Silencing of the microtubule-associated proteins doublecortin-like and doublecortin-like kinase-long induces apoptosis in neuroblastoma cells

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Abstract

Doublecortin-like kinase-long (DCLK-long) and doublecortin-like (DCL) are two splice variants of DCLK gene. DCL and DCLK-long are microtubule-associated proteins with specific expression in proliferative neural progenitor cells. We have tested the hypothesis that knockdown of DCL/DCLK-long by RNA interference technology will induce cell death in neuroblastoma (NB) cells. First, we analyzed the expression of DCL and DCLK-long in several human neuroblastic tumors, other tumors, and normal tissues, revealing high expression of both DCL and DCLK-long in NB and glioma. Secondly, gene expression profiling revealed numerous differentially expressed genes indicating apoptosis induction after DCL/DCLK-long knockdown in NB cells. Finally, apoptosis was confirmed by time-lapse imaging of phosphatidylserine translocation, caspase-3 activation, live/dead double staining assays, and fluorescence-activated cell sorting. Together, our results suggest that silencing DCL/DCLK-long induces apoptosis in NB cells.

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Introduction

Neuroblastoma (NB) is a pediatric tumor arising from immature sympathetic neuroblast cells (Maris & Matthay 1999). It is the most common solid cancer in childhood and the second highest cause of cancer deaths in children (Maris et al. 2007). NB exhibits characteristics of immature sympathetic neuroblasts (Brodeur 2003). NBs contain a mixture of neuroblastic and neuroendocrine cell types that are organized in lobular structures with a central necrotic zone (Jogi et al. 2002, Poomthavorn et al. 2009). This pediatric tumor presents a broad spectrum of clinical behaviors. A subset of tumors undergo spontaneous regression, while others show relentless progression (Tang et al. 2006, Castel et al. 2007, Maris et al. 2007). About half of all cases are classified as high-risk, with overall survival rates below 40%, despite intensive multimodal therapy (Maris et al. 2007). Microtubule-stabilizing agents, such as Vinca alkaloids, are used in NB treatment. However, NB patients develop pharmacoresistance to these chemotherapeutic agents, and systemic toxicity also occurs, which make NB difficult to treat (Don et al. 2004).

Studies have shown that microtubule-stabilizing agents block mitosis primarily by inhibiting the dynamics of spindle microtubules, leading to mitotic arrest (Jordan et al. 1992, Lobert et al. 1999). This arrest induces mitochondrial permeability transition, release of pro-death molecules into the cytosol, and caspase-dependent apoptosis of neoplastic cells (Bhalla 2003). Different mechanisms have been highlighted linking mitotic arrest to the initiating...
events of the mitochondrial apoptosis pathway. These initiating events include either the direct inactivation of the antiapoptotic Bcl2 by phosphorylation, or the activation of the pro-apoptotic molecules Bax and Bad, which in turn inactivate Bcl2 or Bcl-xL (Konishi et al. 2002, Yamaguchi & Wang 2002, Bhalla 2003).

Since NBs derive from proliferating neuroblasts, the study of genes involved in mitotic spindle formation in neuroblast is of specific interest to find new intervention points for NB treatment. We and others have recently identified and characterized one of such genes, doublecortin-like kinase (DCLK). DCLK is a member of the doublecortin (DCX) gene family (Coquelle et al. 2006, Reiner et al. 2006) and regulates neurogenesis (Shu et al. 2006, Vreugdenhil et al. 2007), neuronal migration (Koizumi et al. 2006), and retrograde transport of glucocorticoid receptors (GR; Fitzsimons et al. 2008), and mitotic spindle formation in neuroblasts (Shu et al. 2006, Vreugdenhil et al. 2007). The genomic organization of the DCLK gene is rather complex and gives rise to numerous splice variants. The main splice variants encoded by DCLK gene are doublecortin-like (DCL), DCLK-long, DCLK-short, and calcium/calmodulin-dependent protein kinase (CaMK)-related peptide (CARP; for the functional domains of these splice variants see Supplementary Figure 1, see section on supplementary data given at the end of this article). DCLK-long and DCL contain two DCX domains (Gleeson et al. 1999, Burgess & Reiner 2000, Vreugdenhil et al. 2007), whereas DCLK-long and DCLK-short contain an additional CaMK-like domain (Schenk et al. 2007). CARP lacks both DCX and CaMK-like domains (Vreugdenhil et al. 1999). The microtubule-associated proteins (MAPs), DCL and DCLK-long, exhibit high homology with DCX (Shu et al. 2006, Vreugdenhil et al. 2007). DCX is a MAP involved in the regulation of microtubule dynamics, neuronal migration, and positioning in the neocortex (Bai et al. 2003). During embryonic development, both DCL and DCLK-long are expressed specifically in areas of high neuroblast proliferation but not in other proliferative tissues (Vreugdenhil et al. 2007). Silencing of DCL/ DCLK-long by RNA interference leads to disruption of the mitotic spindles and arrests the cells at prometaphase (Shu et al. 2006, Vreugdenhil et al. 2007). Interestingly, DCLK-long, DCLK-short, and CARP have been linked to apoptosis (Burgess & Reiner 2001, Kruidering et al. 2001, Schenk et al. 2007).

Here, we study the expression and the consequences of DCL/DCLK-long knockdown in NB cells. We show for the first time that there is a specific expression of DCL and DCLK-long in human NBs and profound apoptosis induction after their knockdown, suggesting that these DCLK gene splice products, which are specifically expressed in proliferative neuroblasts, are possible future therapeutic targets for the treatment of NB.

**Materials and methods**

**Cell culture and transfection**

Mouse N1E-115 and human SH-SY5Y NB cells were cultured as described (Vreugdenhil et al. 2007, Molenaar et al. 2008). Cells were grown in 24-well plates (Corning Life Sciences BV, Amsterdam, The Netherlands) coated with 100 ng/μl poly-l-lysine (Sigma–Aldrich, Inc). SH-SY5Y cells were seeded in plates coated with 100 μg/ml poly-d-lysine. For microscopy, both cell lines were grown in 24-well plates with glass bottom (Greiner Bio-One BV, Alphen aan den Rijn, The Netherlands) coated with 200 ng/μl poly-l-lysine (200 μg/ml poly-d-lysine for SH-SY5Y cells) at 80% confluence. The transfection of N1E-115 cells and the siRNAs (siDCL-1, siDCL-2, and siDCL-3) used were described by Vreugdenhil et al. 2007. SH-SY5Y cells were transfected with 200 nM siDCLK-4 (GCCCACUGACCUUCACCTT-sense and GGUAGAAGCUGACUGGGCTT-antisense) and 200 nM siDCLK-5 (UGGAGUACACCAA-GAAUGTT-sense and CAUUCUUGGUACUCATT-antisense) siRNAs using lipofectamine 2000 (Invitrogen) as described in the manufacturer’s protocol. AllStars Negative Control siRNA from Qiagen was used as negative control (NC). A transfection efficiency of 95±5% was obtained, which was determined by quantifying the percentage of transfected cells with a non-targeting siRNA conjugated to FITC (Qiagen).

**Protein extraction and western blots**

Protein extraction, SDS-PAGE, and western blotting were performed as previously (Vreugdenhil et al. 2007). A previously described anti-CAMKLK1 antibody was used to detect DCL (Kruidering et al. 2001, Vreugdenhil et al. 2007, Boekhoorn et al. 2008, Tuy et al. 2008). CPG16 CaM Kinase VI antibody (Becton Dickinson BV, Breda, The Netherlands) was used for detecting DCLK-long. Relative optical densities were analyzed and quantified using ImageJ software (http://rsbweb.nih.gov/ij/; Abramoff et al. 2004) on images obtained from three independent blots. Alkaline phosphatase assay was performed using 30 U of alkaline phosphatase for 30 min at 30 °C, followed by the addition of 10 mM sodium pyrophosphate as inhibitor (Francis et al. 1999).
Development of doxycycline-inducible stable cell line

N1E-115 cells (1×10^6) were transfected with 5 μg of a short hairpin RNA (shRNA) expression vector for DCL (TaconicArtemis, Cologne, Germany; Seibler et al. 2007) by cell electroporation using Amaxa Nucleofactor system (amaxa GmbH, Cologne, Germany) as described in the manufacturer’s protocol. The medium was changed daily and replaced by medium containing 500 μg/ml G418 (Geneticin; Invitrogen). Two weeks after transfection, single clones were picked by standard procedures. The inducible DCL knockdown was tested by western blotting in samples from cells treated with 1 μg/ml doxycycline (Dox) for 3 days, refreshing the medium daily.

RNA isolation

RNA isolation, the concentration measurement, and the integrity determination were performed as described by Dijkmans et al. 2008.

Gene expression profiling from human samples and cell lines

The NB tumor panel used for Affymetrix HG-U133 Plus 2.0 Microarray analysis contains 88 NB samples derived from primary tumors of untreated patients. mRNA isolation and profiling methods were described previously (Molenaar et al. 2008). The expression data were normalized with the MAS5.0 algorithm within the Affymetrix’s GCOS program. Target intensity was set to 100 (α1=0.04 and α2=0.06). The mRNA profiles of two other NB panels, adult tumors, and normal tissues are publicly available and taken from the National Cancer Institute (NCI) Gene Expression Omnibus (GEO) database (GSE2109 (https://exp. intgen.org/geo/listPublicGeoTransactions.do) GSE4290 (http://rembrandt-db.nci.nih.gov), GSE2658 (http://lambertlab.uams.edu). All data were analyzed using the R2 bioinformatic tool (http://r2.amc.nl, J Koster, personal communication). To identify correlating genes with the two DCLK splice variants in the 88-NB panel, the r value is calculated for all genes in the human genome (log2-transformed data are used). The significance of finding a certain correlation (P value) was calculated by the following formula: t = r√((n−1)/n−2), where r corresponds to the correlation value and n denotes the number of samples. Significance of gene enrichment in gene ontology (GO) categories was scored using 2×2 contingency table analysis (χ^2) with continuity correction.

Gene expression profiling from mouse NB N1E-115 cells

Sample preparation, hybridization to microarray, and detection were performed as described (Dijkmans et al. 2008). Raw signals were converted to expression values by Expression Array System Analyzer Software Version 1.1.1 (Applied Biosystems, Nieuwerkerk ad Ijssel, The Netherlands). Out of all 33 012 genes on the array, 14 076 (43%) had signal-to-noise ratio higher than 3, and were regarded as expressed in the N1E-115 cells. Subsequently, a quantile-normalization step was performed, and probe-to-gene annotation release version 12_05 was used for gene annotation. BRB-array software tools (Simon & Lam 2006) were used to identify genes that were differentially expressed among classes using a random-variance t-test (Wright & Simon 2003). Genes were considered statistically significant if their P value was <0.001 and their false discovery rate was lower than 0.015, according to Benjamini & Hochberg (1995). We performed biological pathway analysis for the genes with P<0.001. Differentially expressed GO groups of genes were identified as follows. For each GO group, the number n of genes represented on the microarray in that group was calculated, and subsequently, the Fisher (LS) statistic and Kolmogorov–Smirnov (KS) statistic were performed as described (Simon & Lam 2006). A GO category is regarded significantly differentially regulated if either significance level was <0.01. All GO categories with between 5 and 100 genes represented on the array were considered. Differentially expressed Biocarta pathways were identified using the Hotelling T-square test.

Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) was carried out by TaqMan technology using a Universal Probe Library (Roche) following the manufacturer’s protocol.

Live/dead double staining and caspase-3 activation assays

Forty-eight hours after N1E-115 and SH-SY5Y NB cell transfection with siRNAs or induction for 3 days with Dox, live/dead double staining assay (Calbiochem, San Diego, CA, USA) was performed as described in the manufacturer’s protocol. To detect caspase-3 activation, N1E-115 cells were imaged in the presence of ‘Nuncview’ Alexa-488-labeled caspase-3 substrate (Biotium Inc., Hayward, CA, USA) as indicated in the manufacturer’s protocol. N1E-115 cells treated with 20 nM staurosporine (STS; Sigma–Aldrich, Inc.) for 3 h were used as positive control.
STS is a kinase inhibitor known to induce apoptosis (Lopez & Ferrer 2000). SH-SY5Y human NB cells incubated for 5 h with 500 nM STS were used as positive control. Differential interference contrast (DIC) and fluorescence imaging were performed on a Nikon TE-2000 E system under 37°C and 5% CO₂ controlled conditions.

**Time-lapse imaging of phosphatidylserine translocation**

Forty-eight hours after N1E-115 cells had been transfected, time-lapse imaging of phosphatidylserine (PS) translocation was performed as described recently (Puigvert et al. 2009, 2010) for a period of 19 h. Cells treated with 20 nM STS (Sigma–Aldrich, Inc.) for 3 h were used as positive control. DIC and fluorescence imaging were performed on a Nikon TE-2000 E system under 37°C and 5% CO₂ controlled conditions.

**Image analysis and cell counting**

ImageJ software (Abramoff et al. 2004) was used for image analysis and cell counting. Time-lapse images were processed with Image-Pro Plus (Version 5.1; Media Cybernetics).

**Fluorescence-activated cell sorting**

Fluorescence-activated cell sorting (FACS) analysis was performed as described previously with some changes (Puigvert et al. 2009). Forty-eight hours after transfection, cells seeded in 24-well plates were trypsinized, washed with PBS, resuspended in PBS/EDTA (4 mM), and fixed in 67% ethanol overnight at −20°C. Cells were stained with FACS staining solution (1 mg/ml propidium iodide, 10 mg/ml RNase A, and PBS) for 45 min in the absence of light at room temperature. After resuspension, 5000–10 000 cells were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson). The CellQuest software (Becton Dickinson) was used for data analysis.

**Statistical analysis**

Unless otherwise indicated, assays were carried out for three independent experiments run in triplicates. Results are expressed as mean ± S.E.M. Where appropriate, Student’s t-test was done, and P <0.05 was considered statistically significant.

**Results**

**DCL and DCLK-long mRNAs are highly expressed in human NBs**

To evaluate mRNA expression levels of DCL and DCLK-long in our previously described neuroblastic tumor panel (Molenaar et al. 2008), we used the bioinformatic platform R2 identifying their expression levels in different tumors, in normal tissues, and in cell lines (http://r2.amc.nl, J Koster, personal communication). It also allowed us to predict their involvement in specific signal transduction routes. This application uses a database that contains 19 438 microarrays of 20 845 tumors and normal tissue samples. First, we analyzed the expression of DCLK in neuroblastic tumors (Fig. 1). The R2 platform is linked to the TranscriptView web application (Valentijn et al. 2006), which we used to identify the position of the probesets on transcription variants (Fig. 1A). There are two Affymetrix 133U plus2 microarray probesets annotated to the DCLK gene locus. Probeset 205399_at targets exon 20 of the DCLK gene, which is only transcribed in DCLK-long and DCLK-short variants (Vreugdenhil et al. 2001; Fig. 1A). Probeset 229800_at targets exon 8, which is transcribed in DCL and CaMK-CARP transcripts (Vreugdenhil et al. 2007; Fig. 1A). DCLK-short and CARP have been detected in adult neuronal cells but not in neuroblasts (Vreugdenhil et al. 2001, Burgess & Reiner 2002, Engels et al. 2004). Since exon 8 and exon 20 are mutually exclusive, we can use the corresponding probesets to separately analyze the expression of DCL and DCLK-long transcriptional variants. We compared the expression of both transcripts in the NB datasets with expression in various other tumors and normal tissues. The bar plot of Fig. 1B shows an increased DCLK-long expression in NB compared with various adult tumor types and normal non-nervous tissues. Expression in NBs was in the same range as the expression in adult central nervous system (CNS), as expected, since the probeset also recognizes other DCLK splice variants (Fig. 1A), highly expressed in different areas of the adult brain (Vreugdenhil et al. 2001, Burgess & Reiner 2002, Engels et al. 2004). With the exon 8 probeset, we identified abundant DCL expression in NB compared with other tissue types. Interestingly, only gliomas showed comparable expression levels (Fig. 1C). To analyze a putative correlation between the two probesets, we used gene expression profiles from 88 NBs (see Materials and methods for statistical tests). No significant correlation was found between DCL and DCLK-long expression, as measured with exon 8 or exon 20 probesets, suggesting that both splice variants were expressed independently.
may have different expression profiles as expected from their embryonic expression (Lin et al. 2000, Vreugdenhil et al. 2007, Boekhoorn et al. 2008).

To estimate the signal transduction pathways in which DCLK variants are involved, we searched for genes with correlating expression patterns. This analysis revealed 1206 genes with a significant correlation ($P < 0.01$) with DCLK-long. This gene set exhibits enrichment of genes involved in microtubule-based processes and axon projection (see Supplementary Table 1, see section on supplementary data given at the end of this article). The same analysis for DCL showed 880 genes with a significant correlation ($P < 0.01$). Interestingly, this correlation was most significant for GO clusters involved in mitochondrial respiratory chain processes (see Supplementary Table 2, see section on supplementary data given at the end of this article), suggesting a link between DCL and mitochondria. In silico analysis, using PSORT II software (http://psort.ims.u-tokyo.ac.jp; Nakai & Horton 1999), of the subcellular localization of human DCL also predicts that 17.4% of this MAP is located in mitochondria (see Supplementary Table 3, see section on supplementary data given at the end of this article).

**DCL and DCLK-long proteins are expressed in human NBs and in NB cell lines**

The above-mentioned experiments provided important information on the expression of DCL and DCLK-long mRNA in NBs. Western blotting showed expression of the DCL protein in different human NBs (Fig. 1D), validating the observation done at the mRNA level. Moreover, DCL and DCLK-long proteins were also
detected in mouse and human NB cell lines (Figs 1D and 2), and were not observed in non-NB cell lines (Fig. 1D). In Fig. 1E, we demonstrate that the double band detected in the NG108-15 cell line represents differentially phosphorylated DCL isoforms, as described previously by other authors (Friocourt et al. 2003, Tuy et al. 2008). The molecular weight values estimated for the two DCL bands are in high correlation with those previously described in the literature.

**Synthetic modified siRNAs silence DCL/DCLK-long in mouse NB cells**

To study the consequences of DCL/DCLK-long knockdown, a mouse N1E-115 NB cell line that endogenously expresses these MAPs was used. Three previously described and validated synthetic siRNAs were utilized (Vreugdenhil et al. 2007); two of them, siDCL-2 and siDCL-3, effectively knocked down DCL, while the third one, siDCL-1, was not effective. In parallel, a synthetic non-targeting siRNA (AllStars Negative Control siRNA, Qiagen) was used as an independent NC. Since no significant differences were found between the two NC siRNAs (see Supplementary Figure 2, see section on supplementary data given at the end of this article), we present only the results obtained with the NC siDCL-1 (indicated as NC in the figures). Both siDCL-2 and siDCL-3 silenced DCL more effectively than they silenced DCLK-long at the protein level (Fig. 2). Nevertheless, the knockdown detected by RT-qPCR was ~50% for both MAPs (Fig. 2B), suggesting the existence of posttranslational regulatory mechanisms.

**Apoptotic pathways are affected after DCL/DCLK-long knockdown**

To investigate the effect of the knockdown of DCL/DCLK-long at the molecular level, we have used gene expression profiling of N1E-115 cells. Hierarchical gene cluster analysis showed clustering of siDCL-3 and siDCL-2 samples versus samples of NC (Fig. 3A). Comparing siDCL-2 and NC, samples resulted in the identification of 1034 differentially expressed genes, while 931 differentially expressed genes were identified with siDCL-3. Of these differentially expressed genes, 663 genes were in common (Fig. 3B). The majority of these 663 genes were up-regulated (562) and 101 were down-regulated (Fig. 3B). Pathway analysis resulted in the identification of significant overrepresented pathways related to cell cycle, oxidative stress, and apoptosis (Table 1). Pax6 was one of the most up-regulated genes in our expression profiling studies. The up-regulation of Pax6 has been linked to the inactivation of neuroblast proliferation, apoptosis, and acquisition of neuronal cell fate (Berger et al. 2007). Moreover, Bax is an example of an apoptotic inducer (Nutt et al. 2002) found up-regulated in the affected pathways. Oxidative phosphorylation and ATP synthesis were among the most affected biological processes (see Supplementary Table 5, see section on supplementary data given at the end of this article). Genes such as Ndufa1 (Mamelak et al. 2005) and Cox7c (Lenka et al. 1998) were up-regulated (Fig. 3C and Supplementary Table 4, see section on supplementary data given at the end of this article), indicating an active oxidative phosphorylation process in cells with decreased DCL/DCLK-long expression. Moreover, mitochondria were among the most affected cellular components (see Supplementary Table 5). Differential expression of several selected genes was confirmed by RT-qPCR (see Supplementary Table 6, see section on supplementary data given at the end of this article). Together, these data suggest that DCL/DCLK-long knockdown leads to apoptosis.
The analysis was performed for a negative control group and one of siDCL-3 group were excluded from the analysis because they did not fulfill the microarray quality control criteria. The analysis was performed for a negative control group and one of siDCL-3 group were excluded from the analysis because they did not fulfill the microarray quality control criteria.

In NB cells, we performed biochemical assays to investigate this possibility. Since the above-described microarray results suggest apoptosis induction by DCL/DCLK-long knockdown, we performed biochemical assays to investigate this possibility.

Time-lapse imaging of PS translocation (Puigvert et al. 2009, 2010) showed a significant difference between the NC and cells transfected with the effective siRNAs at the different time points (Fig. 4A and B, Supplementary Video 1, see section on supplementary data given at the end of this article). After counting the number of cells presenting FITC-labeled Annexin-V conjugated to PS at different time points, we identified an increase of PS translocation to the outer membrane in cells with DCL/DCLK-long knockdown (Fig. 4A and B), showing an increase of apoptosis in these cells. At the beginning of the assay (48 h after transfection), 10.33 ± 1.20% apoptotic cells were quantified for siDCL-2, and 16.71 ± 5.07% apoptotic cells were quantified for siDCL-3, while 6.93 ± 0.90% apoptotic cells were detected in the NC (Fig. 4B). Eighteen hours after starting the assay (66 h after transfection), 17.04 ± 3.98% cells transfected with siDCL-2 and 89.18 ± 5.32% cells transfected with siDCL-3 were positive for FITC-labeled Annexin-V conjugated to PS. These values were significantly higher (P < 0.001) than those in the NC (51.82 ± 3.08%; Fig. 4B). Our results indicate that DCL/DCLK-long knockdown leads to apoptosis.
**Table 1** Examples of affected pathways in mouse N1E-115 neuroblastoma cells with doublecortin-like (DCL) and doublecortin-like kinase-long (DCLK-long) knockdown. Sixty-eight pathways (Biocarta pathways) were significant at the nominal 0.0005 level of the Hotelling T-square test.

<table>
<thead>
<tr>
<th>Biocarta pathway</th>
<th>Hotelling’s test</th>
<th>Modulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein kinase A at the centrosome</td>
<td>2.00×10^-7</td>
<td>Ppp2r1a (↓↓); Prkar2a (↓↓); Cyp51 (↓↓); Rhoa (↓↓); Akap9 (↑↑); Prkar2b (↓↓); Prkaca (↑↑); Prkce (↓↓)</td>
</tr>
<tr>
<td>Regulation of Bad phosphorylation</td>
<td>7.40×10^-6</td>
<td>Ywhah (↓↓); Bax (↑↑); Mapk1 (↓↓); Igfl1 (↑↓); Asah1 (↓↓); Bad (↑↑); Pik3r1 (↑↑); Mapk3 (↓↓); Akt1 (↓↓); Rps6ka1 (↓↓); Bcl2l1 (↑↑); Prkaca (↑↑); Bcl2 (↓↓)</td>
</tr>
<tr>
<td>AKAP95 role in mitosis and chromosome dynamics</td>
<td>1.26×10^-5</td>
<td>Ddx5 (↓↓); Ppp2r1a (↓↓); Prkar2a (↓↓); Apk8 (↓↓); Prkar2b (↓↓); Prkaca (↑↑)</td>
</tr>
<tr>
<td>Control of skeletal myogenesis by HDAC and calcium/calmodulin-dependent kinase (CaMK)</td>
<td>2.12×10^-5</td>
<td>Ywhah (↓↓); Igfl1 (↑↑); Ppp3ca (↓↓); Pik3r1 (↑↑); Akt1 (↓↓); Hdc5 (↑↓); Mapk7 (↑↑); Camk2a (↑↑); Mapk14 (↓↓); Mel2a (↓↓); Calm1 (↑↑); Myod1 (↑↑)</td>
</tr>
<tr>
<td>Multiple antiapoptotic pathways from IGF1R signaling lead to Bad phosphorylation</td>
<td>2.43×10^-5</td>
<td>Ywhah (↓↓); Mapk1 (↓↓); Igfl1 (↑↑); Raf1 (↓↓); Asah1 (↓↓); Pik3r1 (↑↑); Grb2 (↓↓); Bad (↑↑); Irs1 (↑↑); Map2k1 (↓↓); Mapk3 (↓↓); Akt1 (↑↑); Rps6ka1 (↓↓); Ppp1r13b (↑↑); Sox1 (↑↑); Hras1 (↑↑); Prkaca (↑↑); Shc1 (↓↓)</td>
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<tr>
<td>Oxidative stress induced gene expression via Nrf2</td>
<td>2.65×10^-5</td>
<td>Hmox1 (↑↑); Sirt7 (↑↑); Mapk1 (↓↓↓); Jun (↑↑↑); Atp4 (↓↓↓); Nfe2l2 (↓↓↓); Malg (↑↑↑); Creb1 (↓↓↓); Crys (↓↓↓); Keap1 (↑↑↑); Fos (↑↑↑); Prkb1 (↑↑↑); Por (↑↑↑); Mapk14 (↑↑↑); Maff (↑↑↑); Makf (↓↓↓)</td>
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<tr>
<td>Cell cycle: G2/M checkpoint</td>
<td>3.10×10^-5</td>
<td>Ywhah (↓↓); Chek1 (↓↓); Cdc34 (↑↑); Myt1 (↓↓); Cdkn1a (↓↓); Wee1 (↓↓); Brcal1 (↑↓); Gadd45a (↑↑); Mdm2 (↓↓); Rps6ka1 (↓↓); Atm (↑↑); Atr (↓↓); Cdc25c (↓↓); Trp53 (↑↑); Cdkn2d (↑↑); Pik1 (↑↑); Prkdc (↓↓); Ywhaq (↓↓)</td>
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<tr>
<td>Role of Ran in mitotic spindle regulation</td>
<td>3.83×10^-5</td>
<td>Kpna2 (↓↓); Aurka (↑↑); Kpnbl1 (↓↓); Tpx2 (↓↓); Kif15 (↑↑); Rcc1 (↑↑); Ranbp1 (↓↓); Rangap1 (↓↓)</td>
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<td>P53 signaling pathway</td>
<td>1.63×10^-4</td>
<td>Cnd1 (↓↓); Bax (↑↑); Cdkn1a (↓↓); Gadd45a (↑↑); Mdm2 (↓↓); Cdk4 (↑↑); Ccne1 (↑↑); Atm (↑↑); Pcna (↓↓); E2f1 (↑↑); Trp53 (↓↓); Apaf1 (↓↓); Bcl2 (↓↓)</td>
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<tr>
<td>Regulation of MAP kinase pathways through dual specificity phosphatases</td>
<td>2.05×10^-4</td>
<td>Dusp2 (↓↓); Dusp6 (↓↓); Dusp1 (↓↓); Mapk3 (↓↓); Dusp9 (↓↓); Mapk14 (↓↓); Dusp8 (↑↑); Dusp4 (↑↑)</td>
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<tr>
<td>Apoptotic signaling in response to DNA damage</td>
<td>3.63×10^-4</td>
<td>Bax (↑↑); Bid (↑↑); Ccys (↑↑); Bad (↑↑); Akt1 (↑↑); Atm (↑↑); Casp3 (↑↑); Tin1 (↑↑); Bcl2l1 (↑↑); Prkb1 (↓↓); Ppp1 (↑↑); Casp9 (↑↑); Trp53 (↑↑); Casp7 (↓↓); Apaf1 (↑↑); Bcl2 (↑↑); Stat1 (↑↑)</td>
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<tr>
<td>Apoptotic DNA fragmentation and tissue homeostasis</td>
<td>4.64×10^-4</td>
<td>Hmgb1 (↓↓); Top2a (↓↓); Df4a (↓↓); Endog (↑↑); Hmgdb2 (↑↑); Casp3 (↑↑); Casp7 (↑↑); Cad (↓↓)</td>
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(↑↑), up-regulated in both comparisons (negative control versus siDCL-2 and negative control versus siDCL-3); (↓↓), down-regulated in both comparisons; (↑↓), up-regulated in the comparison negative control versus siDCL-2 and down-regulated in the comparison negative control versus siDCL-3; (↓↑), down-regulated in the comparison negative control versus siDCL-2 and up-regulated in the comparison negative control versus siDCL-3; (↑NA), up-regulated in the comparison negative control versus siDCL-2 and not altered for the second comparison.

We also performed double staining assays to discriminate between live and dead cells (Fig. 4C; Balcer-Kubiczek et al. 2006). In line with our PS translocation studies, DCL/DCLK-long knockdown leads to a significantly higher (P<0.05) number of dead cells 48 h after transfection with the two effective siRNAs. 22.01±1.62% N1E-115 cells transfected with siDCL-2 and 18.43±1.31% N1E-115 cells transfected with siDCL-3 presented membrane damage, which was indicated by propidium iodide staining. Significantly less (P<0.05; 9.36±0.90%) NC cells were positive for propidium iodide (Fig. 4C). Using an Alexa-488-labeled caspase-3 substrate (Puigvert et al. 2009), caspase-3 activation was also measured. Compared with the NC (11.53±1.53%), a significant increase in percentage of cells with active caspase-3 was detected when transfected with siDCL-2 (16.83±1.37%; P<0.05) and siDCL-3 (29.61±2.41%; P<0.001; Fig. 4D).

FACS corroborated the effects of DCL/DCLK-long silencing (Fig. 5). For FACS analysis, we used cells...
transduced with siDCL-3 due to its higher effectiveness in inducing cell death (Fig. 4). We observed a significantly higher ($P<0.05$) percentage of apoptotic cells ($18.45 \pm 1.00\%$) in cells treated with siDCL-3 than in cells treated with the NC ($10.39 \pm 1.61\%$). At this time point, no significant differences were detected in cell-cycle progression among the different experimental groups. 

Figure 4 Apoptosis studies in mouse N1E-115 neuroblastoma cells at 48 h after transfection. (A and B) Time-lapse imaging of phosphatidylserine translocation. Images were taken at 30 min interval (see Supplementary Video 1). (A) Time-lapse imaging 0, 9 and 18 h after starting the assay. (B) Percentage of cells with translocated phosphatidylserine at different time points for the different treatments. The initial time point of the assay (0 h) corresponds to 48 h after transfection. (C) Live/dead double staining. Viable cells are stained with a cell-permeable green fluorescent cyto-dye and dead cells are stained with both cyto-dye (green) and propidium iodide (red). (D) Caspase-3 activation assay. Bar graph shows the percentage of cells with active caspase-3. STS, staurosporine. NC, negative control. Overlap of DIC and fluorescent imaging were used in the different assays. 20× magnification. Scale bars, 50 μm. Data points and Columns, mean of two independent experiments ($n=6$); bars, s.e.m. *$P<0.05$. ***$P<0.001$. Full color version of this figure available via http://dx.doi.org/10.1677/ERC-09-0301.
To validate the specificity of the observed effects, an inducible stable cell line was developed to express specific shRNAs (Fig. 6). First, we attempted to develop a stable cell line with constitutive expression of shRNA against DCL. However, the cells failed to survive, in agreement with the observed effects of DCL knockdown on cell survival using synthetic siRNAs. Nevertheless, DCL knockdown was possible using a Dox-inducible expression of specific shRNAs against DCL. By western blotting, we detected DCL knockdown in cells treated with Dox (88.67 ± 2.68% in colony 1 and 63.84 ± 5.66% in colony 6), while cells treated with vehicle depicted DCL levels comparable to the parental cell line (Fig. 6A and B). Using these stable cell lines, we observed that DCL knockdown induced a significant increase in cell death ($P < 0.05$; Fig. 6C and D). In Dox-treated cells, 23.44 ± 3.39% (colony 1) and 16.82 ± 3.13% (colony 6) of dead cells were detected. In contrast, 11.77 ± 0.13% (colony 1, no Dox), 7.00 ± 3.25% (colony 6, no Dox), 6.00 ± 3.14% (parental cell line with Dox), and 7.55 ± 0.22% (parental cell line no Dox) of dead cells were detected (Fig. 6C and D). These results showed that a higher percentage of cell death was observed in cells that presented a higher DCL knockdown (colony 1, Dox). Moreover, a similar percentage of knockdown obtained with siRNA, ~90% (Fig. 2), leads to a comparable percentage of cell death, around 20% (Fig. 4C).

Figure 5 Fluorescence-activated cell sorting results of neuroblastoma cells with DCL/DCLK-long knockdown. (A) Histogram representation of cell population 48 h after transfection with siDCL-3 or with the negative control siRNA (NC). Data shown are representative of four independent experiments. (B) Pie graphs of the effect of DCL and DCLK-long knockdown on the distribution of mitotic cells in different phases and on the induction of apoptosis. (C) Bar graphs of cells in apoptosis, in S phase, and in G2-M phase. A significantly higher percentage of apoptotic cells were found in the siDCL-3 group than in the negative control. No significant difference was found between NC and cells with the knockdown (siDCL-3) in the S and G2-M phases of the cell cycling. STS, staurosporine. Columns, mean of four independent experiments ($n = 4$); bars, S.E.M. *$P < 0.05$. Full color version of this figure available via http://dx.doi.org/10.1677/ERC-09-0301.
Together, our results indicate that the knockdown of the MAPs DCL and DCLK-long induces apoptosis in NB cells. In addition, this process might be through a caspase-3 activity-dependent pathway.

**DCL/DCLK-long knockdown in human SH-SY5Y NB cells leads to cell death**

To confirm the results obtained in mouse N1E-115 NB cells, we knocked down DCL/DCLK-long in human NB cells. The expression of DCL and DCLK-long was checked in different human NB cell lines by gene expression profiling. To avoid a possible compensation of DCL/DCLK-long function by other members of the DCX family (Koizumi et al. 2006), SH-SY5Y cells were selected. This cell line presents high expression of DCL and DCLK-long and low expression of DCX (Fig. 7A). In addition, this cell line presents a high rate of cell division, allowing us to perform the studies in the same time frame as with mouse NB cells. Using two effective siRNAs, siDCLK-4 and siDCLK-5, we obtained a significant DCL/DCLK-long knockdown (Fig. 7B and Supplementary Figure 3, see section on supplementary data given at the end of this article). We got 71.04 ± 4.42% DCL knockdown with siDCLK-4 and 65.20 ± 0.79% DCL knockdown with siDCLK-5 (Fig. 7B). 72.56 ± 2.08% DCLK-long knockdown was quantified using siDCLK-4 and 52.84 ± 1.63% DCLK-long knockdown was quantified using siDCLK-5 (Fig. 7B). Using live/dead double staining as with mouse NB cells, we found 27.80 ± 1.38% dead cells using siDCLK-4 and 26.30 ± 2.88% dead cells using siDCLK-5 (Fig. 7C and D), which was significantly higher (P < 0.01 and P < 0.001 respectively) than the 10.64 ± 2.18% detected in cells treated with NC siRNA (Qiagen; Fig. 7C and D). Thus, silencing DCL/DCLK-long by synthetic siRNAs in human SH-SY5Y NB cells induced a significant increase in cell death.
Discussion

In the present work, we demonstrate for the first time the expression of the two MAPs DCL and DCLK-long in human NBs, and using different experimental strategies ranging from gene expression profiling to live-imaging studies, we show that DCL and DCLK-long are crucial for the proliferation and survival of NB cells. Both DCL and DCLK-long, proteins derived from the DCLK gene, are highly expressed in human NBs. Similarly, both are expressed in mouse N1E-115 and human SH-SY5Y NB cells. We demonstrated that silencing of these MAPs by RNA
Microtubule dynamics (Bhat & Setaluri 2007). Never-long may be a potential therapeutic target for NB.

MAPs play a role in tumor cell resistance to microtubule-destabilizing agents by regulating microtubule dynamics (Bhat & Setaluri 2007). Nevertheless, in some cases, NB patients are treated with combination chemotherapy. Therefore, the development of resistance to microtubule-destabilizing agents, such as Vinca alkaloids, cannot be studied in these patients. However, an increase in microtubule-stabilizing proteins leads to resistance to Vinca alkaloids in NB cell lines (Don et al. 2004). This, in addition to the observation that DCL/DCLK-long silencing in NB cell lines leads to microtubule destabilization (Vreugdenhil et al. 2007), suggests that targeting microtubule-stabilizing proteins, such as DCL/DCLK-long, may reduce the development of chemoresistance to microtubule-destabilizing agents.

The mechanisms by which DCL and DCLK-long stabilize microtubules seem similar to that of the highly homologous DCX (Shu et al. 2006). DCL and DCLK-long as well as DCX have been shown to be crucial for neuronal proliferation, migration, and axonal outgrowth (Deuel et al. 2006, Koizumi et al. 2006, Shu et al. 2006, Vreugdenhil et al. 2007). In agreement with this, we detected a significant correlation between DCLK-long and genes involved in microtubule-based processes and axon projection human NB samples.

Our results show higher expression of DCL and DCLK-long in human NBs compared with various other tumor types and with normal non-nervous tissue. At the protein level, different DCL phosphorylated isoforms were detected in NB cell lines. Further research is needed to confirm the presence of phosphorylated DCL isoforms in NB tumors. Interestingly, DCL was also highly expressed in gliomas, in agreement with our previous findings showing high expression of this MAP in radial glial cells (Vreugdenhil et al. 2007). DCL expression in radial glial cells was shown to be crucial for proliferation and stability of the early radial glial scaffold (Vreugdenhil et al. 2007). Consistently, gliomas are a collection of tumors that occur within the CNS and arise from astrocytes, oligodendrocytes, or their precursors, radial glial cells (Holland 2001, Anthony et al. 2004). Therefore, our results indicate that DCL might also be a target of interest for glioma therapy.

Using two different specific siRNAs, siDCL-2 and siDCL-3, targeting completely different regions of the mRNA, we silenced the expression of DCL/DCLK-long in mouse NB cells, which endogenously expressed these MAPs (Vreugdenhil et al. 2007). Gene expression profiling after knockdown revealed an extensive overlap of the gene expression using the two effective siRNAs. The genes identified in non-overlapping groups might be due to off-target effects or due to distinct potencies of the two siRNAs used in these studies. Using the two siRNAs, siDCL-2 and siDCL-3, we observed coherent phenotype. This strongly suggests that the observed effects may not be due to off-targets of the individual siRNAs. Moreover, cell death due to DCL knockdown was confirmed in a shRNA-inducible stable cell line. A similar percentage of DCL knockdown obtained with synthetic siRNA and Dox-inducible shRNA leads to a comparable percentage of cell death in N1E-115 cells. This also indicates that DCL has a more relevant role in cell survival than DCLK-long, therefore suggesting that the activation of the apoptotic process is related to the microtubule-binding domains of these MAPs and not to the kinase domain of DCLK-long.

Analysis of the genes that were modulated in common by using siDCL-2 and siDCL-3 strongly indicates a specific induction of apoptosis. Consistent with this conclusion, the activation of Pax6, one of the most up-regulated genes in our expression profiling studies, leads to inactivation of neuroblast proliferation, apoptosis, and acquisition of neuronal cell fate (Berger et al. 2007). Oxidative stress pathways were also identified, supporting the idea that silencing DCL/DCLK-long leads the cells toward apoptosis. The induction of apoptosis by oxidative stress via mechanisms that involve mitochondria has been extensively documented (Green & Reed 1998, Nazarewicz et al. 2007). Furthermore, oxidative phosphorylation was found among the most over-represented biological processes and mitochondria were found to be one of the cell components most affected. Interestingly, down-regulation of genes involved in mitochondrial function and oxidative phosphorylation pathway has been shown to be a consistent feature of many tumors (Mamelak et al. 2005). One of those genes is NDUFA1, which was up-regulated in mouse NB cells by DCL/DCLK-long knockdown. Moreover, a relation between oxidative phosphorylation, mitochondria, and apoptosis has been suggested (Green & Reed 1998). Disruption of electron transport in the oxidative phosphorylation process has been recognized as an early feature of cell death (Green & Reed 1998).

In agreement with these observations, induction of apoptosis in NB cells by silencing of DCL/DCLK-long was confirmed by several assays. A higher PS translocation to the outer membrane and a higher...
percentage of cells with membrane damage were observed during the knockdown. Also, a higher percentage of apoptotic cells were detected by FACS analysis in the knockdown group. Moreover, an increase in caspase-3 activation was detected in these cells as well.

We have previously shown that cells transfected with siDCL-3 present more disruption of the mitotic spindles (Vreugdenhil et al. 2007), and we identified a higher percentage of cell death in cells transfected with siDCL-3 than in those transfected with siDCL-2. Therefore, our present and previous results suggest that the effectiveness of inducing apoptosis in NB cells may be directly correlated with the level of disruption of mitotic spindles. Previously, we and others have shown that silencing (Shu et al. 2006, Vreugdenhil et al. 2007) or overexpression (Santra et al. 2006, 2009, Shu et al. 2006, Fitzsimons et al. 2008) of MAPs of the DCX family leads to inhibition of cell proliferation. As shown previously, an imbalance in the expression levels of DCL, DCLK-long, or DCX results in aberrant spindle morphology, leading to a comparable disruption in the mitotic progression (Shu et al. 2006, Vreugdenhil et al. 2007). Consistent with this, a link between mitotic arrest due to disruption of mitotic spindles and apoptosis has been described, involving the activation of the pro-apoptotic gene Bax (Bhalla 2003). In our experiments, Bax was up-regulated after DCL/DCLK-long knockdown. In addition, survivin (Bric5) was found down-regulated, suggesting mitotic spindle catastrophe leading to apoptosis (Bhalla 2003).

An alternative explanation for our results might be that intracellular transport of signaling proteins might have been disrupted by DCL/DCLK-long knockdown, which lead the cell toward apoptosis. We have previously shown that DCL regulates GR microtubule-guided intracellular transport in mouse NB cells and in brain neuroblasts (Fitzsimons et al. 2008). GR is known to be transported to mitochondria where it activates specific responsive genes. (Solakidi et al. 2007). Interestingly, mitochondria were the most affected cell components after DCL/DCLK-long knockdown. Furthermore, we found in human NBs a significant correlation between DCL and genes related with mitochondria activity. Interestingly, the relation between DCL and mitochondria function might not be independent from the role of DCL in microtubule stabilization. Mitochondria transport is known to be along microtubules (Morris & Hollenbeck 1995), and connection between microtubules and mitochondrial apoptotic machinery has been proposed (Esteve et al. 2007).

In conclusion, we identified high expression of DCL-derived MAPs in human NBs. We demonstrate for the first time at the gene expression level and by several cell death assays that DCL/DCLK-long knockdown induces profound apoptosis in mouse and human NB cells. Therefore, our results suggest that the MAPs DCL and DCLK-long, which are specifically expressed in proliferative neuroblasts, might be targets for the treatment of NB.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-09-0301.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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