Palmitoylation controls DLK localization, interactions and activity to ensure effective axonal injury signaling

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Dual leucine-zipper kinase (DLK) is critical for axon-to-soma retrograde signaling following nerve injury. However, it is unknown how DLK, a predicted soluble kinase, conveys long-distance signals and why homologous kinases cannot compensate for loss of DLK. Here, we report that DLK, but not homologous kinases, is palmitoylated at a conserved site adjacent to its kinase domain. Using short-hairpin RNA knockdown/rescue, we find that palmitoylation is critical for DLK-dependent retrograde signaling in sensory axons. This functional importance is because of three novel cellular and molecular roles of palmitoylation, which targets DLK to trafficking vesicles, is required to assemble DLK signaling complexes and, unexpectedly, is essential for DLK's kinase activity. By simultaneously controlling DLK localization, interactions, and activity, palmitoylation ensures that only vesicle-bound DLK is active in neurons. These findings explain how DLK specifically mediates nerve injury responses and reveal a novel cellular mechanism that ensures the specificity of neuronal kinase signaling.

MAPK | JNK | regeneration | trafficking | c-Jun

Peripheral nervous system (PNS) axons project long distances to their target tissues, presenting a great challenge for the relay of retrograde signals from distal locations back to neuronal cell bodies. One important requirement for axonal retrograde signaling is to activate transcription following distal nerve injury, a response that is critical for PNS axon regeneration (1, 2). One key mediator of nerve injury responses is dual leucine-zipper kinase (DLK), an evolutionarily conserved upstream activator (a MAP3K) of the mitogen-activated protein kinases (MAPKs) c-Jun N-terminal kinase (JNK) and p38 MAPK (3–6). DLK's importance in retrograde signaling is inferred from in vivo studies, where distal axonal injury triggers DLK-dependent responses in sensory and motor neuron cell bodies (7, 8). Direct evidence for DLK's role in retrograde signaling comes from studies using compartmented chambers, which physically isolate neuronal cell bodies from distal axons. Selective activation of DLK signaling in distal axons results in phosphorylation of the JNK transcription factor substrate c-Jun in cell bodies (9). However, it is unclear how DLK, a predicted soluble, diffusible protein, transfers signals directionally over long distances.

It is also puzzling why genetic loss or pharmacological inhibition of DLK (MAP3K12) causes such striking neuronal phenotypes (5, 7, 9, 10) that are not compensated for by the large family of homologous MAP3Ks [23 enzymes in mammals (11)]. Many of these DLK homologs are also expressed in peripheral neurons (12) and can activate JNK and p38 signaling in vitro and in transfected cells (13). These findings suggest that differential subcellular localization and regulation underlies DLK's unique role in nerve injury signaling, but the nature of any such DLK-specific regulation is unclear.

Interestingly, although bioinformatically predicted to be soluble, DLK localizes to discrete axonal puncta across animal species, including worms, flies, and mice (8, 14, 15). We reasoned that an evolutionarily conserved mechanism might account for DLK's unexpected subcellular localization and thus explain what makes DLK unique among MAP3Ks.

DLK axonal puncta resemble lipid vesicles, raising the possibility that DLK associates with vesicle membranes. One way to target soluble proteins to membranes is by protein–lipid modification. Four such modifications are known for intracellular proteins: palmitoylation, myristoylation, farnesylation, and geranylgeranylation, of which palmitoylation is most frequently observed for neuronal proteins (16, 17). Palmitoylation is best known to target proteins to the plasma membrane, but can also target proteins to vesicles (18–20). However, roles for palmitoylation in axonal retrograde trafficking and signaling have not been described.

Here we report that palmitoylation at a conserved cysteine residue is critical for DLK-dependent retrograde injury signaling. Mechanistically, palmitoylation targets DLK to motile trafficking vesicles and thus provides a way for DLK to traffic retrogradely. In addition, palmitoylation is essential for DLK to bind JNK-interacting protein-3 [JIP3, which couples JNK pathway proteins to microtubule retrograde motors (9, 21)], and to bind the MAP2Ks MKK4 and MKK7, DLK's direct downstream targets in the JNK pathway (11, 22, 23). Strikingly, direct palmitoylation is also essential for DLK kinase activity; preventing DLK palmitoylation eliminates DLK-mediated kinase signaling in heterologous cells and even in vitro. These findings suggest that active, palmitoylated DLK is restricted to trafficking vesicles in neurons, providing an explanation for how DLK-dependent signals are conveyed without affecting basal, physiological JNK activity (9). In addition to providing

Significance

Dual leucine-zipper kinase (DLK) is essential for responses to nerve injury and subsequent neural regeneration by controlling transfer of signals from damaged distal axons to neuronal cell bodies. However, DLK is predicted to be freely diffusible, raising the question of how it conveys long-distance, directional signals. Here we report that direct modification of DLK with the lipid palmitate, a process called palmitoylation, is critical for retrograde injury signaling. At the molecular level, palmitoylation targets DLK to retrograde trafficking vesicles, assembles DLK-dependent signaling complexes, and is also essential for DLK kinase activity. This "multifunctional" palmitoylation explains how DLK mediates injury responses and may be a previously unappreciated mechanism that ensures the specificity of enzymatic signaling in diverse cell types.

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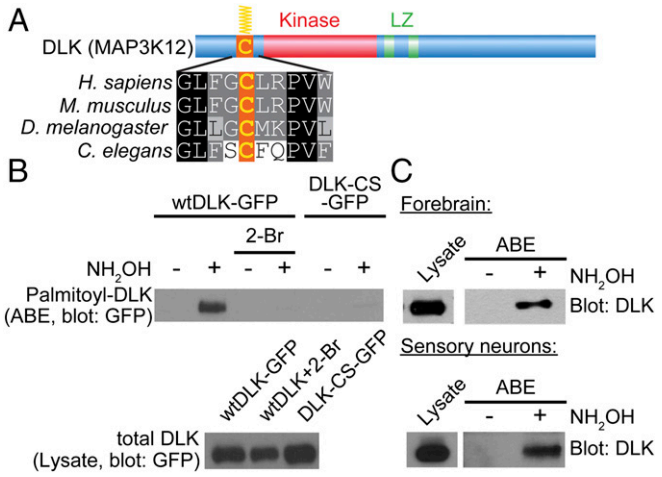

Fig. 1. DLK is palmitoylated at a conserved site. (*A*) A predicted palmitoyl-motif (yellow cysteine) is conserved in DLK orthologs from the indicated species. (*B*) DLK is specifically palmitoylated at C127. HEK293T cells were transfected with GFP-tagged wtDLK (wtDLK-GFP) or DLK-C127S point mutant (DLK-CS-GFP) cDNAs. Palmitoyl-proteins were isolated by ABE. Palmitoyl-DLK is robustly detected in ABE fractions (*Upper*), but no palmitoyl-DLK signal is seen from cells treated with 2-Br, or if Cys-127 is mutated to Ser. DLK protein levels were similar in all conditions (*Lower*). (*C*) ABE assays confirm endogenous DLK palmitoylation in rat forebrain (*Upper*) and cultured sensory neurons (*Lower*).

new insights into axonal DLK signaling, these results reveal palmitoylation as a multifunctional modification that can control the localization, interactions, and activity of signaling kinases. This finding has broad implications for our understanding of how neurons convey specific signals over long times and distances.

Results

DLK Is Palmitoylated at an Evolutionarily Conserved Site. To determine the basis of DLK's reported vesicle-like axonal distribution, we examined DLK's sequence for lipid binding motifs. DLK contains no predicted lipid binding domains and lacks consensus sequences for myristoylation, farnesylation, or geranylgeranylation. However, we bioinformatically identified a predicted palmitoylation site [C127 in mammalian DLK (24)] that is highly conserved across animal species (Fig. 1*A*).

To determine whether DLK is palmitoylated at C127, we expressed GFP-tagged wild-type DLK (wtDLK-GFP) in HEK293T cells and used acyl biotin exchange (ABE), a nonradioactive method, to purify palmitoyl-proteins from lysates (16). WtDLK-GFP was robustly detected in ABE fractions but was absent from controls in which the essential ABE reagent hydroxylamine (NH_2OH) was omitted (Fig. 1*B*). In contrast, no ABE signal was seen if cells were pretreated with the palmitoylation inhibitor 2-Bromopalmitate (2-Br) (25) or if Cys-127 was mutated to nonpalmitoylatable Ser (DLK-CS-GFP) (Fig. 1*B*). In addition, cotransfection of a subset of DHHC family palmitoyl acyltransferases (PATs) markedly increased levels of wtDLK-GFP, but not DLK-CS-GFP, in ABE fractions (Fig. S1 *A*–*C*). DLK palmitoylation at C127 was also detected using a complementary method, metabolic labeling with the palmitate analog 17-octadecynoic acid (17-ODYA), and subsequent coupling to a fluorescent azide reporter tag (Fig. S1*D*) (26, 27).

Palmitoyl-DLK was also robustly detected in ABE fractions from rat forebrain and from cultured sensory neurons, in which DLK-dependent retrograde signaling is well described (9) (Fig. 1*C*). Taken together, these data suggest that DLK is palmitoylated in heterologous cells and in neurons. In contrast, homologous MAP3Ks that are expressed in sensory neurons (12) were not palmitoylated (Fig. S2). In these latter ABE assays, palmitoyl:total signals for DLK were very similar to palmitoyl:total signals of the known palmitoyl-protein PSD-95, suggesting that DLK is a bona fide palmitoyl-protein.

Palmitoylation Targets DLK to Axonal Trafficking Vesicles. We next addressed whether palmitoylation accounts for DLK's previously reported discrete localization in axons. Lentivirally expressed wtDLK-GFP localized to discrete axonal puncta in mammalian cultured sensory neurons (Fig. 2 *A* and *B*), consistent with reports from other neuron types (8, 14, 15). In contrast, only diffuse GFP fluorescent signals were seen in wtDLK-GFP–expressing neurons treated with 2-Br, or in neurons expressing DLK-CS-GFP (Fig. 2 *A* and *B*). These results suggest that palmitoylation controls DLK distribution within axons.

DLK's palmitoyl-motif is conserved in the *Caenorhabditis elegans* ortholog DLK-1 (CeDLK-1) (Fig. 1*A*). Consistent with findings from mammalian neurons, GFP-wt-CeDLK-1 was punctate in *C. elegans* axons but the homologous Cys→Ser point mutant (GFP-CeDLK-1-C104S) was diffuse (Fig. 2*C*). Furthermore, overexpression of wt-CeDLK-1 in *C. elegans* mechanosensory neurons induced extra axonal branching. In contrast, overexpression of CeDLK-1-C104S did not show detectable effects, supporting that palmitoylation at C104 is

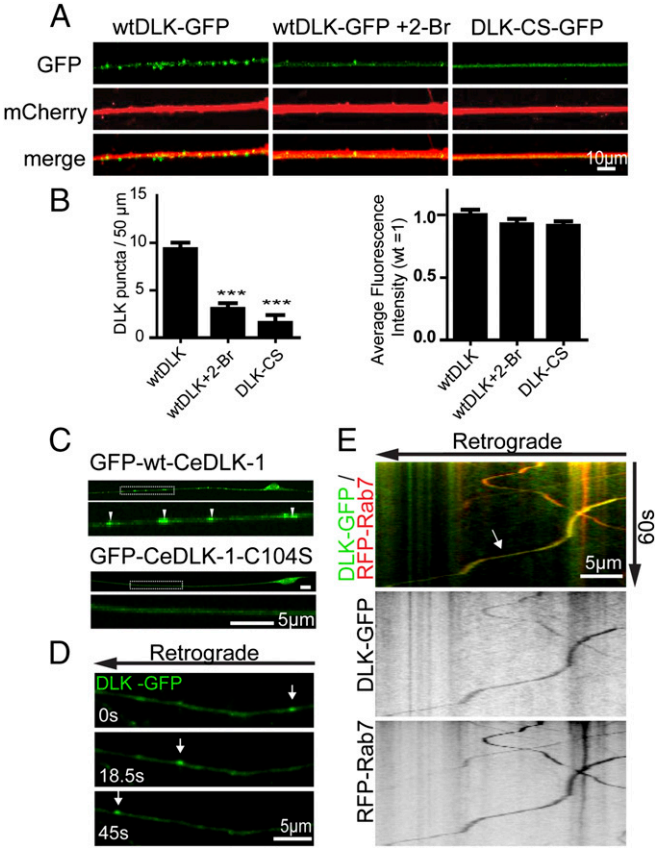

Fig. 2. Palmitoylation targets DLK to motile axonal trafficking vesicles. (*A*) Palmitoylation-dependent localization of DLK to axonal puncta. Sensory neurons cultured in microfluidic chambers were infected with lentiviruses expressing either wtDLK-GFP or DLK-CS-GFP, plus the morphology marker mCherry. Cultures were treated with or without 2-Br, fixed, and immunostained with the indicated antibodies. (*B*) Quantified data confirm that DLK punctate distribution is diffused by 2-Br treatment, or by C127S mutation. Data are mean + SEM for n = 6–11 determinations per condition, $^{***}P < 0.001$ vs. wtDLK-GFP condition, ANOVA with Tukey post hoc test. (*C*) A GFP fusion of the *C. elegans* DLK ortholog DLK-1 (GFP-wt-CeDLK-1) localizes to axonal puncta (arrowheads), but the homologous point mutant (GFP-CeDLK-1-C104S) is diffuse. (*D*) Axonal DLK-GFP puncta are motile. Cultured sensory neurons were infected with lentivirus expressing wtDLK-GFP. GFP-fluorescent images were acquired at the indicated times. (*E*) Cultured sensory neurons were coinfected with lentiviruses expressing wtDLK-GFP and the retrograde trafficking vesicle marker RFP-Rab7. Representative kymographs obtained from live imaging of individual axons reveal DLK puncta that comigrate with Rab7 (arrow).

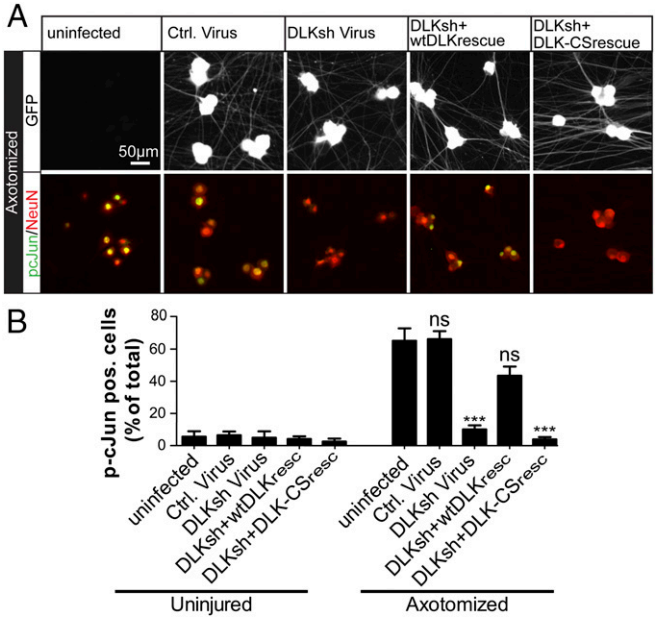

Fig. 3. Retrograde signaling following axotomy requires palmitoyl-DLK. (*A*) Sensory neurons cultured in microfluidic chambers were infected with the indicated lentiviruses, axotomized, and immunostained with the indicated antibodies. Images of axotomized and uninjured sister cultures for all infection conditions are shown in Fig. S5. (*B*) Quantified data reveal that axotomy-induced c-Jun phosphorylation is strongly DLK dependent and is rescued by shRNA-resistant wtDLK. DLK-CS fails to rescue. ***$P < 0.001$ versus uninfected, axotomized condition; ns, not significantly different from uninfected axotomized condition, ANOVA with Tukey post hoc test, $n = 3$–5 determinations per condition.

important for in vivo activity of DLK-1 (Fig. S3). These results suggest that palmitoylation is an evolutionarily conserved mechanism that controls DLK's axonal localization and function.

To define the DLK axonal puncta, we performed time-lapse imaging in sensory axons expressing wtDLK-GFP. A subset of DLK puncta was motile, moving both anterogradely and retrogradely in axons (Fig. 2 *D* and *E*). Dual-color imaging revealed that a subset of motile wtDLK-GFP puncta comigrate with mCherry-tagged Rab7 (mCh-Rab7, a marker of retrograde trafficking vesicles) (28) (Fig. 2*E*). Moreover, a subset of DLK-GFP puncta comigrated with NPY-mCh (a marker of Golgi-derived vesicles) (29) and also with Lysotracker Red, a marker of acidic vesicles (Fig. S4). Taken together, these findings suggest that axonal puncta of palmitoyl-DLK are motile trafficking vesicles.

Direct Palmitoylation Is Required for DLK-Dependent Axonal Retrograde Signaling. This striking effect of palmitoylation on DLK's localization in axons led us to examine whether this modification is required for DLK-dependent axonal retrograde signaling. To address this issue, we first used lentiviral short-hairpin RNA (shRNA) knockdown/rescue to replace endogenous DLK with shRNA-resistant wtDLK or DLK-CS (Fig. S5 *A* and *B*). We then used these lentiviruses to infect cultured sensory neurons in microfluidic chambers, which provide isolated environments for cell bodies and distal axons (Fig. S5*C*). We selectively axotomized distal axons of microfluidic cultures and examined subsequent retrograde signaling to cell bodies (30). Distal axotomy dramatically increased phosphorylation of the DLK-JNK pathway transcription factor target c-Jun in cell bodies of neurons in microfluidic culture (Fig. 3 and Figs. S5*D* and S6). Axotomy-induced c-Jun phosphorylation was almost completely abolished in DLK "knockdown" neurons, and was rescued by shr-DLKwt, but not by shr-DLK-CS (Fig. 3). These findings are consistent with reports that DLK is essential for retrograde signaling (7, 9) but, unexpectedly, reveal that only palmitoylated DLK can perform this role.

Palmitoylation Is Critical to Assemble DLK/JNK Pathway Signaling Complexes. We next sought to define how palmitoylation regulates DLK at the molecular level. In particular, we were struck by the similarity of DLK's axonal vesicular localization to that previously described for the scaffold protein JIP3, which binds DLK and is essential for DLK-dependent retrograde signaling (9, 21). Indeed, wtDLK-GFP clearly colocalized and cotrafficked with mCherry-tagged JIP3 (mCh-JIP3) in axons (Fig. 4*A*). However, given DLK's ability to bind and cotraffic with JIP3, it was striking that the DLK-CS-GFP mutant was diffusely localized in axons (Fig. 2*A*). This finding suggested that DLK might need to be palmitoylated to bind JIP3. Consistent with this notion, wtDLK strongly bound JIP3 in cotransfected cells, as previously reported (9), but C127S mutation completely disrupted DLK-JIP3 binding (Fig. 4*B*). Interestingly, C127S mutation did not affect DLK-DLK homodimerization (31) (Fig. S7*A*). Taken together, these findings suggest that palmitoylation not only targets DLK to vesicles, but also facilitates specific protein–protein interactions. We note that JIP3 still localized to motile, vesicle-like axonal structures in DLK knockdown neurons (Fig. S7*B*), perhaps because of JIP3's known interactions with other axonal cargoes (32).

We next addressed whether palmitoylation is also necessary for additional interactions of DLK, focusing on the MAP2K's MKK4 and MKK7, which are likely DLK's major cellular substrates (11, 22, 23, 33). In cotransfected cells, wtDLK bound MKK4, and this interaction was slightly enhanced in the presence of JIP3. In contrast, DLK-C127S bound MKK4 far more weakly than did wtDLK (Fig. 4*B*). In parallel assays, DLK-C127S also bound MKK7 more weakly than did wtDLK (Fig. S7*C*). Focusing on MKK4, we observed clear cotrafficking of mCh-MKK4 with wild-type (i.e., palmitoylated) DLK-GFP on axonal vesicles and also observed cotrafficking of GFP-MKK4 with mCh-JIP3 (Fig. S8 *A* and *B*). Taken together, these findings suggest that palmitoylation at C127 is essential for DLK to form multiprotein complexes in axons that contain both JIP3 and downstream MAP2Ks. Interestingly, when we examined the turnover of DLK palmitoylation by treating neurons with 2-Br, we observed that >50% of palmitoyl-DLK turns over within 1 h of 2-Br treatment (Fig. S8 *C* and *D*). However, the remaining pool of palmitoyl-DLK was still stable after 4 h of 2-Br treatment, sufficient time to mediate trafficking of DLK-JIP3-MKK complexes over considerable distances. We also found that both palmitoyl and total DLK levels were slightly reduced following in vitro axotomy (Fig. S8*E*), but again note that the pool of palmitoyl-DLK that remains postaxotomy could be critical for retrograde injury signaling.

Palmitoylation Is Critical for DLK to Activate the JNK Pathway in Transfected Cells and in Vitro. Importantly, MKK4 and MKK7 not only bind DLK, but are also direct substrates that are critical for DLK-dependent signaling (11, 22, 23). Our observation that DLK

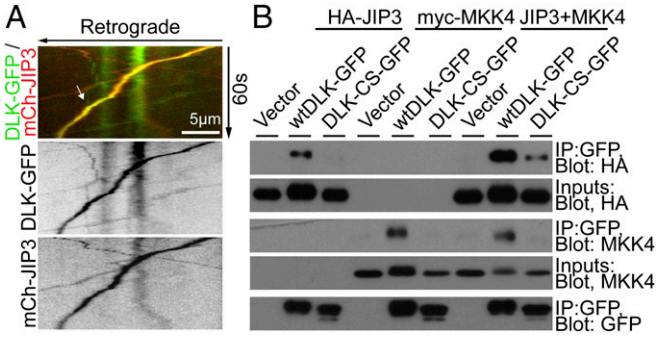

Fig. 4. Palmitoylation is essential for DLK to bind MKK4 and the scaffold protein JIP3. (*A*) Cultured sensory neurons were coinfected with lentiviruses expressing wtDLK-GFP and mCherry-tagged JIP3 (mCh-JIP3). Representative kymographs reveal DLK-positive vesicles that comigrate with JIP3 (arrowed example). (*B*) HEK293T cells were transfected with the indicated constructs. Blots of GFP immunoprecipitates (IPs) reveal that C127S mutation disrupts binding of DLK to both JIP3 and MKK4.

must be palmitoylated to recognize these MKKs (Fig. 4*B* and Fig. S7*C*) and the proximity of DLK's palmitoylation site to its kinase domain suggested that DLK-MKK4/7-JNK signaling per se might be palmitoylation-dependent. To address this possibility, we first assessed whether the palmitoylation inhibitor 2-Br affects signaling via DLK. Consistent with a previous report (3), wtDLK markedly increased phosphorylation of cotransfected JNK3 in HEK293T cells (Fig. 5 *A*–*C*). Strikingly, 2-Br treatment abolished JNK3 activation by DLK, without affecting JNK activation by the nonpalmitoylated MAP3Ks MLK1-3 (Fig. 5 *A*–*C*). The effect of 2-Br was phenocopied by C127S mutation, which prevented DLK-dependent phosphorylation of both JNK3 and of DLK's direct substrates MKK4 and MKK7 (Fig. 5 *D* and *E* and Fig. S9).

These findings suggest that palmitoylation is required for DLK-dependent signaling, irrespective of its role in retrograde trafficking. However, even in nonneuronal cells these results could be because of differential localization of wt-DLK and DLK-CS or, alternatively, could be because DLK must be membrane-localized in cells for activity. We first attempted to distinguish between these possibilities by making a DLK palmitoyl-site mutant with an N-terminal sequence that directs addition of the lipid myristate (Myr-DLK-CS-GFP). We hypothesized that Myr-DLK-CS-GFP would still localize to membranes but that the heterologous lipid attachment would not phenocopy effects of C127 palmitoylation. However, we found that Myr-DLK-CS-GFP was palmitoylated at additional, nonphysiological sites, an unexpected gain-of-function effect that led us not to pursue experiments with this mutant (Fig. S9*B*).

We therefore adopted an alternative approach to rule out effects of cellular localization by assessing the palmitoylation-dependence of DLK activity in an in vitro kinase assay. Strikingly, we found that wtDLK was markedly more active toward GST-tagged MKK4 in vitro, compared with wtDLK from 2-Br–treated cells (Fig. 5*F*), or to DLK-CS (Fig. 5*G*). These results suggest that palmitoylation plays a third role in DLK-dependent signaling, by directly and autonomously regulating DLK kinase activity.

Discussion

Three Complementary Roles of Palmitoylation Govern DLK-Dependent Signaling. DLK is critical for axonal injury responses (7, 8), but how DLK mediates axonal retrograde signaling has been unclear. Here we report that direct palmitoylation exerts three complementary effects on DLK at the cellular and molecular level, which together ensure the fidelity and specificity of DLK-dependent signals.

The first role of palmitoylation is to target DLK to axonal trafficking vesicles (Fig. 2). By allowing DLK to "hitchhike" on vesicles, palmitoylation may provide a way for DLK to transfer signals directionally and more rapidly than would occur by passive diffusion (34). Palmitoylation-dependent vesicular targeting of DLK appears highly conserved across animal species, as palmitoyl-site mutation

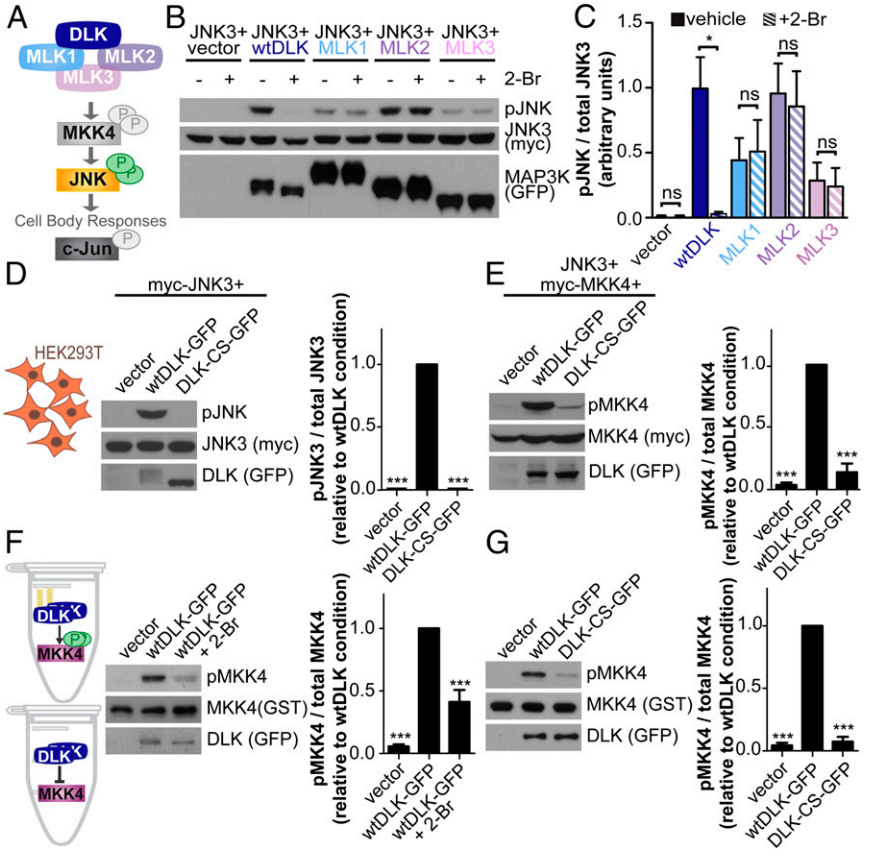

Fig. 5. DLK must be palmitoylated to activate the JNK pathway. (*A*) Scheme of MKK4-JNK activation by DLK and related MAP3Ks. (*B*) HEK293T cells expressing myc-JNK3 plus wtDLK-GFP or related GFP-tagged MAP3Ks were treated with 2-Br or vehicle. Lysates were blotted to detect phospho-JNK (pJNK, *Top*), JNK3 (*Middle*), and MAP3K expression (*Bottom*). (*C*) Quantitation of multiple experiments from *B* confirms that 2-Br specifically prevents JNK3 phosphorylation induced by DLK. $*P <$ 0.05; ns: not significantly different, *t* test, $n = 3$ determinations per condition. (*D*) C127S mutation prevents DLK-dependent JNK3 phosphorylation. Lysates of HEK293T cells expressing myc-JNK3 plus either wtDLK-GFP or DLK-CS-GFP were blotted as in *B*. Histogram shows pJNK:total JNK3 levels (mean ± SEM, relative to wtDLK-GFP). $***P < 0.001$ vs. wtDLK-GFP, ANOVA, Tukey post hoc test, $n = 3$. (*E*) C127S mutation prevents DLK-dependent MKK4 phosphorylation. Lysates of HEK293T cells expressing myc-MKK4 plus either wtDLK-GFP or DLK-CS-GFP were blotted with the indicated antibodies. Histogram shows pMKK4:total MKK4 (mean ± SEM, relative to wtDLK-GFP). $***P < 0.001$ vs. wtDLK-GFP, ANOVA, Tukey post hoc test, $n = 3$. (*F*) Palmitoyl-DLK is more active in vitro. HEK293T cells expressing wtDLK-GFP or vector were treated with 2-Br or vehicle as indicated. GFP immunoprecipitates were assayed for ability to phosphorylate GST-MKK4 in vitro. Samples were blotted and histogram plotted as in *E*. (*G*) As in *F*, except cells expressed wtDLK-GFP or DLK-CS-GFP.

transforms DLK distribution from punctate to diffuse in both mammalian and invertebrate axons (Fig. 2).

Second, palmitoylation is necessary for DLK to form multiprotein complexes with the scaffold protein JIP3 and the downstream kinases MKK4 and MKK7 (Fig. 4 and Figs. S7, S8, and S9*C*). Interestingly, JIP3, which is essential for DLK-dependent retrograde signaling (9), in turn binds microtubule motor proteins (21, 35). Palmitoylation of DLK may thus serve as a master regulatory event to assemble vesicular DLK/JIP3/MKK complexes and to ensure their motility in axons. It is an intriguing possibility that these palmitoyl-DLK complexes also contain the downstream kinase JNK3, which colocalizes with JIP3 on axonal vesicles (21).

Finally, a major finding of this study is that palmitoylation controls the kinase activity of DLK. This novel role for palmitoylation would appear to be independent of any effects on DLK localization in axons, because it is readily demonstrable in heterologous cells and even in in vitro assays (Fig. 5). However, we cannot exclude the possibility that the ability of palmitoylation to increase the local concentration of DLK on membranes also contributes to effects of this modification on DLK intracellular signaling and interactions.

How might palmitoylation exert these effects at the molecular level? DLK interactions with JIP3, MKK4, and MKK7 are all palmitoylation-dependent, but DLK–DLK dimerization is not (Fig. S7*A*). Hence, one possibility is that palmitoylation locally alters DLK conformation without grossly affecting DLK structure. This possibility is enhanced by the observation that DLK's noncatalytic N terminus (residues 1–164), which contains the palmitoyl-site, is Gly- and Pro-rich, and thus predicted to lack extended secondary structure and be flexible (36–38). Moreover, this N-terminal region is highly acidic (pI = 4.58), whereas DLK's kinase domain (residues 165–400) is basic (pI = 8.70). It would thus appear possible that DLK's N-terminal region can loop back and inhibit its kinase domain and that such autoinhibition (a common feature of related MAP3Ks, albeit via different protein–protein interaction motifs) (13) might be sterically impaired by C127 palmitoylation. If this were the case then palmitoylation might act like a phosphorylation event, but in this case the functional moiety would be a hydrophobic lipid rather than a charged phosphate group. However, although intriguing, we appreciate that this is only one possible explanation and that further studies are required to define how palmitoylation controls the interactions and activity of DLK.

We also note that, despite its clear palmitoylation (Fig. 1 and Fig. S1), DLK was not identified in prior palmitoyl-proteomic studies. However, several bona fide palmitoyl-proteins identified by ourselves and others were also not found in such studies (18, 39, 40). One reason for the lack of identification of DLK is likely tissue source; most palmitoyl-proteomic studies were performed from nonneuronal tissues, whereas DLK is largely restricted to the nervous system (41, 42). A second issue is likely detection sensitivity; DLK protein levels in neurons are likely low, in part because DLK protein expression is tightly controlled by ubiquitin-dependent degradation (10, 43, 44). These observations suggest that additional palmitoyl-proteomic studies of neuronal tissues, focusing on low abundance palmitoyl-proteins, may identify other palmitoyl-kinases and other signaling enzymes.

Palmitoylation Regulates DLK but Not Homologous MAP3Ks. If palmitoylation indeed accounts for DLK's unique role in axonal retrograde signaling, then one would strongly predict that related MAP3Ks expressed in sensory neurons (i.e., MLK1-3/MAP3K9-11) are not palmitoylated. Our experiments confirm that this is indeed the case and also reveal that a palmitoylation inhibitor specifically blocks DLK-dependent signaling without affecting signaling by MLK1-3 (Fig. 5 and Fig. S2). Interestingly, the closely related MAP3K13 does contain a homologous palmitoylation site, but MAP3K13 is not detectably expressed in sensory neurons (12), likely explaining its failure to compensate for loss of DLK in this cell type. However, it will be of interest to determine whether MAP3K13 is also palmitoylated and, if so, whether MAP3K13 localization and activity is palmitoylation-dependent in other types of neuron.

Palmitoylation May Explain How DLK Mediates Injury Responses Without Affecting Overall JNK Activity. An additional key feature of DLK is that it mediates axonal retrograde signaling via JNK and c-Jun without affecting basal levels of JNK activity (9). This observation has spurred interest in DLK as a therapeutic target (45), but how DLK plays this specific role has been unclear. Our findings suggest that the triple control of DLK localization, interactions, and activity by palmitoylation holds the key to this issue. This is because active palmitoyl-DLK is tightly associated with vesicles and thus is unlikely to phosphorylate cytosolic pools of MAP2Ks. Conversely, if DLK is depalmitoylated and dissociates from vesicles, it concomitantly loses kinase activity. This elegant security feature may thus minimize aberrant phosphorylation of other JNK pathway substrates (Fig. S9*C*). In situations where DLK signaling drives neurodegeneration (10), then strategies to inhibit DLK palmitoylation could be used in combination with, or as alternatives to, small molecules that directly target DLK's kinase domain. Conversely, in situations in which enhanced DLK signaling might be beneficial (e.g., to increase regeneration of injured adult peripheral nerves), strategies to inhibit the thioesterase(s) that depalmitoylates DLK hold considerable promise, particularly given DLK's high rate of palmitate turnover (Fig. S8*C*). Given their potential therapeutic importance, the identity of DLK PATs and thioesterases is an exciting area for future study.

Broader Roles for Palmitoylation-Dependent Retrograde Signaling and Regulation of Kinase Activity. Finally, the effectiveness of palmitoylation in controlling DLK localization, interactions, and activity suggests that other retrograde signaling proteins or other kinases might use this mechanism. In support of the first possibility, key retrograde signaling proteins, including the transcription factor STAT3 and importins (1), were recently proteomically identified as palmitoyl-proteins (26, 46, 47). These findings suggest that palmitoylation may be critical for retrograde transport of multiple neuronal signaling proteins.

In addition, we recently identified another kinase whose activity in neurons is dependent upon its palmitoylation (48). This raises the possibility that "multifunctional" palmitoylation is broadly used to localize kinases and perhaps other signaling enzymes, and thereby restrict their interactions and activity to specific membrane locations. Multifunctional palmitoylation may thus be critical for the spatial control of kinase signaling across an array of cell types and species.

Methods

Antibodies and cDNAs used in this study are listed in *SI Methods*. Protocols for well-established procedures (immunostaining and immunoprecipitation) and protocols described in prior reports are also fully documented in *SI Methods*.

Chemicals. 2-Bromopalmitate and S-Methyl methanethiosulfonate (MMTS) were from Sigma. All other chemicals were from Fisher Biosciences and were of the highest reagent grade.

Transfection. HEK293T cells were transfected using a calcium phosphate-based method, as described previously (18).

Detection of Palmitoylation by ABE Assay and Metabolic Labeling. ABE was performed as described previously (18) and metabolic labeling using 17-ODYA was performed similarly to prior studies (26, 27). Full details of both assays are provided in *SI Methods*.

Cultured Sensory Neurons. All animal use protocols were approved by Temple University Institutional Animal Care and Use Committee. Dorsal root ganglia were dissected from embryonic day 14.5 rat embryos, digested with 5 mg/mL dispase, and plated on poly-L-lysine/laminin–coated glass coverslips or 12-well dishes. Neurons were cultured in Neurobasal media containing B27, GlutaGro (Mediatech), and 25 ng/mL NGF (BD). Fluorodeoxyuridine (FDU) was added on day in vitro 0 to prevent nonneuronal cell growth.

Microfluidic Cultures. Microfluidic chambers were designed using AutoCAD software, based on a prior description (49). Master molds (fabricated at Stanford University Microfluidics Foundry and Missouri State University Jordan Valley Innovation Center) consisted of three chambers (width of middle chamber: 1,000 μm; width of adjacent chambers: 1,500 μm) connected by 450-μm-long

microgrooves. PDMS devices were cast using Sylgard 184 (Dow Corning). Distal axons in microfluidic chambers were axotomized as described (30, 49). Axotomy of conventionally cultured neurons is described in *SI Methods*.

Lentiviral Infection and shRNA Knockdown. Lentivirus was generated as described previously (18). Full details are in *SI Methods*.

Microscopy. DLK distribution was imaged using a Nikon C2 inverted confocal microscope with an oil immersion objective (40×, 1.3 NA). Maximum intensity projections were generated from *z*-slices (0.25-μm spacing, 1,024 × 1,024 pixel resolution) using NIS Elements software. c-Jun phosphorylation was imaged using a Nikon 80i microscope (10×, 0.3 NA objective). Live images of DLK-GFP were acquired at 0.375-s intervals using a Zeiss Axio Observer Z1 inverted TIRF microscope with dual wavelength laser system, α-plan-apo TIRF objective (100× 1.46 NA) and Orca R2 camera (Hamamatsu). Images were analyzed using either Zeiss Axiovision software or ImageJ.

***C. elegans* Strains.** *C. elegans* strains were maintained on NGM plates at 20° C, as described previously (50). P*mec-4*-GFP::CeDLK-1(wt) (pCZGY2335) and P*mec-4*-GFP::CeDLK-1L(C104S) (pCZGY2336) were made using Gateway cloning (Invitrogen). Details are provided in *SI Methods*. Fluorescent images were collected from young adult animals using a Zeiss LSM710 confocal microscope with a 63× objective (NA = 1.4). Images are maximum-intensity projections from three *z*-stacks separated by 0.5 μm.

In Vitro Kinase Assay. HEK293T cells transfected with DLK-GFP cDNAs were lysed in immunoprecipitation buffer (IPB) (18). After centrifugation, soluble supernatants were immunoprecipitated with anti-GFP antibody precoupled to protein G Sepharose. Beads were washed extensively with IPB and then with kinase assay buffer (10 mM Tris, pH 7.5, 0.2 mM EDTA, 0.1% Tx-100, protease and phosphatase inhibitors). Supernatant was removed and beads resuspended in kinase assay buffer containing 1 μM purified mouse GST-MKK4 and incubated for 10 min with 0.1 mM ATP/10 mM $MgCl_2$ at 30 °C. Assays were stopped with SDS sample buffer, processed for SDS/PAGE and immunoblotted with anti-GST, anti-GFP, anti-pMKK4, or anti-pMKK7 antibodies. GST fusion protein purification is described in *SI Methods*.

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