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Interaction of delta-like 1 homolog (*Drosophila*) with prohibitins and its impact on tumor cell clonogenicity

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Abstract

Cancer stem cell characteristics, especially their self-renewal and clonogenic potentials, play an essential role in malignant progression and response to anti-cancer therapies. Currently, it remains largely unknown what pathways are involved in the regulation of cancer cell stemness and differentiation. Previously, we found that delta-like 1 homolog (*Drosophila*) or DLK1, a developmentally regulated gene, plays a critical role in regulation of differentiation, self-renewal, and tumorigenic growth of neuroblastoma cells. Here, we show that DLK1 specifically interacts with the prohibitin 1 (PHB1) and PHB2, two closely related genes with pleiotropic functions including regulation of mitochondrial function and gene transcription. DLK1 interacts with the PHB1-PHB2 complex via its cytoplasmic domain and regulates mitochondrial functions including mitochondrial membrane potential and production of reactive oxygen species (ROS). We have further found that PHB1 and especially PHB2 regulate cancer cell self-renewal as well as their clonogenic potential. Hence, the DLK1-PHB interaction constitutes a new signaling pathway that maintains clonogenicity and self-renewal potential of cancer cells.

Keywords

cancer cell stemness; self-renewal; delta-like 1 homolog; DLK1; prohibitin; PHB

Introduction

Delta-like 1 homologue (*Drosophila*) or DLK1 is a member of the epidermal growth factor (EGF)-like homeotic supergene family with homologies to members of the *notch/delta/serrate* family (1). Also known as *pref-1*, fetal antigen (FA1), *pG2* and *ZOG*, *DLK1* is predominantly expressed in embryonic and other immature cells but not in differentiated cells (2–4), suggesting an important role of *DLK1* in regulating maintenance and/or differentiation of stem cells and progenitors. Expression of *DLK1* is elevated in a wide range of tumor types, including neuroblastoma, gliomas, breast cancer, colon cancer, pancreatic cancer, small-cell lung carcinoma, and leukemia (5–11). Studies from others and us have shown that DLK1 plays an important role in stem cell maintenance and cellular differentiation. *DLK1* expression *in vitro* inhibits differentiation of mesenchymal progenitor cells (3, 12, 13) and hematopoietic stem cells (8, 9). Our studies have shown that *DLK1* is primarily expressed in undifferentiated neuronal tumor cells (14). Over-expression of DLK1 enhances tumor cell stemness and tumorigenic growth *in vivo* (14, 15). Conversely, *DLK1*-specific RNAi or dominant DLK1 mutants enhance spontaneous neuronal differentiation

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and decrease tumorigenicity of neuroblastoma cells both *in vitro* and *in vivo* (14, 15). We also found that hypoxia strongly induces *DLK1* expression and that *DLK1* is implicated in the regulation of cancer cell stemness within the hypoxic tumor microenvironment (14, 16, 17). In addition, *DLK1* is also implicated in regulating *in vitro* differentiation of glioma cells (7) and hematopoietic tumors (8). However, the mechanisms by which *DLK1* regulates cancer cell stemness and/or differentiation remain largely unknown.

In order to gain mechanistic understanding of the involvement of *DLK1* in intracellular signal transduction, we attempted to identify *DLK1*-interacting proteins using an affinity purification approach. As reported herein, we have found that *DLK1* specifically interacts with the prohibitin (PHB) complex via the *DLK1* cytoplasmic domain. PHB1 and the closely related PHB2 are encoded by evolutionarily conserved genes and possess diverse functions from mitochondrial structural integrity and function to gene transcription in the nucleus (18–20). We have found that *DLK1* regulates mitochondrial membrane potential and production of reactive oxygen species (ROS). Our data further reveal a role of PHBs and especially PHB2 in the regulation of cancer cell self-renewal as well as their clonogenic potential. Hence, the *DLK1*-PHB interaction constitutes a new signaling pathway that promotes the maintenance of cancer cell stemness.

Materials and Methods

Plasmids

Retroviral vectors expressing *DLK1*-FL (full-length *DLK1*), *DLK1*-Ecto, *DLK1*-ΔCyto (deletion of *DLK1* cytoplasmic domain), and *DLK1*-DM (Y339F/S355A mutations in *DLK1* cytoplasmic domain) have been described in our previous publication (14). Flag-tagged *DLK1* (*DLK1*-Flag) was cloned by in-frame fusion of the full-length coding sequence of *DLK1* to the 5' of two tandem repeats of the Flag tag sequence in pCMV-2xFlag. Flag-tagged *DLK1* cytoplasmic domain (*DLK1*Cyto-Flag) was cloned by in-frame fusion of the *DLK1* cytoplasmic domain-coding sequence (amino acids 328–383) to the 3' of two tandem-repeats of the Flag tag sequence in pCMV-2xFlag. The Flag-tagged PHB1 and PHB2 were from Dr. Valerie Bosch of Deutsches Krebsforschungszentrum (21) and sub-cloned into a retroviral vector. The sh*DLK1* constructs were described in our previous publication (14) with the target sequence positions in human *DLK1* mRNA (nm_003836.5) being 1062–1080 for sh*DLK1*-2, 1308–1326 for sh*DLK1*-4, and 1426–1444 for sh*DLK1*-6. All clones were sequence-validated.

Cell Culture and Transfection

Neuroblastoma cell lines SK-N-BE(2)C [BE(2)C] and SK-N-ER (ER) were maintained in Minimum Essential Medium (MEM) and F12 (1:1) with 10% fetal bovine serum (FBS). Cells were transduced with retrovirus (*DLK1*-FL, *DLK1*-ΔCyto, *DLK1*-DM or vector control) and then purified by flow cytometry for the expression of green fluorescent protein (GFP). MCF7 cells (ATCC) were maintained in RPMI1640 medium containing 10% FBS. Human hepatocellular cancer cell line HepG2 and human embryonic kidney cell line 293T (ATCC) cells were cultured in MEM containing 10% FBS. All culture media were supplemented with 25 mM HEPES (pH7.4) to maintain pH stability under hypoxia.

For transfection with siRNA oligos, cells were grown to approximately 80% confluence and then incubated with On-Target SMARTpool siRNAs (Thermo Scientific) according to the manufacture's protocol. After incubation for 48 hr, cells were then trypsinized and plated for further experiments.

Affinity Pull-Down by Co-Immunoprecipitation

Whole-cell lysates (WCL) were prepared by incubating cells expressing different DLK1 constructs or empty vector with the modified RIPA buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.5% sodium deoxycholate, and 1% NP40) for 30 minutes on ice. The lysates were cleared by centrifugation at 4 °C for 15 minutes at 13,200 rpm. Immunoprecipitation was carried out by incubation of WCL (500 µg total protein) with 4 µg of monoclonal anti-N terminus DLK1 antibody (R&D Systems), rabbit anti-PHB2 antibody (Novus), or monoclonal anti-PHB1 antibody (Thermo Scientific, clone II-14-10) overnight at 4 °C. 30 µl of recombinant Protein A agarose beads were then added and incubated for an hour at 4 °C. Immune complexes were eluted in 1x SDS sample buffer and fractionated by SDS-PAGE under reducing conditions. Silver staining was done using a SilverQuest™ Silver Staining Kit (LC6070, Invitrogen) according to the manufacturer's protocol.

Western Blot

Western blots were analyzed as described previously (14) with the following antibodies: polyclonal rabbit anti-DLK1 C-terminus (1:2,000; Millipore, Billerica, MA); polyclonal rabbit anti-PHB2 (1:1,000; Novus), monoclonal mouse anti-PHB1 (1:400; Thermo Scientific), or rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cell Signaling). When necessary, Clean-Blot™ IP Detection Reagents (Thermo Scientific) were used to eliminate cross-reaction with IgG heavy and light chains.

Mass Spectrometry

The immune complex with anti-DLK1 antibody was fractionated using SDS-PAGE under reducing conditions. The protein-containing gel pieces were subjected to mass spectrometry at Taplin Biological Mass Spectrometry Facility of Harvard Medical School.

Trichloroacetic acid (TCA) Precipitation

4 ml of 24 hour-conditioned medium was mixed with 1 ml of TCA (100% (w/v) for 1–2 hours at 4 °C. Precipitates were collected by centrifugation at 13,200 rpm for 10 minutes at 4 °C. After cold acetone wash for 3 times, protein pellets were dried in a 95 °C heat block for 3 minutes and then solubilized in 1x SDS sample buffer. Secreted DLK1 in conditioned media was analyzed using Western blots.

Immunofluorescence and Confocal Microscopy

For immunofluorescence staining of DLK1 and PHBs, BE(2)C cells were fixed with ice-cold methanol for 10 minutes and then washed 3 times with phosphate buffered saline. Non-specific binding was blocked by incubation with 5% horse serum for 30 minutes. Cells were then incubated overnight at 4 °C in an antibody mixture containing mouse anti-DLK1 (1:100, R&D Systems) plus rabbit anti-PHB1 serum or mouse anti-DLK1 plus rabbit anti-PHB1 serum (1:300). The anti-PHB1 and anti-PHB2 antisera were provided by Dr. Valerie Bosch, Krebsforschungszentrum, Heidelberg, Germany. The bound antibodies were visualized by incubation with Alexa 488-conjugated anti-mouse IgG (1:500) and Alexa 555-conjugated goat-anti-rabbit IgG (1:500), all obtained from Invitrogen. Nuclei were stained with TO-PRO3 (1:5,000, Invitrogen). Images were acquired on a Zeiss LSM 510 Meta confocal microscope. Co-localization between DLK1 and PHB was analyzed using Zeiss ZEN2010 software.

JC-1 Staining

BE(2)C cells were stained in the serum-free medium by JC-1 dye (Invitrogen; M-34152, 2 µM) for 30 minutes at 37 °C according to the manufacturer's protocol. Nuclei were stained

with Hoechst 33342 (2 µg/mL). Microscopic examination was done within an hour of staining.

CMH₂XROS Staining and Flow Cytometry

Be(2)C cells were incubated in the serum-free medium with CMH₂XROS dye (400 nM) for 45 min at 37 °C, and the reaction was stopped by washing the cells in ice-cold PBS. The cells were trypsinized and then fixed in 4% paraformaldehyde for 10 minutes at room temperature. After washing in cold PBS, fixed cells were then subjected to FACS (FACS DIVA, BD Bioscience). All samples were analyzed under the same Gain and Amplifier settings.

Tumor Sphere Formation Assay

Tumor cells were trypsinized into single-cell suspension in tumor sphere medium and plated into tissue-culture dishes pre-coated with polyhydroxyethylmethacrylate (polyHEMA; Sigma-Aldrich) as described in our previous publication (14). After incubation for 4–6 days, tumor spheres were counted under the microscope.

Clonogenic Assays

Tumor cells were plated at a clonal density (<1 cell/mm²) in six-well plates and incubated undisturbed for 10 to 14 days. Colonies were stained with crystal violet. Plating efficiency = number of colonies (≥ 50 cells per colony) per input cells × 100%.

Real-Time RT-PCR

Total cellular RNA was isolated with the TRIzol reagent (Invitrogen) and treated with DNase I for 10 minutes before first-strand cDNA was synthesized using Superscript II (Invitrogen). Real-time PCR was performed on StepOne Plus (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) under the following conditions: initiation at 95 °C × 10 min, 40 cycles at 95 °C × 15 sec, 60 °C × 60 sec. 18S rRNA was used as a control for normalization. Specificity of the primers (Table 1) was confirmed by a single peak on the dissociation curve.

Statistics

Statistical differences between two groups were analyzed using the two-tailed, unpaired Student's *t*-test. Comparison among 3 groups was done using one-way ANOVA (Prism Software, Graph Pad Software, Inc.)

Results

DLK1 interacts with PHB1 and PHB2

Previously, we reported that the cytoplasmic domain of DLK1 is necessary for promoting self-renewal and clonogenicity of neuroblastoma cells (14). To delineate the mechanisms of DLK1-mediated signal transduction, we performed co-immunoprecipitation to identify proteins that interact with DLK1 (Figure 1A). Upon electrophoresis separation and mass spectrometry, we identified prohibitin 2 (PHB2) as a potential interacting protein with DLK1. PHB2 has been implicated in a wide range of cellular processes including proliferation, apoptosis, transcription, mitochondrial protein folding, and cell-surface receptor signaling. Its pleiotropic functions are mirrored by its broad subcellular distribution in plasma membrane, nucleus, and cytoplasm, in addition to its predominant localization in the mitochondria (18, 20). PHB2 primarily forms a complex with PHB1 that shares approximately 50% amino acid sequence identity with PHB2. Using a series of co-immunoprecipitation, we found that DLK1 can interact with both PHB1 and PHB2 under

either normoxic or hypoxic (1% O₂) conditions (Figure 1B). It is worth noting that only a small portion of DLK1 is co-immunoprecipitated with PHB1 or PHB2, compared to the levels of DLK1 in whole-cell extract. Consistent with this observation, confocal microscopy analysis showed a low level (<1%) of co-localization between DLK1 and PHBs (Figure 1C). A similarly low level of co-localization was also found under hypoxic conditions (data not shown). The interaction between DLK1 and PHBs is likely regulated spatially and temporally, especially at the plasma membrane. In addition to neuroblastoma cells, DLK1-PHB interaction also occurs in other cell types including the murine non-tumor cell line 3T3-L1 (Figure 3), HepG2 human hepatocellular carcinoma cells and primary human brain tumor cells (Supplemental Figure 1), suggesting an important role of DLK1-PHB interaction in a variety of cell types.

Because DLK1 is a transmembrane protein, we examined whether the DLK1 cytoplasmic domain is involved in interaction with PHBs. Toward this end, we ectopically expressed (Figure 2A) full-length DLK1 (DLK1-FL), DLK1 extracellular domain (DLK1-EC), DLK1 without its intracellular domain (DLK1-ΔIC), and DLK1 with mutations of two putative phosphorylation sites (Tyrosine 339-to-Phenylalanine and Serine 355-to-Alanine) in its cytoplasmic domain (DLK1-DM), respectively, in SK-N-ER (ER) cells with low levels of endogenous DLK1. As shown in Figure 2B, anti-DLK1 antibodies were able to pull down both PHB1 and PHB2 in ER cells transfected with DLK1-FL, DLK1-EC, and vector control, respectively. However, the interaction between DLK1 and PHBs was strongly reduced in ER cells transfected with DLK1-DM and DLK1-ΔIC. Consistent with this observation, anti-PHB2 antibodies failed to pull down DLK1 when DLK1-DM or DLK1-ΔIC was overexpressed (Figure 2C). To determine whether the DLK1 cytoplasmic domain directly interacts with PHBs, we ectopically expressed flag-tagged DLK1 (flag-DLK1-FL) or DLK1 cytoplasmic domain (DLK1-Cyto) in 293T cells and found that the flag-tagged DLK1-Cyto, in addition to flag-DLK1-FL, co-immunoprecipitates with PHB2 (Figure 2D). Collectively, these data strongly indicate that DLK1 cytoplasmic domain is directly involved in the interaction with the PHB complex. Our data further demonstrate an involvement of Tyrosine 339 and Serine 355 of the cytoplasmic domain in the interaction between DLK1 and PHBs.

DLK1 extracellular domain increases DLK1-PHB2 interaction in a paracrine manner

It has been reported that full-length DLK1 can be enzymatically cleaved in its juxta-membrane region by TNF-α-converting enzyme (TACE or ADAM-17) to release the extracellular domain as a soluble form of DLK1 (22). Functionally, the large soluble DLK1 containing the entire extracellular domain potentiates inhibition of adipogenic differentiation (23, 24). We found that DLK1 was cleaved from BE(2)C cells and released into the culture medium (Figure 3A, lane 2). We hypothesized that the soluble DLK1 might exert autocrine/paracrine effects on neuroblastoma cells to regulate their cancer cell stemness. To test this hypothesis, we treated cells with conditioned medium (CM) from ER cells that ectopically expressed the secreted DLK1 extracellular domain (DLK1-EC, Figure 3A, lane 4). Interestingly, the conditioned medium containing soluble DLK1-EC strongly enhanced the interaction between DLK1 and PHB2 in BE(2)C cells, as shown by co-immunoprecipitation (Figure 3B, lane 3 versus lane 5). The same result was also obtained in 3T3-L1 cells (Figure 3B). Furthermore, the DLK1-EC-conditioned medium was able to increase the clonogenic potential of neuroblastoma cells (Figure 3C and D), which is consistent with the role of soluble DLK1 in inhibition of cellular differentiation. Our data suggest that the soluble extracellular domain of DLK1 may facilitate stemness maintenance in part by promoting DLK1-PHB interaction in an autocrine/paracrine manner.

DLK1 modulates mitochondrial functions

Because the PHB complex is predominantly localized in mitochondria and plays a critical role in mitochondrial biogenesis, assembly, and functions (18, 19), we set out to investigate whether interaction between DLK1 and PHBs would have a strong impact on mitochondrial functions. First, we examined the role of DLK1 in the regulation of mitochondrial membrane potential using JC-1, a mitochondrion-specific dye that forms the fluorescent (emission = 590 nm) J-aggregates in mitochondria with high membrane potential (25, 26). Here, we observed that the high DLK1-expressing BE(2)C cells had much fewer J-aggregates-positive (JC-1⁺) cells than did the low DLK1-expressing ER cells (Supplemental Figure 2). Interestingly, when the endogenous DLK1 expression was knocked down in BE(2)C cells using shRNA, the numbers of JC-1⁺ BE(2)C cells were significantly increased (Figure 4A). Consistent with this observation, overexpression of DLK1-DM or DLK1-ΔIC that lacks interaction with PHBs also significantly increased the population of JC-1⁺ BE(2)C cells (Figure 4B). Conversely, overexpression of DLK1-FL or DLK1-EC in ER cells resulted in decreases of JC-1 fluorescence intensity, indicating reduced mitochondrial membrane potential (Figure 4C). Collectively, these data suggest that DLK1 is capable of regulating mitochondrial membrane potential via interaction with PHBs.

It has been shown that high mitochondrial membrane potential may lead to increased production of reactive oxygen species or ROS (27). We therefore investigated the effects of DLK1 on ROS production using the fluorescent dye CMH2XROS as an indicator. Knocking down DLK1 expression in BE(2)C cells led to increases in CMH2XROS fluorescence (Figure 4D), indicating increased mitochondrial ROS production. This result is consistent with increased numbers of JC-1⁺ cells with high mitochondrial membrane potential upon DLK1 knockdown (Figure 4A). However, overexpression of DLK1 in the DLK1-low ER cells did not significantly change CMH2XROS fluorescence (data not shown). It is likely that overexpression of DLK1 is not sufficient to reduce mitochondrial membrane potential in ER cells. Nonetheless, these data collectively demonstrate that DLK1 has the potential to regulate the mitochondrial function.

PHB1 and PHB2 are required for maintaining tumor cell clonogenicity and self-renewal

Previously we demonstrated that DLK1 played a necessary and sufficient role in maintaining neuroblastoma cell stemness, self-renewal, and clonogenic potential (14, 15). The interaction between DLK1 and PHBs suggests that the PHB complex may also play a role in regulation of cancer cell stemness. To test this hypothesis, we investigated the role of PHB1 and PHB2 in the regulation of self-renewal using the tumor sphere formation assay and clonogenic potential using the clonogenic assay, as described in our previous study (14). When treated with siRNAs against the *PHB1* or *PHB2* gene, BE(2)C cells formed significantly fewer tumor spheres in the serum-free suspension culture (Figure 5A, C), indicating reduced self-renewal potential. While the inhibition by siPHB1 was similar to that by siDLK1, siPHB2 resulted in the strongest inhibition of tumor sphere formation. Consistent with these findings, siPHB1 and siPHB2 significantly decreased the clonogenic growth of BE(2)C cells, again with siPHB2 eliciting the strongest inhibition (Figure 5D). Similar observations were also found in the hepatocellular carcinoma HepG2 cells and human breast cancer MCF7 cells (Supplemental Figures 3 & 4). It is worth noting that all siRNA treatments did not reduce cell viability. These data strongly indicate an important role of PHB1 and especially PHB2 in the regulation of stem cell self-renewal and clonogenic growth.

We further found (Figure 5C and Supplemental Figure 5) that ectopic expression of either PHB1 or PHB2 was able to rescue tumor sphere-forming potential of siDLK1-treated BE(2)C cells, although PHB1 appears to negatively affect tumor sphere formation. The

clonogenic potential of the siDLK1-treated cells was also partially improved by ectopically expressed PHB1 or PHB2 (Figure 5D and Supplemental Figure 5). In contrast, DLK1 ectopic expression did not appear to have a strong impact on clonogenic potential of siPHB1- or siPHB2-treated cells. These findings suggest that PHBs likely function downstream of DLK1 to regulate different aspects of stemness.

It is interesting to note that PHB1 protein levels were strongly reduced in siPHB2-treated cells without affecting *PHB1* mRNA levels; and conversely, PHB2 levels were strongly decreased in siPHB1-treated cells with no decrease of *PHB2* mRNA (Figure 5A & B). This observation suggests that formation of the PHB1-PHB2 heterodimeric complex is critical for the stability of each subunit. Even more interestingly, knockdown of PHB1 or PHB2 also resulted in significant decrease of DLK1 (Figure 5A, lanes 4–5). In contrast, siDLK1 did not decrease the levels of PHB1 or PHB2 (Figure 5A, lanes 1–3). Furthermore, the DLK1 cytoplasmic domain, despite its interaction with PHBs, did not affect PHB1 and PHB2 protein expression (Supplemental Figure 6). Because neither siPHB1 nor siPHB2 negatively affected *DLK1* mRNA levels (Figure 5B), the interaction between DLK1 and the PHB complex may exert a strong impact on the stability of DLK1 protein. It is also probable that other PHB-dependent pathways may regulate DLK1 stability via yet unknown post-translational mechanisms.

Discussion

DLK1 is overexpressed in several types of cancers (7–9, 14). However, it remains largely unknown whether and how DLK1, as a transmembrane protein, can influence intracellular signal transduction. In this work, we have discovered that DLK1 interacts with the PHB1-PHB2 complex via its cytoplasmic domain, a previously unknown molecular interaction that is involved in the regulation of cancer cell stemness.

The PHB complex, primarily localized in the inner membrane of mitochondria, plays a critical role in the maintenance of mitochondrial morphology and normal functions (18, 19). Knocking down PHB1 in endothelial cells (28) or knocking down PHB2 in mouse embryonic fibroblasts (29) results in depolarization of mitochondrial membranes. On the other hand, ectopic PHB1 expression facilitates the maintenance of mitochondrial membrane potential in cardiomyocytes (30). In this study, we have found that overexpression of DLK1 reduces mitochondrial membrane potential in neuroblastoma cells, as indicated by decreased formation and, hence, fluorescence of J-aggregates. Conversely, knocking down DLK1 results in increased formation of the J-aggregates and, hence, elevated mitochondrial membrane potential. Consistent with the observations that DLK1 cytoplasmic domain is required for interaction with PHBs, the two dominant-negative mutants, DLK1-ΔIC and DLK1-DM, also increase the J-aggregate formation. Because excessively high mitochondrial membrane potential (>150 mV) can lead to increased formation of free radicals and ROS (27), our data suggest that DLK1, via interaction with PHBs, helps to prevent the development of detrimentally high mitochondrial membrane potential. Consistent with this notion, our data further demonstrate that knocking down DLK1 leads to increased ROS production, as indicated by elevated fluorescence of CMH2XROS.

Interestingly, we have found that both PHB proteins, especially, PHB2, are required for maintaining cancer stem cell characteristics including self-renewal and clonogenic potential. This novel observation is consistent with the findings that both PHB1 and PHB2 are essential for embryonic development (31, 32). Other reports have shown that PHB2 represses myogenic differentiation (33) and PHB1 prevents ROS-induced endothelial cell senescence (28). A very recent report has shown that knocking down PHB1 and/or PHB2 reduces cell growth and colony formation in hepatocellular carcinoma cells (34) and the

cervical cancer HeLa cells (35). Collectively, these data suggest an important role of PHBs in the maintenance of the stem cell state. Our data further demonstrate that the interaction between PHB and DLK1 facilitates self-renewal and enhances clonogenic growth of cancer cells. It is worth noting that the siPHB-induced down-regulation of sphere formation and clonogenicity could potentially be due in part to the decrease of DLK1 protein in siPHB-treated cells. On the other hand, PHBs appear to function downstream of DLK1 because ectopic expression of PHB1 or PHB2 can, to different extents, rescue the siDLK1-dependent decreases in sphere-forming potential and clonogenicity. These observations suggest that interaction between DLK1 and PHB proteins is likely to be complex and each protein may also have independent functions in the regulation of cancer cell stemness.

Although elevated PHB levels have been found in many types of human cancers, it remains controversial whether PHBs promote or suppress tumor growth and/or malignant progression. To a large extent, these controversies may be due to the highly pleiotropic functions of PHB1 and PHB2 in the regulation of proliferation, cell survival, and gene transcription. It is likely that their exact functions are determined by protein-protein interactions in different subcellular locations since PHBs are also found in plasma membrane, cytoplasm, and nucleus (29, 32, 36, 37). Both PHB1 and PHB2 are capable of repressing gene transcription when located in nucleus (32, 36, 38, 39), which may partly explain the tumor suppressive function of PHB. On the other hand, the PHB1-PHB2 complex interacts with c-Raf at the plasma membrane and facilitates the Ras-induced c-Raf activation (37, 40). Our data presented herein suggest that the DLK1 cytoplasmic domain interacts with the PHB1-PHB2 complex at the plasma membrane. Our previous studies have shown that down-regulation of DLK1 expression or its function results in sustained activation of the ERK pathway (14, 15). It will be interesting to determine whether DLK1 interferes with Ras-Raf-PHB interaction. Nonetheless, the DLK1-PHB interaction elicits a broad impact on cellular functions including mitochondrial function, self-renewal, and clonogenic growth of cancer cells. Our study has thus provided a new mechanism underlying the pro-tumorigenic role of DLK1 and PHBs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Implications

This study provides a new mechanistic insight into the regulation of the stem cell characteristics of cancer cells.

Figure 1. DLK1 interacts with the PHB1-PHB2 complex

(A) Whole-cell lysates (WCL) were prepared by solubilizing BE(2)C cells in the modified RIPA buffer and incubated with monoclonal anti-DLK1 N-terminus antibody. Naïve mouse IgG2b was used as the isotype control. Immune complexes were separated by 10% SDS-PAGE and visualized by silver staining. PHB2 was identified by mass spectrometry. Asterisks represent heavy and light chains of IgG. (B) Reciprocal immunoprecipitation to confirm the interaction between DLK1 and PHB proteins using specific antibodies against DLK1 N-terminus, PHB2, and PHB1, respectively. Clean-Blot™ IP Detection Reagents (Thermo Scientific) were used to eliminate cross-reaction with IgG heavy and light chains. BE(2)C cells were either maintained under normal culture conditions (normoxia) or under hypoxia (1% O₂, 16 hr) conditions. (C) Intracellular co-localization of DLK1 and PHBs as revealed by confocal microscopy. BE(2)C cells were fixed in cold methanol and then stained with anti-DLK1 + anti-PHB1 or anti-DLK1+anti-PHB2 as described in Materials and Methods. Nuclei were counterstained with To-PRO3. Images were acquired using a Zeiss LSM 510 Meta confocal microscope.

Figure 2. DLK1 cytoplasmic domain interacts with the PHB1-PHB2 complex

Whole-cell lysates (WCL) were prepared from ER cells overexpressing full-length DLK1 (DLK1-FL), DLK1 extracellular domain (DLK1-EC), DLK1 Y339F/S355A mutant (DLK1-DM), or DLK1 without cytoplasmic domain (DLK1-ΔIC). Expression of the different DLK1 constructs (indicated by arrows) was validated by Western blots (A). Interactions between each DLK1 construct and PHBs were examined by immunoprecipitation with anti-DLK1 N-terminus antibody (B) and anti-PHB2 antibody (C). DLK1 cytoplasmic domain interacts with PHB2 (D). HEK293 cells were transiently transfected with Flag-tagged full-length DLK1 (Flag-DLK1 FL), Flag-tagged DLK1 cytoplasmic domain (Flag-DLK1-Cyto), or the empty vector. WCL was subjected to immunoprecipitation using anti-Flag antibodies. DLK1 and PHB2 were then detected by Western blots.

Figure 3. Secreted DLK1 extracellular domain enhances DLK1-PHB interaction

(A) ER cells expressing DLK1 extracellular domain (ER-EC), ER cells with empty vector (ER-V), or BE(2)C cells were cultured for 48 hours to approximately 80% confluence and maintained in the serum-free medium for 24 hr. Secreted proteins in conditioned media (CM) were precipitated by trichloroacetic acid and then subjected to Western blot analysis. DLK1-EC was detected by anti-DLK1 N-terminus antibodies, and the WCL from BE(2)C was used as a positive control for full-length DLK1. (B) Secreted DLK1 extracellular domain enhances DLK1-PHB interaction. BE(2)C or 3T3-L1 cells were incubated overnight with a 1:1 mixture of the ER cell-conditioned medium (ER-EC or ER-V) and the fresh culture medium containing 10% FBS. WCLs from these CM-treated cells were subjected to immunoprecipitation using anti-DLK1 N-terminus antibodies. The presence of PHB2 was examined by Western blots. (C) Secreted DLK1 extracellular domain enhances clonogenicity. BE(2)C cells were plated at a clonal density (300 cells/well in 6-well plates) in a 1:1 mixture of the ER cell-conditioned medium (ER-EC or ER-V) and the fresh culture medium containing 10% FBS. After incubation for 10 days, tumor colonies were fixed and stained with Crystal Violet. (D) Colonies were counted and Clonogenic Efficiency or Plating Efficiency was calculated as # colonies/# input cells x 100 (mean $\pm$ sem, * p <0.05).

Figure 4. DLK1 modulates mitochondrial functions

(A–C) DLK1 regulates mitochondrial membrane potential. BE(2)C cells (DLK1-high) stably expressing DLK1-DM or DLK1-ΔIC (A) as well as those cells transduced with lentivirus expressing DLK1-targeting shRNA shDLK1-2 or shDLK1-6 (B) were stained with JC-1. Formation of J-aggregates (red fluorescence) indicates high mitochondrial membrane potential. The percentage of cells with J-aggregates (JC-1⁺) was calculated. Data shown are mean ± sem from three independent experiments. ***p<0.0001 versus vector control. Other fluorescence: green = GFP from viral vectors and blue = Hoechst 33342. (C) ER cells (DLK1-low) stably expression DLK1-FL or DLK1-EC were stained with JC-1, and

fluorescence intensity of the J-aggregates was measured by FACS. CCCP treatment reduces mitochondrial membrane potential. **(D)** DLK1 regulates production of reactive oxygen species (ROS). BE(2)C cells were transduced with shDLK1-2, shDLK1-4, shDLK1-6 or vector and stained with mitochondrial ROS indicator CMH₂XROS. Cells were then fixed and analyzed by FACS for CMH₂ROS fluorescence. One of the three independent experiments was shown. **(E)** Validation of DLK1 knockdown by Western blots.

Figure 5. The DLK1-PHB pathway regulates cancer cell stemness

BE(2)C cells were treated with pooled siRNA oligos specific for DLK1, PHB1, PHB2, and control (SMARTpool, Dharmacon). Efficiency of knockdown was examined by Western blots (A) and by quantitative RT-PCR (B), respectively. The qRT-PCR data were analyzed by one-way ANOVA ($p < 0.001$). For pairwise comparison, $*p < 0.01$ versus their respective siCtrl. (C) The DLK1-PHB pathway is required for maintaining self-renewal. BE(2)C cells were treated with siRNAs discussed in (A). Tumor sphere formation assay was performed by plating 5000 cells/well in polyHEMA-coated 12-well plates. Tumor cell spheres were allowed to grow for three days in the serum-free sphere medium. Data shown were mean numbers of spheres per well $\pm$ sem. Data in the left panel were first analyzed by one-way ANOVA ($p < 0.0001$, $n = 6$), followed by pairwise comparison ($*p < 0.0001$ versus siCtrl and $\#p < 0.01$ versus siPHB1 or siDLK1). Data in the right panel were analyzed by unpaired, two-tailed Student's *t*-test of siDLK1 versus siCtrl in each PHB group, $*p < 0.003$, $n = 3$. (D) The DLK1-PHB pathway is required for clonogenicity. siRNA-treated BE(2)C cells were plated at 300 cells/well in 6-well plates and were cultured for 10 days. Colonies were fixed and stained with Crystal Violet and counted. Clonogenic Efficiency = % colonies per input $\pm$ sem ($n = 6$). Data in the left panel were first analyzed by one-way ANOVA ($p < 0.0001$), followed by pairwise comparison ($*p < 0.0001$ versus siCtrl and $\#p < 0.01$ versus siPHB1 or siDLK1). Data in the right panel were also analyzed by one-way ANOVA ($p < 0.03$ for the siDLK1 group). For comparison with Vector/siCtrl, $*p < 0.04$ (Student's *t*-test).

Table 1

Primers for Quantitative Real-Time PCR

Gene	Primer Sequence	Amplicon Size
DLK1	forward: 5'-CTGAAGGTGCCATGAAAGAG-3' reverse: 5'-GCTGAAGGTGGTCATGTCGAT-3'.	273 bp
PHB1	forward: 5'-TGTCATCTTTGACCGATTCCG-3' reverse: 5'-CTGGCACATTACGTGGTCGAG-3'	125 bp
PHB2	forward: 5'-GTGCGCGAATCTGTGTTCAC-3' reverse: 5'-GATAATGGGGTACTGGAACCAAG-3'	135 bp
18S rRNA	forward: 5'-CGGACAGGATTGACAGATTG-3' reverse: 5'-CAAATCGCTCCACCACTAA-3'	83 bp