EMBO reports

review

The DLK signalling pathway—a double-edged sword in neural development and regeneration

Andrea Tedeschi[+] *& Frank Bradke*[++]

Laboratory for Axon Growth & Regeneration, German Center for Neurodegenerative Diseases (DZNE), Bonn, Germany

Dual leucine zipper kinase (DLK), a mitogen-activated protein kinase kinase kinase, controls axon growth, apoptosis and neuron degeneration during neural development, as well as neurodegeneration after various insults to the adult nervous system. Interestingly, recent studies have also highlighted a role of DLK in promoting axon regeneration in diverse model systems. Invertebrates and vertebrates, cold- and warm-blooded animals, as well as central and peripheral mammalian nervous systems all differ in their ability to regenerate injured axons. Here, we discuss how DLK-dependent signalling regulates apparently contradictory functions during neural development and regeneration in different species. In addition, we outline strategies to fine-tune DLK function, either alone or together with other approaches, to promote axon regeneration in the adult mammalian central nervous system.

Keywords: DLK; neural development; degeneration; axon regeneration

EMBO *reports* (2013) **14,** 605–614; published online 17 May 2013; doi:10.1038/embor.2013.64

See the Glossary for abbreviations used in this article.

Introduction

Over the past decades, research involving diverse model organisms has yielded fundamental insights into the molecular and cellular mechanisms of axon growth, degeneration and cell death during neuronal development, as well as neuron degeneration and regeneration failure after injury to the adult nervous system. Neural development is a complex multi-step process, for which a delicate balance exists between cell death against survival and axonal growth against degeneration, which is constantly adjusted from embryonic stages to adult tissue homeostasis. Such a delicate balance also underlies the neuronal response to various insults in the adult. Intracellular signalling cascades integrate a range of extracellular signals to shift the balance in a timely fashion.

The dual leucine zipper kinase (DLK) signalling pathway is one such signalling cascade that regulates several aspects of neural development ranging from axon growth and neuronal migration to apoptosis and axon degeneration in different model organisms [1–6]. An important role in controlling both neurodegeneration [7–10] and regeneration [9,11–16] after injury has also emerged for DLK signalling. Thus, DLK might act as a master sensor that initiates apparently contradictory responses under critical conditions during development and after axonal injury.

DLK functions as a MAP3K activating both JNKs and p38α-δ MAPK pathways [17,18]. The MAPK pathways are organized in three sequential modular cascades: MAP3K, MAP2K and the MAPK. Such modular structure allows for fine-tuning of the DLK signalling in response to a myriad of stimuli, ultimately leading to phosphorylation-dependent modulation of numerous downstream targets including transcription factors, cytoskeleton components, membrane transporters and other protein kinases.

In mice, the DLK (also known as LZK, MAP3K12, MUK and ZPK) protein localizes in several areas of the developing nervous system, such as the brain, spinal cord and sensory ganglia [2,19]. In response to oxidative stress and a limited supply of trophic factors, the activation of DLK-dependent signalling cascades leads to rapid neuron degeneration during development [5]. Conversely, DLK deletion protects several classes of neurons from apoptosis in mouse embryos [5,6]. Earlier observations have shown that genetic disruption of DLK results in reduced JNK activity and decreased phosphorylation of several JNK targets, ultimately causing neuronal migration defects as well as incomplete development of axonal tracts including those of the anterior commissure and corpus callosum [2].

DLK expression is also upregulated in response to axonal injury in mice and rats [10,16]. Absence of DLK protects neurons from apoptosis after nerve injuries and in rodent models of neurodegenerative diseases [10,16,20]. Interestingly, loss of DLK also protects distal axons from Wallerian degeneration, thus providing evidence for a role of the DLK pathway in the axon self-destruction programme after injury [7].

Interestingly, a role for DLK in promoting axon regeneration in diverse model systems has emerged. In this regard, the DLK homologues DLK-1 (*Caenorhabditis elegans*) and Wallenda (*Drosophila*) have been shown to regulate axon regrowth after injury [11,12] and axon regeneration, respectively [9]. In addition, more recent observations have started to highlight the role of DLK in controlling the regenerative response in the mammalian nervous system [13,15,16]. These studies suggest that a MAPK signal pathway that controls the axonal response to injury might be conserved among model organisms.

Laboratory for Axon Growth & Regeneration, German Center for Neurodegenerative Diseases (DZNE), Ludwig-Erhard-Allee 2, 53175 Bonn, Germany
[+]Corresponding author. Tel: +49 228 43302 639; Fax: +49 228 43302 689;
E-mail: andrea.tedeschi@dzne.de
[++]Corresponding author. Tel: +49 228 43302 590; Fax: +49 228 43302 689;
E-mail: frank.bradke@dzne.de

Received 25 January 2013; accepted 23 April 2013; published online 17 May 2013

Glossary

Bad	Bcl2-associated death promoter
Bax	Bcl2-associated X protein
Bcl2	B-cell lymphoma 2
Cdc42	cell division cycle 42
cebp	CCAAT/enhancer-binding protein
CLIP	cytoplasmic linker protein
CNS	central nervous system
CNTF	ciliary neurotrophic factor
DCX	doublecortin
DRG	dorsal root ganglia
efa-6	exchange factor for Arf6
Erk	extracellular signal-regulated kinase
Fos	FBJ osteosarcoma oncogene
GABA	gamma-aminobutyric acid
GEF	guanine exchange factor
gp130	glycoprotein 130
GTP	guanosine triphosphate
hb9	homoebox gene 9
HNRPK	heterogeneous nuclear ribonucleoprotein K
IL-6	interleukin 6
Itch	itchy E3 ubiquitin protein ligase
JAK	janus kinase
JIP1/3	JNK-interacting protein 1/3
JNK	c-Jun amino-terminal kinase
KLP-7	kinesin-like protein 7
LIF	leukaemia inhibitory factor
MAP1B/2/2B	microtubule-associated protein 1B/2/2B
MAPK	mitogen-activated protein kinase
MAP2K	mitogen-activated protein kinase kinase
MAP3K	mitogen-activated protein kinase kinase kinase
MAPKAP	MAP kinase-activated protein kinase
MUK	MAPK-upstream protein kinase
NGF	nerve growth factor
Nmat2	nicotinamide mononucleotide adenylyltransferease 2
phr1	Highwire
PNS	peripheral nervous system
pten	phosphatase and tensin homologue
Rac1	Ras-related C3 botulinum toxin substrate 1
RGC	retinal ganglion cell
RhoA	Ras homologue family member A
RPM-1	regulator of presynaptic morphology 1
SCG10	superior cervical ganglion 10
Shc	Src homology 2 domain-containing transforming protein
STAT3	signal transducer and activator of transcription 3
TIF-IA	transcription initiation factor IA
UPS	ubiquitin-proteasome system
Wld^s	Wallerian degeneration slow

However, our understanding of the upstream and downstream components of the DLK pathway remains fragmentary, and it is unknown whether these components are conserved among different organisms and classes of neurons. Here, we discuss evidence gathered from several models, which together support a multifaceted role for DLK-dependent signalling in regulating aspects of neural development, degeneration and regeneration after injury.

Neural development

The functionality of the nervous system relies on its correct development. Early stages of neural development are characterized by neuronal migration followed by extensive growth of axons and dendrites, and later growth by synapse formation and refinement of functional connections within a neuronal network. Experimental evidence reveals different roles for the DLK–JNK signalling pathway *in vivo* during neural development including axon formation and neuronal migration, as well as neuronal apoptosis and axon degeneration (Fig 1; [1–3,5,6]).

Axon growth and neuronal migration

Axon formation and neuronal polarization are fundamental steps during neural development that allow for directional transmission of information within the fully developed nervous system [21,22]. The DLK protein localizes to axons, and it is found in several areas of the developing mammalian nervous system such as the brain, spinal cord and sensory ganglia [2,19]. *Dlk* null mice have neuronal migration defects and hypoplasia of several axonal tracts including those of the anterior commissure and the corpus callosum [2,3,23]. A reduction in the number of axons is also seen in the lateral olfactory tract, cingulum and internal capsule [2]. Most *dlk* mutant mice eventually die during the perinatal period. Defects similar to those seen in *dlk* mutant mice are also found on conditional deletion of *phr1*, one of the ubiquitin ligases upstream from DLK [3]. However, DLK protein levels are unchanged in *phr1* mutant brains, suggesting that the defects in *phr1* mutants are not due to changes in DLK expression.

DLK induces JNK activity *in vitro* [18]. In mammals, activated JNK phosphorylates a wide range of downstream targets, including nuclear substrates (transcription factors and hormone receptors, HNRPK and TIF-IA) and non-nuclear substrates involved in protein degradation (E3 Itch), apoptosis (Bcl2 family members Bax and Bad), signal transduction (JIP1, Shc) and cell motility (keratin 8, DCX, MAP1B and MAP2B, tau, SCG10, kinesin, paxillin; [24]). In the absence of DLK, JNK activity and the phosphorylation of several JNK targets decreases during mouse brain development [2]. Importantly, forced expression of active JNK1 rescues axon formation defects caused by DLK silencing in cultured mouse cortical neurons [1].

In mammals there are three *jnk* genes (*jnk1*, *jnk2* and *jnk3*), and in total, at least ten splice variants originate from alternative splicing events. Thus, assessing the consequences of *jnk* deletion on axon formation *in vivo* has been problematic [25,26]. Nevertheless, genetic deletion of a single family member, *jnk1*, is sufficient to alter the integrity of the neuronal cytoskeleton, resulting in disrupted axonal tract formation in the mouse developing neocortex [27,28].

Greater defects are seen in *dlk;jnk1* double-mutant mice than *dlk* and *jnk1* single-mutant mice [1], further supporting the hypothesis that DLK–JNK signalling is actively involved in neural development. Several axonal tracts (for example, corpus callosum and anterior commissure), and neuronal structures (for example, internal capsule, hippocampus, plexiform layers and glomerular layer) are either significantly reduced or absent in *dlk;jnk1* double mutants [1]. By contrast, the peripheral nervous system and a few other brain structures develop normally in *dlk;jnk1* double-mutant mice.

Axon formation has been extensively studied by using cultured hippocampal and cortical neurons [22]. Whilst the JNK protein is uniformly distributed, active phospho-JNK localizes to the axon compartment of cultured embryonic rat hippocampal neurons [29]. Importantly, such compartmentalized expression is present through all subsequent stages of development. It is probable that DLK-mediated local activation of MAP2Ks constrains JNK activity to neurites that are beyond the critical length for axon specification in cultured embryonic rat hippocampal neurons [29]. In line with this

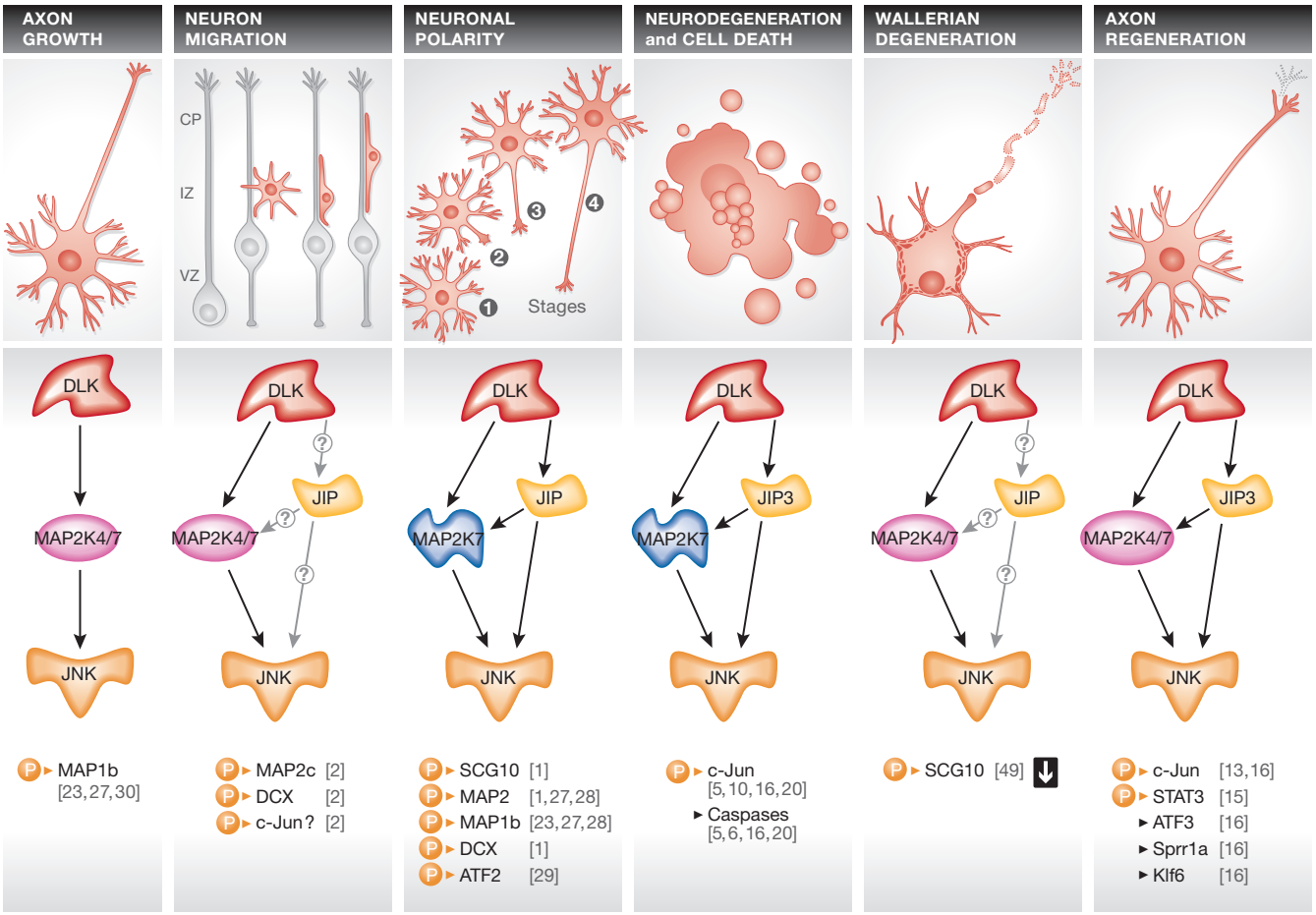

Fig 1 | DLK pathways controlling contradictory responses in mammalian neurons. Under certain circumstances, DLK initiates a coordinated sequence of phosphorylation events culminating in the activation of JNK activity. On activation, JNK can phosphorylate various intracellular targets. Interaction with JIPs directs JNK activity towards specific neuronal responses. ATF2/3, activating transcription factor 2/3; CP, cortical plate; DCX, doublecortin; DLK, dual leucine zipper kinase; IZ, intermediate zone; JIP, JNK-interacting protein; JNK, c-Jun amino-terminal kinase; Klf6, Kruppel-like factor 6; MAP1b/2c, microtubule associated protein 1b/2c; MAP2K4/7, mitogen-activated protein kinase kinase 4/7; SCG10, superior cervical ganglion 10; Sprr1a, small proline-rich protein 1A; STAT3, signal transducer and activator of transcription 3; VZ, ventricular zone.

hypothesis, a study has shed light on how DLK-mediated activation of MAP2K7 might position JNK signalling modules in the neurite shaft to control microtubule bundling in cultured embryonic mouse hippocampal neurons [30]. Moreover, JNK inhibition through pharmacological and dominant-negative approaches results in axon formation defects without affecting dendrites in cultured embryonic rat hippocampal neurons [29]. Thus, in accordance with the *in vivo* findings, activation of DLK–JNK signalling is crucial for axonogenesis, as well as the maintenance of neuronal polarity in cultured cells.

Cytoskeleton components provide structural support for growing axons. Continuous remodelling of the actin-based cytoskeleton, together with changes in microtubule stability, influence neuronal polarization [31–33]. Several microtubule regulators including SCG10, MAP2, tau, MAP1B, CLIPs and DCX influence axon formation [34–37]. Indeed, silencing of SCG10, DCX and MAP2 microtubule modulators, which serve as substrates for the DLK–JNK pathway, causes an accumulation of multi-polar mouse cortical neurons *in vitro* [1]. It is interesting to note that moderate microtubule stabilization can overcome stage-specific defects seen in the polarization of cultured cortical neurons from *dlk;jnk1* mutant mice [1].

In addition to controlling axon formation, the DLK–JNK pathway is recognized as a crucial regulator of radial migration during mouse corticogenesis [2,4,38]. Radial and tangential migration of newly generated neurons contributes to neocortex formation and is often associated with axon formation [39,40]. The expression of DLK and phospho-JNKs is higher in the mouse cortical intermediate zone than in the ventricular zone and cortical plate at embryonic day (E) 16. A reduction in DLK protein levels and JNK activity is seen as soon as differentiating cortical neurons reach the sub-plate zone [4]. Without affecting radial glia cell architecture, *dlk* gene disruption results in incorrect positioning of neurons throughout the cortical plate *in vivo*. Moreover, JNK pharmacological inhibition alters the migration rate of cortical neurons in slice cultures [2]. Absence of DLK as well as JNK inhibition delays radial migration of neuronal cells, disrupting lamination of the mouse cortical plate [2]. Interestingly, DLK overexpression in neural precursor cells leads to an accumulation of neurons in the subventricular zone at E16. By contrast, overexpression of a DLK mutant lacking kinase activity does not have an impact on neuronal migration, indicating that DLK kinase activity is essential for this phenotype [4].

Taken together this collection of results suggests that temporally and spatially controlled DLK–JNK signalling is required for axon growth and corticogenesis during mammalian neural development.

Neuronal apoptosis and axon degeneration

During development, an excess of neurons is generated. However, only those that make stable and functional connections survive. Thus, the accuracy and establishment of functional connections within a neuronal network requires not only axon and dendrite growth, but also neuronal apoptosis and axon degeneration. In fact, axon degeneration is a major mechanism responsible for large-scale elimination of exuberant projections and unstable synaptic contacts [41].

Extracellular factors including NGF are necessary for the survival of sympathetic and sensory neurons. Consistent with an active role in modulating the stress response, the absence of DLK protects cultured mouse embryonic DRG sensory neurons from cell death and axon degeneration induced by NGF withdrawal—a condition that mimics *in vivo* competition for trophic factors [5]. DRG neurons *in vivo* are present in similar numbers in *dlk* mutants and control littermates at E12.5. After developmental apoptosis has occurred, however, a significant decrease in the number of DRG neurons is seen in control mice but not in *dlk* mutants at E17.5. Thus, DLK is a positive regulator of developmental apoptosis in mouse DRG sensory neurons [5]. DLK activates stress-induced JNK signalling without affecting JNK basal activity in these neurons. Such complex regulation is achieved by interaction with the scaffold protein JIP3 to form a specific signalling module together with MAP2K, directing JNK activity towards precise functions (Fig 1; [5]). In projecting axons, the DLK protein is also found at the growth cone [42]. Therefore, DLK might be transported retrogradely to activate stress pathways in the nucleus [5]. It has been shown that DLK–JNK positively regulates neuronal apoptosis and axon degeneration in a c-Jun-dependent and -independent manner, respectively [5].

When approaching target cells, multiple neurons compete to form synapses. In particular, the establishment of neuromuscular connections controls the survival of spinal motor neurons. As a result of competition, about 50% of spinal motor neurons die during embryonic development. More than twice as many spinal motor neurons are found in *dlk* mutant mice compared with control animals [5,6]. As the number of hb9-positive cells committed to becoming spinal motor neurons is comparable to that in control mice at E12.5, it is probable that the absence of DLK reduces apoptosis rather than enhancing motor neuron specification. Interestingly, a similar number of spinal motor neurons to that observed during embryonic development is also found at six months of age in *dlk* mutants, with no signs of neural atrophy [6]. Genetic deletion of *bax*, a proapoptotic member of the Bcl2 family, significantly suppresses apoptosis during mouse neural development [43]. Although rescued from apoptosis, *bax*-deleted motor neurons show clear signs of atrophy during the perinatal period. By contrast, *dlk* mutant motor neurons are apparently healthy, supporting the hypothesis that DLK promotes additional cell-autonomous responses including axon degeneration in conditions within which apoptosis does not occur [5,6]. Collectively, these results provide evidence for a role of DLK in controlling developmental apoptosis and axon degeneration in different classes of neurons.

Degenerative responses to insults

Neuronal degeneration occurs not only during development, but also in response to various insults including neurotoxicity, demyelination, ischaemia and trauma as well as in neurodegenerative diseases. Morphological changes in neuronal cell bodies in response to stress include displacement of the nucleus towards the periphery of the pericaryon, swelling of the cell body and spreading of large Nissl bodies due to fragmentation of rough endoplasmic reticulum. Axons are vulnerable, highly specialized structures that require maintenance through their entire lifespan. In fact, changes in body size, body movement and ageing constantly challenge the integrity of axonal structures. As a consequence of axonal lesion, loss-of-maintenance factors probably trigger an axonal self-destruction programme called Wallerian degeneration in the distal stump. It is worth noting that Wallerian degeneration after axonal injury should be distinguished from developmental axon pruning. Despite the fact that the two phenomena share several substrates, the mechanisms differ [44]. Studies have underscored a role for DLK-dependent signalling in promoting apoptosis and axon degeneration under different experimental conditions including models of Wallerian degeneration and neurodegeneration (Fig 1).

Cell body response

Recent findings offer an intriguing basis for a possible involvement of DLK-mediated signalling in the pathophysiology of neurodegenerative diseases such as Parkinson disease and optic neuropathies.

By using a viral-mediated delivery approach, dominant-negative forms of DLK suppress neuronal apoptosis in dopamine neurons in a 6-OHDA mouse model of Parkinsonism [20]. After DLK inhibition, neuroprotective and trophic support seems to correlate with inhibition of c-Jun phosphorylation in dopamine neurons [20]. Thus, DLK might activate JNK to induce neuronal cell death through phosphorylation of c-Jun in this model. Given that DLK inhibition has no effect on nigrostriatal projections, these results support the idea that molecular pathways responsible for neuronal apoptosis differ from those mediating axonal degeneration.

More recently, a functional genomic screen has identified DLK as a crucial mediator of neuronal cell death in mammalian models of optic neuropathies, such as glaucoma and after optic nerve injury. After screening an extensive library of 1,869 short interfering RNAs, targeting more than 600 kinases, knockdown of DLK and its downstream substrate MAP2K7 have been found to protect cultured mouse RGCs from cell death [10]. Injuries to the optic nerve cause considerable RGC death after two weeks. DLK conditional deletion promotes mouse RGC survival after optic nerve injury *in vivo* [10,16]. Once again, the increase in survival correlates with a decrease in JNK, c-Jun phosphorylation and activated caspase 3. In response to optic nerve lesion, DLK seems to be upregulated within one day in RGC axons and within three days in the RGC body, raising the possibility that DLK might trigger a stress response leading to cell death [10,16]. In line with this hypothesis, whilst DLK overexpression accelerates RGC death, overexpression of kinase-dead DLK promotes RGC survival [10]. Moreover, DLK pharmacological inhibition using tozasertib, a protein kinase inhibitor, results in rat RGC survival after optic nerve transection and in a glaucoma model induced by increasing intra-ocular pressure [10]. However, the broad action of tozasertib might result in inhibition of other kinases in addition

to DLK. Therefore, developing more specific inhibitors should be a priority in order to validate and extend these highly relevant findings into more clinically applicable strategies.

Altogether, these observations suggest that activation of a DLK stress-induced pathway leads to proapoptotic JNK activation in models of CNS degeneration.

Axonal response

In addition to changes in neuronal cell bodies, axonal injuries trigger an active self-destruction programme called Wallerian degeneration in the distal part of the axon. Preventing Ca^{2+} influx, inhibiting protein degradation and overexpressing Nmnat2 and the chimaeric Wld^{s} delay Wallerian degeneration [45–47]. Despite progress, our understanding of the molecular pathways that regulate this process remains limited [48]. Absence of the DLK homologue Wallenda preserves axons from degeneration in a *Drosophila* model of olfactory receptor–neuron axotomy [7]. In cultured mouse embryonic DRG neurons, DLK-deficient axons have a marked decrease in degeneration when compared with controls after axotomy. Similar results are also seen after *in vivo* sciatic nerve transection in adult mice [7]. As already mentioned, axon degeneration occurs in response to various insults including neurotoxicity. Absence of DLK also protects cultured DRG axons from vincristine-induced fragmentation, further supporting the hypothesis that DLK signalling might function in an axon self-destruction programme [7]. Although DLK can activate both JNK1–3 and p38α-δ MAPK pathways in response to injury, pharmacological inhibition of JNK within the first three hours after axotomy is sufficient to significantly inhibit axon fragmentation in cultured DRG neurons [7]. These results suggest that early JNK activity is necessary for the commitment to degenerate after injury, before breakdown occurs. A more recent study provides a better understanding of a JNK-controlled downstream mechanism that commits axons to degenerate after injury. Experimental evidence has shown that the JNK substrate SCG10, a microtubule-binding protein, is important in axon maintenance. Early after injury, SCG10 protein levels dramatically decrease in the distal axon compartment of both cultured mouse DRG neurons and in adult sciatic nerves. Of note, a decrease in SCG10 expression occurs within the first few hours after injury, before any sign of axon fragmentation *in vitro*. Moreover, SCG10 knockdown using short hairpin RNA lentiviral constructs accelerates degeneration of cultured DRG neurons after axonal injury [49]. Unlike NMNAT2, absence of SCG10 does not trigger axon degeneration, supporting the idea that loss of SCG10 might represent a functionally important step during the commitment to axon degeneration [49].

Compared with DLK inhibition, Wld^{s} expression and Ca^{2+} chelation are more effective in protecting injured axons, suggesting that activation of DLK-mediated signalling is acting in parallel with other responses. A striking loss-of-function phenotype has been characterized in fruit flies carrying mutations in *Highwire* (*rpm-1* in *C. elegans* and *phr1* in mice), a gene encoding an E3 ubiquitin ligase that is also an upstream regulator of Wallenda. Highwire mutations markedly inhibit axon degeneration in a *Drosophila* model of Wallerian degeneration [8]. Intriguingly, NMNAT has been identified as a target downstream of Highwire. At post-transcriptional levels, Highwire promotes NMNAT downregulation in the distal stump of injured axons independently from an effect on Wallenda [8]. Even though earlier studies have demonstrated that inhibition of the UPS results in delayed axon degeneration in *Drosophila* [44,50,51], findings suggest that Highwire downregulates NMNAT in a UPS-independent manner [8]. In the absence of Highwire, increased NMNAT protein levels are indeed required and are sufficient to inhibit axon degeneration in several classes of neurons and developmental stages in *Drosophila* [8]. The mechanism by which Highwire regulates NMNAT remains to be explored in future studies.

Taken together, these results suggest that DLK functions in an internal neuronal signalling pathway to promote axon degeneration after injury.

Regenerative response to injury

Injuries in the adult mammalian CNS lead to permanent functional impairments, severely hindering daily activities and decreasing the quality of life. Adult CNS neurons not only fail to regenerate, but also have a reduced sprouting ability, both of which contribute to the limited functional recovery after injury [52]. However, regeneration spontaneously occurs in most invertebrates, cold-blooded animals including amphibians, fish, reptiles and in the mammalian PNS. Why do some species successfully regenerate whilst others fail? What are the molecular pathways accounting for the differences in the growth ability of PNS and CNS neurons?

Animal models such as *C. elegans* offer several experimental advantages for studying axon growth and regeneration failure [53,54]. Given the short life cycle, large progeny number, detailed genetic map and low maintenance costs, more and more laboratories use *C. elegans* as a model organism for extensive genetic screening and to study signal transduction pathways. Several candidate genes identified in worm, fruit fly and zebrafish models, with a role in promoting or inhibiting regeneration, await testing in rodents. Although some signalling pathways might be conserved, injury-related changes to the neuronal environment and immune system differ among species. Nonetheless, translational studies using mammalian models have resulted in exciting findings, highlighting a crucial role for DLK-dependent mechanisms in regulating the regenerative response to injury [15,16]. In the following paragraphs, we provide an overview of the DLK-dependent regenerative response to injury and discuss its implications.

MAPK signalling in axon regeneration

Many studies have demonstrated a role for a conserved MAPK pathway in axon regeneration using different animal models [11–15]. Although *C. elegans* and *Drosophila* neurons spontaneously regenerate after laser axotomy, growth cones never form in *dlk-1* and *wallenda* mutants [9,11,12]. The transformation of severed axonal ends into growth cone-like structures is one of the crucial steps in mounting a successful regenerative response [55]. Growth cones are specialized structures at the leading edge of developing and regenerating axons. Continuous polymerization and depolymerization of actin filaments, together with changes in microtubule dynamics, allows growth cones to guide axons to reach their specific cellular targets during neural development and axonal regeneration. Whether signalling pathways required for neuronal development are also activated during axon regeneration is still a matter of debate [56,57].

Numerous building blocks are necessary during the assembly of a new growth cone after injury. Raw materials are recycled from axonal debris, transported along the axon or synthesized locally. The DLK-1 pathway regulates *cebp-1* mRNA stability through the MAPKAP kinase MAK-2 in *C. elegans* [12]. It has been shown that growth cones do not form in *C. elegans cebp-1* mutants, suggesting

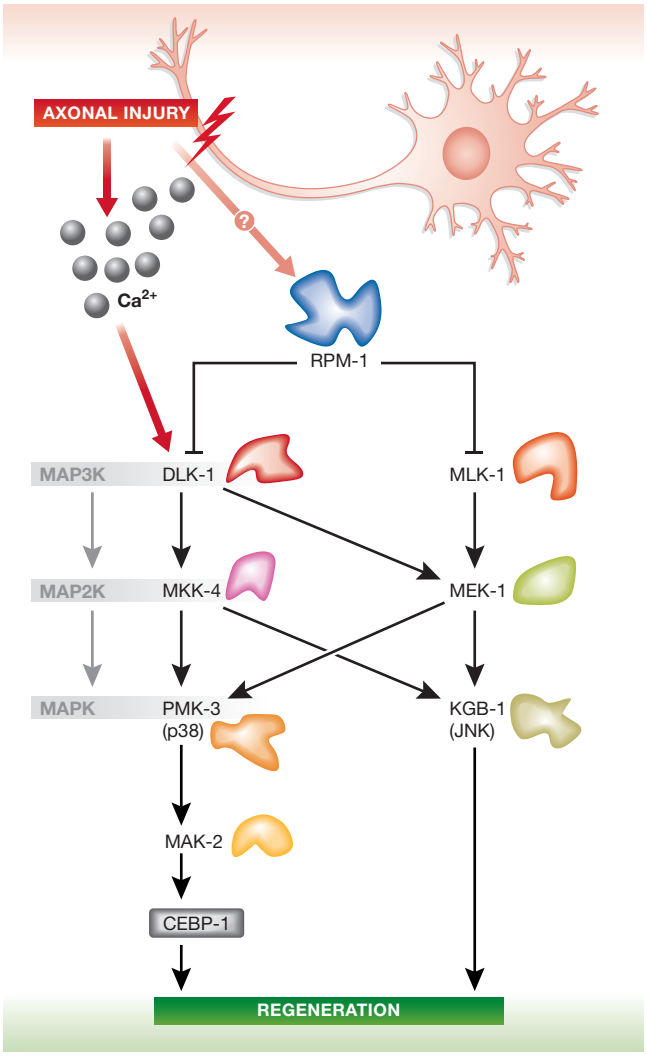

Fig 2 | Two MAPK pathways promoting axon regeneration in *Caenorhabditis elegans*. Injury signals including Ca^{2+} influx trigger activation of DLK-1 and the DLK-1–MKK-4–PMK-3 pathway. In parallel to the DLK-1 pathway, the MLK-1–MEK-1–KGB-1 pathway is also activated. Whilst DLK-1 can activate both MKK-4 and MEK-1, MLK-1 can only activate MEK-1. CEBP-1, CCAAT/enhancer-binding protein 1; DLK-1, dual leucine zipper kinase 1; JNK, c-Jun amino-terminal kinase; MAPK, mitogen activated protein kinase; MAP2K, mitogen-activated protein kinase kinase; MAP3K, mitogen-activated protein kinase kinase kinase; MKK-4, MAP kinase kinase 4; RPM-1, regulator of presynaptic morphology 1.

that regulation of *cebp-1* mRNA stability and translation is an important step to promote axon regrowth after injury (Fig 2; [12]). When axons successfully regenerate, a dynamic growth cone replaces transient filopodia that emerge from the axonal stump within hours after injury. Specialized structures such as filopodia and lamellipodia emerge from growth cones, serving as anchor points both to sustain growth and actively integrate guidance cues of the extracellular environment. Real-time imaging experiments suggest that DLK-1 signalling might be required for the filopodia to growth cone transition in *C. elegans* [11]. Furthermore, restricted expression of DLK-1 in GABA motor neurons rescues regenerative failure seen in *C. elegans dlk-1* null mutants, suggesting that DLK-1 functions in a cell-autonomous manner [11]. Through highly conserved ubiquitin-mediated protein degradation, the DLK-1 pathway is negatively regulated by RPM-1 [42,58,59]. Consistently, defects similar to those found in *dlk-1* mutants are also seen after RPM-1 overexpression [11]. Loss-of-function mutations in the DLK-1 downstream targets MAP2K *mkk-4* and p38 MAPK homologue *pmk-3* result in regeneration defects in *C. elegans*, further supporting the finding that activation of the entire signalling pathway is required for axon regeneration in *C. elegans* [11]. The MAP3K MLK-1 pathway has also been found to control axon regeneration of *C. elegans* GABA motor neurons. In fact, mutations in *mlk-1* or its downstream target *mek-1* cause regeneration defects [14]. Moreover, neurons fail to regenerate when carrying mutations in the MAPK *kgb-1/jnk*, a MEK-1 downstream target [14]. Crosstalk between DLK-1- and MLK-1-signalling pathways can explain differences in phenotype severity. In fact, results suggest that a coordinated activation of JNK and p38 MAPK pathways is required for axon regeneration in *C. elegans* (Fig 2; [14]).

Interestingly, delayed DLK-1 expression results in limited regeneration [11], indicating that DLK-1 must function shortly after injury, presumably in close relation to other phenomena. In fact, a successful regenerative response requires concomitant activation of multiple events. For example, axotomy triggers rapid entry of extracellular ions through opening of the plasma membrane [60]. In *Aplysia*, Ca^{2+} influx represents an important step to activate programmes leading either to the formation of a new growth cone or a retraction bulb [61]. Recent work has shed light on how DLK-1 activity is triggered in response to injury in *C. elegans* [62,63]. Changes in Ca^{2+} concentration regulate the switch between an inactive and active DLK-1 protein complex [63]. In mammals, however, DLK lacks the domain that allows activity regulation through Ca^{2+} binding. LZK, another mammalian orthologue, has such a domain, but its role in axon growth and regeneration is not known and deserves attention for future studies.

Taken together, these results demonstrate that DLK-1 initiates a regenerative response to axonal injury by simultaneous activation of JNK and p38 MAPK pathways in non-mammalian model systems.

Microtubule dynamics

Considerable attention has been focused on understanding how the actin and microtubule cytoskeleton network regulates axon growth during neural development as well as after axonal injuries in model organisms. The microtubule network of mature axons is composed of stable and labile microtubules [64], and is normally maintained in a stabilized steady state. In mammals, microtubules disassemble after axonal injury, hindering the regrowth of injured CNS axons [65]. When severed, most mammalian CNS axons form retraction bulbs and die-back, retracting from the site of injury. Dystrophic end bulbs are filled with a disorganized microtubule network [65]. Of note, moderate microtubule stabilization results in axon regeneration within the injured CNS [66,67].

Although progress has been made in temporally characterizing the axonal response to injury, the molecular pathways underlying the morphological changes remain elusive.

Taking advantage of versatile animal models and femtosecond laser axotomy, real-time imaging approaches have begun to explore the role of specific pathways in both growth cone formation and axon regeneration after injury. By activating microtubule growth, *C. elegans* D-type larval motor neurons rapidly transform proximal

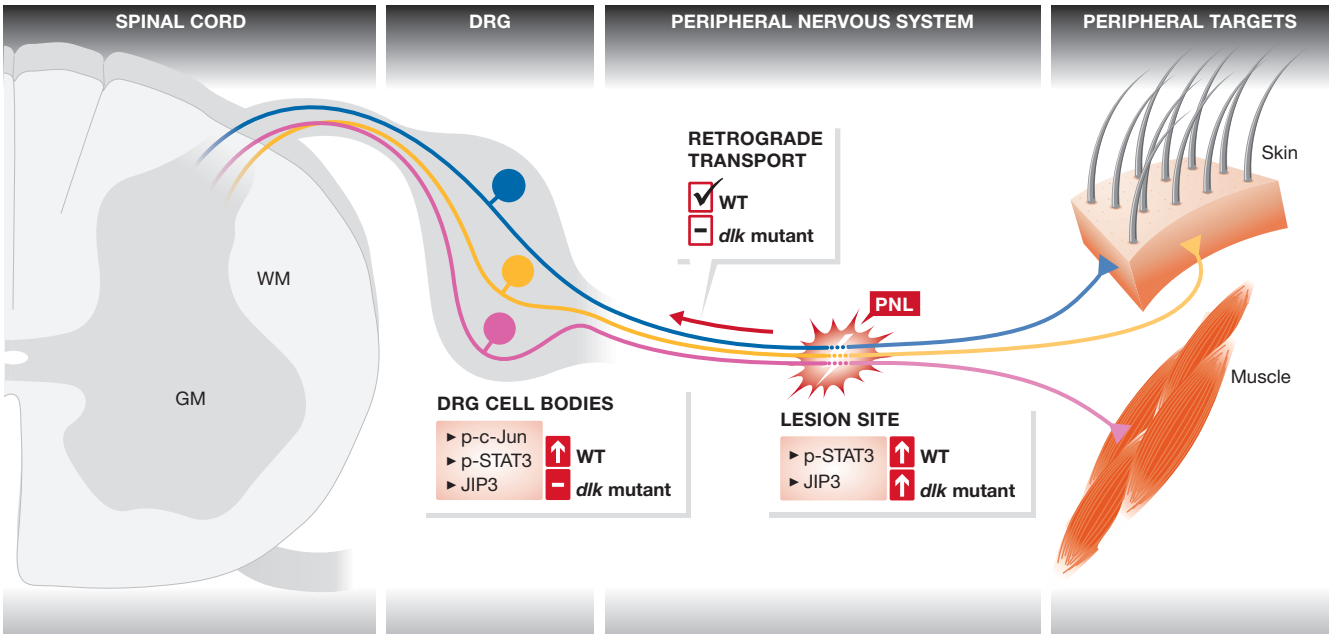

Fig 3 | Retrograde transport of injury-activated signals. After peripheral nerve lesion, locally activated regulators are retrogradely transported from the site of injury back to the mouse DRG cell body. Absence of DLK prevents the correct translocation of pro-regenerative signals including phospho-STAT3 and c-Jun needed to activate the intrinsic regeneration programme. p-c-Jun, phosphorylated c-Jun; DRG, dorsal root ganglion; DLK, dual leucine zipper kinase; GM, grey matter; JIP3, JNK-interacting protein 3; PNL, peripheral nerve lesion; STAT3, signal transducer and activator of transcription 3; WT, wild-type; WM, white matter.

axonal stumps into growth cone-like structures in response to laser axotomy [68]. Activation of microtubule growth follows distinct phases in *C. elegans* mechanosensory axons. First, an increase of dynamic microtubules at the site of injury is accompanied by local downregulation of KLP-7, a depolymerizing kinesin 13 family member that controls steady state conditions [69]. Thereafter, the cytosolic carboxypeptidase CCPP-6A is required for the formation of more stable microtubules during a period of sustained growth [69]. Interestingly, the DLK-1 signalling pathway controls both aspects of microtubule growth in *C. elegans* mechanosensory axons, as discussed below.

In addition to the DLK-1 pathway, an extensive functional screen has identified several clusters of genes required for axon regrowth in *C. elegans*, many of which regulate microtubule dynamics [70]. Interestingly, *C. elegans* GEF *efa-6* mutants display increased axon growth within a few hours of injury [70]. GEFs are known to activate several GTPases, including members of the Ras superfamily. Cdc42, Rac1 and RhoA are members of the Rho family of GTPases (a subfamily of Ras), and they control many aspects of cytoskeletal dynamics in mammals [33,71,72]. Morphological analysis of *efa-6* mutants in *C. elegans* has revealed a substantial increase in the number of dynamic microtubules as well as extended periods of persistent growth, all resulting in enhanced axon regrowth compared with wild-type worms [70]. In line with this, earlier work has suggested that EFA-6 limits microtubule growth by decreasing microtubule dynamics in *C. elegans* [73]. Of note, the *efa-6* mutation rescues, at least in part, axon regeneration defects seen in *C. elegans dlk-1* mutants, indicating that EFA-6 functions downstream of and/or in parallel to DLK-1 [70]. Other studies had further indicated that a DLK–MAP2K7–JNK1 signalling module regulates microtubule bundling during neurite elongation in cultured mouse hippocampal neurons [30]. The existence of locally activated DLK–JNK modules is intriguing and should stimulate future investigation about the possible relationship of these modules with microtubule cytoskeleton dynamics in more complex mammalian models.

In conclusion, these observations demonstrate that DLK-dependent signalling controls several aspects of microtubule growth in diverse model organisms.

Retrograde signals

Axonal injuries trigger signals that must travel from the site of injury back to the neuronal cell body [74–76]. In addition to controlling survival pathways, retrograde transport of injury-activated factors leads to profound changes in the expression of genes related to cytoskeletal dynamics and axon growth [76–78]. Therefore, for a neuron, sensing the degree of damage is one of the first priorities in the process of making a decision whether to regenerate or undergo apoptosis. As part of the activation mechanism at the axon terminal, several factors including JNK and Erk are phosphorylated and transported to the nucleus through retrograde motor complexes [75,79,80]. In *Drosophila* motor neurons, Wallenda-dependent signalling mediates the nuclear response to injury. It has been shown that a Wallenda–JNK–Fos-signalling module controls the sprouting ability of injured *Drosophila* motor neurons [9]. Most *Drosophila* motor neurons show signs of growth within 14 h of injury. However, the extent of growth significantly drops after knocking down Wallenda expression and when expressing JNK and Fos dominant-negative mutants [9].

Neuronal cell bodies are often located far away from the damaged area, thus injury-activated signals need to travel considerable distances to trigger a cell body response. When the axonal transport machinery is mutated, the motor neuron response is inhibited, suggesting that Wallenda requires the axonal transport machinery to shuttle injury-activated signals to the nucleus [9].

Sidebar A | In need of answers

(i) How is temporal and spatial activation of DLK controlled in response to cellular stresses and signals in mammals?
(ii) What are the context-dependent mechanisms that shift the balance between contradictory DLK-dependent responses?
(iii) Does pharmacological inhibition of DLK positively affect the neuron response in models of inflammatory, metabolic, stroke and vascular diseases?
(iv) Does the level of DLK activity change in systems that do and do not spontaneously regenerate after injury?

Several positive post-traumatic signals have been identified in mammals. Injury-induced cytokines including LIF, CNTF and IL-6 act through the gp130 receptor complex, upstream from the pro-regenerative JAK–STAT signalling pathway. Recent work has shed light on how mammalian sensory and motor axon regeneration is impaired in the absence of DLK (Fig 3). Data suggest that DLK might be involved in the correct translocation of pro-regenerative signals including phospho-STAT3 and phospho-c-Jun from the injury site to the neuronal cell body [13,15]. At the site of injury, phospho-STAT3 levels are increased normally in *dlk* null mice. However, retrograde transport of phospho-STAT3 and the adaptor protein JIP3 is abolished in the absence of DLK [15]. In rodents, impairment in retrograde transport does not seem to affect the local assembly of a new growth cone. Hence, absence of DLK might have an impact on growth cone performance rather than on growth cone assembly [15].

Absence of phospho-STAT3 nuclear accumulation is constantly seen in model systems in which regeneration fails [81]. Conversely, increasing experimental evidence suggests that STAT3 activation is required for CNS regrowth [82,83]. It is most probable that activation of gp130–JAK–STAT3 signalling relays injury-activated signals to the nucleus, in which they potentially turn on an intrinsic regenerative response through regulation of gene transcription [84].

After optic nerve injury, DLK-dependent signalling triggers a rapid transcriptional response in mouse RGCs [16]. Interestingly, DLK-induced changes in the expression of proapoptotic and pro-regenerative genes provide further evidence for the contradictory actions of DLK [16]. Although DLK simultaneously activates pro-apoptotic and regenerative programmes in response to optic nerve injury, the dominant response in regenerative incompetent neurons such as RGC is cell death [10].

Collectively, these results suggest that the ability to activate nuclear responses, through retrograde transport of injury-related signals, might be a major mechanism by which DLK promotes axon regeneration in regeneration-competent neurons.

Strategies to fine-tune DLK signalling

Data from several research groups demonstrate that DLK activation early after injury participates in the nuclear response to injury [9,11,15,16]. How is DLK regulated in response to injury? In *C. elegans* and *Drosophila*, RPM-1 and Highwire negatively regulate DLK-1 and Wallenda, respectively. In *C. elegans*, it is unknown if RPM-1 is regulated in response to injury. However, Highwire expression decreases along the injured axons of *Drosophila* motor neurons, revealing a potential mechanism for Wallenda activation [9].

In *C. elegans*, regeneration is modestly enhanced in *rpm-1* mutants as well as after DLK-1 overexpression, suggesting that an increase in DLK-1 expression improves the regeneration ability of GABA motor neurons [11]. Given that many factors influence the regenerative response in *C. elegans* neuron, it is not known whether these findings can be generalized to other classes of neurons and injury paradigm [54,85]. Nonetheless, an absence of Highwire accelerates regeneration within a few hours of injury in *Drosophila* motor neurons [9]. Together, these results suggest that increasing DLK protein levels directly, or indirectly by inhibiting RPM-1/Highwire, boosts nerve regeneration in both *C. elegans* and *Drosophila*.

In contrast to the above-mentioned model organisms, mammalian CNS neurons have a more limited ability to regenerate and a higher probability of undergoing apoptosis when regeneration fails. What are the consequences of an increase in DLK expression in mammalian CNS neurons? DLK overexpression results in increased RGC death in response to injury [10]. Given that injured CNS neurons must survive to regenerate their axons, it is probable that increasing survival might be sufficient to induce them to regenerate [86]. Although absence or inhibition of DLK protects mouse and rat RGC from injury-induced cell death, no signs of regeneration are noticed [10,16]. Furthermore, *pten* conditional deletion—a strategy to promote RGC regeneration [87]—fails to promote axon regeneration in the absence of DLK [16], thus DLK seems to be a crucial player in the initiation of the regenerative response. Taken together, these results indicate that an increase in DLK expression is not sufficient to induce regeneration in mammalian RGC neurons, however DLK is required for RGC regeneration under specific conditions.

Conclusion

Here we have presented and discussed evidence supporting a role for DLK-dependent signalling in regulating apparently contradictory responses during both neural development and after various insults to the adult nervous system. The ability of DLK to induce apoptosis and axon degeneration is in contrast with its role in promoting axon growth and regeneration. Although progress has been made, our understanding of the mechanisms underlying spatial and temporal activation of DLK in response to a multitude of stresses is still fragmentary (Sidebar A). It is not clear whether the upstream and downstream components of the DLK signalling pathway are conserved in different model organisms. Thus far, our knowledge of the role of DLK in controlling axon regeneration is mostly limited to studies performed in model organisms and systems in which injured axons spontaneously regenerate. It is probable that a context-dependent variability might have an impact on the translation of previous findings to more complex model systems and different injury paradigms. Mammalian CNS neurons, for example, have a limited ability to regenerate. Abortive responses and cell death often lead to permanent neurological deficits due to the failure to re-establish functional connections after CNS trauma, stroke and other types of neurodegenerative disease. Failure of neuroregeneration and repair is partly due to the presence of a hostile environment in the CNS [88,89]. Therefore, future work should aim at understanding how modulation of DLK activity alone, and in combination with other approaches, might aid neuronal survival, CNS repair and regeneration. We believe that progress in this direction will support the development of more applicable strategies to promote repair of the adult mammalian CNS following various insults.

ACKNOWLEDGEMENTS
We would like to thank Farida Hellal, David Elliott and Charlotte Coles for critical reading of the manuscript. We apologize to all colleagues whose contributions to this field could not be cited due to space restrictions. The Deutsche Forschungsgemeinschaft, IRP and Wings for Life support research in the Bradke Laboratory.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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Andrea Tedeschi & Frank Bradke