Interaction of Delta-like 1 Homolog (*Drosophila*) with Prohibitins and Its Impact on Tumor Cell Clonogenicity

Asma Begum, Qun Lin, Chenye Yu, Yuri Kim, and Zhong Yun

Abstract

Cancer stem cell characteristics, especially their self-renewal and clonogenic potentials, play an essential role in malignant progression and response to anticancer 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 the 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. 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.

Implications: This study provides a new mechanistic insight into the regulation of the stem cell characteristics of cancer cells. *Mol Cancer Res;* 12(1); 155–64. ©2013 AACR.
Materials and Methods

Plasmids

Retroviral vectors expressing DLK1-FL (full-length DLK1), DLK1-Ecto, DLK1-Δcyto (deletion of DLK1-cyto domain), and DLK1-DM (Y339F/S355A mutations in DLK1-cyto domain) have been described in our previous study (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. The Flag-tagged DLK1-cyto domain (DLK1-cyto-Flag) was cloned by in-frame fusion of the DLK1-cyto domain–coding sequence (amino acids 328-383) to the 3′ of two tandem repeats of the Flag-tag sequence in pcMV-2xFlag. The Tagged PHB1 and PHB2 were from Dr. Valerie Bosch of Deutsches Krebsforschungszentrum (DKFZ; Heidelberg, Germany; ref. 21) and subcloned into a retroviral vector. The shDLK1 constructs were described in our previous study (14), with the target sequence positions in human DLK1 mRNA (nm_003836.5) being 1062-1080 for shDLK1-2, 1308-1326 for shDLK1-4, and 1426-1444 for shDLK1-6. All clones were sequence validated.

Cell culture and transfection

Neuroblastoma cell lines SK-N-BE(2)C [abbreviated as BE(2)C] and SK-N-ER (abbreviated as ER) were maintained in minimum essential medium (MEM) and F12 (1:1) with 10% 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 GFP. MCF7 cells (American Type Culture Collection, ATCC) were cultured in MEM containing 10% FBS. The human hepatocellular cancer cell line HepG2 and human embryonic kidney cell line 293T (ATCC) were cultured in MEM containing 10% FBS. The human hepatocellular cancer cell line HepG2 and human embryonic kidney cell line 293T (ATCC) were cultured in MEM containing 10% FBS. All culture media were supplemented with 25 mmol/L HEPES (pH 7.4) 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 manufacturer’s protocol. After incubation for 48 hours, cells were then trypsinized and plated for further experiments.

Affinity pull-down by coimmunoprecipitation

Whole-cell lysates (WCL) were prepared by incubating cells expressing different DLK1 constructs or empty vector with the modified radioimmunoprecipitation assay buffer (RIPA; 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L 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. Thirty μL of recombinant protein A agarose beads were then added and incubated for an hour at 4°C. Immune complexes were eluted in 1 × SDS sample buffer and fractionated by SDS–PAGE under reducing conditions. Silver staining was done using the SilverQuest Silver Staining Kit (LC6070; Invitrogen) according to the manufacturer’s protocol.

Western blot analysis

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

Mass spectrometry

The immune complex with anti-DLK1 antibody was fractioned 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 (Boston, MA).

Trichloroacetic acid precipitation

About 4 mL of 24-hour conditioned medium was mixed with 1 mL of trichloroacetic acid [TCA; 100% (w/v)] for 1 to 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 three times, protein pellets were dried in a 95°C heat block for 3 minutes and then solubilized in 1 × SDS sample buffer. Secreted DLK1 in conditioned media was analyzed using Western blot analysis.

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 three times with PBS. Nonspecific 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 (DKFZ). 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. Colocalization 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 μmol/L) 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 nmol/L) for 45 minutes at 37°C, and the reaction was stopped by washing the cells in ice-cold PBS. The cells were trypsinized and then fixed with 4% paraformaldehyde for 10 minutes at room temperature. After washing in cold PBS, fixed cells were then subjected to fluorescence-activated cell sorting (FACS; FACS DIVA; BD Biosciences). 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 precoated with polyhydroxyethylmethacrylate (polyHEMA; Sigma-Aldrich) as described in our previous publication (14). After incubation for 4 to 6 days, tumor spheres were counted under the microscope.

Clonogenic assays
Tumor cells were plated at a clonal density (<1 cell/mm²) in 6-well plates and incubated undisturbed for 10 to 14 days. Colonies were stained with Crystal Violet. Plating efficiency = number of colonies (≥50 cells/colony) per input cells × 100%.

Real-time reverse transcription 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 minutes, 40 cycles at 95°C × 15 seconds, and 60°C × 60 seconds. Of note, 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.

Table 1. Primers for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Amplicon size</th>
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<tr>
<td>DLK1</td>
<td>Forward: 5'-CTGAAGGTGCTCCATGAAAGAG-3'</td>
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<tr>
<td></td>
<td>Reverse: 5'-GCTGAGGTGCTGATCGAT-3'</td>
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<td>PHB1</td>
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<td>18S rRNA</td>
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<td>83 bp</td>
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<tr>
<td></td>
<td>Reverse: 5'-CAAATGCGTCCACCAAAT-3'</td>
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Statistical analysis
Statistical differences between two groups were analyzed using the two-tailed, unpaired Student t test. Comparison among ≥3 groups was done using one-way ANOVA (Prism Software; GraphPad 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 coimmunoprecipitation to identify proteins that interact with DLK1 (Fig. 1A). Upon electrophoresis separation and mass spectrometry, we identified 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 coimmunoprecipitation, we found that DLK1 can interact with both PHB1 and PHB2 under either normoxic or hypoxic (1% O₂) conditions (Fig. 1B). It is worth noting that only a small portion of DLK1 is coimmunoprecipitated with PHB1 or PHB2, compared with the levels of DLK1 in whole-cell extract. Consistent with this observation, confocal microscopy analysis showed a low level (<1%) of colocalization between DLK1 and PHBs (Fig. 1C). A similarly low level of colocalization 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 nontumor cell line 3T3-L1 (Fig. 3), HepG2 human hepatocellular carcinoma cells and primary human brain tumor cells (Supplementary Fig. S1), 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-cyto domain is involved in interaction with PHBs. Toward this end, we ectopically expressed (Fig. 2A) DLK1-FL, DLK1 extracellular domain (DLK1-EC), DLK1 without its intracellular domain (DLK1-DIC), and DLK1 with mutations of two putative phosphorylation sites (tyrosine 339-to-phenoalanine 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 Fig. 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-DIC. Consistent with this observation, anti-PHB2 antibodies failed to pull-down DLK1 when DLK1-DM or DLK1-DIC was overexpressed (Fig. 2C). To determine whether the DLK1-cyto domain directly interacts with PHBs, we ectopically expressed Flag-tagged DLK1 full-length (Flag-DLK1-FL) or DLK1-cyto domain in 293T cells and found that the Flag-tagged DLK1-Cyto, in addition to Flag-DLK1-FL, coimmunoprecipitates with PHB2 (Fig. 2D). Collectively, these data strongly indicate that DLK1-cyto 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.

**Figure 1.** DLK1 interacts with the PHB1–PHB2 complex: A, WCLs 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. The 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 hours) conditions. C, intracellular colocalization 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.
Endogenous DLK1 expression was knocked down in BE(2)C cells (Supplementary Fig. S2). Interestingly, when the endogenous DLK1-expressing BE(2)C cells had much fewer J-aggregates potential (25, 26). Here, we observed that the high DLK1-expressing BE(2)C cells with high membrane potential using JC-1, a mitochondrial membrane potential indicator. Knocking down DLK1 expression in BE(2)C cells were significantly increased (Fig. 4A). Consistent with this observation, overexpression of DLK1-DM or DLK1-AIC that lacks interaction with PHBs also significantly increased the population of JC-1+ BE(2)C cells (Fig. 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 (Fig. 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 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 (Fig. 4D and E), indicating increased mitochondrial ROS production. This result is consistent with increased numbers of JC-1+ cells with high mitochondrial membrane potential upon DLK1 knockdown (Fig. 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.
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 (Fig. 5A and 5C), indicating reduced self-renewal potential. Although 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 (Fig. 5D). Similar observations were also found in the hepatocellular carcinoma HepG2 cells and human breast cancer MCF7 cells (Supplementary Figs. S3 and S4). 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 (Fig. 5C and Supplementary Fig. S5) 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 seems to negatively affect tumor sphere formation. The clonogenic potential of the siDLK1–treated cells was also partially improved by ectopically expressed PHB1 or PHB2 (Fig. 5D and Supplementary Fig. S5). In contrast, DLK1 ectopic expression did not seem 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 (Fig. 5A and 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 (Fig. 5A, lanes 4 and 5). In contrast, siDLK1 did not decrease the levels of PHB1 or PHB2 (Fig. 5A, lanes 1–3). Furthermore, the DLK1–cyto domain, despite its interaction with PHBs, did not affect PHB1 and PHB2 protein expression (Supplementary Fig. S6). Because neither siPHB1 nor siPHB2 negatively affected DLK1 mRNA levels (Fig. 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 posttranslational mechanisms.
DLK1-PHB Interaction and Cancer Cell Stemness.

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.

![Figure 4](image_url). 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. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) treatment reduces mitochondrial membrane potential. D, DLK1 regulates production of 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 CMH2XROS fluorescence. One of the three independent experiments was shown. E, validation of DLK1 knockdown by Western blot analysis.
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-cyto domain is required for interaction

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), respectively. Efficiency of knockdown was examined by Western blot analysis (A) and quantitative RT-PCR (B), respectively. The quantitative RT-PCR data were analyzed by one-way ANOVA ($P < 0.001$). For pairwise comparison, $^\wedge$, $P < 0.01$ 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 5,000 cells per well in polyHEMA-coated 12-well plates. Tumor cell spheres were allowed to grow for 3 days in the serum-free sphere medium. Data shown were mean numbers of spheres per well ± SEM. Data in the left were first analyzed by one-way ANOVA ($P < 0.0001$; $n = 6$), followed by pairwise comparison ($^\wedge$, $P < 0.0001$ vs. siCtrl and $^\#$, $P < 0.01$ vs. siPHB1 or siDLK1). Data on the right were analyzed by an unpaired, two-tailed Student t test of siDLK1 versus siCtrl in each PHB group; $^\wedge$, $P < 0.003$; $n = 3$. D, the DLK1–PHB pathway is required for clonogenicity. siRNA-treated BE(2)C cells were plated at 300 cells per well in 6-well plates and were cultured for 10 days. Colonies were fixed and stained with Crystal Violet and counted. Clonogenic efficiency = percentage of colonies per input ± SEM ($n = 6$). Data on the left were first analyzed by one-way ANOVA ($P < 0.0001$), followed by pairwise comparison ($^\wedge$, $P < 0.0001$ vs. siCtrl and $^\#$, $P < 0.01$ vs. siPHB1 or siDLK1). Data on the right were also analyzed by one-way ANOVA ($P < 0.03$ for the siDLK1 group). For comparison with vector/siCtrl, $^\wedge$, $P < 0.04$ (Student t test).
with PHBs, the two dominant-negative mutants, DLK1-DIC 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 downregulation 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 seem to function downstream of DLK1 because ectopic expression of PHB1 or PHB2 can, to different extents, rescue the siDLK1-dependent decrease 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 pleotropic 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 because 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-cyto 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 protumorigenic role of DLK1 and PHBs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: Z. Yun
Development of methodology: A. Begum, Q. Lin, Y. Kim, Z. Yun
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Begum, Q. Lin, C. Yu, Z. Yun
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Begum, Q. Lin, Z. Yun
Writing, review, and/or revision of the manuscript: A. Begum, Q. Lin, Z. Yun
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Yun
Study supervision: Q. Lin, Z. Yun

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