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The proteins DLK1 and DLK2 modulate NOTCH1-dependent proliferation and oncogenic potential of human SK-MEL-2 melanoma cells ☆

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ABSTRACT

NOTCH receptors regulate cell proliferation and survival in several types of cancer cells. Depending on the cellular context, NOTCH1 can function as an oncogene or as a tumor suppressor gene. DLK1 is also involved in the regulation of cell growth and cancer, but nothing is known about the role of DLK2 in these processes. Recently, the proteins DLK1 and DLK2 have been reported to interact with NOTCH1 and to inhibit NOTCH1 activation and signaling in different cell lines. In this work, we focused on the role of DLK proteins in the control of melanoma cell growth, where NOTCH1 is known to exert an oncogenic effect. We found that human DLK proteins inhibit NOTCH signaling in SK-MEL-2 metastatic melanoma cells. Moreover, the proliferation rate of these cells was dependent upon the level of NOTCH activation and signaling as regulated by DLK proteins. In particular, high levels of NOTCH inhibition resulted in a decrease, whereas lower levels of NOTCH inhibition led to an increase in melanoma cell proliferation rates, both *in vitro* and *in vivo*. Finally, our data revealed additive NOTCH-mediated effects of DLK proteins and the γ-secretase inhibitor DAPT on cell proliferation. The data presented in this work suggest that a fine regulation of NOTCH signaling plays an important role in the control of metastatic melanoma cell proliferation. Our results open the way to new research on the role of DLK proteins as potential therapeutic tools for the treatment of human melanoma.

1. Introduction

The evolutionarily conserved NOTCH signaling pathway plays a critical role in many cell processes, including cell proliferation. In mammals, NOTCH signaling is triggered by the interaction of a membrane receptor (NOTCH1-4) with a DELTA or JAGGED canonical ligand. This induces metalloproteinase-mediated and γ-secretase-mediated cleavage of the NOTCH receptor, which releases an active NOTCH intracellular domain (NICD). NICD translocates to the nucleus and forms a complex with CBF-1/RBP-Jκ, a factor mediating the transcription of target genes, such as the HES/HEY family of transcription factors, among others [1].

NOTCH deregulation appears to exert oncogenic effects in a wide range of tumor types. However, NOTCH-dependent tumor suppressor effects have been also reported. Today it is becoming clear that the NOTCH1 receptor can act both as an oncogene and as a tumor suppressor depending on the cellular context, its level of expression, and cross-talking with other signaling pathways [2–6]. However, how activation of a single pathway gives rise to these opposite outcomes is not fully understood.

It has been reported that NOTCH1 signaling plays a dual opposite role in some skin cancers. Several studies support a tumor suppressor role of NOTCH1 signaling in non-melanoma skin cancer [5]. In contrast, active NOTCH1 is expressed in human melanoma and it is able to transform primary melanocytes *in vitro* and to promote progression of primary melanoma towards an advanced stage [7–10]. Previous studies have revealed several potential downstream pathways, including MCAM, N-cadherin, B-catenin, MAPK, and PI3K–AKT pathways, mediating the oncogenic effects of NOTCH signaling in melanoma cells [9–12]. These data support an oncogenic role for NOTCH signaling in melanoma and highlight the potential of inhibiting the NOTCH pathway as a therapeutic approach for the treatment of this prevalent tumor [13–15].

DLK1 and DLK2 are also members of the EGF-like repeat-containing family of proteins, which includes NOTCH receptors and their ligands. DLK1 functions both as a transmembrane and as a secreted protein. An increasing body of evidence indicates that both, membrane or

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secreted, forms of DLK1 interact with NOTCH1 and act as negative regulators of NOTCH activation and signaling [16,17]. Recently, it has been reported that DLK2 also interacts with NOTCH1 and inhibits NOTCH signaling [18]. DLK1 is involved in a variety of processes, including differentiation, cell proliferation and tumorigenesis [19–28]. In fact, it has been reported that DLK1 may also function both as an oncogenic and as an anti-oncogenic factor [20,29–31]. DLK2 also participates in the regulation of adipogenic differentiation, as DLK1 does [32]. The possible functional effects of DLK2 expression in tumorigenesis and cell growth are still unknown.

The role of NOTCH signaling in melanoma prompted us to analyze the effects of DLK proteins on melanoma NOTCH signaling and cell proliferation. In this study, we provide extensive evidence supporting that, in agreement with previous reports involving other cell types, DLK proteins can regulate also NOTCH signaling in melanoma cells. Interestingly, this can lead to opposite effects in metastatic melanoma growth, depending upon the final NOTCH activation levels. Our results suggest that both DLK proteins could be potentially interesting to be used, alone or together with other NOTCH inhibitors, such as DAPT, to regulate the growth of metastatic human melanoma cells by modulating NOTCH activation and signaling.

2. Materials and methods

2.1. Plasmids

The plasmid HDLK1S contains the entire human *DLK1* cDNA from plasmid HDLK1-AG1 [33]. HDLK1-AG1 was digested with *Hind*III-*Not*I restriction enzymes and the purified cDNA-containing fragment was cloned into the *Hind*III-*Not*I restriction sites of the pLNCX2 expression vector (Clontech, Palo Alto, CA, USA). Plasmid HDLK2S contains the entire human *DLK2* cDNA (MGC Full-Length clone IMAGE ID 54954558). *HDLK2* cDNA from the MGC clone was isolated by digestion with *Hind*III-*Not*I restriction enzymes and subsequently cloned into the *Hind*III-*Not*I restriction sites of pLNCX2. HDLK2aS derives from the same IMAGE clone. In this case, *HDLK2* cDNA was isolated by digestion with *Not*I-*Sal*I restriction enzymes and cloned into the *Not*I-*Sal*I restriction sites of pLNCX2 in antisense orientation.

2.2. Cell culture and transfection conditions

The human metastatic melanoma cell line SK-MEL-2 was acquired from American Type Culture Collection (HTB-68) and cultured at 37 °C in a 5% CO_2 humidified atmosphere, with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) (Lonza, Verviers, Belgium). Transfections were performed with 80% confluent cells, using a 6:2 ratio of FUGENE HD Transfection Reagent and plasmid DNA, respectively, following the manufacturer's recommendations (Roche Diagnostics GmbH, Mannheim, Germany). Stable transfectants were selected under standard culture conditions in a selective medium containing 750 μg/ml of G418 (Sigma, St. Louis, MO, USA). Conditioned media were obtained either from HDLK1S or pLNCX2 stably transfected SK-MEL-2 cells. The conditioned media were dialyzed against complete new medium by using Centricon YM-100 (Millipore, Carrigtwohill, IRL). In some cases, the cells were treated with increasing concentrations of γ-secretase inhibitor IX (DAPT) (Calbiochem, San Diego, CA, USA) or DMSO, as a control.

2.3. Cell growth assays

Cell proliferation was measured by MTT assays according to the manufacturer's protocol (Sigma). We seeded 1000 cells per well in a 96-well plate, and the medium was replaced every 2 days. In some cases, the cells were cultured in conditioned medium from either HDLK1S or pLNCX2 stably transfected SK-MEL-2 cells. To study the effects of the γ-secretase inhibitor IX (DAPT) on cell proliferation, cells were treated with the indicated concentrations of DAPT or DMSO, as a control. Proliferation assays were carried out for the days indicated in the figures.

2.4. RNA extraction and RT-qPCR

For gene expression analysis, cell monolayers were washed twice with PBS and detached with Trypsin/Versene (Lonza). Cells were then collected by centrifugation (180 ×*g*, 5 min, at 4 °C) and washed twice with PBS. Total RNA was isolated by using the RNeasy Kit (Qiagen, Chatsworth, CA, USA). Following DNase treatment (Qiagen), RNA (1 μg) was reverse-transcribed by using the cDNA RevertAid™ Minus First Strand kit (Fermentas, Madrid, Spain), according to the manufacturer's recommendations. To perform RT-qPCR, cDNA was PCR-amplified by using the SYBR-GREEN Master Mix and ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The PCR conditions used were: initial denaturation step at 95 °C, followed by 30 s at 60 °C. *GADPH* expression was used as a control to compare the C_T from the different samples. The primers used for determining the expression level of *HES1*, *HEY1*, and *HEY2* were previously described [8]. The primers used for determining the expression level of $p21^{WAF1/Cip1}$ (H_CDKN1A_1) and *cyclin D1* (H_CCND1_1) were the predesigned SYBR® Green I Primers used for RT-qPCR (Sigma): FH1_CDKN1A: 5′ CAG CAT GAC AGA TTT CTA CC 3′, as the *p21* upper primer, and RH1_CDKN1A: 5′ CAG GGT ATG TAC ATG AGG AG 3′, as the *p21* lower primer, FH1_CCND1: 5′ GCC TCT AAG ATG AAG GAG AC 3′ as the *cyclin D1* upper primer, and RH1_CCND1: 5′ CCA TTT GCA GCA GCT C 3′ as the *cyclin D1* lower primer. The primers employed for determining the expression level of *GAPDH* were: GAPDHup; 5′ CAA TGA CCC CTT CAT TGA CC 3′, as the upper primer, and GAPDHlow; 5′ GAT CTC GCT CCT GGA AGA TG 3′, as the lower primer.

2.5. Western blot

For protein expression, cell pellets were lysed in RIPA buffer and incubated at 4 °C for 30 min. Protein content of cleared lysates was quantified, and 100 μg of total protein extract or conditioned medium were loaded into 12% (w/v) SDS-PAGE gels. Western blot was performed as described previously [34] by using the following antibodies: anti-DLK1 [16], diluted 1:2000; anti-DLK2 (Abnova, Heidelberg, Germany), diluted 1:500; anti-cleaved NOTCH1 (Val 1744, Cell Signaling, Beverly, MA, USA), diluted 1:500; anti-NOTCH1 (C-20; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), diluted 1:1000: and anti-tubulin (Sigma), diluted 1:5000. Densitometry analyses of Western blot signals were made by using Quantity One 1D analysis software (Bio-Rad, Munchen, Germany).

2.6. Luciferase assays

To measure NOTCH transcriptional activity, SK-MEL-2 cells were co-transfected with pGLucWT and combinations of the *DLK1* or *DLK2* expression plasmids under study. pGlucWT was used for determining CBF1-dependent promoter activity, using luciferase as a reporter gene. pGlucWT contains the luciferase reporter gene under the control of four copies of the CBF1 recognition site sequence cloned upstream of the SV40 promoter in the vector pGL3 [34]. To analyze NOTCH basal transcriptional activity in stable transfectants, these cells were only transfected with plasmid pGLucWT and the luciferase activity was determined. To measure the inhibition of NOTCH transcriptional activity caused by DAPT, pLNCX2-transfected cells were transfected with pGLucWT and incubated in the presence of different concentrations of DAPT for 24 h, and then, the luciferase activity was measured. To normalize the data obtained, cells were also transfected with pRLTK (renilla expression plasmid). In all these luciferase assays, cells were lysed and processed using the dual luciferase kit (Dual-Luciferase

Reporter Assay System, Promega, Madison, WI, USA) 24–48 h after transfection. These assays were repeated at least three times.

2.7. Animal experiments

Female Athymic Nude-Foxn1^{nu} mice (5-week-old) were supplied by Harlan Laboratories (Barcelona, Spain). 5×10^6 SK-MEL-2 cells stably transfected with empty vector or with plasmids HDLK1S or HDLK2S were injected subcutaneously in the dorsal flanks of mice for a total of 5 mice per group. Mice were sacrificed 45 days after injection. Tumors were measured and tumor size was calculated using the following formula: length (mm) × width (mm). *In vivo* experiments were performed in accordance with Spanish and European regulations, and approved by the University of Castilla–La Mancha Animal Care and Use Committee.

2.8. Statistical analysis

Data are presented as mean ± SD of two different cell transfectants for each construct, in at least three independent assays performed by triplicate. Data were also analyzed with GraphPad and/or SPSS software to perform the two-tailed Student's t test or the Mann–Whitney U test to determine statistical significance relative to pLNCX2 (empty vector) transfected cells or non-treated cells. A P value of ≤ 0.05 was considered statistically significant (*); a P value ≤ 0.01 was considered highly statistically significant (**); and a P value of ≤ 0.001 was considered extremely statistically significant (***).

3. Results

3.1. Overexpression of human DLK1 or DLK2 proteins is associated with increased SK-MEL-2 melanoma cell growth

To study the hypothetical involvement of DLK proteins on metastatic melanoma cell proliferation, we first modified DLK1 or DLK2 expression levels in SK-MEL-2 cells, which lack endogenous *DLK1* expression, but express *DLK2*. We stably transfected SK-MEL-2 cells with the empty vector (pLNCX2), sense *DLK1* (HDLK1S), or sense or antisense *DLK2* cDNA (HDLK2S, HDLK2aS) expression constructs. A minimum of two different stable transfectants from each construct was used in all experiments. We analyzed *DLK1* and *DLK2* expression in our stable transfectants by RT-qPCR (data not shown). By using Western blot, we confirmed that SK-MEL-2 cells transfected with sense *DLK1* (HDLK1S) or *DLK2* (HDLK2S) expression constructs overexpressed DLK1 or DLK2 proteins, respectively, and that SK-MEL-2 cells stably transfected with antisense *DLK2* (HDLK2aS) showed decreased DLK2 expression levels (Fig. 1A). To test the effects of DLK protein expression on melanoma growth, we performed cell proliferation assays. Overexpression of DLK1 or DLK2 in SK-MEL-2 melanoma cells significantly enhanced their proliferation rates, whereas diminished expression of DLK2 inhibited the proliferation of these cells compared to non-transfected parental cells or empty vector-transfected cells (Fig. 1B).

Cell proliferation is tightly regulated by expression and modulation of cell cycle-dependent cyclins, CDKs, and CDK inhibitors. To explore the underlying mechanism of increased cell proliferation induced by DLK1 and DLK2 proteins, we analyzed their effects on the expression levels of the cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ and *cyclin D1*, since it has been previously reported that DLK1 modulates their expression in some cell types [25,30]. As shown in Fig. 1C, a significant decrease in $p21^{WAF1/Cip1}$ expression and an increase in *cyclin D1* expression, relative to control cells transfected with the empty vector, were detected in SK-MEL-2 cells stably transfected with HDLK1S or HDLK2S plasmids. As expected, downregulation of *DLK2* expression increased $p21^{WAF1/Cip1}$ expression and decreased *cyclin D1* expression. These results support a role of DLK proteins in melanoma cell proliferation.

Taking into consideration our data indicated that DLK proteins modulate *in vitro* cell growth of SK-MEL2 melanoma cells, we therefore analyzed whether they could affect *in vivo* SK-MEL2 melanoma growth. To investigate this, we injected in nude mice, subcutaneously, 5×10^6 SK-MEL-2 cells stably transfected with the empty vector or with plasmids HDLK1S or HDLK2S, and monitored the tumor size on the days indicated in Fig. 2. The results showed that, as expected, SK-MEL-2 cells stably transfected with the empty vector did not develop significant tumors *in vivo*. However, an increase in tumor cell growth was observed in HDLK1S or HDLK2 stable SK-MEL-2 transfectants, as compared to the control (Fig. 2A). Comparison of tumor size on day 45 after inoculation also indicated that the tumors that finally developed from DLK1 or DLK2-overexpressing melanoma cells were significantly larger than those generated by the control cells (Fig. 2B). These results demonstrate an *in vivo* oncogenic effect of DLK proteins in melanoma cells.

As DLK1 functions both as a transmembrane and as a secreted protein, we decided to analyze the effect of soluble DLK1 protein on SK-MEL-2 melanoma cell proliferation. SK-MEL-2 cells stably transfected with empty vector or with HDLK1S or HDLK2S plasmids were grown in HDLK1S conditioned medium or vector (pLNCX2) conditioned medium (Materials and methods; Fig. 3A). As expected, the proliferation rate of empty vector-transfected control cells was enhanced in the presence of DLK1-enriched medium as compared to the same cells grown in conditioned medium from control cells (Fig. 3B). However, adding DLK1-enriched conditioned medium to HDLK1S or HDLK2S stable transfectants significantly inhibited their proliferation rates (Fig. 3C and D). These results demonstrate that the levels of DLK2 and soluble or transmembrane DLK1 proteins are able to modulate melanoma cell proliferation.

3.2. DLK1 and DLK2 proteins inhibit NOTCH activation and signaling in human metastatic SK-MEL-2 melanoma cells

DLK1 and DLK2 have been reported to interact with NOTCH1 and inhibit NOTCH signaling [18]. Considering that NOTCH signaling may play an important role in melanoma progression, we studied whether human DLK1 and DLK2 also acted as NOTCH signaling inhibitors in metastatic melanoma cells. As shown in Fig. 4A, overexpression of DLK1 or DLK2 in SK-MEL-2 melanoma cells significantly inhibited NOTCH1 activation, whereas a decrease in DLK2 expression resulted in an increase of NOTCH1 activation. As a control, we treated vector-transfected cells with the γ-secretase inhibitor DAPT (10 μM) for 24 h, a treatment known to suppress NOTCH activation. In addition, we analyzed NOTCH-dependent transcriptional activity, as measured by luciferase assays, in SK-MEL-2 stably transfected with plasmids HDLK1S, HDLK2S or HDLK2aS, and transiently transfected with plasmid pGLucWT. Overexpression of either DLK1 or DLK2 resulted in the inhibition of NOTCH signaling (Fig. 4B). However, we did not detect significant changes in NOTCH activation levels in SK-MEL-2 cells with decreased levels of DLK2. We also confirmed the effects of DLK proteins on NOTCH signaling by analyzing the expression levels of the NOTCH target genes *HES1*, *HEY1* and *HEY2* by RT-qPCR (Fig. 4C). The expression of these genes by HDLK1S or HDLK2S stably transfected cells was significantly reduced, whereas their expression levels in HDLK2aS transfectants were increased, thus confirming the inhibitory effects of DLK1 and DLK2 on NOTCH signaling.

The potential effects of DLK1 or DLK2 on NOTCH activation and signaling were analyzed also by co-transfecting SK-MEL-2 cells with plasmid pGLucWT and different amounts of plasmids HDLK1S and/or HDLK2S. As shown in Fig. 5A, overexpression of DLK1 and/or DLK2 proteins decreased NOTCH signaling. Besides, overexpression of DLK1 inhibited NOTCH signaling in a dose-dependent manner. Finally, we further analyzed NOTCH transcriptional

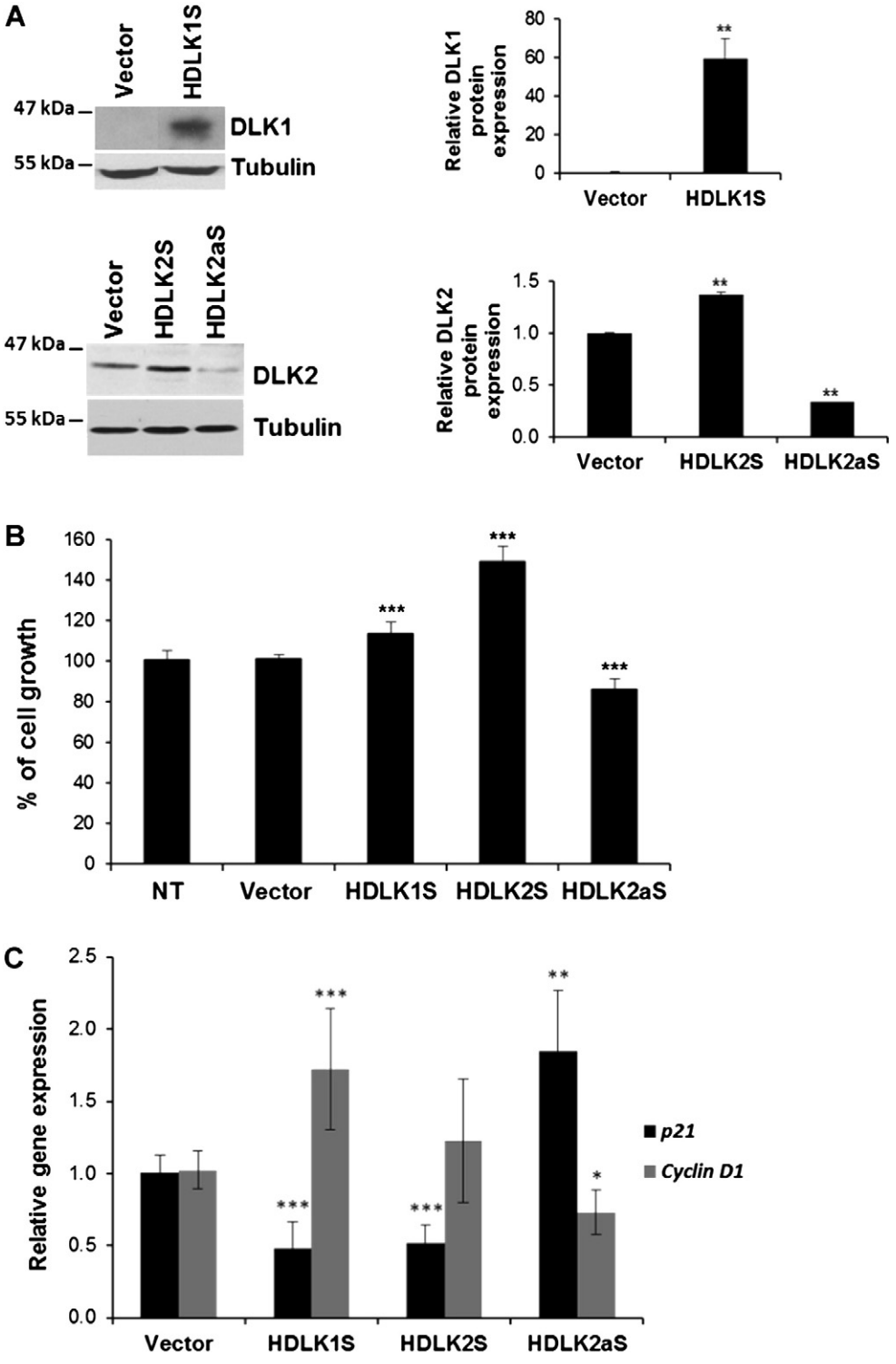

Fig. 1. Overexpression of human DLK1 or DLK2 proteins promotes SK-MEL-2 cell proliferation. (A) SK-MEL-2 cells were stably transfected with empty vector or plasmids HDLK1S, HDLK2S or HDLK2aS. Representative Western blot analysis (left) and densitometry analysis (right) for human DLK1 (~42 kDa) and DLK2 (~42 kDa) protein expression are shown. Data were normalized with tubulin (~50 kDa) expression levels, used as a loading control. (B) Cell growth measured by MTT assays. The graph shows the percentage of cell growth (mean ± SD) of the indicated transfectants as compared with that of vector cells (adjusted to 100%) after 7 days of culture. NT: non-transfected cells. Data were obtained from two different stable transfectants in three independent experiments. (C) Level of expression of the cell cycle genes $p21^{WAF1/Cip1}$ and cyclin D1 in SK-MEL-2 cells stably transfected with HDLK1S, HDLK2S or HDLK2aS. Data were normalized to GAPDH mRNA levels in RT-qPCR assays. Student's t-test results relative to vector cells: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$).

activity in SK-MEL-2 cells stably transfected with DLK1 or DLK2-expressing plasmids, transiently transfected with plasmid pGLucWT, and cultured in conditioned medium from vector or HDLK1S-transfected cells. As expected, DLK1-enriched conditioned medium inhibited NOTCH signaling. Moreover, this inhibition was stronger in cells overexpressing DLK1 or DLK2 proteins, as compared to control cells transfected with the empty vector (Fig. 5B). These results demonstrate that DLK proteins are able to inhibit NOTCH activation and signaling in a dose-dependent manner in metastatic melanoma cells.

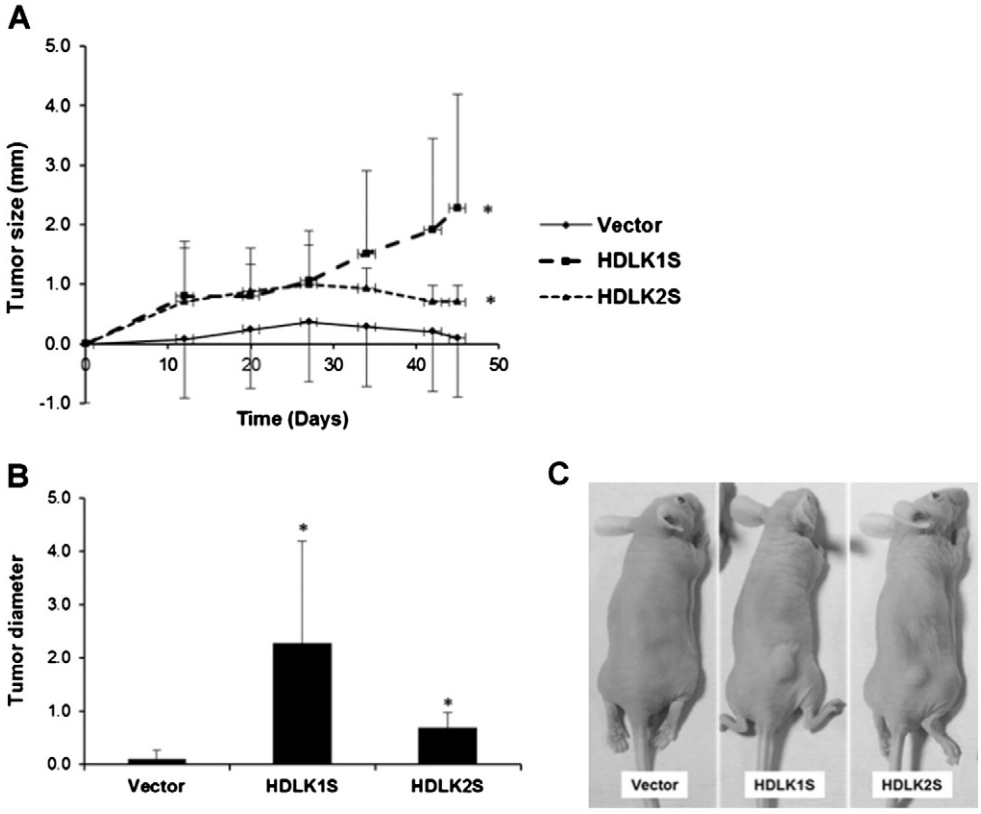

Fig. 2. SK-MEL2 cells overexpressing human DLK1 or DLK2 promote tumor cell growth *in vivo*. (A) SK-MEL-2 cells stably transfected with empty vector or plasmids HDLK1S or HDLK2S were injected s.c. into nude mice. Tumor size was measured at the indicated times (days). Results indicated are the mean ± SD. (B) The graph shows the mean ± SD of the tumor diameters at the end point of the experiment. Mann–Whitney U test results relative to vector cells injected mice: *($P < 0.05$). (C) Mice were sacrificed after 45 days and photographed. Representative photographs of skin tumor formed in mice from each group are shown.

3.3. DLK1 and DLK2 cooperate with DAPT to decrease NOTCH activation in human metastatic melanoma SK-MEL-2 cells

Previous studies have shown that the suppression of NOTCH activation and signaling with γ-secretase inhibitors (GSIs) decreases the proliferation rate of melanoma cell lines in a dose-dependent manner, leaving melanocyte growth unaltered [8]. Moreover, it has been described that treatment of melanoma cell lines with a low dose of DAPT (0.2 μM), suppressed primary melanoma cell proliferation, but did not affect the proliferation of metastatic melanoma cells [9]. To further investigate the role of DLK proteins on NOTCH1 activation and melanoma cell proliferation, we examined the effects of different concentrations of DAPT in our different transfected cells. As shown in Fig. 6A, treatment of control SK-MEL-2 cells with a high dose of DAPT (2.5 μM) led to a drastic decrease of active NOTCH1, as compared to the levels of active NOTCH1 in cells treated with lower doses of DAPT (0.1 μM or 1.0 μM). We also analyzed NOTCH-dependent transcriptional activity in SK-MEL-2 cells transiently co-transfected with plasmid pGLucWT and empty vector, or with plasmids HDLK1S, HDLK2S or HDLK2aS, and treated with different concentrations of DAPT. As shown in Fig. 6B, any dose of DAPT, and either DLK1 or DLK2 overexpression, significantly decreased, but downregulation of DLK2 expression increased, NOTCH-dependent transcriptional activity as measured by luciferase activity assays. Moreover, our results indicate that treatment of SK-MEL-2 cells with a low dose of DAPT (0.1 μM or 1.0 μM) led to a similar level of NOTCH signaling inhibition as that shown by SK-MEL-2 cells overexpressing DLK1 or DLK2.

To investigate the dose-dependent effects of DAPT and DLK protein levels on NOTCH activation, we treated SK-MEL-2 cells stably transfected with DLK1 or DLK2-expressing plasmids with two different concentrations of DAPT and analyzed the active NOTCH1 levels by Western blot. As shown in Fig. 6C, the treatment of HDLK1S or HDLK2S stable transfected cells with either a high (2.5 μM), or a low (1.0 μM) dose of DAPT led to complete NOTCH1 inhibition. We also analyzed these effects by measuring the NOTCH-dependent transcriptional activity in SK-MEL-2 cells overexpressing DLK1 or DLK2, and treated, or not, with 2.5 μM or 1.0 μM DAPT. As shown in Fig. 6D, the inhibition of NOTCH-dependent transcriptional activity in cells overexpressing DLK1 or DLK2 and treated with DAPT was stronger than that obtained in control cells or in non-treated HDLK1S or HDLK2S stable transfectants.

3.4. Effects of DAPT treatment and increased DLK protein expression on SK-MEL-2 cell proliferation

To investigate the dose-dependent effects of the above treatments on metastatic melanoma cell proliferation, we first analyzed by MTT assays the cell proliferation rate of SK-MEL-2 cells in the presence of increasing concentrations of DAPT. The treatment of these cells with a high dose of DAPT (2.5 μM) significantly reduced their proliferation rate. Surprisingly, low doses of DAPT (0.1 μM or 1.0 μM) significantly enhanced their proliferation rate (Fig. 7A). Then, we analyzed the proliferation rate of SK-MEL-2 cells stably transfected with plasmids HDLK1S or HDLK2S and treated, or not, with 2.5 μM or 1.0 μM DAPT. As shown in Fig. 7B and C, treatment of HDLK1S or HDLK2S stable transfectants with either dose of DAPT significantly inhibited their proliferation rates. Taken together these results suggest that treatment of SK-MEL-2 melanoma cells overexpressing DLK1 or DLK2 with a low dose of DAPT leads to a more effective inhibition of cell proliferation than that obtained in cells transfected with empty vector and treated with any dose of DAPT, or cells overexpressing DLK1 or DLK2 not treated with DAPT. Thus, our data suggest that treatment of human metastatic melanoma

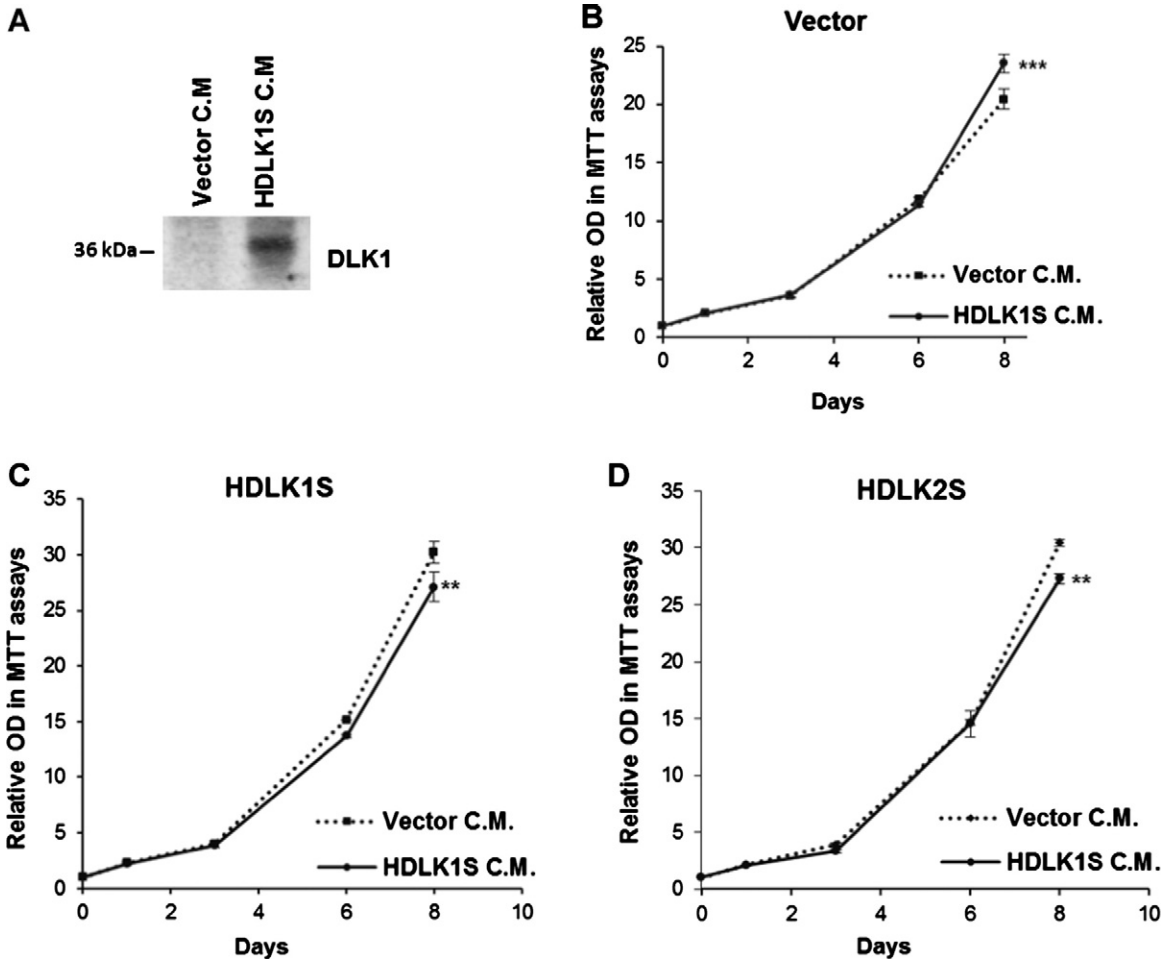

Fig. 3. Conditioned medium containing human DLK1 promotes the proliferation of SK-MEL-2 cells, but inhibits that of SK-MEL-2 cells overexpressing DLK1 or DLK2 proteins. (A) Western blot analysis of soluble DLK1 protein (~38 kDa) in conditioned medium from SK-MEL-2 cells stably transfected with empty vector (Vector C.M) or HDLK1S plasmid (DLK1S C.M). The proliferation rate of SK-MEL-2 cells stably transfected with empty vector (B), plasmid HDLK1S (C) or plasmid HDLK2S (D), in the presence of Vector C.M. or HDLK1S C.M., at the indicated times (days), was measured by MTT assays. Each graph shows the mean ± SD of two different transfectants, in at least three independent experiments. The number of cells at the different time points was normalized to the number of cells at time zero, set arbitrarily at 1. Student's t-test results relative to vector cells: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$).

tumors with a combination of DLK proteins and DAPT could be more effective at inhibiting NOTCH signaling and cell proliferation than treating these cells with each one of these inhibitory agents separately.

4. Discussion

In this report, we show that the effects of the proteins DLK1 and DLK2 on metastatic melanoma cell growth depend upon their levels of expression and the levels of NOTCH signaling inhibition generated by them or their combined treatment with NOTCH signaling inhibitors, such as DAPT. In our first assays, we observed that ectopically expressed DLK1 or DLK2 significantly enhanced the proliferation rate of human metastatic melanoma SK-MEL-2 cells, as determined by MTT assays, compared to empty vector-transfected cells. Related to these results, the effects of DLK1 on SK-MEL-2 cell proliferation appear to be consistent with recent studies indicating that overexpression of DLK1 promotes proliferation of some tumor cell lines [25,29,30]. However, our findings contradict other studies where DLK1 has been shown to exert inhibitory effects on the proliferation of other cell types [20,27,28,35]. On the other hand, SK-MEL-2 cells with diminished levels of DLK2 expression, obtained by antisense transfection, showed decreased proliferation rates by MTT assays, compared to empty vector-transfected cells. To our knowledge, this is the first report in the literature describing a role of DLK2 in the control of cell proliferation.

It has been previously reported that the upregulation of p21 and the decrease in cyclin D1 expression contribute to G1 cell cycle arrest in SK-MEL-2 cells [36,37]. Similarly, we have observed that the overexpression of DLK1 or DLK2 in these cells significantly decreased $p21^{WAF1/Cip1}$ expression and increased *cyclin D1* expression, whereas lower levels of DLK2 expression resulted in an increase in $p21^{WAF1/Cip1}$ expression and a decrease in *cyclin D1* expression. These results agree with other published reports showing that DLK1-overexpressing human hepatocellular carcinoma cells decreased $p21^{WAF1/Cip1}$ expression and induced cell proliferation [30]. Yin and colleagues [25] found that DLK1 promoted glioblastoma cell proliferation and increased the expression of proliferation-enhancing proteins, such as cyclin D1. In contrast, forced expression of DLK1 in human erythroid leukemia cells enhanced their proliferation rates, but no difference was detected in levels of Rb, cyclin D1, and p21 proteins [38]. Our results suggest that DLK proteins could induce melanoma cell proliferation through changes in the expression of key cell cycle regulators, such as $p21^{WAF1/Cip1}$ and cyclin D1.

Although SK-MEL-2 cells do not form tumors well in nude mice [39], we decided to perform a tumor growth assay in these animals to analyze whether DLK proteins could promote melanoma cell growth *in vivo*, as they do *in vitro*. In the present study we observed that SK-MEL-2 cells overexpressing either DLK1 or DLK2 and subcutaneously implanted in nude mice, increased tumor growth *in vivo*, compared to mice inoculated with cells transfected with the empty vector. This finding agrees with other authors who showed that the overexpression of

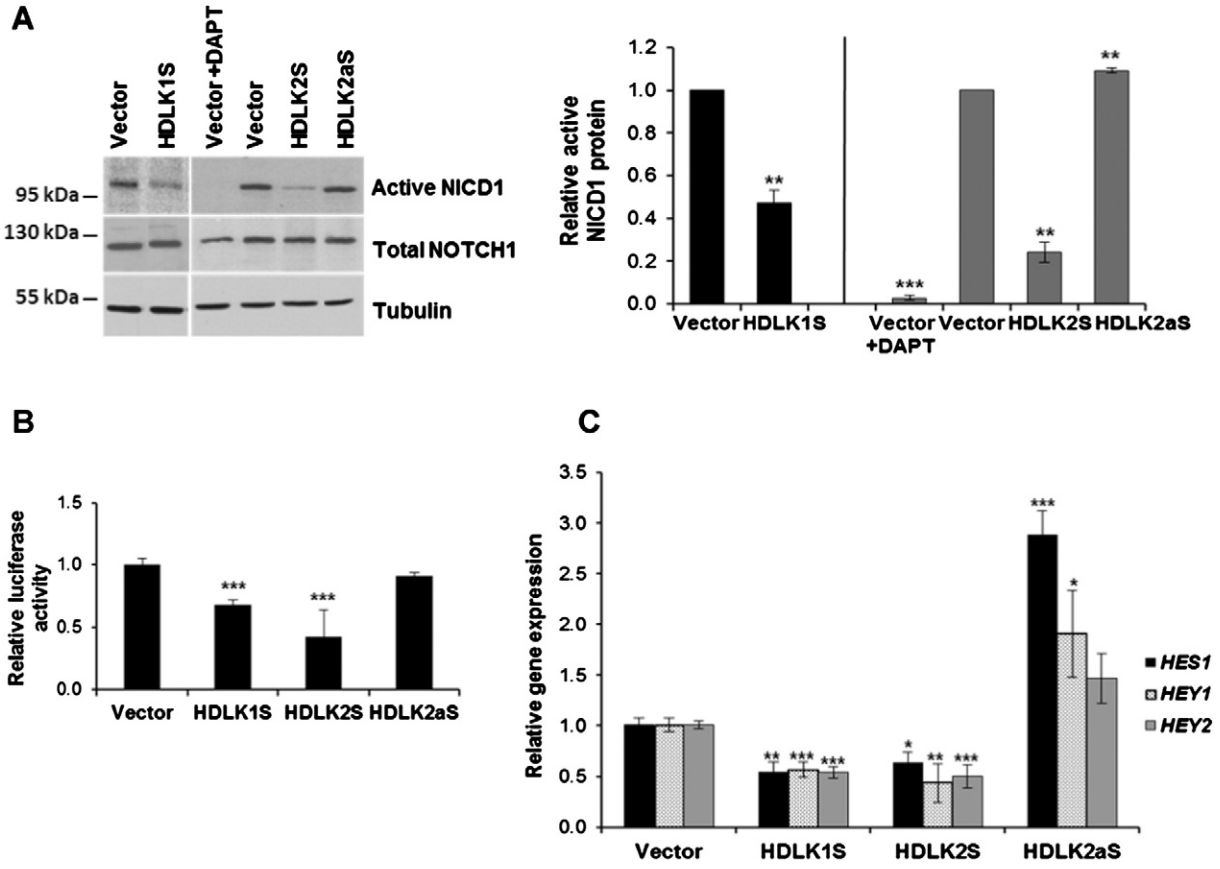

Fig. 4. Overexpression of human DLK1 or DLK2 proteins inhibits NOTCH1 activation and signaling in SK-MEL-2 cells. (A) Representative Western blot analysis (left) for active NOTCH1 (active NICD1 ~ 110 kDa) in SK-MEL-2 cells stably transfected with empty vector or plasmids HDLK1S, HDLK2S or HDLK2aS. SK-MEL-2 cells treated with 10 μM DAPT for 24 h were used as a control. NICD1 expression was normalized to total NOTCH1 expression (~120 kDa) and data were finally normalized to those of cells transfected with the empty vector. These data were represented in the graph (right) as the mean ± SD of two different transfectants for each construct, in at least three independent experiments. The empty vector transfectants are the reference control for cells transfected both with DLK1 and with DLK2 expressing plasmids. (B) Analysis of NOTCH transcriptional activity, as measured by luciferase assays, in SK-MEL-2 stably transfected with empty vector or plasmids HDLK1S, HDLK2S or HDLK2aS, and transiently transfected with plasmid pGLucWT, which expresses a NOTCH-dependent luciferase reporter gene. The relative luciferase activity was calculated by normalizing data to those obtained from cells transfected with the empty vector and it is represented as the mean ± SD of two different transfectants for each construct, in at least three independent experiments. (C) Level of expression of the NOTCH target genes *HES1*, *HEY1* and *HEY2*, in HDLK1S, HDLK2S or HDLK2aS stably transfected SK-MEL-2 cells. Data were normalized to GADPH mRNA levels in quantitative RT-PCR assays. Student's t-test results relative to vector cells: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$).

DLK1 promoted tumorigenic cell growth in mice [30,40,41]. On the contrary, other studies have shown that expression of exogenous DLK1 could inhibit *in vivo* tumor growth [20,42]. Our current findings support that DLK could be oncogenic in some tumor types and tumor suppressor in others, probably depending on their protein expression levels and the effects of DLK on NOTCH signaling in each tumor type.

DLK1 was previously shown to act both as a transmembrane and as a soluble protein [16,25], but nothing is known so far about whether DLK2 is also secreted or not. As expected, the results obtained by using conditioned media are consistent with DLK1 being released to the extracellular medium of SK-MEL-2 cells stably transfected with a DLK1-expressing plasmid. As we observed in DLK1 or DLK2 stable transfectants, we found that the growth rate of metastatic melanoma control cells (transfected with the empty vector) was significantly increased in the presence of conditioned medium from DLK1-expressing cells. Surprisingly, we detected that the addition of conditioned medium, containing soluble DLK1 protein, to HDLK1S or HDLK2S stable transfectants led to a significant inhibition of cell proliferation. These results indicate that the proliferation rate of SK-MEL-2 cells is highly sensitive to changes in the concentration or expression levels of DLK proteins.

The versatile effects of NOTCH signaling on cell differentiation, cell proliferation, survival, and tumorigenesis have been widely described. It has been also described that DLK1 and DLK2 proteins act as inhibitors of NOTCH signaling in other cellular systems [16–18,25]. In this work, we have observed that melanoma cell growth rates depend on the NOTCH signaling levels. Therefore, we studied the effects of DLK1 and DLK2 on NOTCH signaling in these cells to investigate whether the effects of DLK proteins on cell growth are a consequence on their modulation of NOTCH signaling. We have focused on NOTCH1, which is overexpressed in melanomas, but our data do not exclude the potential involvement of other NOTCH family members, also expressed in human melanoma cells [8,43]. We have found that forced expression of DLK1 or DLK2 in these cells led to a decrease in NOTCH1 activation, and *HES1*, *HEY1* and *HEY2* NOTCH-target gene expression. Moreover, the decrease of DLK2 expression levels in SK-MEL-2 cells led to an increase in both NOTCH1 activation and the expression of the NOTCH-target genes analyzed. These data demonstrate the inhibitory effect of DLK1 and DLK2 on NOTCH activation and signaling in human melanoma cells. In addition, our data also show that both, human soluble and transmembrane DLK1 variants, are able to inhibit NOTCH signaling in melanoma cells in a dose-dependent manner. To our knowledge, this is the first report describing the NOTCH inhibitory properties of human DLK proteins in tumorigenic cells.

Moreover, very important data obtained in this work are that different concentrations of DLK proteins can lead to an increase or to a decrease of SK-MEL-2 cell proliferation, most likely by inducing different levels of NOTCH signaling inhibition. Consistent with our findings, it has been reported that different levels of NOTCH signaling exert different effects [2,44]. More surprising is the fact that downregulation of

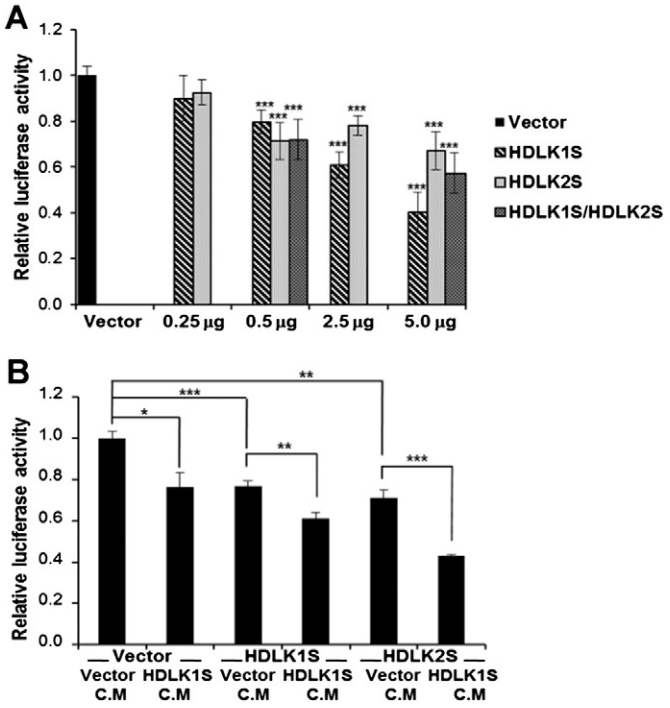

Fig. 5. NOTCH signaling is inhibited by DLK1 and DLK2 proteins in a dose-dependent manner. (A) Analysis of NOTCH transcriptional activity, as measured by luciferase assays, in SK-MEL-2 cells transiently co-transfected with plasmid pGlucWT and empty vector, or the indicated amounts of plasmids HDLK1S and/or HDLK2S. The relative luciferase activities, represented as the mean ± SD of three independent experiments, were calculated by normalizing the data to those obtained from cells transfected with the empty vector. Student's t-test results are also relative to vector cells values. (B) Analysis of NOTCH transcriptional activity, as measured by luciferase assays, in DLK1 or DLK2 stable SK-MEL-2 transfectants, transfected with plasmid pGlucWT and cultured with HDLK1S conditioned medium (C.M.) or Vector C.M. The relative luciferase activities were calculated by normalizing the data to those obtained from cells transfected with the empty vector and are represented as the mean ± SD of two different transfectants for each construct, in at least three independent experiments. Student's t-test results relative to cell samples are indicated in the figure: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$).

DLK2 expression, which implied an increase in NOTCH activation and signaling, led to an inhibition of the growth rate of SK-MEL-2 cells. We do not have an explanation for this effect, but DLK2 could also participate in the regulation of apoptosis, as DLK1 does [20,45]. Besides, we cannot discard that DLK proteins could target other NOTCH receptors, and be involved in the regulation of NOTCH receptors, promoting other effects not described yet. Further studies will be necessary to determine the role of DLK proteins in the inhibition or not of each individual NOTCH receptor. In any case, the data presented here support that the inhibition of NOTCH activation by DLK proteins modulates NOTCH signaling and leads to variations in the cell proliferation rates of human metastatic melanoma cells.

Activation of NOTCH signaling is able to either stimulate or inhibit proliferation by regulating the cell cycle in a cell type-specific and context-dependent manner [46]. The effect of NOTCH signaling on the regulation of $p21^{WAF1/Cip1}$ and cyclin D1 expression may differ in various tumor cell types. Our results suggest that a slight NOTCH signaling inhibition may be associated with the downregulation of $p21^{WAF1/Cip1}$ expression and upregulation of cyclin D1 expression, which could result in an increase of melanoma cell proliferation rate. It has been previously described that the activation of NOTCH signaling induces $p21^{WAF1/Cip1}$ expression and downregulates cyclin D1 expression which correlates with growth inhibition [47]. However, it has also been described that the cell cycle arrest through upregulation of $p21^{WAF1/Cip1}$ expression and/or downregulation of cyclin D1 expression was promoted by inhibition of NOTCH1 signaling in melanoma tumor cells and other cell types [11,48–52].

Interestingly, in this work we have also found that different levels of NOTCH signaling may cause opposite effects on SK-MEL-2 proliferation rate. In particular, high levels of NOTCH inhibition resulted in a decrease of metastatic melanoma cell growth, whereas lower levels of NOTCH inhibition led to an increase in their cell proliferation rates. As mentioned above, we found that DLK proteins, while moderately decreasing NOTCH signaling levels, promoted the growth of SK-MEL-2 cells, whereas decreased expression of DLK2 protein, leading to increased NOTCH signaling, inhibited their proliferation. However, we observed that high levels of DLK protein expression, associated with a strong inhibition of NOTCH signaling, led to an inhibition of SK-MEL-2 proliferation. These data indicate that human DLK1 and DLK2 proteins may modulate the cell proliferation rate of human metastatic melanoma cells through the modulation of NOTCH signaling levels.

Recently, enthusiasm has increased in targeting the NOTCH pathway using γ-secretase inhibitors (GSIs) because a number of preclinical studies have shown that GSIs hold promise as a new target-based therapy for tumors in which activated NOTCH signaling is important for their growth [4,6,13,15,53]. However, one of the major challenges is to eliminate unwanted toxicity associated with the GSIs. It is important to note that γ-secretase is a protease enzyme complex with up to one hundred potential substrates, including NOTCH receptors [13,54,55]. Thus, these side effects may be minimized if agents selective for NOTCH receptors could be developed [56]. In this regard, our work suggests that products derived from DLK proteins could be used in the future as novel anti-NOTCH agents with higher specificity to target NOTCH signaling than the current γ-secretase inhibitors.

In addition, we show here that low inhibition of NOTCH signaling may promote cell growth, which implies an unexpected negative effect that could be a potential additional risk for the patients treated with NOTCH signaling inhibitors in inadequate doses. Our data indicate that a high dose of DAPT, a high amount of DLK proteins, or a low DAPT dose combined with a low amount of DLK proteins leads to an inhibition of the proliferation rate of melanoma SK-MEL-2 cells due to a strong NOTCH inhibition; however, the treatment of these cells with a low dose of DAPT or a low amount of DLK proteins leads to a lower reduction of NOTCH activation and to an increase in their proliferation rate. Thus, the level of NOTCH signaling appears to be critical for the proliferation outcome in response to the extracellular signals triggering this process. These findings highlight the necessity of a careful evaluation of GSI dose levels and therapeutic windows to determine the optimal design of human clinical trials for candidate drugs targeting NOTCH signaling. Besides, the data presented here suggest that an effective therapeutic approach could involve GSIs treatment in combination with DLK proteins or derived peptides. In fact, initial trials have shown that GSI toxicity was dose-limiting [15,57]. Thus, this strategy may offer the advantage of allowing specific NOTCH inhibition and potentially minimizing side effects caused by highly non-specific GSIs. Finally, recent investigations in melanoma research have led to the identification of several molecular pathways and specific gene products that are often deregulated during melanoma initiation and progression to locally advanced and metastatic disease states. The combination of different drugs targeting distinct pathways or gene products altered during melanoma development may constitute more promising therapeutic strategies than the use of a single therapeutic approach [6,14,58]. Therefore, balancing efficacy and toxicity of the use of an appropriate combination of DLK1 and/or DLK2 proteins with conventional chemotherapy might be considered as a research avenue to improve the treatment of melanoma in the future.

In summary, our findings indicate that both DLK1 and DLK2 proteins regulate metastatic melanoma cell proliferation in a dose-dependent manner. Besides, our data revealed that the level of NOTCH signaling, which can be regulated by DLK proteins and their combined treatment with NOTCH signaling inhibitors, such as DAPT, can lead to opposite effects in metastatic melanoma cell growth. Interestingly, we show that insufficient inhibition of NOTCH signaling may promote tumor

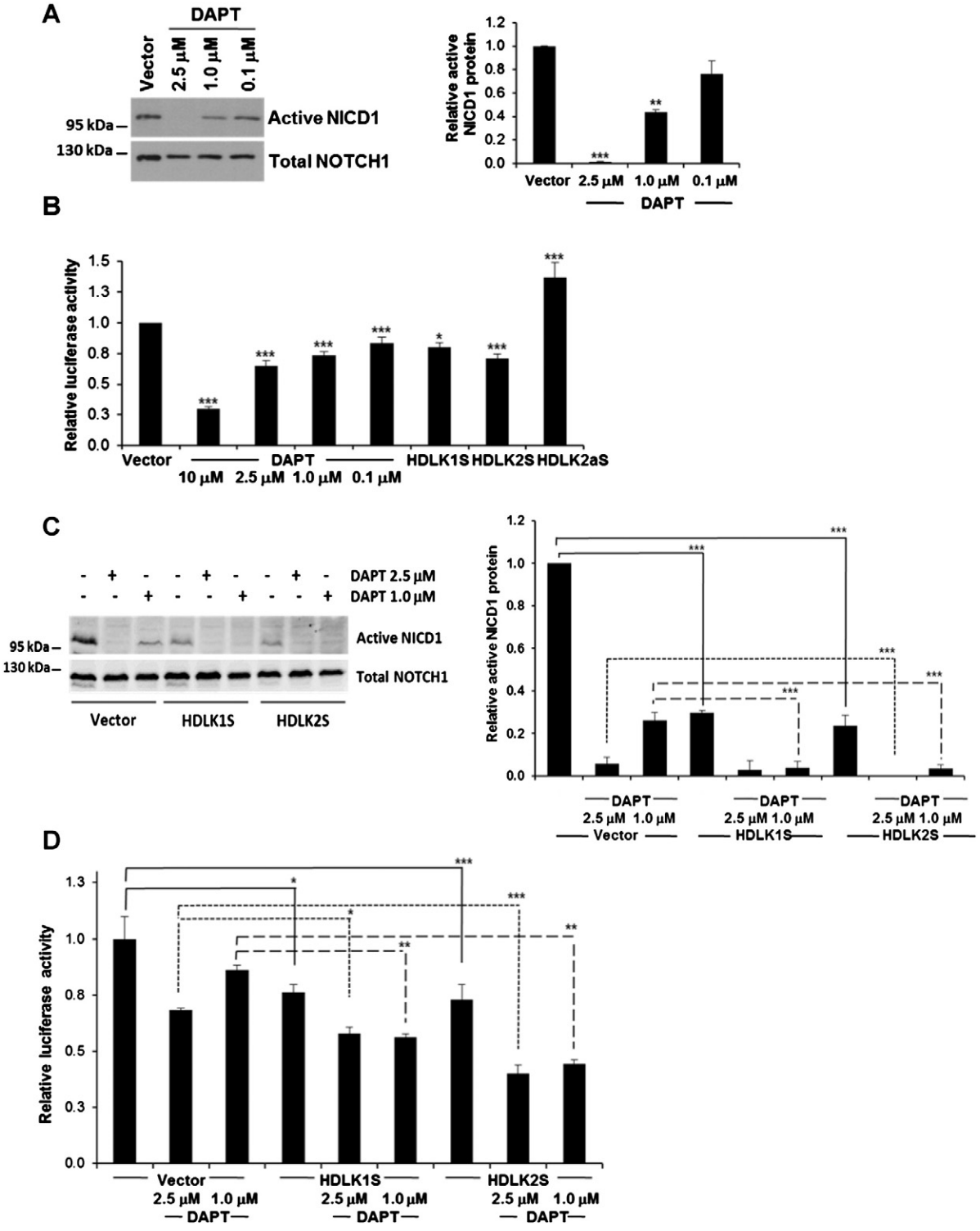

Fig. 6. NOTCH activation and signaling in SK-MEL-2 cells treated with the γ-secretase inhibitor DAPT and/or DLK proteins. (A) Analysis of active NOTCH1 protein (active NICD1 ~ 110 kDa) in the presence of the γ-secretase inhibitor DAPT at the indicated concentrations. A representative Western blot assay is shown (left). NICD1 expression was normalized to total NOTCH1 expression (~120 kDa) and data were finally normalized to those obtained from cells non-treated with DAPT. These data were represented in the graph (right) as the mean ± SD of three independent experiments. (B) Analysis of NOTCH transcriptional activity, as measured by luciferase assays, in SK-MEL-2 cells transiently co-transfected with pGLucWT and empty vector or HDLK1S, HDLK2S or HDLK2aS plasmids. Empty vector transfectants were treated with DAPT at the indicated concentrations. These data were represented in the graph as the mean ± SD of three independent experiments. Student's t-test results relative to vector cells without DAPT treatment. (C) Representative Western blot analysis (left) of active NICD1 protein in stable SK-MEL-2 transfectants overexpressing DLK1 or DLK2 and treated, or not, with DAPT at the indicated concentrations. NICD1 expression (~110 kDa) was normalized to total NOTCH1 expression (~120 kDa) and data were finally normalized to those obtained from cells transfected with the empty vector. These data were represented in the graph as the mean ± SD of two different transfectants for each construct, in at least three independent experiments. Student's t-test results relative to cell samples are indicated in the figure. (D) Analysis of NOTCH transcriptional activity, as measured by luciferase assays, in SK-MEL-2 cells transiently co-transfected with pGLucWT and empty vector or plasmids HDLK1S or HDLK2S, and treated, or not, with the γ-secretase inhibitor DAPT at the indicated concentrations. The relative luciferase activities were calculated by normalizing the data to those obtained from cells transfected with the empty vector and treated, or not, with DAPT, and they were represented as the mean ± SD of two different transfectants for each construct, in at least three independent experiments. Student's t-test results relative to cell samples are indicated in the figure: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$).

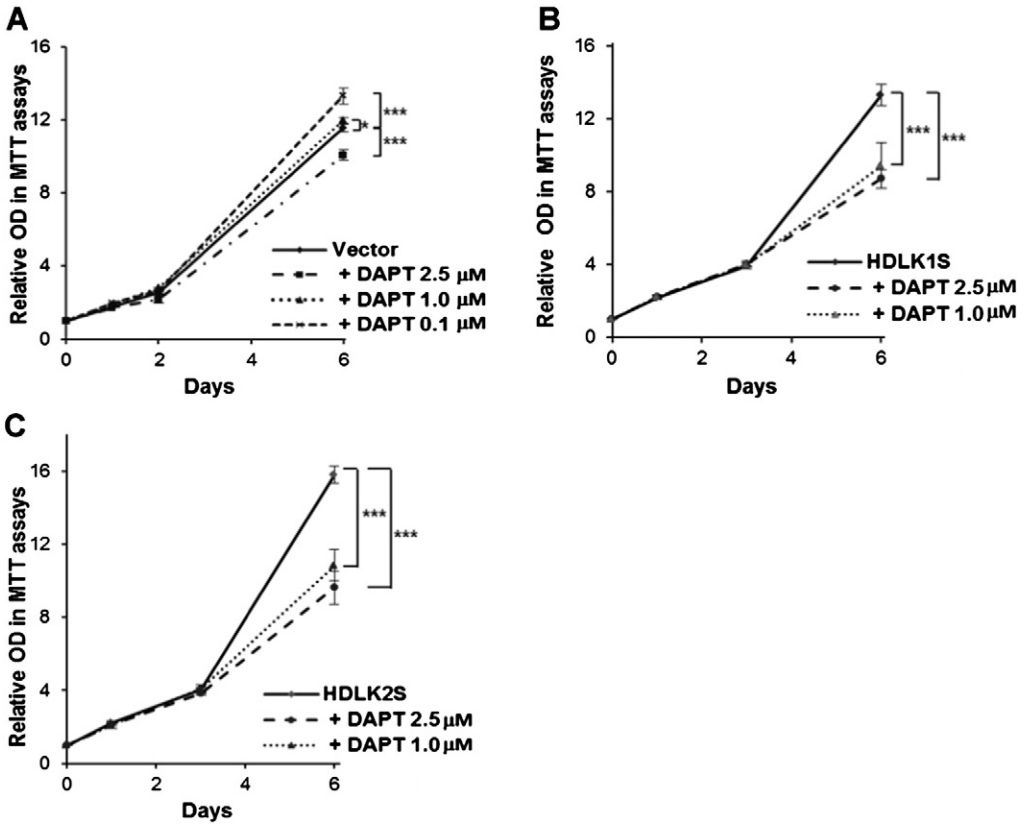

Fig. 7. Effects of DLK1 or DLK2 overexpression and/or DAPT treatment in SK-MEL-2 cell proliferation. Proliferation rate, as measured by MTT assays, of SK-MEL-2 cells stably transfected with the empty vector (A), HDLK1S (B), or HDL2S (C), in the presence of the γ-secretase inhibitor DAPT at the indicated concentrations. The means ± SD of two different transfectants for each construct in at least three independent experiments are plotted. The number of cells in the different time points was normalized to the number of cells at time zero, set arbitrarily at 1. Student's t-test results relative to non-treated cells: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$).

cell growth, an effect that needs to be considered in the design of clinical trials and patient treatment strategies. Finally, the data presented here suggest that the use of a correct combination of GSIs and DLK proteins or related peptides might be a favorable strategy for NOTCH-targeted cancer therapies in the future.

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