terminator [2-3]), and pDEST [4-3]. pPN61 (P*unc-47:mlk-1*:GFP): The construct was obtained in a multisite Gateway reaction combining pMH522 (P*unc-47* [4-1]), pPN59 (*mlk-1* [1-2]), pGH50 (GFP:*unc-54* terminator [2-3]), and pDEST [4-3].

Transgenic animals were obtained as described (47). *basEx1* was generated in a previous study (18). basIs4 was the result of X-ray integration of *oxEx968* (*Punc-47*:GFP at 20 ng/μL, *Pmyo-2*:GFP at 1 ng/μL). *basEx30* was generated by injecting pPN60 DNA at 30 ng/μL along with pCFJ90 (P*myo-2*:mCherry) at 2 ng/μL as a coinjection marker. *basEx37* was generated by injecting pPN61 at 30 ng/μL along with pCFJ90 (P*myo-2*:mCherry) at 2 ng/μL as a coinjection marker.

**Axotomy.** Axotomy and time-lapse microscopy was performed as described (18). All animals were subjected to axotomy at the L4 stage (unless noted), recovered 18–24 h, then prepared for confocal imaging. Regeneration was quantified by scoring the percentage of severed axons that formed a new growth cone and/or grew a distance of 5 μm or more.

**Coimmunoprecipitation.** For coimmunoprecipitation assay, COS7 cells were transfected with HA-tagged PMK-3 and T7-tagged VHP-1(PN) constructs (32). After 48 h, cells were lysed and the protein extracts were subjected to coimmunoprecipitation assay as described (32).

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