New therapeutic targets in the intrinsic apoptotic pathway in neuroblastoma
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Identification and Validation of BIRC6 as a Novel Drug Target for Neuroblastoma Therapy
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Abstract

Neuroblastoma are pediatric tumors of the sympathetic nervous system with a poor prognosis. Apoptosis is often deregulated in cancer cells, but only few defects in apoptotic routes have been identified in neuroblastoma. Here we investigated genomic aberrations affecting genes of the intrinsic apoptotic pathway in neuroblastoma. We analyzed DNA profiling data (CGH and SNP arrays) and mRNA expression data of 31 genes of the intrinsic apoptotic pathway in a dataset of 88 neuroblastoma tumors using the R2 bioinformatic platform (http://r2.amc.nl). We observed frequent gain of the BIRC6 gene on chromosome 2, which resulted in increased mRNA expression. BIRC6 is an inhibitor of apoptosis protein (IAP), that can bind and degrade the cytoplasmic fraction of the pro-apoptotic protein DIABLO. DIABLO expression was exceptionally high in neuroblastoma but the protein was only detected in the mitochondria. Upon silencing of BIRC6 by shRNA, DIABLO protein levels increased and cells went into apoptosis. Co-immunoprecipitation confirmed interaction between DIABLO and BIRC6 in neuroblastoma cell lines. Our findings indicate that BIRC6 may have an essential role in neuroblastoma by inactivating cytoplasmic DIABLO. BIRC6 inhibition may therefore provide a means for therapeutic intervention in neuroblastoma.
Introduction

BIRC6 (also known as BRUCE or APOLLON) is a cytoplasmic protein with a dual role. Firstly, BIRC6 has an anti-apoptotic function in the intrinsic apoptotic pathway. BIRC6 antagonizes the pro-apoptotic DIABLO protein. BIRC6 can bind the cytoplasmic DIABLO fraction and induce ubiquitination and proteasomal degradation of this protein.\(^1,2\) BIRC6 thereby protects against the pro-apoptotic function of DIABLO. DIABLO is a mitochondrial protein which is released into the cytoplasm upon an apoptotic stimulus. This release is regulated by the levels of the BH3 family proteins, which induce pore formation in the mitochondrial membrane.\(^3,5\) Cytoplasmic DIABLO can bind to the BIR domains of BIRC2 (cIAP1), BIRC3 (cIAP2) and BIRC4 (XIAP), thereby inhibiting the anti-apoptotic function of these proteins.\(^6\) A second function of BIRC6 has been shown in recent studies where BIRC6 was required for abscission and membrane delivery during the midbody ring formation during cell division.\(^7,8\)

BIRC6 is highly expressed in several types of cancer. BIRC6 over-expression in acute myeloid leukemia correlated to a poor outcome.\(^9\) A genome wide screening of chromosomal aberrations in Burkitt’s lymphoma showed that a region of 2p including the BIRC6 gene was gained in a few samples.\(^10\) Also, high BIRC6 expression in colon cancer stem cells was related to drug resistance.\(^11\)

Neuroblastoma are pediatric tumors that originate from the embryonal precursor cells of the sympathetic nervous system. High stage tumors have a poor prognosis with 20 to 40% overall survival.\(^12,13-14\) BIRC6 is located on chromosome 2p in the region, which shows frequent gain in neuroblastoma.\(^12\) This region includes MYCN and ALK, the two best known oncogenes in neuroblastoma. MYCN is amplified in 20-30% of neuroblastoma, which strongly correlates to a poor prognosis.\(^12,13-15\) The other oncogene on 2p is ALK, which was recently found to be mutated in 6-10% of primary neuroblastoma.\(^16-20\) MYCN amplification and ALK mutation seem to occur independent of the gain of chromosome 2p.\(^21\) Therefore other additional tumor driving genes could be located on this frequently gained region.

The apoptotic pathway has been widely investigated in neuroblastoma and only few tumor driving events have been described. TP53 is mostly intact in primary neuroblastoma although functional defects in the TP53 pathway have been described.\(^22\) Caspase 8 (CASP8) is hypermethylated and thereby inactivated in some neuroblastoma resulting in an inactive extrinsic apoptotic pathway.\(^23\) The IAP
**BIRC5 (Survivin)** is located on the chromosome 17q region frequently gained in neuroblastoma and high BIRC5 expression correlates to a poor prognosis.\textsuperscript{24-26} Finally, the anti-apoptotic mitochondrial *BCL2* protein is highly expressed in neuroblastoma. Targeted inhibitors against BIRC5\textsuperscript{27-32} and *BCL2*\textsuperscript{33} are currently tested for further clinical implementation. The poor prognosis of high grade neuroblastoma urges to identify additional targets for therapeutic intervention.

To identify patterns in the aberrations of genes involved in intrinsic apoptotic signaling we combined high throughput analysis of DNA copy number and mRNA expression of these genes in a dataset of 88 neuroblastoma tumors. *BIRC5* and *BIRC6* were frequently gained and *CASP9* was often lost. *BIRC6* was not previously evaluated in a neuroblastoma model. Therefore we studied the potency of BIRC6 as a new drug target for neuroblastoma therapy. Silencing of BIRC6 induced apoptosis and BIRC6 physically interacted with DIABLO. Also BIRC6 knockdown induced DIABLO up-regulation, indicating that BIRC6 can degrade DIABLO very effectively. BIRC6 might therefore be a suitable target for therapeutic intervention in neuroblastoma.

**Methods**

*Patient samples*

We used a neuroblastoma tumor panel for Affymetrix Microarray analysis containing 88 primary neuroblastoma tumor samples of untreated patients, which were all included for mRNA analyses and of which 87 neuroblastoma tumor samples were used for CGH analysis and SNP array.\textsuperscript{34} Material was obtained during surgery and immediately frozen in liquid nitrogen. Some samples were excluded because DNA quality was too low for high throughput analysis. Public available neuroblastoma datasets we used were of Delattre\textsuperscript{35} and Lastowska (geo ID: gse13136). Public available datasets were used for comparing neuroblastoma with normal tissues (Roth dataset, geo ID: gse3526) and adult tumors (EXPO dataset, geo ID: gse2109).

*Affymetrix expression analysis*

Total RNA of neuroblastoma tumors was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. RNA concentration and quality were determined using the RNA 6000 nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). Fragmentation of cRNA, hybridization to hg-u133 plus 2.0
microarrays and scanning were performed according to the manufacturer’s protocol (Affymetrix inc).

**CGH analysis**

High-molecular-weight DNA was isolated from tumor tissue by a standard salt-chloroform extraction method. For reference DNA we obtained healthy tissue. We used a custom 44K Agilent aCGH chip, enriched for critical regions of loss/gain for neuroblastoma (10 kb resolution), miRNAs/T-UCRs (5 oligos/gene) and cancer gene census genes (5 oligos/gene) (Agilent Technologies). A total of 150 ng of tumor and reference DNA was labeled with Cy3 and Cy5, respectively (BioPrime ArrayCGH Genomic Labeling System, Invitrogen). Further processing was done according to the manufacturer’s guidelines. Features were extracted using the feature extraction v10.1.0.0.0 software program. Data were further analyzed using the R2 web application (see below). Circular binary segmentation was used for scoring the regions of gain, amplification and deletion.

**Whole-Genome Genotyping**

Tumor DNA was extracted as previously described, quantified with NanoDrop and the quality was determined by the Abs 260/280 and 230/260 ratio. SNP arrays were processed for analysis of copy number variations according to the manufacturer’s recommendations with the Infinium II assay on Human370/660-quad arrays containing > 370 000/ > 660 000 markers and run on the Illumina Beadstation (Swegene Centre for Integrative Biology, Lund University – SCIBLU, Sweden) according to the manufacturer’s recommendations. Raw data were processed using Illumina’s BeadStudio software suite (Genotyping module 3.0), producing report files containing normalized intensity data and SNP genotypes. Subsequently, log 2 Ratio and B-allele frequency data were imported into the R2 web application for detailed analysis and comparison with the CGH and expression data.

**Bioinformatics**

All data were analyzed using the R2 web application, which is publicly available at http://r2.amc.nl. The expression data were normalized with the MAS5.0 algorithm within the GCOS program of Affymetrix Inc. Target intensity was set to 100. For scoring genomic aberrations of the 31 included genes, we considered CGH aberrant if the logfold value was more than 0.45 for gain or less than -0.45 for loss and if a breaking point was clearly visible. We excluded whole chromosome gains or losses. Also the detected gains or losses had to be confirmed by SNP array.
Cell lines
All cell lines were grown in Dulbecco Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum, 10 mM L-glutamine, 10 U/ml penicillin/streptomycin, Non Essential Amino Acids (1x) and 10 μg/ml streptomycin. Cells were maintained at 37 °C under 5% CO₂. For primary references of these cell lines, see Molenaar et al.³⁷

Lentiviral shRNA production and transduction
Lentiviral particles were produced in HEK293T cells by cotransfection of lentiviral vector containing the short hairpin RNA (shRNA) with lentiviral packaging plasmids pMD2G, pRRE and pRSV/REV using FuGene HD. Supernatant of the HEK293T cells was harvested at 48 and 72 hours after transfection, which was purified by filtration and ultracentrifuging. The concentration was determined by a p24 ELISA. Cells were plated in a 10% confluence. After 24 hours cells were transduced with lentiviral DIABLO shRNA (Sigma, ‘E8’: TRCN0000004511 and ‘E9’: TRCN0000004512) or BIRC6 shRNA (Sigma, ‘C7’: TRCN0000004157 and ‘C11’: TRCN0000004161) in various concentrations (Multiplicity of infection (MOI): 1 - 3). SHC-002 shRNA (non-targeting shRNA: CAACAAGATGAAGAGCACCAA) was used as a negative control. 24 hours after transduction medium was refreshed and puromycin was added to determine the efficacy of transduction. Protein was harvested 72 hours after transduction and analyzed by Western blot.

Compounds
ABT263, a small molecule BCL2 inhibitor, was dissolved in DMSO with a concentration of 20 mM for stock solution. A final concentration of 200 nM ABT263 was used. For the experiment using this compound we chose SJNB12 instead of IMR32 or SKNSH because this cell line highly expresses BCL2, which makes it very sensitive to this compound.³³ Z-Val-Asp(OMe)-Val-Ala-Asp(OMe)-FMK (ZVDVADVFMK, a widely used CASP2 inhibitor; R&D systems) was added to the cells following manufacturer’s protocol in a concentration of 20 μM.

Western Blotting
72 hours after transduction with shRNA, attached and floating cells were harvested on ice. Cells were lysated with Laemmli buffer (20% glycerol, 4% SDS, 100 mM Tris HCl pH 6.8 in mQ). Protein was quantified with RC-DC protein assay (Bio-Rad). Lysates were separated on a 10 % SDS-Page gel and electroblotted on a transfer membrane (Millipore, IPFL00010). Blocking and incubation were performed in OBB according to manufacturer’s protocol (LI-COR). Primary antibodies used were
BIRC6 (Abcam, ab19609), DIABLO (Abcam, ab32023), PARP (Cell Signaling: 9542) and BCL2 (Cell Signalling; 2872). Protein loading was checked by β-actin (Abcam, ab6276) or α-tubulin (Sigma, T5168). The secondary antibodies used were provided by LI-COR. Proteins were visualized with the Odyssey bioanalyzer (LI-COR).

**In cell western**

48 hours after transduction with BIRC6 shRNA, cells were fixed with 4% paraformaldehyde for 20 minutes. Blocking and incubation were performed in OBB according to manufacturer’s protocol (LI-COR). Primary antibodies used were BIRC6 (BD Biosciences, 611193) and β-actin mouse monoclonal (Abcam, ab6276). Proteins were visualized with the Odyssey bioanalyzer (LI-COR) and quantified and corrected for actin using the Odyssey software.

**Immunofluorescence**

Cells were grown on glass slides in 6-well plates. Cells were fixed with 4% paraformaldehyde in PBS 48 hours after transduction. We used DIABLO (Abcam, ab32023) as a primary antibody, and anti-rabbit (Alexa, 11012) as a secondary antibody. Mitochondria were stained using Mitotracker (Invitrogen, M22426). Antibodies were dissolved in 5% ELK in PBS/0.2% tween-20. Slides were stained with DAPI (1:1000) in vectashield (Vector Laboratories).

**Cell fractionation**

Protein was harvested and fractionated using the Subcellular Proteome Extraction Kit according to manufacturer’s protocol (Novagen, 539790). Fraction I (cytosol) and II (cell organelle) were used for Western blot.

**Co-immunoprecipitation**

Cells were lysed in a buffer containing 150 mM NaCl, 50 mM Hapes, 5 mM EDTA, 0.3% NP-40, 10 mM β-glycerophosphate, 6% glycerol, protease inhibitors (Complete mini, Roche) and Phosphatase inhibitors (5 mM NaF, 1 mM Na2VO3). The antibody used for IP was BIRC6 (Abcam, ab19609); negative controls were flag (Cell Signaling, 2368) and protein without antibody. Other negative controls were for every antibody a sample without protein (data not shown). Protein-G agarose beads (Roche) and antibody have been incubated for pre-coupling overnight after which lysate was added and incubated overnight. Immunocomplexes were washed, heated at 95°C for 10 minutes and put on a gel for Western blot. Primary antibodies used were anti-BIRC6 and anti-DIABLO (Abcam; ab32023). Blots were incubated overnight with
primary antibodies, after which a one hour incubation step with anti-rabbit IgG (Cell Signaling; 3678) was performed followed by incubation with the secondary antibody that was provided by LI-COR.

**MTT-assay**

Cells were plated in 30% confluence in a 48-well plate and transduced with BIRC6 shRNA and treated with ZVDVAD after 24 hours. 72 hours after treatment, 25 µl of Thiazolyl blue tetrazolium bromide (MTT, Sigma M2128) was added. After 4-6 hours of incubation 250 µl of 10% SDS, 0.01 M HCl was added to stop the reaction. The absorbance was measured at 570 nm and 720 nm using a platereader (Biotek).

**Results**

*Gain of BIRC5 and BIRC6 and loss of CASP9 in neuroblastoma tumors*

To identify patterns in the aberrations of genes involved in intrinsic apoptotic signaling we combined high throughput analysis of DNA copy number and mRNA expression of these genes. We included all 31 genes that are directly involved in the mitochondrial apoptotic pathway and their downstream target genes (table 1). Array CGH data of 87 primary neuroblastoma tumor samples were analyzed for DNA copy number variations of the 31 genes included in our intrinsic apoptotic gene panel. Binary segmentation data was used to score the DNA copy number variations and they were subsequently confirmed using log fold data from SNP array analyses of the same tumors. Only three genes showed DNA copy number aberrations at a frequency above 10% (fig 1a). The *BIRC5* gene, which is located in the smallest region of overlap (SRO) of gain of chromosomal band 17q25\(^{12}\), was gained in 49% of the tumors. *CASP9* is located at the SRO of deletions of 1p36\(^{12}\) and it was lost in 30% of the tumors. *BIRC6*, which is located on 2p22, was gained in 24% of the neuroblastoma tumors. Distal chromosome 2p is a known region of gain in neuroblastoma. *BIRC6* is not located in the SRO of this gained region, but is gained in 84% of the tumors with 2p gain (fig 1b). Of these three genes, *BIRC6* was not studied before in neuroblastoma. We therefore investigated whether the gain of BIRC6 resulted in aberrant expression. We compared *BIRC6* expression in tumors with and without *BIRC6* gain, which showed that tumors with BIRC6 gain have significantly higher *BIRC6* RNA levels (Student T-test: P = 3.1*10\(^{-6}\)) (fig 1c). Moreover, *BIRC6* is also highly expressed compared to several adult tumors and various normal tissues (fig 1d). These findings suggest that the aberrant expression
of BIRC6 is at least partially caused by genomic aberrations that often occur in neuroblastoma tumors.

**BIRC6 knockdown induces apoptosis in neuroblastoma cells**

We investigated whether the high BIRC6 levels indeed counteract apoptosis in neuroblastoma. We used 2 lentiviral shRNAs targeting different parts of the coding sequence of BIRC6. SKNSH, one of the neuroblastoma cell lines with the highest BIRC6 expression, was transduced with these vectors (fig 2a). BIRC6 is a 528 kD protein and difficult to assess on Western blot. Therefore we first analyzed BIRC6 protein levels by in cell western. For this method cells are fixed directly in the culture well and stained with a BIRC6 antibody. Analysis showed concentration dependent down-regulation of BIRC6 protein with both BIRC6 shRNAs at 48 hours after transduction (fig 2b). Although less optimal, we could confirm BIRC6 down-regulation using Western blot (fig 2c). We then investigated whether BIRC6 silencing induced apoptosis. Light microscopy showed a decreased cell number and cell death at 72 hours after BIRC6 silencing (fig 2d). This was confirmed by MTT assays, which showed strongly reduced cell viability after transduction with both BIRC6 shRNAs (fig 2e, dark grey bars). This phenotype was caused by an apoptotic response as western blot demonstrated PARP cleavage at 72 hours after transduction with both BIRC6 shRNAs (fig 2f). These findings confirm an anti-apoptotic role for BIRC6 in neuroblastoma cells.

**High BIRC6 levels keep cytoplasmic DIABLO levels low**

The BIRC6 protein functions both by silencing of DIABLO and in the formation of the midbody ring during cell division. Inhibition of each of these functions can result in cell death. We therefore investigated which process caused apoptosis after BIRC6 silencing in neuroblastoma. Apoptosis induced after inhibiting the midbody-related function of BIRC6 has been found to be mediated by CASP2.7,8,38 We inhibited CASP2 by ZVDVAD, a widely used CASP2 inhibitor39-41 that we have previously used to show that apoptosis induced by silencing of BIRC5 is mediated by CASP2.26 ZVDVAD however did not inhibit apoptosis induced by BIRC6 knockdown in SKNSH and IMR32 cells (fig 2e). This indicates that this process is not CASP2-mediated, implying that the role of BIRC6 in neuroblastoma is not essential for completion of cell division during midbody ring formation.

Alternatively, BIRC6 functions as an IAP that binds DIABLO in the cytoplasm and thereby induces ubiquitination and degradation of DIABLO.1,2 DIABLO mRNA expression levels in neuroblastoma tumors were surprisingly high compared to other
Fig 1: Gain of BIRC5 and BIRC6 and loss of CASP9 in neuroblastoma tumors

(A) The percentage of genomic aberrations is presented on the Y-axis and all selected genes in the intrinsic apoptotic pathway on the X-axis. Red bars indicate gained genes and green bars indicate lost genes. When a bar is green/red combined, it means that both gains and losses in that gene occurred. The red horizontal line represents the cut-off for further analysis. (B) Chromosome 2 is represented with the regions of 2p that are gained in our dataset of 88 neuroblastoma tumors. The BIRC6 locus is indicated with a red arrow. (C) Boxplots of BIRC6 mRNA expression in tumors with or without gain of BIRC6. (D) Boxplots of BIRC6 mRNA expression in 3 neuroblastoma datasets (red), adult tumors (blue) and various normal tissues (green). The boxes represent the 25th to 75th percentile with the median depicted as a horizontal line. Extremes are indicated by the whiskers, and the presence of outliers is indicated by (o).
Fig 2: Knockdown of BIRC6 in SKNSH induces apoptosis
(A) BIRC6 mRNA expression in 24 neuroblastoma cell lines. (B) In cell western of SKNSH 48 hours after transduction. The Y-axis represents the ratio between BIRC6 and Actin protein expression as determined by the Odyssey bioanalyzer. The X-axis represents the concentration BIRC6 shRNA that was added. Black bars are cells transduced with control virus (SHC002), dark grey: C7 BIRC6 shRNA and light grey: C11 BIRC6 shRNA. MOI = Multiplicity of Infection. (C) Western blot of SKNSH 48 hours after transduction with no virus (NV), control virus SHC002 (C) or BIRC6 shRNA (C11 and C7). Blots were incubated with BIRC6 and actin antibodies. (D) Pictures were made 72 hours after transduction before protein harvest with a 100x magnitude. (E) MTT-assay of SKNSH and IMR32 transduced with control virus (SHC) or BIRC6 shRNA (C7 and C11). The dark grey bars represent cells transduced with virus alone; the light grey bars represent cells that are treated with BIRC6 shRNA combined with ZVDVAD, a CASP2 inhibitor. Fig 2f: Western blot of SKNSH 72 hours after transduction with no virus (NV), control virus SHC002 (C) or BIRC6 shRNA (C11 and C7). Blots were incubated with PARP, DIABLO and actin antibodies.
types of tumors and compared to normal tissue (fig 3a). Moreover, we confirmed an
amplification of a region on the chromosome 12q arm in the neuroblastoma cell line
NGP, which included the DIABLO locus (fig 3b). We therefore investigated whether
the high BIRC6 expression allows neuroblastoma cells to survive the high levels of the
pro-apoptotic protein DIABLO. DIABLO is a mitochondrial protein, which translocates
to the cytoplasm after apoptotic stimuli where it can be degraded by BIRC6. We
therefore investigated the cellular localization of DIABLO. Immunofluorescence
indeed showed a clear localization of DIABLO within the mitochondria (fig 3c), which
was confirmed by cell fractionation showing that the majority of DIABLO is localized
in the cell organelle fraction (fig 3d). To test whether DIABLO can be functionally
activated upon an apoptotic stimulus, we treated neuroblastoma cells with the BCL2
inhibitor ABT263, which results in stimulation of pore formation in the mitochondria.
Cell fractionation indicated that the cytosolic DIABLO levels increased 24 hours
after ABT263 treatment (fig 3d). This shows that DIABLO can be released from the
mitochondria and stimulate apoptosis.
Untreated cells show low cytoplasmic DIABLO levels (fig. 3d). These levels are
probably restricted by BIRC6 activity. To test this hypothesis, we first investigated
whether BIRC6 and DIABLO physically interact in neuroblastoma cells by a co-
immunoprecipitation analysis. Cell lysates of SKNSH and IMR32 cells were
immunoprecipitated with a BIRC6 antibody. Western blot analysis of these
precipitates with a DIABLO antibody showed a strong signal at the correct position for
DIABLO. This confirms a physical interaction between both proteins (fig 3e). Finally,
we investigated whether silencing of BIRC6 results in increased DIABLO protein
levels. Western blot analysis of SKNSH cells treated with two different shRNAs for
BIRC6 showed that the silencing of BIRC6 induced by both viruses resulted in a
clear increase of DIABLO protein levels (fig 2f).
These experiments suggest that BIRC6 effectively inactivates cytoplasmic DIABLO
in neuroblastoma cells and thereby prevents an apoptotic response. Since BIRC6
knockdown induced apoptosis, BIRC6 might represent an interesting new target for
targeted inhibition in neuroblastoma.

Discussion

In this paper we analyzed aberrations in gene copy number and mRNA expression
of genes directly involved in the intrinsic apoptotic pathway in neuroblastoma.
BIRC6, known as an inhibitor of the pro-apoptotic protein DIABLO, showed gene
Fig 3: DIABLO mRNA and protein expression

(A) Boxplots of DIABLO mRNA expression in 3 neuroblastoma datasets (red), adult tumors (blue) and various normal tissues (green). The boxes represent the 25th to 75th percentile with the median depicted as a horizontal line. Extremes are indicated by the whiskers, and the presence of outliers is indicated by (o). (B) Array CGH of NGP of the region of chromosome 12q24.31 in which DIABLO is located (arrow). Both the B allele frequency (top) and the Log R ratio (bottom) are shown. The chromosome region is shown underneath the picture.

(C) Immunofluorescence of untreated IMR32 cells. Blue is DAPI, red is mitotracker, green is DIABLO antibody. In the right lower corner the merged pictures are shown. (D) Cell fractionation of SJNB12 cells 24 hours after addition of ABT263. The cytoplasmic fraction (cyto) and organelle fraction (organelle) are shown. Blots were incubated with DIABLO, BCL2 and a-tubulin antibodies. (E) Co-immunoprecipitation of IMR32 (top) and SKNSH (bottom) with BIRC6 and DIABLO antibodies. Negative control was the immunoprecipitation antibody Flag. Also a protein sample without antibody and for every antibody a sample without protein was used as negative control. Both blots were incubated with DIABLO antibody. IP antibodies are indicated above the blots.
copy number gains and increased expression. Silencing of BIRC6 with two shRNAs targeting different parts of the coding sequence resulted in increased cytoplasmic DIABLO levels and triggered apoptosis. BIRC6 indeed interacted with DIABLO proteins. Targeted inhibition of BIRC6 therefore might offer new therapeutic openings in neuroblastoma treatment.

The apoptotic response in neuroblastoma cells upon BIRC6 silencing occurs in a background of surprisingly high DIABLO expression levels. High cytoplasmic levels of DIABLO can be tumor inhibitory through binding of IAPs in the cytoplasm. We suggest two mechanisms why high DIABLO expression does not induce apoptosis in neuroblastoma. Firstly, we show that the major fraction of DIABLO protein has a mitochondrial localization. This mitochondrial sequestration is probably mediated by the exceptionally high levels of BCL2 that we have recently described.33 Targeted inhibition of BCL2 caused an increase in cytoplasmic DIABLO levels, suggesting that sequestration of DIABLO in the mitochondria occurs by inhibition of pore formation through BCL2. Secondly, we show that DIABLO is effectively bound by BIRC6 in neuroblastoma and that DIABLO levels increase upon silencing of BIRC6. This suggests effective degradation of cytoplasmic DIABLO by BIRC6. The function of high mitochondrial DIABLO levels in neuroblastoma remains elusive. Other pro-apoptotic proteins in the mitochondria, like cytochrome C, have shown to be involved in energy metabolism but no such mechanism has been found for DIABLO yet.

Release of the strongly increased mitochondrial DIABLO levels would offer a therapeutic potential in neuroblastoma. This suggests that the proteins that impair the pro-apoptotic function of DIABLO could be effective drug targets. The previously shown efficacy of targeted BCL2 inhibitions could relate to high DIABLO levels.33 Moreover, direct BIRC6 inhibition would also increase the cytoplasmic pro-apoptotic function of DIABLO. BIRC6 inhibitors are not available at this moment but targeted drug development might be worth to consider. BIRC6 is not located on the SRO of 2p, but it is frequently gained and functionally active and we therefore consider BIRC6 as an important player in the dysfunction of apoptosis in neuroblastoma. The BIRC6 gene is validated as a potential therapeutic target. If BIRC6 inhibitors are developed, a combined inhibition of BIRC6 and BCL2 could yield synergistic effects.
Acknowledgements

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