DLK induces developmental neuronal degeneration via selective regulation of proapoptotic JNK activity

Arundhati Sengupta Ghosh, Bei Wang, Christine D. Pozniak, Mark Chen, Ryan J. Watts, and Joseph W. Lewcock

Neurodegeneration Laboratories, Department of Neuroscience, Genentech, Inc., South San Francisco, CA 94080

The establishment of peripheral innervation during development requires axonal outgrowth to target regions and subsequent re-finement of connectivity through the removal of exuberant neuronal processes and the elimination of excess neurons via apoptosis (Oppenheim, 1991; Luo and O’Leary, 2005). Developmental apoptosis has been extensively studied in sympathetic and dorsal root ganglion (DRG) neurons that depend on NGF for their survival (Levi-Montalcini and Booker, 1960; Crowley et al., 1994). In these neurons, loss of NGF signaling results in rapid degeneration (Gorin and Johnson, 1979). Regulators of the intrinsic apoptosis pathway including Bcl-2–associated X (BAX) protein and Bcl-2 have been implicated in this process (Garcia et al., 1992), and mice lacking a functional BAX gene lose significantly fewer neurons during development (Deckwerth et al., 1996; White et al., 1998). A c-Jun–dependent transcriptional program is also required for apoptosis to proceed, which is initiated after c-Jun phosphorylation by the JNK family of MAPKs (Ham et al., 1995; Whittfield et al., 2001; Palmada et al., 2002; Besirli et al., 2005). This parallels what has been observed after neuronal injury, in which phosphorylation of c-Jun and other downstream targets by JNK is necessary for neuronal cell death (Bogoyevitch, 2006).

The pathways that underlie the selective degeneration of neuronal processes in development and disease are less well defined, though a growing body of literature suggests that this degeneration is an active process that can be separated from neuronal apoptosis. This idea is supported by data demonstrating that expression of Wld<sup>e</sup>, a gene fusion between UFD2/E4 and NMAT (nicotinamide nucleotide adenyllytransferase), is able to strongly protect axons but not cell bodies from degeneration (Mack et al., 2001). Recently, components of the intrinsic pathways that regulate axonal degeneration have also been identified. JNK signaling as well as the ubiquitin proteasome system and apoptotic caspases are essential for degeneration in certain experimental paradigms, though some model system–dependent differences have been observed (Watts et al., 2003; Miller et al., 2009; Nikolaev et al., 2009; Vohra et al., 2010).

The JNK pathway is required for both neuronal apoptosis and axon degeneration but also functions to regulate neuronal interaction of DLK with the scaffolding protein JIP3 to form a specialized JNK signaling complex. Local activation of DLK-based signaling in the axon results in phosphorylation of c-Jun and apoptosis after redistribution of JNK to the cell body. In contrast, regulation of axon degeneration by DLK is c-Jun independent and mediated by distinct JNK substrates. DLK-null mice displayed reduced apoptosis in multiple neuronal populations during development, demonstrating that prodegenerative DLK signaling is required in vivo.
growth and homeostasis (Chang et al., 2003; Björkblom et al., 2005). Neurons contain high levels of activated JNK even in the absence of stress but have the ability to discriminate this basal activity from proapoptotic JNK signaling (Coffey et al., 2000). Studies using JNK-null mice have demonstrated that each of the three mammalian JNK genes has specific functions, which explains at least in part how this selectivity is achieved. For instance, mice lacking JNK2 and/or JNK3 are protected from stress-induced neuronal apoptosis and display reduced phosphorylation of stress-specific downstream targets such as c-Jun, whereas JNK1-null mice show no protection (Chang et al., 2003; Hunot et al., 2004; Yang et al., 1997). Additional selectivity is likely to be mediated via interaction of JNKs with JNK-interacting proteins (JIPs), which are thought to facilitate formation signaling complexes comprised of JNKs and upstream kinases (Whitmarsh, 2006). It has been hypothesized that specific combinations of JNK, JIP, and upstream kinases can lead to highly specific JNK signaling complexes with defined outputs (Waetzig and Herdegen, 2005), but few such complexes have been identified.

Experiments using the pan-mixed lineage kinase (MLK) inhibitor CEP-1347 have suggested that this family of kinases is a major upstream regulator of JNK activation in neurons (Maroney et al., 1998), yet the specific MLKs that control neuronal degeneration are not well defined. Recently, the MLK dual leucine zipper kinase (DLK) has been shown to play a role in neuronal injury–induced axonal degeneration, a function that is likely JNK mediated (Miller et al., 2009). In other contexts, however, DLK does not mediate degeneration and is instead required for axonal regeneration after injury (Hammarlund et al., 2009; Xiong et al., 2010). During development, DLK is a component of a pathway that regulates axon outgrowth and synapse formation via regulation of JNK and/or P38 MAPks (Nakata et al., 2005; Collins et al., 2006; Hirai et al., 2006; Lewcock et al., 2007), and reduced DLK expression either directly or indirectly leads to increased numbers of spinal motor neurons (Itoh et al., 2011).

In this study, we sought to understand the mechanisms of DLK-based signaling in the context of nervous system development. Using an in vitro NGF withdrawal paradigm that mimics the competition for trophic factors encountered by peripherally projecting sensory neurons in vivo, we discovered that DLK is required for both axonal degeneration and neuronal apoptosis. DLK-mediated degeneration is based on specific regulation of stress-induced JNK activity in axons that is achieved via interaction of DLK with the scaffolding protein JIP3. These results are further supported by the observation that developmental apoptosis is significantly reduced in multiple neuronal populations in vivo. Collectively, this suggests that DLK-based regulation of the JNK signaling pathway is essential for the neuronal apoptosis and axon degeneration that occur during development.

Results

DLK is required for neuronal apoptosis and axon degeneration in DRG neurons

DLK is specifically expressed in postmitotic neurons during development, including neurons of the DRG and spinal cord (Hirai et al., 2005). We generated DLK-null animals through excision of exons 2–5, which resulted in no expression of DLK protein in the embryonic nervous system (Fig. S1). In the presence of NGF, DRG neurons from DLK−/− mice in culture appeared morphologically normal and displayed comparable growth with neurons from wild-type (wt) littermates, indicating no major defects in axon outgrowth in this neuronal population (Fig. S2). To ascertain whether DLK regulates neuronal apoptosis, we cultured DRG neurons in the presence of NGF to elicit growth and then withdrew NGF from the culture media to induce neuronal degeneration. Levels of apoptosis after NGF withdrawal were measured by counting the number of neuronal cell bodies staining positive with an antibody against the activated form of caspase 3, which is elevated during apoptosis in this cell population. Interestingly, the presence of activated caspase 3 in neuronal cell bodies was strikingly reduced in DLK−/− neurons as compared with controls, indicative of a significant protection of DLK−/− neurons from apoptosis induced by NGF withdrawal (Fig. 1, A–D).

NGF deprivation has also been shown to induce axonal degeneration independent of cell death in NGF-dependent cell populations (Campenot, 1977); therefore, we next explored whether DLK is also required for axon degeneration using DRG explant cultures. Interestingly, whereas axons grown from wt DRG explants completely degenerated by 18 h, DLK-null neurons displayed minimal degeneration at this time point (Fig. 1, E–H). The axonal protection observed in explant cultures could be a secondary result of the antiapoptotic effects of DLK removal, so we next examined whether DLK affects local axon degeneration using compartmentalized chambers that separate axons from cell bodies. When NGF is removed only from the axonal compartment in this experimental setup, degeneration of axons proceeds on a similar timeline to that observed in explants, but no significant apoptosis occurs during this time period (Campenot, 1977; Mok et al., 2009). Similar to what was observed in explants, DLK−/− axons displayed significantly reduced degeneration after NGF deprivation as compared with axons from wt littermates (Fig. 1, I–L). These data argue that DLK is critical for both axon degeneration and cell death in response to growth factor deprivation. Importantly, loss of DLK is also able to protect against local axon degeneration, arguing that it has an essential role in this process even in conditions in which neuronal apoptosis does not occur.

DLK activates a JNK-mediated stress response pathway

To identify pathways modulated by DLK in the context of developmental degeneration in mouse, the activation of MAPK pathways was measured in cultured DRG neurons after 3 h of NGF deprivation. This early time point is before significant degeneration but is sufficient to cause a fourfold reduction in the levels of phosphorylated extracellular signal-regulated kinase (ERK; p-ERK) resulting from the loss of NGF/TrkA-based survival signaling. Levels of p-ERK were similar in wt and DLK−/− neurons, arguing that the removal of DLK does not protect neurons via maintaining ERK activity in the absence of NGF (Fig. 2, A and B). Levels of phosphorylated JNK (p-JNK) and phosphorylated P38-α were unchanged at this time point, though...
Interestingly, NGF deprivation resulted in a redistribution of p-JNK from axons to cell bodies over a period of 4 h, which did not occur in DLK−/− neurons (Figs. 2 [C and D] and S3). Staining of cultures with an antibody directed to Tuj1 confirmed that the lack of p-JNK labeling in axons was not a result of the axons degenerating but rather a specific relocalization of p-JNK to the cell body (Fig. 2, G and H). The timing of p-JNK relocalization strongly correlated with the number of neurons that stained positive for p-c-Jun (Fig. 2, I–K), consistent with the hypothesis that nuclear localization of p-JNK is required for c-Jun phosphorylation and neuronal apoptosis (Björkblom et al., 2008).

To better understand the mechanism of JNK activation induced by NGF withdrawal, we next examined p-JNK localization by immunostaining to determine the subcellular distribution of p-JNK protein. Under normal culture conditions, DRG neurons showed punctate p-JNK staining throughout the cell body and neuronal processes in both wt and DLK−/− neurons (Figs. 2 [A and B] and S3). These data suggest that the withdrawal of NGF induces JNK-based stress response pathways in DRG neurons and that this activation is DLK dependent.

Figure 1. **Apoptosis and axon degeneration are significantly reduced in DLK−/− neurons.** (A–C) Cultured DRG neurons from E13.5 embryos stained with antibodies for activated caspase 3 (green) and Tuj1 (red). Neurons grow robustly in the presence of NGF (A) and display negligible activated caspase 3 staining. Caspase 3 is activated in many neurons after 8 h of NGF withdrawal in wt (DLK+/+) neurons (B) but is reduced in DLK−/− neurons (C). Bar, 50 µm. (D) Quantification of cultures shown in A–C reveals significantly less activation of caspase 3 in DLK−/− neurons (wt = 79 ± 17% and DLK−/− = 5 ± 4.5% of neurons; n = 3; ***, P < 0.001). (E–G) Tuj1 staining of DRG explants from wt and DLK−/− embryos in the presence or absence of NGF. NGF results in robust axon outgrowth from explants (E). Withdrawal of NGF from explant cultures results in the degeneration of axons in a period of 18 h in wt explants (F) but not in DLK−/− explants (G). Bar, 100 µm. (H) Quantification of cultures shown in F and G using a scoring system designed to measure the amount of axon degeneration [0 = no degeneration, and 5 = complete degeneration] reveals significantly less degeneration in DLK−/− axons (wt = 4.7 ± 0.1 and DLK−/− = 0.8 ± 0.2; n = 5; ***, P < 0.001). (I–K) Tuj1 staining of DRG axons from E13.5 embryos from wt and DLK−/− embryos grown in compartmentalized (Comp.) chambers that separate distal axons from cell bodies. NGF elicits robust growth (I), and removal of NGF from the axonal compartment only results in rapid local degeneration of wt axons (J) but not DLK−/− axons (K) in 28 h. Bar, 50 µm. (L) Quantification of compartmentalized chamber cultures shown in J and K using the aforementioned scoring system reveals reduced axon degeneration in DLK−/− (wt = 4.25 ± 0.1 and DLK−/− = 1.3 ± 0.1; n = 2; ***, P < 0.001). Error bars represent SEM.
Figure 2. **DLK is required for activation of stress-induced JNK signaling in neurons but does not affect basal JNK activity.** (A and B) Phosphorylation levels of ERK, JNK, and c-Jun in E13.5 DRG neuron cultures from wt (DLK+/+) and DLK−/− embryos in the presence or absence of NGF by Western blotting (A). Quantification of A reveals that levels of p-ERK are reduced in both DLK−/− and wt neurons 3 h after NGF withdrawal (wt = 26.2 ± 19% and DLK−/− = 19.1 ± 13% of NGF control; n = 4; *, P < 0.05), whereas no change in p-JNK is observed at this time point. At 1 h, p-JNK levels are increased in wt neurons but not DLK−/− neurons after NGF withdrawal (wt = 289 ± 100% and DLK−/− = 143 ± 16% of NGF control; n = 4; P < 0.05). wt neurons displayed a large increase in p-c-Jun 3 h after NGF withdrawal, which is significantly reduced in DLK−/− neurons (wt = 717 ± 146% and DLK−/− = 201 ± 58% of NGF control; n = 4; **, P < 0.01; B). Molecular mass is indicated in kilodaltons. (C–F) Cultured DRG neurons from E13.5 embryos stained with antibodies for activated p-JNK and NeuN. p-JNK is largely relocalized from the axon (white arrow) to the nucleus (open arrow) after 4 h of NGF withdrawal in wt neurons but not in DLK−/− neurons. (G and H) DRG neurons stained with Tuj1 show that loss of p-JNK in axons is not a result of axonal degeneration at this time point. (I) Quantification of cultures shown in J and K reveals significantly less p-c-Jun staining in DLK−/− neurons (wt = 93 ± 11.5% and DLK−/− = 9 ± 9% of neurons; n = 2; **, P < 0.01). (J and K) DRG neurons stained with activated p-c-Jun and NeuN. In wt cultures, the majority of neurons are p-c-Jun positive after 4 h of NGF withdrawal, whereas in DLK−/− cultures, only a few neurons show dim staining for p-c-Jun. Error bars represent SEM. Bars, 10 µm.
To determine whether JIP3 and DLK can form a signaling complex, we tested whether these two proteins interact when coexpressed in HEK-293 cells. Immunoprecipitation (IP) of Flag-tagged DLK was able to pull down coexpressed Myc-tagged JIP3 but not a GFP control (Fig. 4 H), indicating that these proteins can interact. To investigate whether this JIP3–DLK complex was functionally relevant, we next assessed the ability of JIP3 to enhance the DLK-dependent activation of JNK and c-Jun. Transfection of DLK into HEK-293 cells resulted in increased phosphorylation of JNK and c-Jun, even in the absence of any extrinsic stress on these cells (Fig. 4 I). This phosphorylation did not occur after transfection of a kinase-dead DLK construct, arguing that it is a specific signaling event (unpublished data). Transfection of JIP3 alone did not result in significant phosphorylation of JNK, but when JIP3 was cotransfected with DLK, it resulted in notably higher levels of p-JNK and p-c-Jun than DLK alone (Fig. 4 I). This demonstrates that DLK activity is sufficient to stimulate the phosphorylation of JNK, and JIP3 enhances this activation.

Selective activation of JNK by DLK requires JIP3

The observation that DLK−/− neurons retain normal localization and levels of p-JNK when cultured in the presence of NGF, yet display deficiencies in p-JNK relocalization and attenuated phosphorylation of c-Jun in NGF deprivation paradigms, suggested that DLK is able to selectively modulate the prodegenerative aspects of JNK signaling. We hypothesized that this may be achieved through the interaction of DLK with a specific JIP to form a signaling complex that would allow for restricted JNK activation. To test this possibility, we examined whether siRNA-based knockdown of individual JIPs was able to phenocopy the protective effects observed in DLK−/− neurons. Interestingly, siRNA-based knockdown of JIP3 provided similar levels of protection to those observed after knockdown or knockout of DLK, whereas JIP1 siRNAs provided negligible protection despite efficient knockdown of JIP1 protein (Figs. 4 [A–G] and S4).

To determine whether JIP3 and DLK can form a signaling complex, we tested whether these two proteins interact when coexpressed in HEK-293 cells. Immunoprecipitation (IP) of Flag-tagged DLK was able to pull down coexpressed Myc-tagged JIP3 but not a GFP control (Fig. 4 H), indicating that these proteins can interact. To investigate whether this JIP3–DLK complex was functionally relevant, we next assessed the ability of JIP3 to enhance the DLK-dependent activation of JNK and c-Jun. Transfection of DLK into HEK-293 cells resulted in increased phosphorylation of JNK and c-Jun, even in the absence of any extrinsic stress on these cells (Fig. 4 I). This phosphorylation did not occur after transfection of a kinase-dead DLK construct, arguing that it is a specific signaling event (unpublished data). Transfection of JIP3 alone did not result in significant phosphorylation of JNK, but when JIP3 was cotransfected with DLK, it resulted in notably higher levels of p-JNK and p-c-Jun than DLK alone (Fig. 4 I). This demonstrates that DLK activity is sufficient to stimulate the phosphorylation of JNK, and JIP3 enhances this activation.

To determine whether a DLK–JIP3 complex regulates stress-induced JNK activity in neurons, we next examined whether the endogenous DLK and JIP3 genes interact as was observed after overexpression in HEK-293 cells. Sufficient protein for IP studies could not be obtained from DRG neurons, observed in DLK−/− neurons (Fig. 1, A–H). As small molecule inhibitors can often inhibit multiple kinases in addition to their desired target, this experiment was repeated with two additional structurally distinct JNK inhibitors, which yielded similar results (SP600125 and JNK VIII; unpublished data). These data support a mechanism in which DLK is required for activation of the JNK–c-Jun stress response pathway that occurs in neurons as a result of NGF deprivation, and this JNK activity results in neuronal apoptosis and degeneration of axons.

Selective activation of JNK by DLK requires JIP3

The observation that DLK−/− neurons retain normal localization and levels of p-JNK when cultured in the presence of NGF, yet display deficiencies in p-JNK relocalization and attenuated phosphorylation of c-Jun in NGF deprivation paradigms, suggested that DLK is able to selectively modulate the prodegenerative aspects of JNK signaling. We hypothesized that this may be achieved through the interaction of DLK with a specific JIP to form a signaling complex that would allow for restricted JNK activation. To test this possibility, we examined whether siRNA-based knockdown of individual JIPs was able to phenocopy the protective effects observed in DLK−/− neurons. Interestingly, siRNA-based knockdown of JIP3 provided similar levels of protection to those observed after knockdown or knockout of DLK, whereas JIP1 siRNAs provided negligible protection despite efficient knockdown of JIP1 protein (Figs. 4 [A–G] and S4).

To determine whether JIP3 and DLK can form a signaling complex, we tested whether these two proteins interact when coexpressed in HEK-293 cells. Immunoprecipitation (IP) of Flag-tagged DLK was able to pull down coexpressed Myc-tagged JIP3 but not a GFP control (Fig. 4 H), indicating that these proteins can interact. To investigate whether this JIP3–DLK complex was functionally relevant, we next assessed the ability of JIP3 to enhance the DLK-dependent activation of JNK and c-Jun. Transfection of DLK into HEK-293 cells resulted in increased phosphorylation of JNK and c-Jun, even in the absence of any extrinsic stress on these cells (Fig. 4 I). This phosphorylation did not occur after transfection of a kinase-dead DLK construct, arguing that it is a specific signaling event (unpublished data). Transfection of JIP3 alone did not result in significant phosphorylation of JNK, but when JIP3 was cotransfected with DLK, it resulted in notably higher levels of p-JNK and p-c-Jun than DLK alone (Fig. 4 I). This demonstrates that DLK activity is sufficient to stimulate the phosphorylation of JNK, and JIP3 enhances this activation.

To determine whether a DLK–JIP3 complex regulates stress-induced JNK activity in neurons, we next examined whether the endogenous DLK and JIP3 genes interact as was observed after overexpression in HEK-293 cells. Sufficient protein for IP studies could not be obtained from DRG neurons,
so whole-brain lysate from neonatal mice (postnatal day 1) was used as a substitute. Consistent with our previous observations, IP with an anti-DLK antibody was also able to pull down JIP3 protein, which was not observed in an IgG control (Fig. 4 J).

The functional relevance of this interaction was then examined by measuring the phosphorylation of JNK, c-Jun, and ERK in DRGs after siRNA knockdown of JIP3 in the presence of NGF. The results observed were nearly identical to those observed with DLK−/− neurons, i.e., the increase in levels of p-c-Jun seen in control cultures was not observed in neurons electroporated with a JIP3 siRNA after 3 h of NGF deprivation, and the modest increase in p-JNK at 1 h was not observed after JIP3 knockdown (Fig. 4, K and L). siRNA-based knockdown of JIP3 also inhibited relocalization of p-JNK in dissociated DRG cultures (unpublished data). Although these data cannot distinguish between a direct JIP3–DLK interaction and one that requires additional binding partners, it strongly suggests that DLK and JIP3 are components of a signaling complex that is required for JNK and c-Jun phosphorylation induced by NGF withdrawal.
DLK activation in distal axons initiates a retrograde stress response

Our previous work demonstrated that a significant portion of DLK protein was localized to the growth cone in projecting axons (Lewcock et al., 2007). This raises the possibility that regulation of neuronal apoptosis by DLK originates in the periphery and is retrogradely transported back to the nucleus. To test this hypothesis, we again used DRG neurons grown in compartmentalized culture chambers to separate axons from cell bodies (Fig. 5 A). In this setup, removal of NGF selectively from distal axons does not result in rapid neuronal apoptosis but is sufficient to induce phosphorylation of c-Jun in the nucleus within 6 h, a similar timeline to what is observed in dissociated cultures (Fig. 5, B and C; Mok et al., 2009). Interestingly, when this experiment was conducted in neurons electroporated with siRNAs directed against either DLK or JIP3 before plating, a significant reduction in the number of p-c-Jun–positive cells was observed (Fig. 5, D–H), arguing that the DLK–JIP3 signaling complex is essential for c-Jun phosphorylation. Experiments using siRNA-based knockdown were unable distinguish between DLK–JIP3 acting in the distal axon or in the central compartment in response to a distinct peripherally derived signal. To address this, a complementary experiment was performed in which NGF was removed from all compartments, and JNK inhibitors were added to the distal axons only (Fig. 5 I). JNK inhibitors used as specific inhibitors of DLK were not available, and our data suggest that DLK-induced degeneration is mediated largely by JNK (Fig. 3). Removal of NGF from all compartments of the chamber results in neuronal apoptosis equivalent to that seen in dissociated cultures (Mok et al., 2009) and allows assessment of whether inhibition of DLK–JNK in the distal axon is sufficient to prevent cell death. We again
examined p-c-Jun levels as a readout, as previous studies have shown that it is an essential step toward neuronal apoptosis under conditions of global NGF deprivation (Ham et al., 1995; Whitfield et al., 2001; Palmada et al., 2002). Interestingly, the addition of JNK inhibitors to distal axons alone was able to significantly reduce numbers of p-c-Jun–positive cells in the central compartment to levels similar to those seen when JNK inhibitors were added to all compartments (Fig. 5, J–M). These observations suggest that DLK–JNK activity in distal axons is necessary though not sufficient for NGF withdrawal–induced apoptosis.

JNK but not c-Jun is required for axonal degeneration

Next, we addressed whether regulation of axon degeneration by DLK is also c-Jun dependent. To do this, we measured levels of axon degeneration in c-Jun conditional–null mice crossed to a Nestin-Cre (Behrens et al., 2002), which eliminates c-Jun expression in nearly all DRG neurons by E13.5 (embryonic day 13.5; abbreviated c-Junlox/lox; unpublished data). NGF was withdrawn from explants for 14, 16, or 18 h to assess the rate of axon degeneration in each genotype. Surprisingly, axons from c-Junlox/lox explants degenerated at similar rates to axons examined p-c-Jun levels as a readout, as previous studies have shown that it is an essential step toward neuronal apoptosis under conditions of global NGF deprivation (Ham et al., 1995; Whitfield et al., 2001; Palmada et al., 2002). Interestingly, the addition of JNK inhibitors to distal axons alone was able to significantly reduce numbers of p-c-Jun–positive cells in the central compartment to levels similar to those seen when JNK inhibitors were added to all compartments (Fig. 5, J–M). These observations suggest that DLK–JNK activity in distal axons is necessary though not sufficient for NGF withdrawal–induced apoptosis.

JNK but not c-Jun is required for axonal degeneration

Next, we addressed whether regulation of axon degeneration by DLK is also c-Jun dependent. To do this, we measured levels of axon degeneration in c-Jun conditional–null mice crossed to a Nestin-Cre (Behrens et al., 2002), which eliminates c-Jun expression in nearly all DRG neurons by E13.5 (embryonic day 13.5; abbreviated c-Junlox/lox; unpublished data). NGF was withdrawn from explants for 14, 16, or 18 h to assess the rate of axon degeneration in each genotype. Surprisingly, axons from c-Junlox/lox explants degenerated at similar rates to axons
from wt or heterozygous littersmates (Fig. 6, A–F and I). However, when JNK inhibitors were added to c-Jun-lox/lox explants during NGF deprivation, a strong protection of axons was observed (Fig. 6, G–I). To confirm that the loss of c-Jun is sufficient to rescue neuronal apoptosis of DRG neurons, we examined the activation of caspase 3 in neuronal cell bodies after the removal of NGF. Consistent with previous studies in sympathetic neurons (Ham et al., 1995; Palmada et al., 2002), a significantly reduced number of c-Jun-lox/lox neurons stained with an antibody specific for the activated form of caspase 3 (Fig. 6, K and L). This implies that, although c-Jun is essential for neuronal apoptosis after NGF withdrawal, downstream targets of JNK activity other than c-Jun regulate axon degeneration after NGF deprivation.

Activation of caspases is downstream of JNK–c-Jun activity in apoptosis of sympathetic neurons (Whitfield et al., 2001) and has more recently been demonstrated to be essential for axon degeneration in the context of NGF withdrawal (Nikolaev et al., 2009; Schoenmann et al., 2010). Based on these findings, we sought to determine whether caspases were activated in DLK–/– axons. To do this, we monitored the activity of caspase 9, as this is the primary initiator caspase in the intrinsic cell death pathway and downstream of BAX, which is also required for axon degeneration (Nikolaev et al., 2009). Using a cleaved caspase 9–specific antibody, activation of this protease could be observed after 8 h of NGF withdrawal in axons of wt explant cultures, but no activation was observed in axons of DLK–/– explants, indicating that DLK is upstream of axonal caspase activity (Fig. 6, M–P). To determine whether c-Jun is required downstream of DLK for caspase 9 activation, we conducted a similar experiment using c-Jun-lox/lox neurons. Consistent with the timeline of degeneration observed in c-Jun-lox/lox explants, c-Jun-lox/lox axons had similar levels of active caspase 9 present in axons as compared with wt control cultures (Fig. 6, M and O), whereas treatment of wt cultures with JNK inhibitors yielded similar levels of caspase 9 activation to what was seen in DLK–/– neurons (not depicted). This suggests that, unlike what has been reported in the context of neuronal apoptosis after NGF withdrawal, caspase activation and subsequent degeneration of axons are not dependent on c-Jun transcriptional activity.

**DLK is required for developmental apoptosis in vivo**

To determine the relevance of DLK for neuronal apoptosis and axon degeneration in normal development, we examined the phenotype of DLK–/– mice during the period of axon projection and refinement in DRG neurons (E12.5–17.5). At E12.5, a developmental stage before any significant developmental apoptosis in DRG neurons (White et al., 1998), DLK-null mice were grossly indistinguishable from wt littermates and displayed normal patterns of motor and sensory axon outgrowth in vivo, consistent with our in vitro observations (Fig. S5). However, examination of E17.5 embryos revealed notable increases in the number of DRG neurons in DLK-null animals, with a 1.8-fold increase in the total number of pan-Trk–stained DRG neurons compared with wt littermates in the lumbar region (Fig. 7, A–C). When the number of pan-Trk–stained neurons was normalized to the total DRG area, a 1.5-fold increase in neuronal number/DRG area was still observed in DLK–/– embryos, indicative of more neurons being packed into individual DRGs (Fig. 7 D). The phenotype of DLK–/– neurons we observed in culture suggested that the increase in Trk-positive cell number observed at later stages was likely a result of reduced developmental apoptosis in DLK–/– embryos. To test this hypothesis, E15.5 embryos were stained for the activated form of caspase 3, which revealed a 1.7-fold decrease in the amount of cells per area undergoing apoptosis in DLK–/– DRGs as compared with wt littermate controls (Fig. 7, E–G). We were unable to identify in vivo axon degeneration phenotypes in DRG neurons as a result of two main limitations. First, no measurable axonal degeneration/pruning events in DRG neurons have been identified that occur in the absence of a secondary mutation (Schoenmann et al., 2010). Second, it would be impossible to discriminate between true axon degeneration defects and axonal misprojection as a result of excess DRG neurons in DLK–/– mice.

DLK is broadly expressed in the nervous system, so we next examined whether reductions in developmental apoptosis also occurred in spinal motor neurons, another neuronal population in which excess neurons are lost between E13.5 and 17.5 (White et al., 1996, 1998). To do this, we stained lower thoracic spinal cord sections from DLK–/– mice with an antibody to HB9, a spinal motor neuron–specific marker (Arber et al., 1999). Normal numbers of HB9-positive motor neurons were present in DLK–/– embryos at E13.5, yet by E15.5, the number of motor neurons in DLK–/– embryos was roughly double that of wt littermates (186% of control; Fig. 7, H–K and N). This increase in cell number was sustained at E17.5, the latest time point examined as a result of neonatal lethality of DLK-null animals (180% of control; Fig. 7, L–N). As initial numbers of motor neurons were generated in DLK–/– embryos, this phenotype is likely a result of reduced developmental apoptosis in motor neurons during later stages of development, similar to what was observed in DRGs. In addition, our results are comparable with changes in the motor neuron cell number observed in animals lacking choline acetyltransferase or BAX, both of which also display defects in developmental loss of motor neurons at similar developmental stages (White et al., 1998; Brandon et al., 2003). Collectively, these data suggest that DLK-dependent signaling pathways are essential to developmental apoptosis in multiple neuronal types.

**Discussion**

**DLK is required for neuronal degeneration during development**

In this study, we identify a role for DLK as a critical regulator of neuronal degeneration in multiple peripherally projecting neurons during development. DLK functions in this context by activating JNK-based stress response signaling in a JIP3-dependent fashion without affecting basal JNK activity. The phenotypes observed in DLK–/– mice suggest that DLK is essential for prodegeneration signaling in response to developmental cues in
both motor and sensory neurons. Previous work has established that 50–60% of motor neurons are lost by apoptosis during development (Oppenheim, 1991); therefore, the near doubling of DRG and motor neurons observed in DLK^−/− mice implies that these embryos lose few neurons during this time period. This level of protection is surprising, given the amount of cross talk that is often observed within MAPK pathways. Multiple MAPKKKs have been shown capable of activating JNK via MKK4/MKK7 in various contexts (Xu et al., 2001), which leads to the prediction that stress-induced JNK activation is a major neuronal degeneration pathway used during development.

Mechanisms of DLK-dependent degeneration

Our data suggest that DLK regulates neuronal degeneration largely via modulation of the JNK signaling pathway. In contrast to many other cell types, neurons maintain relatively high levels of active JNK even in the absence of stress (Coffey et al., 2000). This high level of p-JNK does not lead to the phosphorylation of proapoptotic downstream targets such as c-Jun and has been hypothesized to phosphorylate a distinct set of downstream targets involved in neuronal growth and function (Coffey et al., 2000; Waetzig and Herdegen, 2005). Interestingly, the removal of DLK does not appear to significantly affect the nonstress levels of p-JNK as judged by Western blotting and staining of neuronal cultures, and the alterations in p-JNK levels even after NGF withdrawal are relatively small compared with the changes observed in stress-specific JNK targets such as p-c-Jun (Fig. 2). The same is not true when neuronal MAPKKKs are broadly circumvented.
require c-Jun and is mediated by distinct JNK targets. The observation that JIP1 does not provide similar neuronal protection implies that signaling specificity may be mediated by this interaction. It has been hypothesized that the binding of specific combinations of MAPKs to scaffolding proteins can generate diverse signaling complexes with distinct sets of downstream targets (Waetzig and Herdegen, 2005), though few examples of such complexes exist for which a specialized function has been identified. We propose that DLK–JIP3–JNK is an example of such a complex, which is able to selectively regulate stress-induced JNK activity in the context of NGF deprivation (Fig. 8). The observation that JIP1 does not provide additional neuronal protection adds to support the idea that this is a specific function of DLK bound to JIP3. Redistribution of p-JNK observed after NGF withdrawal likely also plays an important role in degeneration and may be required to position p-JNK proximal to substrates such as c-Jun. Indeed, nuclear localization of JNK has been shown to be required for neuronal apoptosis (Björkblom et al., 2008), and a similar relocalization has been observed in the context of axonal injury (Middlemas et al., 2003). We show that both DLK and JIP3 are required for p-JNK relocalization in response to NGF withdrawal, arguing that it too is dependent on the DLK–JIP3 signaling complex. This is consistent with previous results that demonstrated that JIP3 can mediate retrograde transport of JNK in response to axonal injury through interactions with the P150-glued subunit of the dynactin motor protein complex (Cavalli et al., 2005), and it is conceivable that DLK–JNK interaction with JIP3 mediates retrograde transport of JNK after NGF withdrawal as well.

It is also possible that the signaling specificity downstream of DLK is mediated by activation of only a subset of the three JNK genes in mouse, all of which are expressed in embryonic neurons. The phenotypes observed in DLK-null mice argue that JNK2 and JNK3 are largely responsible for the JNK-mediated neuronal degeneration, at least in the context of injury (Bogoyevitch, 2006). Furthermore, JIP3 has been shown to preferentially interact with JNK3 over other JNK isoforms (Ito et al., 1999), raising the possibility that a significant amount of DLK–JIP3 signaling after NGF withdrawal could occur via JNK3. On the other hand, experiments in primary neurons have demonstrated that pan-JNK inhibition is sometimes required to provide complete rescue from degeneration (Björkblom et al., 2008), arguing that other JNK genes can also contribute to this process. Our data demonstrate that phosphorylation of both the 46- and 55-kD JNK bands is increased after NGF withdrawal and implies that multiple JNKs become activated, though it is possible that this pattern represents phosphorylation of different splice forms of a single JNK gene (Gupta et al., 1996). However, we also observed that knockout or siRNA-based knockdown of any individual JNK gene was not sufficient to provide protection after NGF withdrawal (unpublished data). This suggests that degeneration is likely mediated by a combination of JNK genes and that additional components of the pathway such as DLK and/or JIPs are necessary for regulation of prodegeneration-specific JNK activity.

**c-Jun-independent functions of DLK–JNK in degeneration**

The c-Jun–independent regulation of axon degeneration by DLK–JNK makes a strong case that phosphorylation of additional downstream targets is required for DLK-dependent neuronal degeneration. Several transcription factors can be phosphorylated by JNKs, including ATF2 (Widmann et al., 1999), and may contribute to the breakdown of axons. The DLK-dependent relocalization of p-JNK to the nucleus after NGF withdrawal agrees with this hypothesis. However, the observation that local axon degeneration is modulated by DLK–JNK suggests a possible alternative scenario in which this process is regulated via phosphorylation of axonal JNK targets. A local nontranscriptional role in axons would be consistent with the observation that both loss of DLK and pharmacological JNK inhibition protect from Wallerian degeneration after axotomy (Miller et al., 2009), in which the involvement of transcription is not possible. Several cytosolic JNK targets have been identified in neurons that may contribute to this degeneration, including doublecortin, SCG10, and Tau (Goedert et al., 1997; Gdalyahu et al., 2004; Tararuk et al., 2006). In addition, evidence exists in other systems that JNK is able to phosphorylate late members of the intrinsic apoptotic machinery, including Bcl-2–associated death promoter and Bcl-2–like protein 11 (Donovan et al., 2002; Putcha et al., 2003). Phosphorylation of these substrates in axons may also contribute to degeneration, which is consistent with our finding that caspase activity in the axon can be modulated by DLK–JNK independent of c-Jun.

In summary, we have demonstrated that DLK is required for neuronal degeneration in peripherally projecting neuronal populations during development and is the primary MAPKKK upstream of c-Jun activation in this context. Although first described in developmental NGF withdrawal paradigms, the proapoptotic functions of c-Jun have since been shown to be conserved in neuronal injury and neurodegenerative disease. If DLK is required for JNK–c-Jun activation in the disease setting as well, targeting this kinase may represent an attractive approach for therapeutic intervention.
Materials and methods

Model mice

DLK knockout mice were generated by homologous recombination using a phosphoglycerate kinase–neomycin cassette flanked by homology arms of 5.1 and 2.8 kb. The 5′ arm contained a LoxP site 1.5 kb away from the neomycin cassette. Embryonic stem (ES) cells were screened via PCR with the following primers, which amplified over both homology arms: 5′-AGCGGCGAGATGTTTGTGC-3′ and 5′-CTTCGTCGGAATGTTTGTGC-3′ (5′ arm), 5′-CTTCGTCAGCAGGTATGTGC-3′ and 5′-CTTCGTCGATTTGACATCG-3′ (3′ arm). The primers were confirmed via Southern blotting. Transfection of ES cells with a Cre-expressing plasmid resulted in recombination, which was assessed with the following primers: 5′-CATACTCTAGGCAAGTCCTCATA-3′, 5′-ATTCGCTTGGTTAAGTTGGA-3′, and 5′-CTTGCGGATATGCTGACG-3′. H9B2GF mice were obtained from S. Paff (Salk Institute, La Jolla, CA) and have been previously described (Lee et al., 2004). c-Jun knockout mice were obtained from E. Wagner (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain), have been previously described (Behrens et al., 2002), and were crossed to Nestin-Cre (Jackson ImmunoResearch Laboratories, Inc.) to eliminate c-Jun expression in neurons.

Primary neuron culture

E13.5 DRG cultures were dissected and cultured in F12 media containing N3 supplement, glucose, and 25 ng/ml NGF on precoated poly-lysin and laminin chamber slides (BioCoat; BD). In DRG explant experiments 24 h after plating, media were replaced with media containing no NGF and 25 µg/ml anti-NGF antibody (Genentech) for various time periods and were then fixed for staining. For dissociated cultures, DRGs were digested in 0.05% trypsin for 30 min at 37°C and were plated as described above. 24 h after plating, mitotic inhibitor (cytosine arabinofuranoside [AraC]) was added to the culture and then removed 24 h later. NGF was withdrawn from the culture 4–5 d after plating as described above. In experiments using JNK inhibitor AS601245 (also known as JNK inhibitor V; EMD), 10 mM stock solution was made in DMSO and diluted to 10 µM working concentration in media. Compartmentalized chamber assays were performed essentially as previously described (Nikolaev et al., 2009). In brief, 35-mm tissue culture dishes were coated with poly-lysin and laminin and scratched with a pin rake (Tyler Research) to generate tracks for axonal growth. 50 ml of culture media containing 4 mg/ml methylene-cellulose was placed on the scratched area so that axons could grow within the track. A Teflon divider (Camp; Tyler Research) that creates a central cell body chamber flanked by two axon chambers was then seated on silicone grease and placed on the culture dish as such that the cell body chamber was in the middle of the scratched area. Dissociated DRGs from E13.5 mouse embryos were suspended in methylene-cellulose-thickened medium and loaded in the cell body compartment, and both axon compartment chambers were filled with culture media with 4 mg/ml methylene-cellulose. 1 d after plating, media containing 7 mM AraC were added to the cell body compartment for a period of 24 h. 3–5 d after plating, NGF was withdrawn from different compartments by replacing media containing 4 mg/ml methyl-cellulose and 25 mg/ml anti-NGF antibody.

For siRNA experiments, dissociated DRGs were transfected with siRNA using a nucleofection system (Lonza). DIV 2 siRNA (sense 5′-GCACTGAAATGCGAACAAGCTCT-3′ and antisense 5′-GAGTGGTCCCAATGCTGCTG-3′) was synthesized at Genentech, and JIP1 (sc-35723; Santa Cruz Biotechnology, Inc.) and two siRNAs targeted to different regions of JIP3 (S013000789 and S013000796; Qiagen) were purchased. Levels of knockdown were tested by quantitative PCR at 5 d after plating using the Syber green qPCR kit (Qiagen) and verified primer sets (Qiagen) for JIP1, JIP3, and DLK. The control siRNA used was an siRNA directed against luciferase. Glyceraldehyde 3-phosphate dehydrogenase expression level was used as a control for all samples. Quantitative PCR was analyzed by the ΔΔCT method comparing expression levels to the level of expression in control siRNA. Quantitative PCR was performed in triplicate (n = 2 experiments for each siRNA).

Immunocytochemistry and immunohistochemistry

Cultured neurons were fixed with 4% PFA and 15% sucrose for 30 min at room temperature, were blocked and permeabilized in PBS with 5% BSA and 0.2% Triton X-100 for 1 h, and were then stained overnight in blocking buffer, which contained the following antibodies: p-JNK, p-c-Jun serine 63 total JNK, ERK, p-ERK, cleaved caspase 3, cleaved caspase 9 (Cell Signaling Technology), activated caspase 3 (R&D Systems), HB9 (a gift from S. Paff), and Alexa Fluor–conjugated secondary antibodies (Invitrogen). For whole-mount embryo neurofilament staining, embryos were eviscerated, fixed in 4% PFA, and stained with rabbit anti-Neurofilament antibody (Covance) using the same protocol as described above, except that all antibody incubations were overnight, and buffers included 0.4% Triton X-100.

Western blotting and IP

DRG cultures were lysed in 100 µl Triton X-100 lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and protease and phosphatase inhibitors) for 30 min at 4°C. Because of the limited amount of protein harvested from DRGs, protein was precipitated using TCA and then washed with acetone three times to remove the residual TCA. The pellet was dried and resuspended in 1× SDS NuPage loading buffer containing a reducing agent. The amount of protein in samples was quantified by Western blotting for tubulin. Similar amounts of protein were then loaded on 4%–12% Bis-Tris gels and subjected to standard immunoblot procedures. Primary antibodies used for Western blotting were the same as those used for immunocytochemistry. Blot images were taken and quantified using the VersaDoc system (Bio-Rad Laboratories), p-JNK (upper band) and p-ERK (lower band) were quantified by normalizing to total levels of JNK and ERK, respectively, and were then compared with wt control or control siRNA with NGF. p-c-Jun quantification was also normalized to wt/control siRNA with NGF present. Each experiment for Western blots on DLK −/− neurons was performed with more than or equal to three embryos for each condition and repeated three times, whereas siRNA knockdown Western blots used electroporated DRG neurons from five embryos for each condition and were repeated more than or equal to two times. The p-JNK and p-c-Jun time course blots were performed with more than or equal to two embryos for each genotype at each time point.

IP studies in HEK-293 cells used a full-length mouse coding sequence of N-terminal Flag-tagged DLK, N-terminal Myc-tagged JIP3, and GFP expressed using Fugene 6. 20 h after transfection, cells were washed with cold PBS and were lysed in 100 µl Triton X-100 lysis buffer (see above) for 30 min at 4°C. The amount of protein was quantified using bicinchoninic acid protein assay reagent (Thermo Fisher Scientific), and 200 µg of protein was taken for IP using a Flag IP kit (Sigma-Aldrich). 5% of protein was run as input, whereas 30% of the IP was run on Western blots. The IP experiment was repeated three times and showed similar results. For IP from mouse brain, whole brain was harvested from postnatal day 1 mice and lysed in buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris/ HCl, and 1 mM EDTA at 30 min at 4°C. IP was conducted using primary Anti-Neuropilin-1 (Abcam) or a rabbit IgG antibody. Beads were then washed twice in the lysis buffer followed by two washes in SDS buffer without Triton X-100, and protein was then eluted in 1× SDS loading buffer containing a reducing agent. Equal amounts of brain lysate were added to each IP condition. Approximately 2% of the protein was run as input, whereas 30% of the pull-down was run in each lane of the Western blots and blotted with DLK (Genentech) or JIP3 (Novus Biologicals) antibody.

Imaging and quantification

Images of cultured neurons were acquired using a fluorescent microscope (DM5500; Leica) with a camera (DFC360) using a 20 or 40× objective, whereas whole-mount embryos and Flag-positive DRGs were imaged on a confocal microscope (LSM710; Carl Zeiss) using a 10 or 20× objective, respectively. Whole mounts were imaged as a flattened z stack and presented as maximum intensity projections. γ was altered to weak signal in compartmentalized chamber images shown in Fig. 5 and to more easily visualize neurits in Figs. 6 K and M and S3 C using Photoshop (Adobe), but data within a panel were identically imaged and modified. For all quantifications, values represent the mean of multiple experiments, and error bars represent SEM.

Axon degeneration in DRG explants and compartmentalized cultures was quantified blindly on a scale of 0–5, in which 0 equals no degeneration [equivalent to control cultures containing NGF] and 5 equals complete degeneration. Representative images were used to define intermediate stages of degeneration. For explant experiments, n = 5 embryos using recombinant protein consisting of DLK AS597-P888; provided by S. Hirai (Yokohama City University, Japan) or Genentech; Hirai et al., 2005). Slides were washed three times in PBS, incubated for 1 h at room temperature with Alexa Fluor–conjugated secondary antibodies (Invitrogen) followed by 3x PBS washes, and mounted in Fluoromount-G. Staining of tissue was performed using the protocol above but with PBS containing 5% normal goat serum and 0.1% Triton X-100 on 20-µm transverse sections cut on a cryostat. The antibodies used were pan-Trk (Cell Signaling Technology), activated caspase 3 (R&D Systems), HB9 (a gift from S. Paff), and Alexa Fluor–conjugated secondary antibodies (Invitrogen). For whole-mount embryo neurofilament staining, embryos were eviscerated, fixed in 4% PFA, and stained with rabbit anti-Neurofilament antibody (Covance) using the same protocol as described above, except that all antibody incubations were overnight, and buffers included 0.4% Triton X-100.
with more than three explants scored per embryo. For compartmentalized chamber experiments, more than four chambers were quantified in two independent experiments. Axon degeneration quantification in dissociated DRG neurons was conducted using MetaMorph software (Molecular Devices). A journal that quantifies intact axons only was written and used to quantify all images, giving a total neurite length as a final readout for neuronal death. J. Neurosci. 25:169–179. doi:10.1523/JNEUROSCI.00026-X

Björkblom, B., J.C. Vainio, V. Hongisto, V. Komarovski, J.J. Filén, T.A. Nyman, T. Kallioniemi, M.J. Courtneay, and E.T. Coffey. 2004. All JNKs can kill, but nuclear localization is critical for neuronal death. J. Biol. Chem. 279:51706–51713. doi:10.1074/jbc.M401656200

Björkblom, B., N. Ostman, V. Hongisto, V. Komarovski, J.J. Filén, T.A. Nyman, T. Kallioniemi, M.J. Courtneay, and E.T. Coffey. 2005. Constitutively active cytoplasmic c-Jun N-terminal kinase 1 is a dominant regulator of dendritic architecture: role of microtubule-associated protein 2 as an effector. J. Neurosci. 25:6530–6536. doi:10.1523/JNEUROSCI.1517-05.2005

Björkblom, B., J.C. Vainio, V. Hongisto, T. Herdegen, M.J. Courtneay, and E.T. Coffey. 2008. All JNKs can kill, but nuclear localization is critical for neuronal death. J. Biol. Chem. 283:19704–19713. doi:10.1074/jbc.M707741420

Bogoyevitch, M.A. 2006. The isoform-specific functions of the c-Jun N-terminal kinases (JNKs): differences revealed by gene targeting. Bioessays. 28:934–938. doi:10.1002/bies.20458

Brandon, E.W., P. Lin, K.A. D’Amour, D.P. Pizzo, B. Dominguez, Y. Suguita, S. Thode, C.P. Ko, L.J. Thal, F.H. Gage, and K.F. Lee. 2003. Aberrant patterning of neuromuscular synapses in choline acetyltransferase- deficient mice. J. Neurosci. 23:539–549.

Campenot, R.B. 1977. Local control of neurite development by nerve growth factor. Proc. Natl. Acad. Sci. USA. 74:4516–4519. doi:10.1073/pnas.74.10.4516

Cavalli, V., P. Kujala, J. Klumperman, and L.S. Goldstein. 2005. Sunday dinner links axonal transport to damage signaling. J. Cell Biol. 168:775–787. doi:10.1083/jcb.200410136

Chang, L.-Y., J. Jones, M.H. Ellisman, L.S. Goldstein, and M. Karin. 2003. JNK1 is required for maintenance of neuronal microtubules and controls phosphorylation of microtubule-associated proteins. Dev. Cell. 4:521–533. doi:10.1016/S1534-5807(03)00094-7

Coffey, E.T., V. Hongisto, M. Dickens, R.J. Davis, and M.J. Courtneay. 2000. Dual roles for c-Jun N-terminal kinase in developmental and stress responses in cerebellar granule neurons. J. Neurosci. 20:7602–7613.

Collins, S.A., Y.P. Watanabe, S.B. McMahon, and D.A. DiAntonio. 2006. Highwire restrains synaptic growth by attenuating a MAP kinase signal. Neuron. 51:57–69. doi:10.1016/j.neuron.2006.05.026

Crawley, C., S.D. Spencer, M.C. Nishimura, K.S. Chen, S. Pitts-Meek, M.P. Armanini, H.L. Ling, S.B. McMahon, D.L. Shelton, A.D. Levinson, et al. 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell. 76:1001–1011. doi:10.1016/0092-8674(94)90378-6

Deckwerth, T.L., J.L. Elliott, C.M. Knudson, E.M. Johnson Jr., W.D. Snider, and S.J. Korsmeyer. 1996. BAX is required for neuronal death after trophic factor deprivation and during development. Neuron. 17:401–411. doi:10.1016/0896-6273(96)90817-3

Donovan, N., E.B. Becker, Y. Konishi, and A. Bonni. 2002. JNK phosphorylation and activation of BAD couples the stress-activated signaling pathway to the cell death machinery. J. Biol. Chem. 277:40494–40499. doi:10.1074/jbc.M206113200

E.T. Coffey. 2008. All JNKs can kill, but nuclear localization is critical for neuronal death. J. Biol. Chem. 283:19704–19713. doi:10.1074/jbc.M707741420

Garcia, I., I. Martinou, Y. Tsujimoto, and J.C. Martinou. 1992. Prevention of procaspase activation by c-Jun NH(2)-terminal kinase 1 is a dominant regulator of dendritic architecture. Science. 258:302–304. doi:10.1126/science.1411528

Gdalyahu, A., I. Ghosh, T. Levy, T. Sapriss, S. Sapoznik, Y. Fishler, D. Azoulay, and O. Reiner. 2004. DCX, a new mediator of the JNK pathway. EMBO J. 23:823–832. doi:10.1038/sj.emboj.7600079

Goedert, M., M. Hasegawa, R. Jakes, S. Lawler, A. Cuenda, and P. Cotin. 1997. Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases. FEBS Lett. 409:57–62. doi:10.1016/S0014-5793(97)00483-3

Gorin, P.D., and E.M. Johnson. 1979. Experimental autoimmune model of nerve growth factor deprivation: effects on developing peripheral sympathetic and sensory neurons. Proc. Natl. Acad. Sci. USA. 76:5382–5386. doi:10.1073/pnas.76.10.5382

Gupta, S., T. Barrett, A.J. Whitmarsh, J. Cavanagh, H.K. Sluss, B. Dérijard, and R.J. Davis. 1996. Selective interaction of JNK protein kinases isoforms with transcription factors. EMBO J. 15:2760–2770.

Ham, J., C. Babij, J. Whitfield, C.M. Pfair, D. Lallemend, M. Yaniv, and L.L. Rubin. 1995. A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. Neuron. 14:927–939. doi:10.1016/0896-6273(95)90331-3

Hammarsland, M., P. Nix, L. Hauth, E.M. Jorgensen, and M. Bastiani. 2009. Axon regeneration requires a conserved MAP kinase pathway. Science. 323:802–806. doi:10.1126/science.1155277

Hirai, S., A. Kawaguchi, J. Suenaga, M. Ono, D.F. Cui, and S. Ohno. 2005. Expression of MUK/DLDKZPK, an activator of the JNK pathway, in the nervous systems of the developing mouse embryo. Gene Expr. Patterns. 5:517–523. doi:10.1016/j.modgep.2004.12.002

Hirai, S., F. Cui, T. Miyata, M. Ogawa, H. Kiyonari, Y. Suda, S. Aizawa, Y. Bamba, and S. Ohno. 2006. c-Jun N-terminal kinase activator dual leucine zipper kinase regulates axon growth and neuronal migration in the developing cerebral cortex. J. Neurosci. 26:11992–12002. doi:10.1523/JNEUROSCI.2277-06.2006

Hunot, S., M. Vila, P. Teismann, R.J. Davis, E.C. Hirsch, S. Prezbdorski, P. Rakić, and R.A. Flavell. 2004. JNK-mediated induction of cyclooxygenase 2 in

DLK required for JNK-dependent neuronal degeneration • Sengupta Ghosh et al.
required for neurodegeneration in a mouse model of Parkinson’s disease. Proc. Natl. Acad. Sci. USA. 101:665–670. doi:10.1073/pnas.0307453101

Ito, M., K. Yoshokawa, M. Akechi, S. Yamashita, N. Takamatsu, K. Sugiyama, M. Hibii, Y. Nakabeppu, T. Shiba, and K.I. Yamamoto. 1999. JSAP1, a novel jun N-terminal protein kinase (JNK)-binding protein that functions as a Scaffold factor in the JNK signaling pathway. Mol. Cell. Biol. 19:7539–7548.

Itoh, A., M. Horiechi, K. Wakayama, J. Xu, P. Bannerman, D. Pleasure, and T. Itoh. 2011. ZPK/DLK, a mitogen-activated protein kinase kinase kinase, is a critical mediator of programmed cell death of motoneurons. J. Neurosci. 31:7223–7228. doi:10.1523/JNEUROSCI.5947-10.2011

Lee, S.K., L.W. Jurata, J. Funahashi, E.C. Ruiz, and S.L. Pfaff. 2004. Analysis of embryonic motoneuron gene regulation: derepression of general activators function in concert with enhancer factors. Development. 131:3295–3306. doi:10.1242/dev.011179

Levi-Montalcini, R., and B. Booker. 1960. Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve-growth protein. Proc. Natl. Acad. Sci. USA. 46:384–391. doi:10.1073/pnas.46.3.384

Lewcock, J.W., N. Genoud, K. Lettieri, and S.L. Pfaff. 2007. The ubiquitin ligase Phrl regulates axon outgrowth through modulation of microtubule dynamics. Neuro. 56:604–620. doi:10.1523/JNEUROSCI.0709-2007

Luo, L., and D.D. O’Leary. 2005. Axon retraction and degeneration in development and disease. Annu. Rev. Neurosci. 28:127–156. doi:10.1146/annurev.neuro.28.061604.135632

Mack, T.G., M. Reiner, B. Beirowski, W. Mi, M. Emanuelli, D. Wagner, D. Thomson, T. Gillingwater, F. Court, L. Conforti, et al. 2001. Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nimmtl promoter construct. Nat. Neurosci. 4:1199–1206. doi:10.1038/nn779

Moroney, A.C., M.A. Glicksman, A.N. Basma, K.M. Walton, E. Knight Jr., C.A. Murphy, B.A. Bartlett, J.P. Finn, T. Angeles, Y. Matsuda, et al. 1998. Motoneuron apoptosis is blocked by CEP-1347 (KT 7515), a novel inhibitor of the JNK signaling pathway. J. Neurosci. 18:104–111.

Middlemas, A., J.D. Delcroix, N.M. Sayers, D.R. Tomlinson, and P. Fernyhough. 2013. Enhanced activation of axonally transported stress-activated protein kinases in peripheral nerve in diabetic neuropathy is prevented by neurotrophin-3. Brain. 126:1671–1682. doi:10.1093/brain/awg150

Miller, B.R., C. Press, R.W. Daniels, Y. Sasaki, J. Milbrandt, and A. DiAntonio. 2009. A dual leucine kinase-dependent axon self-destruction program promotes Wallerian degeneration. Nat. Neurosci. 12:387–389. doi:10.1038/nn.2290

Mok, S.A., K. Lund, and R.B. Campenot. 2009. A retrograde apoptotic signal originating in NGF-deprived distal axons of rat sympathetic neurons in compartmental cultures. Cell Res. 19:546–560. doi:10.1038/cr.2009.11

Nakata, K., B. Abrams, B. Grill, A. Goncharov, X. Huang, A.D. Chisholm, and Y. Jin. 2005. Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. Cell. 120:407–420. doi:10.1016/j.cell.2004.12.017

Nikolaev, A., T. McLaughlin, D.D. O’Leary, and M. Tessier-Lavigne. 2009. Palmada, M., S. Kanwal, N.J. Rutkoski, C.Y. Kuan, A.J. Whitmarsh, M. Rincón, T.S. Zheng, R.J. Davis, P. Rakic, and R.A. Flavell. 1997. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature. 389:865–870. doi:10.1038/39699

Oppenheim, R.W. 1991. Cell death during development of the nervous system. Annu. Rev. Neurosci. 14:453–501. doi:10.1146/annurev.ne.14.030191.003221

Palmada, M., S. Kanwal, N.J. Rutkoski, C. Gustafson-Brown, R.S. Johnson, B. Abrams, and A. DiAntonio. 2009. JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. Neuron. 38:899–914. doi:10.1016/j.neuron.2009.11.015

Putcha, G.V., S. Le, F. Frank, C.G. Besirli, K. Clark, B. Chu, S. Alis, R.J. Youle, A. LaMarche, A.C. Moroney, and E.M. Johnson Jr. 2003. JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. Neuron. 38:899–914. doi:10.1016/j.neuron.2003.01.055-6

Schoenmann, Z., E. Assa-Kunin, S. Tionmy, A. Minis, L. Hakaki-Topper, E. Aranina, and A. Yaron. 2010. Axonal degeneration is regulated by the apoptotic machinery or a NAD+-sensitive pathway in insects and mammals. J. Neurosci. 30:6375–6386. doi:10.1523/JNEUROSCI.0292-10.2010

Taronet, T., N. Ostman, W. Li, B. Björkblom, A. Padzik, J. Zdrojewska, V. Dongisto, T. Herdegen, W. Konopka, M.J. Courtney, and E.T. Coffey. 2000. JNK1 phosphorylation of SCG10 determines microtubule dynamics and axodendritic length. J. Cell Biol. 173:265–277. doi:10.1083/jcb.200511055

Vohra, B.P., Y. Sasaki, B.R. Miller, J. Chang, A. DiAntonio, and J. Milbrandt. 2010. Amyloid precursor protein cleavage-dependent and -independent axonal degeneration programs share a common nicotinamide mononucleotide adenlytransferase 1-sensitive pathway. J. Neurosci. 30:13729–13738. doi:10.1523/JNEUROSCI.2939-10.2010

Waetzig, V., and T. Herdegen. 2005. Context-specific inhibition of JNKs: overcoming the dilemma of protection and damage. Trends Pharmacol. Sci. 26:455–461.

Watts, R.J., E.D. Hoopfer, and L. Luo. 2003. Axon pruning during Drosophila metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. Neuron. 38:871–885. doi:10.1016/S0896-6273(03)00295-2

White, F.A., I. Silos-Santiago, D.C. Molliver, M. Nishimura, H. Phillips, M. Barbacid, and W.D. Snider. 1996. Synchronous onset of NGF and TrkA survival dependence in developing dorsal root ganglia. J. Neurosci. 16:4662–4672.

Whitefield, J., S.J. Neame, L. Paquet, O. Bernard, and J. Ham. 2001. Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. Neuron. 29:629–643. doi:10.1016/S0896-6273(01)00239-2

Whitmarsh, A.J. 2006. The JIP family of MAPK scaffold proteins. Biochem. Soc. Trans. 34:828–832. doi:10.1042/BST0340828

Widmann, C., S. Gibson, M.B. Iarpe, and G.L. Johnson. 1999. Mitogen-activated protein kinase conservation of a three-kinase module from yeast to human. Physiol. Rev. 79:143–180.

Xiong, X., X. Wang, R. Ewanek, P. Bhat, A. DiAntonio, and C.A. Collins. 2010. Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. J. Cell Biol. 191:211–223. doi:10.1083/jcb.201006039

Xu, Z., A.C. Maroney, P. Dobranzski, N.V. Kukobook, and L.A. Greene. 2001. The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. Mol. Cell. Biol. 21:4713–4724. doi:10.1128/MCB.21.14.4713-4724.2001

Yang, D.D., C.Y. Kuan, A.J. Whitmarsh, M. Rincón, T.S. Zheng, R.J. Davis, P. Rakic, and R.A. Flavell. 1997. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature. 389:865–870. doi:10.1038/39699