

Hypoxia-Regulated Delta-like 1 Homologue Enhances Cancer Cell Stemness and Tumorigenicity

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Abstract

Reduced oxygenation, or hypoxia, inhibits differentiation and facilitates stem cell maintenance. Hypoxia commonly occurs in solid tumors and promotes malignant progression. Hypoxic tumors are aggressive and exhibit stem cell-like characteristics. It remains unclear, however, whether and how hypoxia regulates cancer cell differentiation and maintains cancer cell stemness. Here, we show that hypoxia increases the expression of the stem cell gene *DLK1*, or *delta-like 1 homologue (Drosophila)*, in neuronal tumor cells. Inhibition of *DLK1* enhances spontaneous differentiation, decreases clonogenicity, and reduces *in vivo* tumor growth. Overexpression of *DLK1* inhibits differentiation and enhances tumorigenic potentials. We further show that the *DLK1* cytoplasmic domain, especially Tyrosine339 and Serine355, is required for maintaining both clonogenicity and tumorigenicity. Because elevated *DLK1* expression is found in many tumor types, our observations suggest that hypoxia and *DLK1* may constitute an important stem cell pathway for the regulation of cancer stem cell-like functionality and tumorigenicity. [Cancer Res 2009;69(24):9271–80]

Introduction

How a tumor is initiated, sustains its growth, and progresses toward malignancy remains an ardently pursued topic. The cancer stem cell model posits that tumor growth is sustained by a small population of cancer cells that, like normal stem cells, are capable of self-renewal and differentiation (1). In contrast, the stochastic or clonal evolution model states that most of the tumor cells within a growing tumor mass are capable of self-renewal and that heterogeneity results from interclonal variations (2). For the most part, the majority of tumors, especially solid tumors, may be more appropriately explained by a combination of these two prevailing models (2). In either case, the tumor microenvironment may have profound impact on how cancer stem cells are maintained or how subclones with growth and survival advantages evolve and are selected. Currently, little is known about the role of the tumor microenvironment on the maintenance of stem cell characteristics, the cell-fate decision, and tumorigenic potential of poorly differentiated cancer cells.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Solid tumors contain regions with oxygen deficiency or hypoxia, and tumor hypoxia predicts poor clinical outcomes (3). Hypoxic tumor cells appear to be highly tumorigenic and poorly differentiated with expression of stem cell markers (4, 5). Expression of hypoxia-inducible factor (HIF)-1 α is elevated in poorly differentiated pancreatic cancers (6). HIF-2 α appears to be preferentially expressed in stem cell-like populations in neuroblastoma (NB) and brain tumors (7, 8). These observations reveal a strong correlation between the hypoxia pathways and cancer stem cell characteristics. We and others have shown that hypoxia can inhibit differentiation of embryonic stem cells and progenitor cells (9–12). Interestingly, we found (11) that hypoxia arrested adipogenic progenitor cells in an undifferentiated state and increased expression of *DLK1*, or *delta-like 1 homologue (Drosophila)*, a type I transmembrane protein preferentially expressed in embryonic tissues and immature cells (13). Elevated expression of *DLK1* is also reported in several tumor types, especially neuronal tumors such as NB and glioma (14–19). However, the role of *DLK1* in tumor development or progression remains to be examined. Using NB cells as a model, we show that expression of *DLK1* is increased by hypoxia and plays an essential role in the maintenance of the undifferentiated NB phenotype, their clonogenic potential, and their tumorigenicity *in vivo*. This previously unknown hypoxia-regulated stem cell pathway may have the potential to regulate the maintenance of cancer stem cell characteristics and to promote tumor progression.

Materials and Methods

Cell culture and hypoxia. SK-N-BE(2)C [BE(2)C], SK-N-ER (ER), and SH-SY5Y (SY5Y) cells were maintained in MEM and F12 (1:1), and IMR32 cells, in MEM, supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, and 25 mmol/L HEPES at pH 7.4. Retroviral infection was described previously (11, 12). All-*trans*-retinoic acid (RA) or bromodeoxyuridine (BrdUrd) was used to induce differentiation. Hypoxia (1% O₂) experiments were performed in a hypoxia chamber (Invivo₂ 400, Ruskinn Technology) and anoxia experiments were performed in a Bactron Anaerobic Chamber (Sheldon MFG, Inc.). Deferoxamine mesylate (Sigma-Aldrich) was used to mimic hypoxia effects at 21% O₂ (11, 12). Culture media were replaced every other day inside the hypoxia chamber.

Tumor sphere culture. Cells were plated at 30,000 cells/mL in the sphere medium (see Supplementary Materials and Methods for details). To facilitate sphere formation, tissue culture dishes were coated with polyhydroxyethylmethacrylate (polyHEMA; Sigma-Aldrich) as described in Supplementary Materials and Methods. Cells were dissociated by repeated pipetting or with 0.05% trypsin to obtain single-cell suspension for passages or for cell counting.

Plasmids. The retroviral *DLK-FL* (full-length *DLK1*) and *DLK-ΔCyto* (without the cytoplasmic domain) constructs containing the *GFP* gene were provided by Dr. R. Bhatia (City of Hope National Medical Center, Duarte, CA; ref. 19). The mutations in the *DLK1* cytoplasmic domain, Y339F, S355A, and Y339F/S355A, were created using the QuikChange site-directed mutagenesis kit (Stratagene). The constitutively active HIF-1 α mutant (P402G/P564A) was described previously (20). The constitutively active HIF-2 α mutant

(Pro531A) was obtained from Dr. F. Lee (University of Pennsylvania School of Medicine, Philadelphia, PA; ref. 21) and was subcloned into the pLZRS retroviral vector.

The lentiviral short hairpin RNA (shRNA) constructs (shDLK-2H and shDLK-4H) were cloned into CS-CDF-EG-PRE-K1f (22), and the siRNA oligonucleotides (siDLK-05 and siFLK-07) were from Dharmacon. Detailed cloning information and nucleotide sequences are shown in Supplementary Materials and Methods.

Real-time reverse transcription-PCR. First-strand cDNA was synthesized from total RNA. Real-time PCR was performed on StepOne Plus (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's recommended protocol. The primer sequences can be found in Supplementary Materials and Methods.

Chromosome immunoprecipitation. BE(2)C cells were incubated at 1% O₂ for 16 to 18 h and were used for chromosome immunoprecipitation (ChIP) according to our previously published protocol (20). The detailed procedure and ChIP primer sequences can be found in Supplementary Materials and Methods.

Northern blot. Total RNA was fractionated in 1% agarose gel and hybridized at 65°C overnight in Church's Buffer with an [α -³²P] dCTP-labeled DLK1/pref-1 cDNA probe prepared from pCMV-Sport6.1-pref-1 (IMAGE 6393667). The radioactive blots were visualized on a Storm 860 Phosphor-Imager (GE Healthcare).

Western blot. Western blot was done as described previously (11) with the following antibodies: polyclonal rabbit anti-DLK1 (1:3,000; Chemicon International); anti-Sox2 (1:1,000; Chemicon International); c-kit (1:500; Zymed Laboratories); CD-133 (1:100; Abgent.); Notch1 (1:2,000), HIF-1 α (1:2,000), and HIF-2 α (1:1,000; Novus Biologicals); proliferating cell nuclear antigen (PCNA; 1:500; Santa Cruz Biotechnology); and phospho-extracellular signal-regulated kinase (ERK) and total ERK (1:1,000; Cell Signaling), or β -actin (1:20,000; Sigma-Aldrich).

Cell proliferation assay (MTS). BE(2)C cells were transduced with lentivirus for 48 h and then reseeded at a density of 2×10^4 cells per well in 96-well plates. Cell growth was then analyzed every day using the MTS assay (Promega).

Immunofluorescence. Cells were fixed for 10 min with -20°C methanol and then incubated with mouse anti-DLK1 (1:100; R&D Systems) or rabbit anti- β -tubulin III (1:100; Sigma-Aldrich), followed by incubation with Alexa 594-conjugated donkey-anti-mouse (1:500; Invitrogen) or Alexa 488-conjugated goat-anti-rabbit (1:500; Invitrogen). Hoechst 33342 (2 μ g/mL) was used for nuclear staining.

Clonogenic assays. NB cells were plated at 300 to 500 cells per well in six-well plates for 10 to 14 d, and colonies were stained with crystal violet. Plating efficiency = number of colonies (≥ 50 cells per colony) per input cells $\times 100\%$.

Tumor xenografts. NB cells were harvested by trypsinization, resuspended in cold PBS, then mixed with an equal volume of Matrigel (BD Bioscience), and then injected (100 μ L/site) s.c. on both sides of the anterior backs of a group of five *NU/J* mice (6-wk-old males; Jackson Laboratory). Tumor growth was monitored twice weekly. Tumor take was recorded when a palpable tumor was detected. Tumor size was measured using a precision caliper as the longest surface length (mm; *L*) and width (*W*). Tumor volume (*V*; mm³) = $LW^2/2$ (23). All protocols were reviewed and approved by the Yale Institutional Animal Care and Use Committee.

Statistics. The statistical difference between two groups was analyzed by the two-tailed, unpaired Student's *t* test using Prism 3.0 (GraphPad Software, Inc.). All results are expressed as mean \pm SEM. For statistical analysis of tumor growth, the general statistical model used is:

$$\log(\text{volume}) = \alpha + \beta \log(t)$$

where α and β are constants, and *t* is the elapsed time. Large values of α correspond to uniformly larger tumor sizes across all dates. The slope β measures the rate of growth on this log scale. Statistical analysis was performed using linear regression in time.

Results

Hypoxia increases DLK1 expression. DLK1 was strongly increased at both mRNA and protein levels (Supplementary Fig. S1A and Fig. 1A) either at 1% O₂ (hypoxia) or in the presence of deferoxamine, a hypoxia-mimicking compound in a panel of NB cell lines with *MYCN* amplification [BE(2)C and IMR32] or without *MYCN* amplification (ER and SY5Y). DLK1 expression can also be induced under anoxia (Supplementary Fig. S1B). We chose 1% O₂ because it is close to the pO₂ threshold (5–10 mmHg or 0.7–1.3% O₂) widely used in clinical studies to define tumor hypoxia *in vivo* (3). We further found that hypoxia also increased DLK1 expression in glioblastoma cells as well as normal neuronal progenitor cells (Supplementary Fig. S1B and C), suggesting a common regulatory mechanism for *DLK1* expression by hypoxia in neuronal progenitor cells and neuronal tumors. DLK1 is predominantly expressed as a full-length protein with a relative motility of around 50 kDa (Fig. 1A, left). An ~45 kDa form of DLK1 was found in ER cells (Fig. 1A, lanes 5–6), likely due to the enzymatic cleavage of its extracellular domains (24).

Hypoxia induced the expression of HIF-1 α protein in all the NB cell lines (Fig. 1A, left). Other hypoxia-inducible genes, such as vascular endothelial growth factor (VEGF) and glucose transporter 1 (GLUT1), were also induced by hypoxia (Supplementary Fig. S2A and B). However, only BE(2)C and SY5Y cells expressed HIF-2 α (Supplementary Fig. S2C and D), and the HIF-2 α expression was independent of the *MYCN* status. It is likely that a combination of HIF- α expression and the *MYCN* status could potentially explain different levels of DLK1 expression in NB cells.

Importantly, HIF-1 α or HIF-2 α alone is sufficient to enhance DLK1 expression (Fig. 1B, left), whereas deletion of both HIF-1 α and HIF-2 α is needed to abolish hypoxia-dependent increase of DLK1 expression (Fig. 1B, right). The proximal 5' promoter/enhancer region of the *DLK1* gene contains three putative hypoxia responsive elements (HREs) with the conserved motif of 5'-ACGTG-3' (25) at -758, -402, and -248 bp, respectively. To ascertain HIF binding by ChIP assays, we designed two sets of primers flanking the HRE at -758 bp (DLK1-3D) and -248 bp (DLK1-4P), respectively. Both HIF-1 α and HIF-2 α were coimmunoprecipitated with this proximal *DLK1* promoter/enhancer region in hypoxia-treated cells as determined by amplicons with the predicted sizes (Fig. 1C, left) and by qRT-PCR (Fig. 1C, right). No promoter binding was found using the control IgG. Neither anti-HIF-1 α nor anti-HIF-2 α antibody precipitated the *RAD51* promoter, a HIF-independent gene (26), further demonstrating the specificity of HIFs toward the *DLK1* promoter. Consistent with the literature (7, 27), HIF-1 α appeared to bind the *VEGFA* promoter more efficiently than HIF-2 α did. These data clearly show that hypoxia increases *DLK1* transcription via the HIF-dependent mechanism.

DLK1 facilitates the maintenance of an undifferentiated NB phenotype. We focused on BE(2)C cells because they have a high level of DLK1 expression (Fig. 1A) and possess cancer stem cell characteristics (28, 29). We found that *DLK1* mRNA (Fig. 1D, lane 3) and protein (Fig. 1D, lane 7) were strongly decreased upon RA-induced differentiation. Interestingly, *DLK1* expression was maintained by hypoxia (1% O₂) even in the presence of RA (Fig. 1D, lanes 4 and 8). This result was consistent with the observation that hypoxia decreased RA-induced neurite growth, a hallmark of neuronal differentiation (Supplementary Fig. S3). In addition to RA, BrdUrd, an inducer of non-neuronal differentiation (28, 29), also dramatically decreased DLK1 expression as

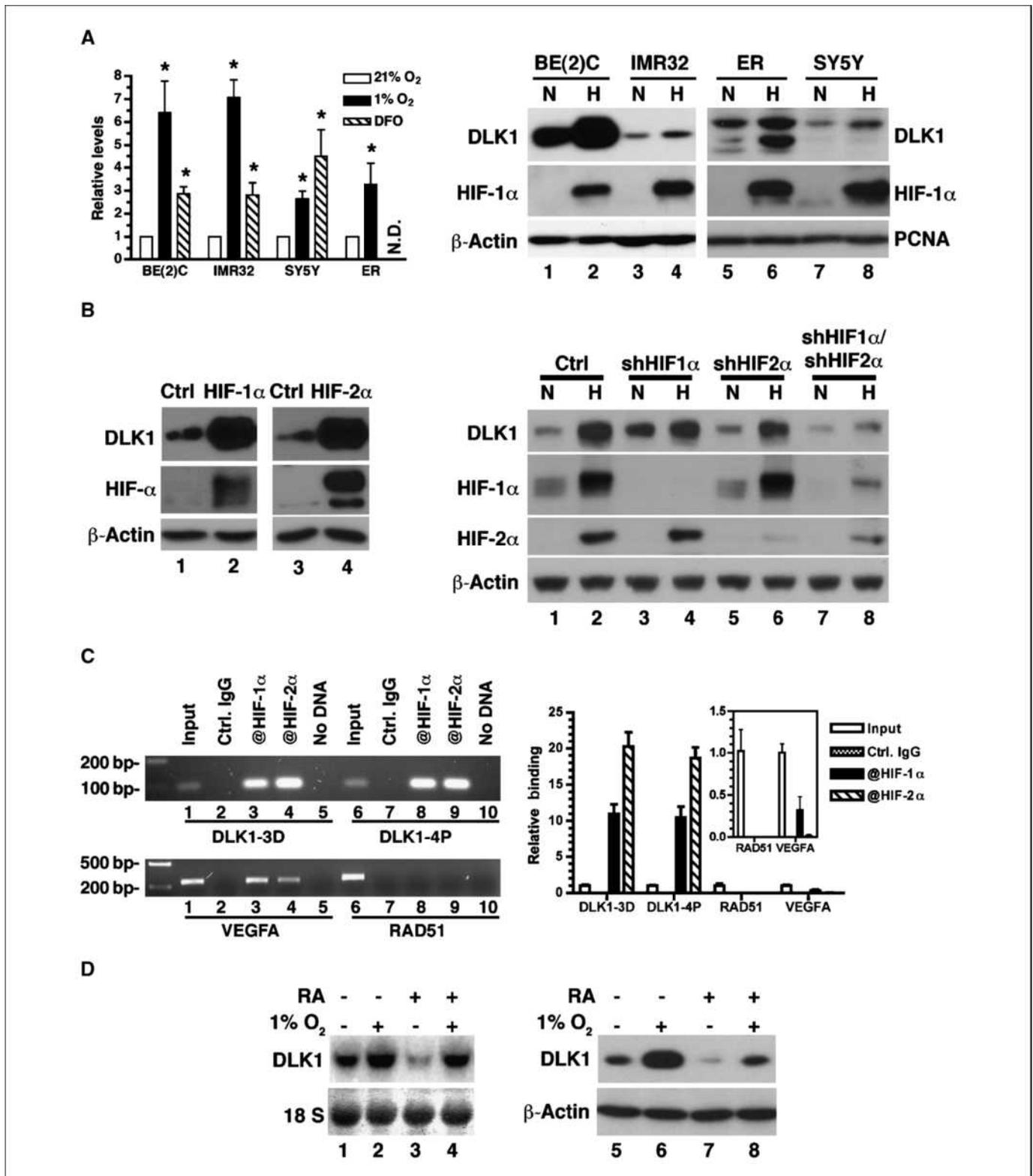


Figure 1. Hypoxia induces DLK1 expression. **A**, quantitative reverse transcription-PCR (left) for *DLK1* mRNA. Total RNA was prepared from NB cells after 16 h of incubation at 1%, 21% O₂, or in the presence of 100 μmol/L deferoxamine. Data were normalized to *HPRT* mRNA that did not change under hypoxia (columns, mean from ≥3 experiments; bars, SEM; *, *P* < 0.05). **B**, Western blot for DLK1 (right) with HIF-1α as a control for hypoxia and β-actin or PCNA as a loading control. *N.D.*, not determined. **C**, ChIP using @HIF-1α or @HIF-2α antibodies. BE(2)C cells were incubated at 1% O₂ for 16 h to induce the HIF-α subunits. Two different sets of primers (DLK1-3D and DLK1-4P) were used to detect the *DLK1* promoter gene with *RAD51* primers as negative control and *VEGFA* primers as positive control. Amplicons with the predicted sizes were validated using the end-point PCR for 28 cycles (left). Relative levels of promoter binding were analyzed by qRT-PCR (right). **D**, Northern (left) and Western blot (right) for DLK1 in BE(2)C cells cultured at 1% (+) or 21% (-) O₂ with or without 1 μmol/L RA for 24 h.

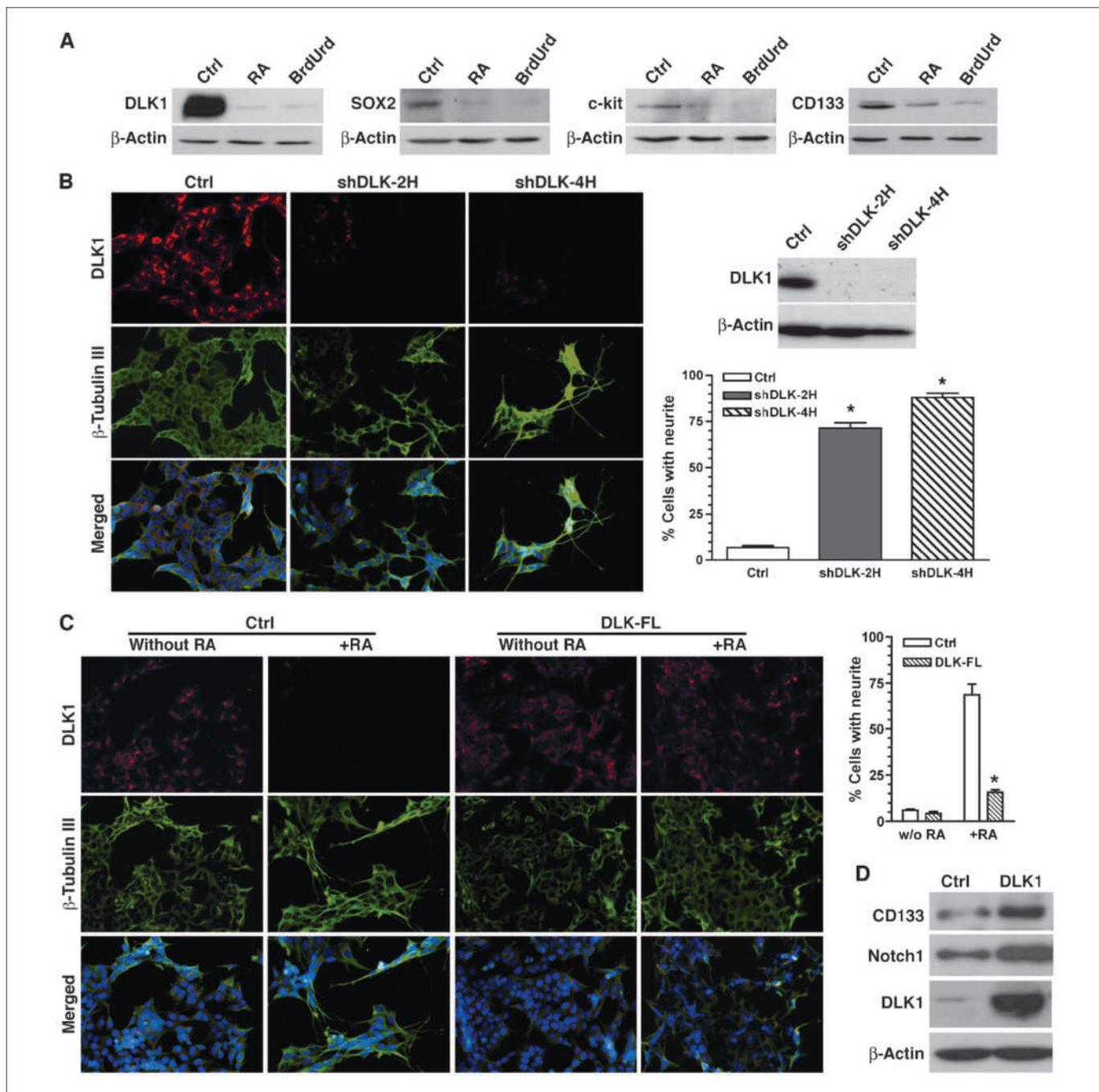


Figure 2. DLK1 maintains an undifferentiated NB phenotype. *A*, BE(2)C cells were differentiated with 1 μ Mol/L RA or 10 μ Mol/L BrdUrd for 5 d. DLK1, Sox2, c-kit, and CD-133 were detected by Western blot. *B*, BE(2)C cells were infected with lentivirus expressing shDLK-2H, shDLK-4H, or empty lentiviral vector (*ctrl*). *Left*, Infected cells were stained using antibodies against DLK1 (red) or neuron-specific β -tubulin III (green) with Hoechst 33342 as nuclear stain (blue). Magnification, $\times 200$. *Right*, expression of DLK1 was analyzed by Western blot, and cells with β -tubulin III-positive neurites were enumerated from five to six random fields (columns, mean; bars, SEM; *, $P < 0.0001$ versus control). *C*, *left*, fluorescence-activated cell sorting (FACS)-selected BE(2)C cells expressing the full-length DLK1 (DLK-FL) or empty retroviral vector (*ctrl*) were cultured for 3 d with or without 1 nmol/L RA and then stained as in *B*. Magnification, $\times 200$. *Right*, neurite-positive cells were counted from five to six random fields (columns, mean; bars, SEM; *, $P = 0.0001$ versus RA-treated control). *D*, Western blot for CD133 and Notch1 in ER cells expressing the full-length DLK1 with the empty retroviral vector as control (*ctrl*).

shown by indirect immunofluorescence (Supplementary Fig. S4A). We further found (Fig. 2A) that DLK1 expression was highly correlated with the expression of other neuronal stem cell markers including c-kit, CD-133, and SOX2 (29, 30). These observations show that DLK1 is a bona fide stem/progenitor cell marker for immature NB cells.

To examine the role of *DLK1* in NB cell differentiation, we designed two lentivirus-based shRNA interference constructs, shDLK-2H and shDLK-4H, which target two separate regions of *DLK1* mRNA. Both shDLK constructs inhibited expression of endogenous *DLK1* in BE(2)C cells (Fig. 2B), although shDLK-4H appeared to be more effective (up to 2-fold more efficient based

on qRT-PCR analysis) than shDLK-2H in decreasing *DLK1* mRNA. Interestingly, BE(2)C cells with *DLK1* knockdown spontaneously developed neuronal processes under normal culture conditions, as revealed by immunofluorescence of the neuron-specific β -tubulin III (Fig. 2B). Spontaneous differentiation was observed only in cells expressing shDLK-2H or shDLK-4H but not in control-infected cells (Supplementary Fig. S4B). We further confirmed this observation using two separate siRNA oligonucleotides (Supplementary Fig. S4C). We also found that expression of the sympathetic neuronal peptide neurotransmitter gene neuropeptide tyrosine (*NPY*; ref. 5) was increased in BE(2)C cells either treated with RA or transduced with shDLK-4H (Supplementary Fig. S5A and B). However, expression of *HASH-1* (*ASCL1*), a gene involved in early sympathetic lineage commitment (5), was decreased during RA-induced differentiation (Supplementary Fig. S5A), but not upon transduction of the lentiviral shDLK-4H (Supplementary Fig. S5B), suggesting nonoverlapping pathways may be involved between RA- and shDLK-induced differentiation. These results clearly show that loss of *DLK1* predisposes NB cells to spontaneous differentiation.

On the other hand, overexpression of DLK-FL significantly suppressed RA-induced neurite formation ($P < 0.0001$ versus RA-treated control; Fig. 2C) and resulted in increased levels of the stem cell markers CD133 and Notch1 (Fig. 2D), suggesting enhanced stem cell characteristics. Collectively, these data suggest a critical role of *DLK1* in the maintenance of undifferentiated NB cell phenotype.

In addition, we found that DLK1 may have an impact on other cytoplasmic pathways actively involved in neural stem cells including epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), and Notch (31–36). Levels of EGFR, FGFR1, and Notch were strongly reduced (Supplementary Fig. S6A, C, and E) in cells treated with RA or BrdUrd, as well as *DLK1* knockdown (Supplementary Fig. S6B, D, and F). On the other hand, ERK phosphorylation was strongly increased in BE(2)C cells treated by RA or BrdUrd (Supplementary Fig. S6G), as well as by shRNA (Supplementary Fig. S6H), which is consistent with increased ERK phosphorylation during differentiation of neuronal progenitor cells (37). Together, these observations show that multiple signaling pathways are potentially involved during shDLK-induced spontaneous differentiation of NB cells. However, it remains to be determined

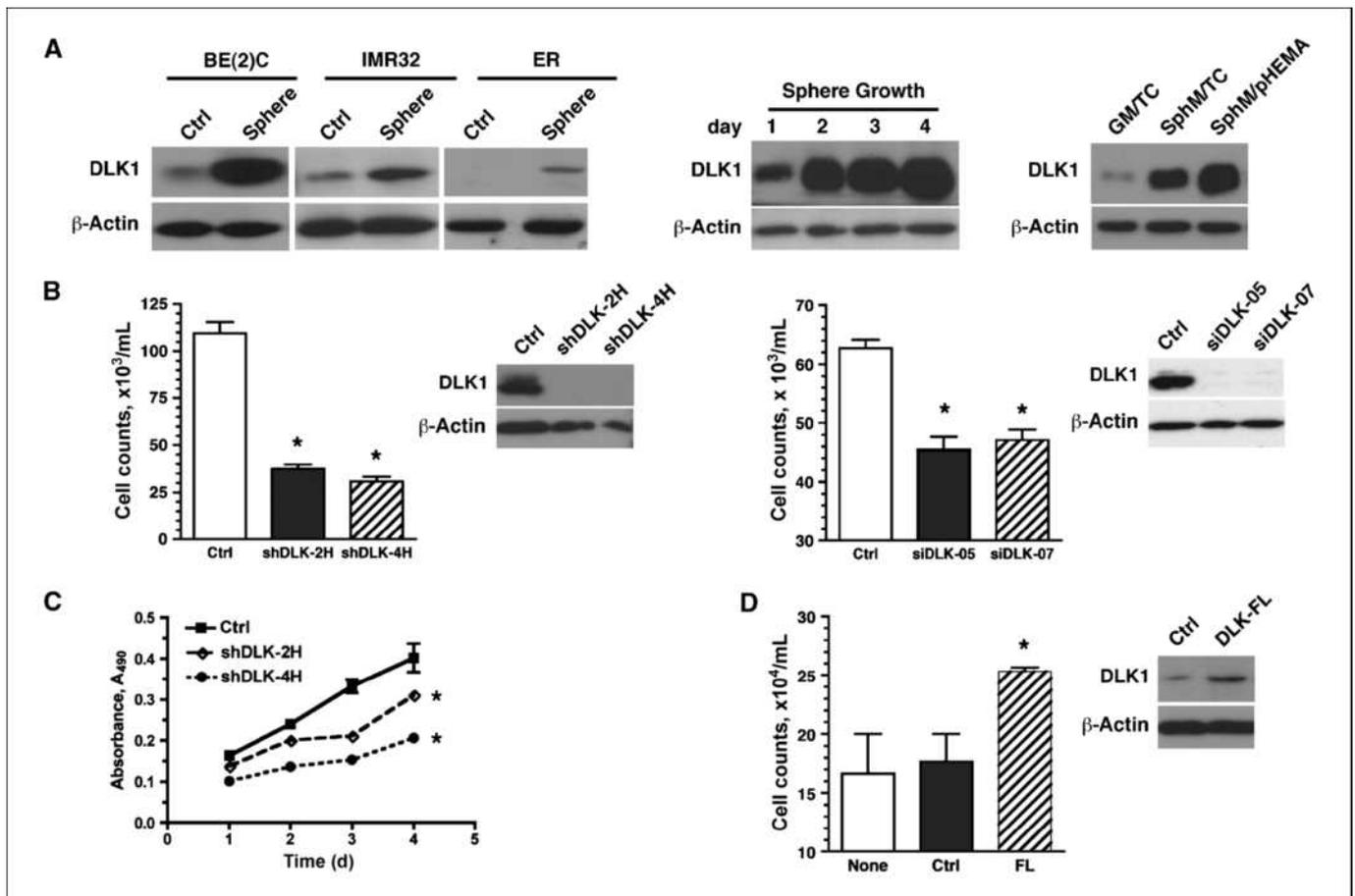


Figure 3. DLK1 regulates tumor sphere formation. *A, left*, DLK1 expression in NB cell lines cultured in sphere medium (*sphere*) or in standard growth media (*ctrl*). *Middle*, kinetics of DLK1 expression during sphere formation in BE(2) cells cultured in sphere media (*SphM*) for 1 to 4 d. *Right*, DLK1 expression in BE(2) cells cultured for 2 d in tissue culture dishes with growth medium (*GM/TC*), tissue culture dishes with sphere media (*SphM/TC*), or polyHEMA-coated dishes with sphere media (*SphM/pHEMA*). *B*, BE(2)C cells were infected with lentivirus [shDLK-2H, shDLK-4H, or vector (*ctrl*); *left*] or transfected with siRNA oligos [siDLK-05, siDLK-07, or a nontargeting siRNA (*ctrl*); *right*]. Cells were plated at 48 h into polyHEMA-coated six-well plates and cultured for 4 d in sphere medium. Spheres were collected and then trypsinized for cell counting (*columns*, mean; *bars*, SEM; $n = 6$; *, $P < 0.0003$ versus control). DLK1 knockdown was confirmed by Western blot. *C*, BE(2)C cells were infected with lentivirus expressing shDLK-2H, shDLK-4H, or vector (*ctrl*). Cell growth was measured daily using the MTS assay (*points*, mean; *bars*, SEM; $n = 4$; *, $P < 0.05$ versus control, one-way ANOVA). *D*, FACS-sorted SY5Y cells [DLK-FL or empty vector (*ctrl*)] and uninfected cells (*none*) were cultured in the sphere medium for 5 d. Cells in tumor spheres were counted (*columns*, mean; *bars*, SEM; $n = 6$; *, $P < 0.04$ versus control). DLK1 expression was confirmed by Western blot.

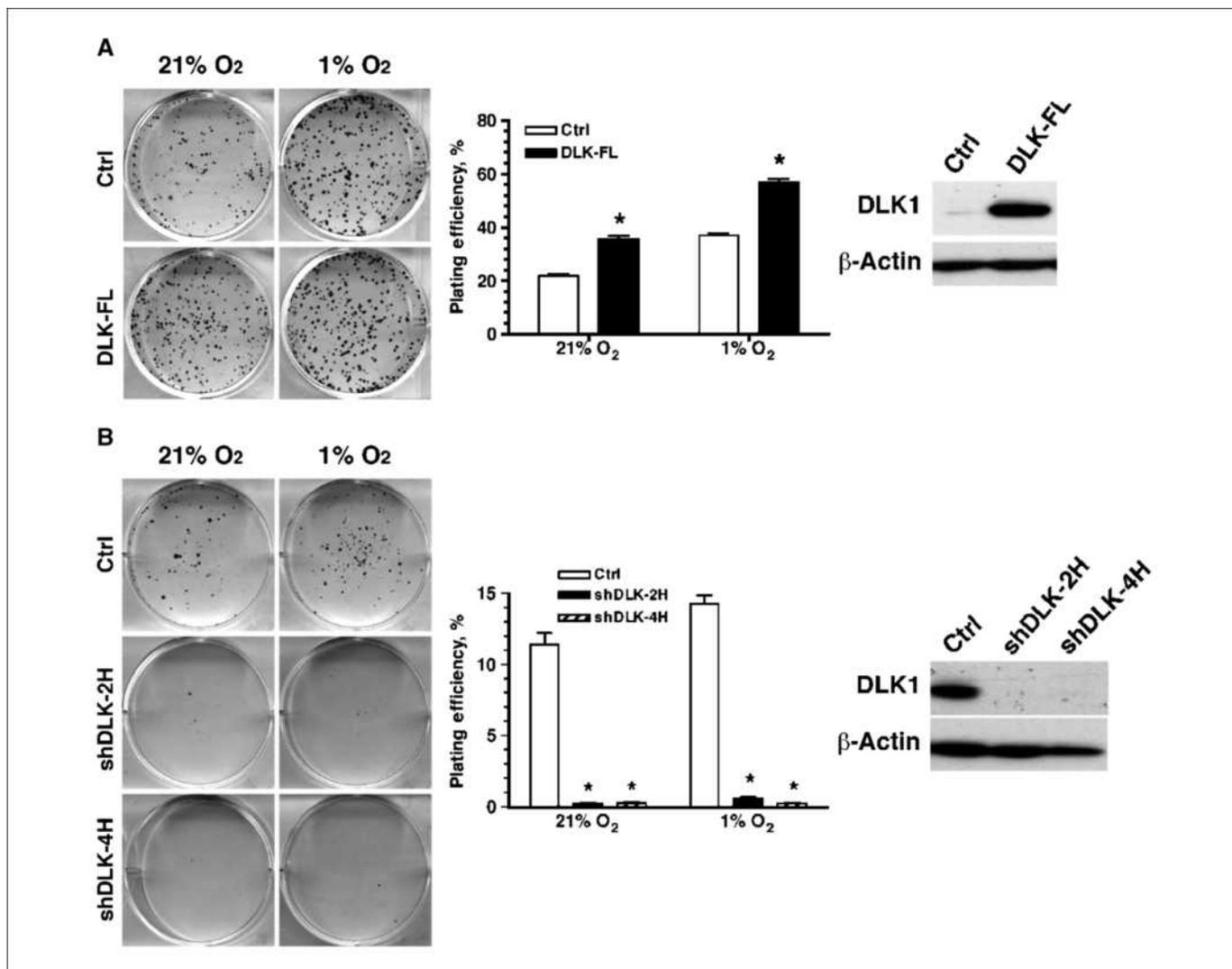


Figure 4. DLK1 regulates clonogenic growth *in vitro*. *A*, ER cells were FACS-sorted for stable expression of DLK-FL or empty vector (*ctrl*). *B*, BE(2)C cells were infected with lentivirus expression shDLK-2H, shDLK-4H, or empty vector (*ctrl*). *Left*, representative images of colonies. *Middle*, plating efficiency = % colonies per input \pm SEM ($n = 6$; *, $P < 0.0001$ versus respective control). *Right*, Western blot.

whether DLK1 directly regulates these different pathways. Furthermore, it is worth noting that elevated *NPY* expression (Supplementary Fig. S5B) and ERK phosphorylation (Supplementary Fig. S5C) still occurred in shDLK-transduced cells under hypoxia, suggesting that hypoxia is not able to completely block the shDLK-induced spontaneous differentiation.

DLK1 regulates tumor sphere formation. The ability to grow as nonadherent spheroids in the sphere medium has been widely used to assess cancer stem cell characteristics (30, 38). All four NB cell lines used in this study were able to form tumor spheres (Supplementary Fig. S7A), albeit to different degrees. DLK1 expression was strongly increased in tumor spheres compared with adherent cells maintained in the serum-containing medium (Fig. 3A, *left*). When plated in tissue culture dishes, BE(2)C cells formed tumor spheres gradually in the sphere medium (Supplementary Fig. S7B). Interestingly, DLK1 expression increased as the tumor spheres grew (Fig. 3A, *middle*), further suggesting a relationship between enhanced DLK1 expression and tumor sphere formation.

To ascertain that increased *DLK1* expression results from sphere formation and not just the effects of the sphere medium, we compared DLK1 expression under three different conditions: (a) as a monolayer in tissue culture dishes with the regular growth medium (GM/TC), (b) as a mixture of adherent and nonadherent cells in tissue culture dishes with the sphere medium (*SphM/TC*), or (c) as completely nonadherent culture in polyHEMA-coated dishes with the sphere medium (*SphM/pHEMA*). As shown in Fig. 3A (*right*), the sphere medium generally enhanced DLK1 expression over the GM/TC control. More robust increases of DLK1 expression occurred under the *SphM/pHEMA* condition that forced formation of tumor spheres. We further determined whether hypoxia developed in the growing tumor spheres using the hypoxia marker pimonidazole hydrochloride (hypoxyprobe-1). Only $\leq 16\%$ cells became hypoxic in the 6-day-old tumor spheres (Supplementary Fig. S7C), suggesting that the increased DLK1 expression in tumor spheres is unlikely to be driven by hypoxia. Because the "sphere culture" condition facilitates the expansion or enrichment of stem cells and/or progenitors, NB cells growing as spheroids likely possess enhanced stem

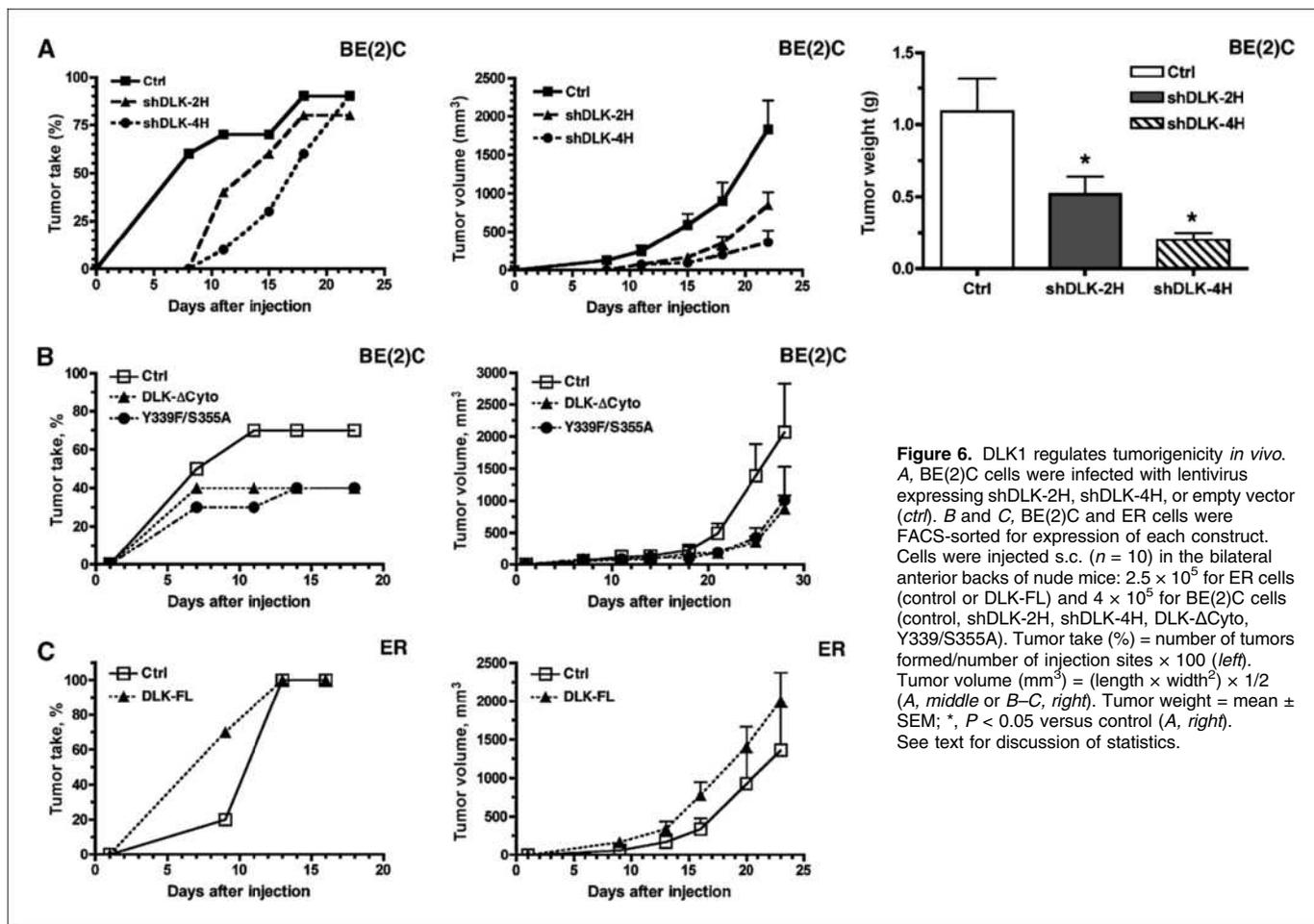


Figure 6. DLK1 regulates tumorigenicity *in vivo*. A, BE(2)C cells were infected with lentivirus expressing shDLK-2H, shDLK-4H, or empty vector (*ctrl*). B and C, BE(2)C and ER cells were FACS-sorted for expression of each construct. Cells were injected s.c. ($n = 10$) in the bilateral anterior backs of nude mice: 2.5×10^5 for ER cells (control or DLK-FL) and 4×10^5 for BE(2)C cells (control, shDLK-2H, shDLK-4H, DLK-ΔCyto, Y339F/S355A). Tumor take (%) = number of tumors formed/number of injection sites $\times 100$ (left). Tumor volume (mm^3) = (length \times width 2) $\times 1/2$ (A, middle or B–C, right). Tumor weight = mean \pm SEM; *, $P < 0.05$ versus control (A, right). See text for discussion of statistics.

(a) DLK1 cytoplasmic domain deletion (DLK-ΔCyto), (b) full-length DLK1 with a single mutation, Y339-to-phenylalanine(F) or S355-to-alanine(A), and (c) full-length DLK1 with both mutations (Y339F/S355A). BE(2)C cells with stable expression of DLK-ΔCyto, Y339F, S355A, or Y339F/S355A exhibited significantly reduced clonogenicity under both normoxia and hypoxia (Fig. 5B), as well as decreased growth of tumor spheres (Fig. 5C). These results suggest that the DLK1 cytoplasmic domain may play an essential role in facilitating clonogenic growth and tumor sphere formation. However, DLK-ΔCyto and Y339F/S355A did not appear to have significant impact on either spontaneous differentiation or cell growth under conventional tissue culture conditions. It is likely that the two DLK1 mutants exert their dominant-negative effects only under certain stress conditions, such as in the tumor sphere media or under the clonogenic conditions.

DLK1 promotes tumorigenicity *in vivo*. Using the subcutaneous xenograft assay in athymic mice, we found that BE(2)C cells with shRNA-mediated downregulation of *DLK1* expression exhibited longer tumor delay (Fig. 6A, left). Both shDLK-2H and shDLK-4H tumors were significantly smaller in volume during growth ($P < 0.002$ for each group versus control; Fig. 6A, middle) and by weight at the end of the experiment ($P < 0.05$ for each group versus control; Fig. 6A, right). Interestingly, overexpression of both DLK-ΔCyto and Y339F/S355A mutants in BE(2)C cells decreased overall tumor take rate and retarded tumor growth (Fig. 6B). Statistically, the Y339F/S355A tumors were smaller ($P = 0.044$) and grew more slowly ($P = 0.022$) than the vector control within

the first 21 days of growth. Tumor growth rate appeared to be similar during the late stage of tumor growth. The mean growth rate of the DLK-ΔCyto tumors was nearly identical to that of the Y339F/S355A tumors. However, the differences between the DLK-ΔCyto and the control group were not statistically significant, likely due to the variability among individual tumor sizes. Nonetheless, these data suggest that the DLK-ΔCyto and Y339F/S355A mutants may possess dominant-negative functions.

On the other hand, overexpression of DLK-FL in ER cells (DLK1-low) accelerated tumor take or shortened tumor latency (Fig. 6C). Tumors developed from ER cells with DLK-FL were larger ($P < 0.01$) and grew faster ($P < 0.04$) than the control cells (Fig. 6C). However, the difference in the growth rate between the DLK-FL and the control group appears to narrow after day 16. Nevertheless, results from our additional xenograft experiments also showed that DLK-FL enhanced tumorigenicity, whereas DLK-ΔCyto and Y339F/S355A decreased tumorigenicity (Supplementary Fig. S9). These results indicate that DLK1 plays an important role in the regulation of tumorigenicity *in vivo*.

Discussion

Hypoxia is an important environmental factor that seems to favor undifferentiated stem or progenitor cells, including human embryonic stem cells, neuronal, and other mesenchymal progenitor cells (9, 10, 12, 20). Jögi and colleagues (5) have shown that hypoxic NB cells acquire an immature phenotype and that hypoxia-pretreated

NB cells grow slightly faster *in vivo*. Recent genomics studies have further revealed that poorly differentiated human tumors display a gene expression signature similar to that found in normal embryonic stem cells (39) or lineage-committed progenitor cells (40). However, it remains largely unknown how stem cell genes regulate tumor progression, and how their expression is regulated by tumor microenvironment.

DLK1, a member of the *notch/delta/serrate* family, is preferentially expressed in immature cells with regenerative potentials (13, 15). *DLK1* inhibits adipogenesis (24) and also seems to regulate the differentiation of hematopoietic stem cells (17, 19) and lymphoid progenitors (41, 42). Elevated expression of *DLK1* is found in a variety of tumor cells, including NB (18), gliomas (16), small-cell lung carcinoma (43), and leukemia (17, 19). Other evidence suggests that *DLK1* may inhibit tumor cell differentiation and increase proliferation (16, 19). Nonetheless, the role of *DLK1* in tumor progression remains poorly understood.

In this report, we have shown that hypoxia increases *DLK1* expression via the HIF-dependent mechanism. *DLK1* knockdown results in enhanced spontaneous differentiation of NB cells, reduced tumor sphere growth, decreased clonogenicity *in vitro*, and decreased tumorigenicity *in vivo*. On the other hand, overexpression of *DLK1* inhibits differentiation and promotes tumorigenicity. Our data have further revealed an important role of the *DLK1* cytoplasmic domain, especially the conserved putative phosphorylation sites Y339 and S355. Consistent with our findings, Li and colleagues (19) have found that the *DLK1* cytoplasmic domain is required for blocking RA-induced differentiation of human promyelocytic HL-60 cells. Our observations have provided strong evidence establishing *DLK1* as a new class of stem cell genes that play a significant role in the regulation of cancer stem cell characteristics and tumorigenicity.

Our data suggest that *DLK1* can exert potential impact on several membrane-associated signaling pathways including EGFR, FGFR, and Notch that facilitate maintenance or self-renewal of neural stem/progenitor cells (31–36), as well as ERK phosphorylation (37). Obviously, the mechanisms of *DLK1* function likely involve multiple pathways. We cannot rule out the possibility that changes in these signaling pathways result from shDLK-induced spontaneous differentiation, rather than directly from the loss of interaction with *DLK1*. The exact mechanisms of *DLK1* function warrant further investigation.

Recent studies have shown that a small population of immature NB tumor cells is localized in a perivascular space *in vivo* and shows strong immunochemical staining for HIF-2 α (7, 44). These HIF-2 α ⁺ cells also seem to be positive for *MYCN* amplification (44). However, we have found that HIF-2 α is differentially expressed in NB cell lines, although HIF-1 α is ubiquitously expressed and does not correlate with *MYCN* amplification. It is possible that HIF-1 α and HIF-2 α are regulated by different mechanisms, especially under *in vivo* conditions. Consistent with this notion, our previous study (11) has found that HIF-2 α is expressed only in the differentiated adipocytes, but not in the progenitor cells, and is stabilized at 21% O₂. It seems that elevated HIF-2 α expression is preferentially associated with a stem cell-like population from NB tumors (7, 44) or gliomas (8). Since our data have shown that both HIF-1 α and HIF-2 α are capable of enhancing *DLK1* transcription, it will be of great interest to determine whether elevated HIF-2 α expression, especially in such perivascular niches, has an effect on *DLK1* expression *in vivo*. Our observations, together with others, support a new paradigm that hypoxia signaling promotes tumor progression by upregulation of stem cell genes including *DLK1* that facilitate the maintenance or selection of cancer cells with stem cell characteristics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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