Regulation of Activity of Dual Leucine Zipper Kinase

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The dual leucine zipper kinase (DLK) alias mitogen-activated protein 3 kinase 12 (MAP3K12) has gained much attention. DLK belongs to the mixed lineage kinases, characterized by homology to serine/threonine and tyrosine kinase, but exerts serine/threonine kinase activity. DLK has been implicated in many diseases, including several neurodegenerative diseases, glaucoma, and diabetes mellitus. As a MAP3K, it is generally assumed that DLK becomes phosphorylated and activated by upstream signals and phosphorylates and activates itself, the downstream serine/threonine MAP2K, and, ultimately, MAPK. In addition, other mechanisms such as protein–protein interactions, proteasomal degradation, dephosphorylation by various phosphatases, palmitoylation, and subcellular localization have been shown to be involved in the regulation of DLK activity or its fine-tuning.

Keywords: dual leucine zipper kinase ; phosphorylation ; protein–protein interaction ; proteasomal degradation ; palmitoylation

1. (Patho)physiological Actions of DLK

The dual leucine zipper kinase (DLK) alias mitogen-activated protein 3 kinase 12 (MAP3K12) has gained much attention in recent years due to its involvement in several neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Parkinson's disease and Alzheimer's disease [1][2][3][4][5][6], and glaucoma [7]. In an insulin-producing beta-cell line, DLK was shown to become activated by prodiabetogenic signals, inhibit insulin synthesis and secretion, and induce apoptosis, thus promoting the pathogenesis of diabetes mellitus type 2 [8][9][10][11][12][13]. Furthermore, in the next-generation sequencing data set GSE81608, the upregulation of *MAP3K12* was observed in the islets of type 2 diabetic patients in comparison to healthy donors [14], and in a genome-wide association study, the SNV rs77511173-C (location 12.53489753) within *MAP3K12* was associated with body mass index in a Japanese population [15]. However, in a rat model, DLK was required for postnatal beta-cell proliferation [16]. Thus, DLK might be important for the development of the neuronal system [17] and early postnatal beta-cells [16].

The reactivation of latent neuronal herpes simplex virus (HSV) infections can cause, besides blisters and sores, severe encephalitis, and it is triggered by neuronal stress. By inducing the initial wave of the increased expression of lytic genes, DLK mediates the reactivation of latent HSV infection in different model systems [18][19][20]. In addition, an association of DLK with different kinds of cancer was found, whereby DLK mRNA expression was positively correlated with lung adenocarcinoma, pancreatic duct adenocarcinoma, sarcoma, and thymoma. The survival rates in kidney renal clear cell carcinoma, kidney renal papillary cell carcinoma, pheochromocytoma, paraganglioma, stomach adenocarcinoma, and uterine corpus endometrial carcinoma were all negatively associated with DLK mRNA expression [21]. In prostate cancer cells, DLK was shown to regulate proliferation and invasion [22]. Thus, the inhibition of DLK is supposed to be a promising drug target for the treatment of several neurodegenerative diseases and, possibly, diabetes. Indeed, some DLK inhibitors have been developed and already tested in vitro and in vivo, and sunitinib and tozasertib have been re-purposed as DLK inhibitors but are in fact multi-kinase inhibitors. However, in a phase-1 with patients suffering from ALS treated with GDC-0134, an unexpected increase in a putative ALS biomarker was observed, and no tolerable dose could be found [4]. The development of the DLK inhibitor IACS-52825 was stopped due to dose-independent reversible optic nerve swelling in monkeys [23].

DLK belongs to the class of the mixed lineage kinases (MLK), characterized by sequence homology to both serine/threonine and tyrosine kinases in their primary structure, but functioning as serine/threonine kinase [20][21][22]. The class of the MLK can be subdivided into three subclasses: The largest class consists of MLK1-4 (MAP3K9-11 and 21, respectively), sharing 75% sequence identity in their kinases domains and displaying an amino-terminal SH3 domain, followed by the kinase domain, a leucine zipper, the Cdc42/Rac Interacting Binding (CRIB) motif, and a large C-terminal region. Another subgroup consists of the leucine zipper and sterile-alpha motifs (SAM) ZAK or MLK7 (MAP3K20), containing the dimerizing SAM in addition to the leucine zipper domain. DLK forms another MLK subgroup with the

leucine zipper kinase (LZK; MAP3K13). The kinases share 90% amino acid sequence identity within their enzymatic and dual leucine zipper domain (**Figure 1**).

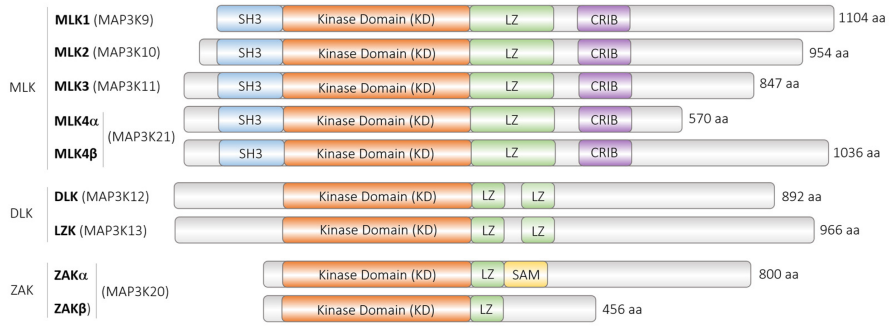

Figure 1. Mixed-lineage kinase (MLK) subfamilies. Schematic depiction of the functional domains of different MLK family members (not to scale). MLK (mixed-lineage kinase), DLK (dual leucine zipper kinase), LZK (leucine zipper kinase), ZAK (sterile alpha motif and leucine zipper containing kinase AZK), SH3 (Src homology 3 domain), LZ (Leucine Zipper), CRIB (Cdc42/Rac interactive-binding), and SAM (sterile-α motif). NCBI RefSeq accession numbers (if applicable): MLK1/MAP3K9 (NP_001271159.1, Isoform 2), MLK2/MAP3K10 (NP_002437.2), MLK3/MAP3K11 (NP_002410.1), MLK 4/MAP3K21 (CAC84639.1, Isoform 1/α; NP_115811.2, Isoform 2/β), DLK/MAP3K12 (NP_001180440.1, Isoform 1), LZK/MAP3K13 (NP_004712.1, Isoform 1), and ZAK/MAP3K20 (NP_057737.2, Isoform 1/α; NP_598407.1, Isoform 2/β).

DLK and LZK are highly conserved orthologues of Wallenda/DLK in *Drosophila melanogaster* and DLK-1 in *Caenorhabditis elegans*, suggesting an important role for DLK in evolution and development. Indeed, while mice lacking *Mlk1*, *Mlk2*, *Mlk3*, or *Lzk* are viable [21][23][24], mice lacking *Dlk* die perinatally and show signs of impaired neuronal development [17]. In line with this finding, the genome aggregation database (gnomAD v 2.1), a repository of data on human genes and their single-nucleotide variants (SNV), calculated for the *MAP3K12* (gene) 41.3 loss of function (LoF) SNV but observed none of them in their different cohorts. Thus, in species as diverse as mice and humans, intact DLK appears to be essential for prenatal development. Yet, the conditional deletion of *Dlk* in mice aged 10 to 12 weeks did not result in gross phenotypic changes, suggesting that in adults, the absence of DLK or its function does not interfere with vital functions under normal conditions [25]. The inhibition of DLK has been proposed as a promising drug target to treat neurodegenerative diseases like amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease [1][2][4], glaucoma [7], and diabetes mellitus type 2 [8][9][10]. This suggests that abnormal DLK activity in adults contributes to pathological signaling in various tissues. However, DLK signaling is required for the induction of the pro-regenerative transcriptional program in peripheral nerves after injury [26][27], and it has been shown to be constitutively active in the adult mouse brain, exerting both homeostatic and stress-induced functions [3]. These findings suggest that DLK indeed acts as a "double-edged sword" [28].

2. Regulation of DLK

2.1. Regulation of DLK at the Transcriptional Level

Not much is known about the regulation of the DLK gene expression: The TATA-box—the less core promoter regions of human and mouse DLK gene upstream of exon 1 share 88% identity with completely conserved xenobiotic responsive element-like sites, GC-boxes, and exon 1 in between both species. Using electrophoretic mobility shift assays (EMSA) and reporter gene assays with 5′-deleted promoter fragments, the transcription Sp3 factor was shown to bind to and activate the core promoter in the human neuroblastoma cell line SH-SY5Y [29]. In the 3T3-L1 cell line, the ligand of the peroxisome-proliferator-activated receptor γ (PPARγ) rosiglitazone increased DLK expression, whereas the inhibition of PPARγ either by small hairpin RNA or the receptor antagonist GW9662 suppressed DLK protein and mRNA expression. Two binding-sites for PPARγ and its heterodimer retinoic X receptor were identified via EMSA and chromatin immunoprecipitation assays [30]. In human adipose stromal/stem cells, precursors of mature adipocytes, and bisphenol A increased *DLK* expression, presumably after binding to estrogen receptors [31], but it was not investigated whether the *DLK* promoter contained an estrogen receptor responsive element. Thus, DLK gene expression is regulated by Sp3, the nuclear receptors PPARγ, and the estrogen receptor. Of note, DLK itself has been shown to increase PPARγ gene expression in 3T3-L1 and adipose stromal/stem cells [31][32]. Finally, reduced *Dlk* expression was observed in the lenses of mice deficient in the transcription factors *Mafg* and *Mafk* [33], suggesting that these transcription factors may be involved in *Dlk* expression.

2.2. Regulation of DLK at the Post-Transcriptional Level

The human MAP3K12 gene spans 21.871 nucleotides (nt) and is located on the complementary strand of chromosome 12, GRCh38.p14 Primary Assembly. It is flanked by the gene encoding for the TARBP2 subunit of the RISC loading complex (TARBP2) and the poly(rC) binding protein 2 (PCBP2) gene, both located on the positive strand in opposite directions relative to the DLK gene. The transcripts of the human DLK Isoforms 1 (NM_001193511.2) and 2 (NM_006301.4) differ in a 99 nt stretch that is absent in Isoform 2, resulting in a slightly shorter protein of 859 amino acids (aa) and a calculated molecular weight of 93.2 kDa, instead of 892 aa and 96.3 kDa (NCBI, https://www.ncbi.nlm.nih.gov/gene/7786, 30 June 2023, 14:33. conserved) (**Figure 2**).

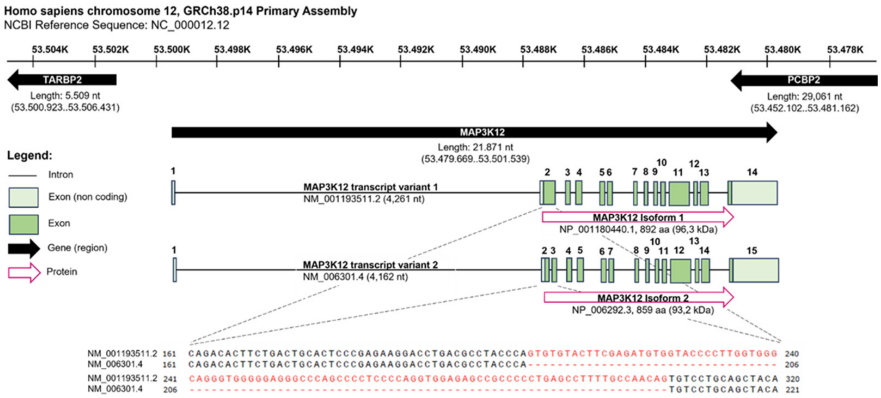

Figure 2. The human MAP3K12 gene spans 21.871 nucleotides (nt) and is located on the complementary strand of chromosome 12, GRCh38.p14 Primary Assembly. It is flanked by the gene encoding for the TARBP2 subunit of RISC loading complex (TARBP2) and the poly(rC) binding protein 2 (PCBP2) gene, both located on the positive strand in opposite directions relative to the DLK gene. The transcripts of the human DLK Isoforms 1 (NM_001193511.2) and 2 (NM_006301.4) differ in a 99 nt stretch absent in Isoform 2, resulting in a slightly shorter protein of 859 amino acids (aa) and a calculated molecular weight of 93.2 kDa instead of 892 aa and 96.3 kDa. Data were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/gene/7786, 30 June 2023, 14:33). The figure created using BioRender.

MicroRNAs (miRNAs) provide another mechanism for regulating gene expression at the post-transcriptional level. MiRNAs are small non-coding RNAs of about 22 nucleotides. By directing the RNA-induced silencing complex (RISC) to specific target mRNAs, miRNA can repress target genes and affect various biological responses [34]. In turn, the expression of miRNAs is regulated by several physiological and pathophysiological conditions. *DLK* mRNA is predicted to be a target for miRNAs (TargetScan v8.0; targetscan.org) [35], and several interactions have been validated experimentally (miRTarBase) [36]. In neuroblasts, the downregulation of the entire miR-17 family during neuronal differentiation and upregulation of *DLK* mRNA was observed, and the overexpression of miR-17 and miR-20a reduced *DLK* mRNA [37]. In endothelial progenitor cells (EPC) from type 2 diabetic patients, the expression of miRNA-130a was reduced, and increased *DLK* expression was observed compared to EPC from healthy controls. In line with this finding, the overexpression of miRNA-130a decreased DLK protein expression in EPC, suggesting that DLK expression is regulated by miRNA-130a [38]. The regulation of DLK via miRNA was also demonstrated in a mouse model of Alzheimer's disease (AD). MicroRNA-191-5p was shown to target the 3′-untranslated region of *MAP3K12*, downregulating DLK expression and alleviating microglial cell injury in the AD mouse model [39]. Yu et al. found, in various prostate cancer cell lines, that the tumor suppressor miR-150-5p downregulates MAP3K12 [40]. These studies show that DLK expression is subject to miRNA regulation. Of note, miRNAs commonly target mRNAs of several proteins, so it is to be expected that a given miRNA downregulates more proteins than DLK only.

2.3. Regulation of DLK at the Post-Translational Level

2.3.1. Phosphorylation of DLK

Already, the first studies on DLK showed that this kinase is heavily phosphorylated, and the overexpression of DLK alone is sufficient to activate this kinase [20][41]. At least some phosphorylation sites and DLK phosphorylating kinases have been identified in the meantime. Upon homodimerization via its leucine zipper domains, DLK becomes auto-phosphorylated in trans at Ser-302 (**Figure 3**B) [11][42][43].

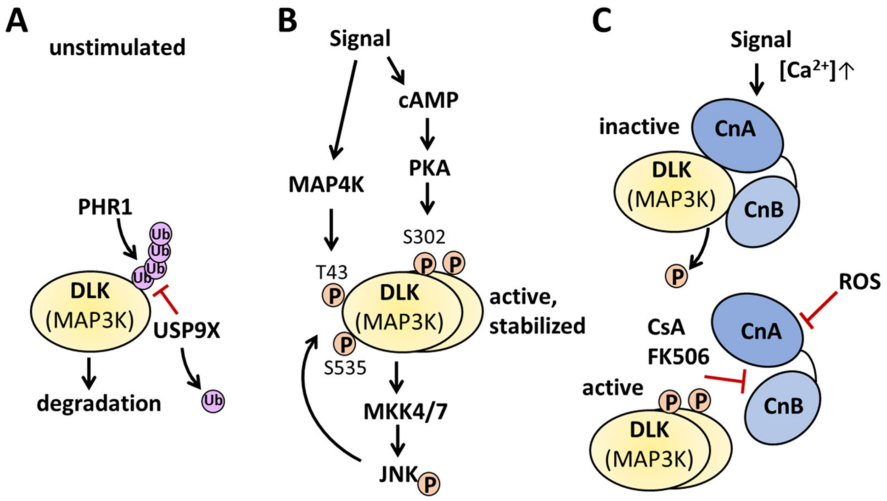

Figure 3. Examples for regulation of DLK activity. (**A**) Under basal unstimulated conditions, unphosphorylated DLK protein abundance is regulated by the E3 ubiquitin ligase PHR1 and the deubiquitinase USP9X. (**B**) Signals activating cAMP and PKA phosphorylate dimerized DLK on Ser-302, leading to the activation of MKK4/7 and JNK. JNK, in turn, phosphorylates DLK on Thr-43 and Ser-535, preventing the interaction with PHR1, thereby stabilizing DLK. Other signals activating MAP4K phosphorylate DLK on Thr-43 and stabilize DLK. (**C**) Upon an increase in the intracellular calcium concentration, calcineurin interacts with monomeric or dimeric DLK and dephosphorylates the kinase. The inhibition of calcineurin by ROS prevents the dephosphorylation of DLK, whereas the interaction of immunophilin-bound CsA or FK506 displaces DLK from the calcineurin interaction site, and DLK dimerizes and autophosphorylates in trans. For further information, please see the text.

2.3.2. Dephosphorylation of DLK

Kinase–phosphatase interactions are a well-known component of cellular responses and signaling pathways, affecting kinase phosphorylation, expression levels, and interactions with other proteins. In invertebrates, DLK activity has been shown to be regulated by the protein phosphatases Mg^{2+}/Mn^{2+} dependent (PPM)-1 and PPM-2, as well as the protein phosphatase 2A (PP2A) [5][44][45]. In mammals, the inhibition of protein phosphatases 2A [5][41][46] and 2B [10][12][41][47] affected DLK activity.

Several studies described the roles of the PHR proteins as key regulators of presynaptic differentiation and function, thereby fundamentally affecting neuronal development [48][49][50]. Among the PHR proteins, the Regulator of Presynaptic Morphology (RPM)-1 has been shown to negatively regulate DLK-1 as part of an ubiquitin ligase complex in *C. elegans* [51][52]. In 2011, Tulgren et al. provided additional evidence that PPM-1, a serine/threonine phosphatase homologous to human PPM1A, acts as a second negative regulatory mechanism downstream of RPM-1 to control the DLK-1 pathway in *C. elegans* [44]. However, the involvement of PPM-1 was shown to act at the level of PMK-3 (p38 MAPK) and not directly at the level of DLK-1 (MAPKKK), as previously described by Takekawa et al. in mammalian cells [53]. In contrast, the serine/threonine Protein Phosphatase Magnesium/Manganese-dependent 2 (PPM-2) has been described in transgenic animals, genetic, and biochemical approaches for directly regulating DLK-1 [45]. Baker et al. (2014) demonstrated that PPM-2 acts on DLK-1 at the Ser-874, regulating its phosphorylation and activation. However, the authors note that the activation of RPM-1 is a prerequisite for the activity of PPM-2 on DLK-1, and this PHR protein employs ubiquitination and phosphatase-based mechanisms to inhibit DLK-1. This observation is based on immunoprecipitation approaches, followed by mass spectrometry, immunoblot, and immunofluorescence analysis, in which Baker et al. showed that RPM-1 binds to and positively regulates PPM-2. As RPM-1 has previously been shown to be a part of a neuronal complex involving multiple proteins [51][52], more precise approaches are lacking to confirm that RPM-1 directly binds to PPM-2. In addition, the small segment of the *C. elegans* DLK-1 containing Ser-874 is not conserved with mammalian DLK, and no functional orthologue of PPM-2 is known in vertebrates (Yan et al., 2012 [54]).

2.3.3. Palmitoylation of DLK

The dual leucine zipper kinase is crucial for retrograde signaling after injury in neurons and important in brain development, but the activation of DLK can also lead to apoptosis and neuronal degeneration in different disease models, such as ALS or Alzheimer's disease. Due to the importance of the DLK for cell fate, the activity of DLK, at least in neurons, needs to be highly restricted in temporal and spatial manners and confined to local events. In a search for an explanation on how a bioinformatically predicted soluble protein could travel from distant axonal regions back to the

nucleus in a controlled way, Holland et al. found and validated an evolutionary conserved cysteine residue (C127, in human DLK) in the DLK as a site for post-translational modification via palmitoylation (**Figure 4**) [55].

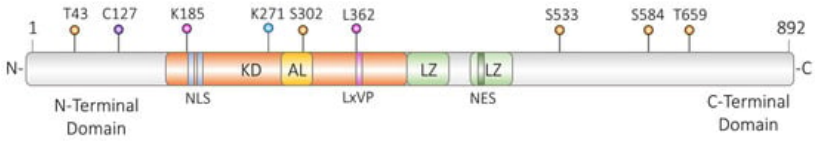

Figure 4. DLK amino acid residues are modulated at the post-translational level. Isoform 1 of human DLK is depicted; T—threonin; C—cystein; K—lysin; S—serin; orange dots—phosphorylation and, possibly, dephosphorylation sites; violet dot—palmitoylation site; blue dot—ubiquitylation and SUMOylation site. KD—kinase domain; AL—activation loop within the kinase domain; LZ—leucine zipper for homodimerization; NLS—bipartite nuclear localization site; NES—nuclear export site; K185—ATP binding site; L—leucine; x—any amino acid; V—valine; P—proline; LxVP—interaction site with calcineurin. For further information, please see the text.

2.3.4. Regulation via Protein–Protein Interactions

DLK protein content and, therefore, activity is regulated via the interaction with diverse proteins. The Regulator of Presynaptic Morphology 1 (RPM-1) in *C. elegans* and Highwire (Hiw) in *D. melanogaster* were the first proteins demonstrated to interact with DLK-1 and Wallenda, respectively. Together with the human protein associated with Myc (PAM), these proteins are termed PHR (PAM, Highwire, RPM-1) proteins, which are huge proteins with more than 3700 aa containing diverse enzymatic activities and mainly regulate synapse formation and axon termination [56]. In *C. elegans*, *D. melanogaster*, and the dorsal root ganglia of mammals, RPM-1, Highwire, and PHR, respectively, ubiquitinate DLK-1/Wallenda/DLK, leading to the kinase's proteasomal degradation, thus terminating kinase activity [56]. However, in mammals, PHR does function independently of DLK in some neuronal contexts [56]. Using primary dorsal root ganglia cells as a model, Huntwork-Rodriguez et al. (2013) showed that the interaction between DLK and the E3 ubiquitin ligase PHR is regulated via the JNK-induced phosphorylation of DLK: the phosphorylation of DLK on Thr-43 and Ser-533 stabilizes DLK, presumably preventing the interaction with PHR1. Under basal conditions, when DLK is not phosphorylated, its abundance is regulated by a balance between PHR1 and the deubiquitinase ubiquitin-specific peptidase 9, X-linked (USP9X) [43]. In addition to PHR, the FK506-binding protein-like (FKBPL) and the FK506-binding protein 8 (FKBP8) were identified as DLK-interacting proteins [57]. By interacting with the N-terminus of DLK, containing its kinase domain, the N-terminus of FKBPL, containing its peptidyl-prolyl isomerase domain, inhibited DLK activity and reduced its protein stability. Both FKBPL and FKBP8 induced DLK degradation via the lysosomal pathway. Additionally, FKBP8-mediated DLK degradation was prevented when Lys-271 was mutated to Arg, which was shown to function as an ubiquitination and SUMOylation site. Thus, FKBP8 induced DLK lysosomal and proteasomal degradation [57].

The heat-shock proteins (HSP) are another group of proteins regulating DLK protein abundance and, therefore, enzymatic activity. The HSP90 acts as a chaperone, which, in contrast to other HSPs, facilitates the maturation, complex assembly, localization, and ligand binding of signal transduction proteins like kinases and nuclear receptors [58][59]. Using HSP90 and DLK inhibition and co-immunoprecipitation assays, an interaction between DLK and HSP90 was shown to occur in *D. melanogaster*, embryonic DRG neurons and the HEK cell line, whereby the inhibition or loss of HSP90 (or its fly ortholog Hsp83) reduced DLK protein content [58]. Hence, the interaction between HSP90 and DLK preserves DLK, possibly by preventing the interaction between DLK and PHR1 leading to DLK proteasomal degradation. In contrast, in the cell line COS7, the interaction of activated DLK with HSP70 results in DLK proteasomal degradation, which was dependent on the HSP70 co-chaperone CHIP (C-terminus of HSP70 interacting protein), an E3 ubiquitin ligase [60]. Notably, both chaperones, HSP90 and HSP70, can interact with the co-chaperone CHIP and induce the proteasomal degradation of their respective clients [61]. This suggests that the outcome of the interaction of DLK with HSP chaperones does depend on the expression of the co-chaperone CHIP.

In addition, a direct interaction between DLK and calcineurin has been demonstrated [10].

2.3.5. Regulation of DLK via Its Oligomerization

The homodimerization of DLK via its leucine zipper has been shown to be essential for DLK activity [42]. Experiments conducted in different cell lines (NIH3T3, COS-1) indicated that the formation of high-molecular DLK polymers occurred in response to the apoptosis-inducing agent Calphostin C, independent of its ability to inhibit protein kinase C (PKC) [62][63][64]. DLK oligomerization was abolished by the tissue-transglutaminase (tTG) inhibitor monodansylcadaverine, indicating that a tTG-catalyzed protein crosslinking reaction was the underlying cause. A model emerged whereby the Calphostin-C-induced intracellular rise of Ca^{2+} stimulates Ca^{2+}-dependent tTG2 crosslinking-activity, leading to an increase in the DLK polymers. Oligomerization then increases DLK activity and, hence, activates the JNK-Pathway. After JNK-activation, the

apoptosis regulator Bax translocates to mitochondria and induces caspase activation, ultimately leading to apoptosis [62][63][64]. It remains unknown which domains of DLK mediate the oligomerization. Nevertheless, bringing single DLK molecules together, either as homo- or oligomers, leads to increased DLK activity.

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