New therapeutic targets in the intrinsic apoptotic pathway in neuroblastoma

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Knockdown of Survivin (BIRC5) Causes Apoptosis in Neuroblastoma via Mitotic Catastrophe
Knockdown of Survivin (BIRC5) Causes Apoptosis in Neuroblastoma via Mitotic Catastrophe

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Abstract

*BIRC5* (Survivin) is one of the genes located on chromosome arm 17q in the region that is often gained in neuroblastoma. BIRC5 is a protein in the intrinsic apoptotic pathway that interacts with XIAP and DIABLO leading to CASP3 and CASP9 inactivation. BIRC5 is also involved in stabilizing the microtubule-kinetochore dynamics. Based on Affymetrix mRNA expression data, we here show that BIRC5 expression is strongly up-regulated in neuroblastoma compared to normal tissues, adult malignancies and non malignant fetal adrenal neuroblasts. The over-expression of BIRC5 correlates with an unfavorable prognosis independent of the presence of 17q gain. Silencing of BIRC5 in neuroblastoma cell lines by various antisense molecules resulted in massive apoptosis as measured by PARP cleavage and FACS analysis. As both the intrinsic apoptotic pathway and the chromosomal passenger complex can be therapeutically targeted, we investigated in which of them BIRC5 exerted its essential anti-apoptotic role. Immunofluorescence analysis of neuroblastoma cells after BIRC5 silencing showed formation of multinucleated cells indicating mitotic catastrophe, which leads to apoptosis via TP53 and CASP2. We show that BIRC5 silencing indeed resulted in activation of TP53 and we could rescue apoptosis by CASP2 inhibition. We conclude that BIRC5 stabilizes the microtubules in the chromosomal passenger complex in neuroblastoma and that the apoptotic response results from mitotic catastrophe, which makes BIRC5 an interesting target for therapy.
Introduction

Neuroblastoma are pediatric tumors that originate from the embryonal precursor cells of the sympathetic nervous system. MYCN amplification, gain of 17q and deletion of 1p are frequently occurring genetic abnormalities in neuroblastoma and all correlate with a bad prognosis. Risk stratification is based on tumor stage according to the ‘International Neuroblastoma Staging System’ (INSS), age of the patient and genetic risk factors as MYCN amplification and deletion of 1p. Patients with low-risk tumors can be treated by surgery alone and have a very good prognosis. However, patients with high risk disease are treated with intensive chemotherapy, surgery and high-dose myeloablative therapy to eradicate minimal residual disease. Despite extensive treatment, children with high stage neuroblastoma have a poor prognosis with 20 to 35% overall survival.1-3

Chromosome 17q is gained in the majority of neuroblastoma and BIRC5 (Survivin) is one of the genes located in the SRO (Smallest Region of Overlap) of 17q. BIRC5 is a member of the family of Inhibitor of Apoptosis Proteins (IAPs), which correlates to a bad prognosis.4,5 It functions in the intrinsic apoptotic pathway and it is released from the mitochondria into the cytosol after cell death stimuli, where it can interact with HBXIP, XIAP or DIABLO. If the BIRC5-HBXIP complex is formed, it binds and inhibits CASP9 (Caspase-9), thus preventing apoptosome formation and CASP3 (Caspase-3) cleavage. If BIRC5 is not bound to HBXIP, it can form a complex with and stabilize XIAP, an IAP that inhibits CASP3 cleavage. BIRC5 can also bind and inactivate DIABLO (SMAC), a pro-apoptotic protein that binds and inhibits XIAP.6-9

More recent studies showed a second function of BIRC5 outside the intrinsic apoptotic pathway. BIRC5 was found to be a chromosomal passenger protein that forms a complex with CDCA8 (Borealin), AURKB (Aurora Kinase B) and INCENP by which it regulates microtubule dynamics at the kinetochores. Independent of the chromosomal passenger complex, BIRC5 can stabilize the microtubules by binding directly to them.10-12 If the microtubule-kinetochore dynamics are disturbed, mitotic catastrophe occurs. This results in TP53 and CASP2 (Caspase-2) activation after which the mitochondrial apoptotic pathway is activated.13,14 BIRC5 has five isoforms, generated by alternative splicing. Three variants are anti-apoptotic (BIRC5, BIRC5 ΔEx3, BIRC5 3B) and two variants may be pro-apoptotic (BIRC5 2B and BIRC5 2α).15,16
We hypothesized that over-expression of BIRC5, observed in high risk neuroblastoma tumors, is involved in preventing apoptosis. Analysis of Affymetrix expression data showed that BIRC5 is over-expressed in neuroblastoma compared to various normal tissues, adult tumors and compared to its tissue of origin (fetal adrenal medulla). High expression of BIRC5 in neuroblastoma correlated to a bad prognosis independently of 17q-gain. Targeted inhibition of BIRC5 in neuroblastoma cell lines resulted in a strong induction of apoptosis. We analyzed whether this results from a role of BIRC5 in the intrinsic apoptotic pathway or a role in the chromosomal passenger complex. We did not detect an interaction between BIRC5 and DIABLO or XIAP. BIRC5 silencing resulted in multinucleated cells as shown by immunofluorescence and inhibition of CASP2 could rescue these cells from apoptosis. Also TP53 was activated after BIRC5 silencing. These data strongly suggest that apoptosis after BIRC5 knockdown is caused by mitotic catastrophe.

Methods

Patient Material
The neuroblastic tumor panel used for Affymetrix microarray analysis contains 88 neuroblastoma samples. All samples were derived from primary tumors of untreated patients. Material was obtained during surgery and immediately frozen in liquid nitrogen.

RNA extraction and Affymetrix profiling
For profiling total RNA of neuroblastoma cell lines and tumors was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. RNA concentration was determined using the NanoDrop ND-1000 and quality was determined using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). For Affymetrix Microarray analysis, fragmentation of RNA, labeling, hybridization to HG-U133 Plus 2.0 microarrays and scanning was carried out according to the manufacturer’s protocol (Affymetrix Inc.). The expression data were normalized with the MAS5.0 algorithm within the GCOS program of Affymetrix. Target intensity was set to 100 (α1=0.04 and α2 0.06). If more than one probe set was available for one gene the probe set with the highest expression was selected, considered that the probe set was correctly located on the gene of interest. Public available neuroblastoma datasets we used were of Delattre17 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). For the comparison between neuroblastoma and adrenal neuroblasts a public available dataset of 18 neuroblastoma (13 high stage neuroblastoma and 5 low stage neuroblastoma), 3 samples of the adrenal cortex and 3 samples of adrenal neuroblasts were used. The correlation between BIRC5 expression and prognosis and the difference in expression between neuroblastoma and other tumors or normal tissue was analyzed using the bioinformatic platform R2.

Tissue array
Paraffin-embedded tumors were cut into 4-μm sections, mounted on aminoalkylsaline-coated glass slides, and dried overnight at 37°C. Sections were dewaxed in xylene and graded ethanol, and endogenous peroxidase was blocked in a 0.3% H2O2 solution in 100% methanol. Subsequently, the slides were rinsed thoroughly in distilled water and pretreated with a boiling procedure for 10 minutes in 10/1 mM Tris/EDTA pH 9 in an autoclave. After rinsing in distilled water and PBS, slides were incubated with primary antibody against BIRC5 (abcam, ab469). Slides were incubated for 1 hour in room temperature in a 1:5000 solution (diluted in an antibody diluent). Slides were then blocked with a postantibody blocking (Power Vision kit, ImmunoLogic) 1:1 diluted in PBS for 15 minutes, followed by a 30-minute incubation with poly–horseradish peroxidase (HRP)–goat α mouse/rabbit IgG (Power Vision kit, ImmunoLogic) 1:1 diluted in PBS. Chromogen and substrate were 3,3′-diaminobenzidine (DAB) and peroxide (1% DAB and 1% peroxide in distilled water). Nuclear counterstaining was done with hematoxylin. After dewatering in graded ethanol and xylene, slides were coated with glass. As a negative control we used liver tissue.

Cell lines
SHEP-21N was grown in RPMI-1640 medium (GIBCO) supplemented with 10% fetal calf serum, 4 mM l-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. The other cell lines were grown in Dulbecco Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum, 10 mM L-glutamine, 10 U/ml penicillin, 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.

EZ-N-3042 and transfection procedures
Cells were transfected 24 hours after plating in 10-30% confluence. The Locked Nucleic Acid-Antisense Oligonucleotide (LNA-ASO; provided by Santaris Pharma) was dissolved in PBS and was transfected with Lipofectamin 2000 (invitrogen)
following manufacturer’s procedures. The sequence of the BIRC5 LNA-ASO (EZN-3042) used was: CTCAatccatggCAGc. The sequence of Scrambled LNA-ASO (EZN-3046) was: CGCAgattagaaACCt. The LNA nucleotids are depicted in capitals; the small letters represent DNA nucleotids. Dose efficacy curves were made to calculate the IC50 levels (concentration drug needed for 50% cell survival).

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 BIRC5 shRNA (Sigma, TRCN0000073720) in various concentrations (Multiplicity of infection (MOI): 0.5 - 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. Cells were harvested 48 and 72 hours after transfection for FACS analysis.

Compounds
ZM447439, a small molecule AURKB inhibitor, was dissolved in DMSO with a concentration of 100 mM for stock solution. For synergy assays a concentration series was made from 0 – 6250 nM.

CASP2 inhibition
24 hours after plating IMR32 cells in 10% confluence, Z-Val-Asp(OMe)-Val-Ala-Asp(OMe)-FMK (ZVDVAD-FMK, a widely used CASP2 inhibitor; R&D systems) was added to the cells following manufacturer’s protocol in concentrations between 10 and 50 µM. The cells were transduced with BIRC5 shRNA at the same time. Both immunofluorescence and the MTT assay were performed 48 hours after treatment.

MTT-assay
Cells were plated in a 10-30% confluence in a 96-well plate and transfected after 24 hours with EZN-3042 and EZN-3046 as described above. 48 hours after transfection, 10 µl of Thyazolyl blue tetrazolium bromide (MTT, Sigma M2128) was added. After 4-6 hours of incubation 100 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 (biotech). The IC50 (concentration drug needed for 50% cell viability reduction) was calculated using concentration vector curves. The Combination Index (CI) was calculated by the Chou Talalay method\textsuperscript{20} using the CalcuSyn software.

**RT-PCR**
SKNBE cells were harvested 24 hours after transfection with EZN-3042. For RNA extraction Trizol reagent (invitrogen) was used according to the manufacturer’s protocol and the RNA concentration was determined using the NanoDrop ND-1000. cDNA was made from 1 µg of the extracted RNA with 12.5 pM t12 primer in mQ at 70 °C for 10 minutes. A mix was added with final concentrations of 2 mM MgCl2, 0.5 mM dNTP, 1x Fs-buffer and superscript III (Invitrogen, 100 U) in mQ. The reaction was performed at 50 °C for 60 minutes and 70 °C for 15 minutes. The primers (Biolegio) used for PCR of BIRC5 were: Forward: 5’-GCATGGGTGCCCCGACGTTG-3’, Reverse: 5’-GCTCCGGCCAGAGGCCTCAA-3’. RT-PCR reactions were performed in a final concentration of: 312.5x diluted cDNA, 1 ng/µl forward primer and reverse primer and 2x diluted reddymix (ABgene) in mQ. After activation of Taq at 94 °C, PCR followed with 35 cycles of denaturation at 95 °C for 1 minute, annealing at 50 °C for 1 minute and extension at 72 °C for 2 minutes with a final extension at 72 °C for 5 minutes. Equal volumes of PCR products were electrophoresed through a 1 % agarose gel in TBE-buffer.

**Western Blotting**
24 hours after transfection with EZN-3042, attached and floating cells were harvested on ice. Cells were lysated with Laemmlibuffer (20% glycerol, 4% SDS, 100mM 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 2.5 - 5% ELK in TBS using standard procedures. Primary antibodies used were BIRC5 rabbit polyclonal (abcam ab469), PARP mouse monoclonal (BD-biosciences, 556494), P53 (Neomarkers, BP53-12), and β-actin mouse monoclonal (abcam, ab6276). The secondary antibodies used were a secondary sheep anti-mouse or anti-rabbit horseradish peroxidase linked antibody (Amersham) or secondary antibodies provided by LI-COR. Proteins were visualized using an ECL detection kit (Amersham), or with the Odyssey bioanalyzer (LI-COR).
**FACS analysis**

24 and 48 hours after transfection with EZN-3042 both the attached and the floating cells were fixed with 100% ethanol at -20 °C. After fixing, the cells were stained with 0.05 mg/ml propidium iodide and 0.05 mg/ml RNAse A in PBS. After 1 hour incubation, DNA content of the nuclei was analyzed using a fluorescence activated cell sorter. A total of 20,000 nuclei per sample were counted. The cell cycle distribution and apoptotic sub G1 fraction was determined using Flowjo version 7.2.2.

**Co-immunoprecipitation**

Cells were untreated or treated with ABT263 (Toronto Research Chemicals) at IC50 levels (4.4 μM for IMR32 and 7.9 μM for SKNSH). Cells were lysed in a buffer containing 150 mM NaCl, 50 mM Hepes, 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). Antibodies used for IP were BIRC5 rabbit monoclonal (Cell Signaling, 2808) and DIABLO rabbit monoclonal (abcam, ab-32023). 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 rabbit polyclonal anti-BIRC5, rabbit monoclonal anti-XIAP (Cell signaling, 2045) and rabbit monoclonal anti-DIABLO. Blots were incubated overnight with primary antibodies, after which a one hour incubation step with anti-rabbit IgG was performed followed by incubation with the secondary antibody that was provided by LI-COR.

**Immunofluorescence**

Cells were grown on glass slides in 6-well plates. Cells were fixed with 4% paraformaldehyde in PBS 48 hours after treatment. We used mouse anti-α tubulin (1:1000, Sigma) as a primary antibody and goat anti-mouse (Alexa, A11029) for secondary antibody. Antibodies were dissolved in 5% ELK in PBS/0.2% tween-20. Slides were stained with DAPI (1:1000) in vectashield (Vector Laboratories). At least 5 pictures were made per slide and quantified for number of aberrant cells. Significance between groups was calculated using the Student’s T-test.
Results

*BIRC5 expression in neuroblastoma tumors*

*BIRC5* is up-regulated in several kinds of tumors and is widely investigated as a drug target.\(^{21}\) We therefore evaluated the in vivo expression of *BIRC5* mRNA in neuroblastoma series analyzed by Affymetrix arrays. We used the R2 bioinformatics platform for data analysis (see methods). First, we analyzed the expression of *BIRC5* in 3 independent neuroblastoma data sets of different research groups comprising 88, 64 and 33 tumors. We also analyzed the *BIRC5* expression in various normal tissues and adult tumor types. Neuroblastoma have a significantly increased expression of *BIRC5* compared to normal tissues and also compared to various adult tumor types which are known to have increased *BIRC5* expression (fig 1a). Many neuroblastoma originate in the adrenal medulla, which largely consists of cells from the adrenal sympathetic neuronal lineage. *BIRC5* expression in high stage neuroblastoma tumors was also strongly increased compared to the fetal neuroblasts in normal adrenal tissue (fig 1b) and a higher *BIRC5* expression was found in high stage neuroblastoma than in stage 1, 2 and 4s tumors (fig 1c). We analyzed the prognostic value of *BIRC5* in our series of 88 neuroblastoma used for expression profiling and as expected we found that a high *BIRC5* expression correlated with a worse patient outcome (the lowest P-value of 6.8x10\(^{-7}\) was observed at a cut-off expression value of 400) (fig 1d). The poor prognosis was also significant independent of chromosome 17q gain (p= 4.0x10\(^{-3}\) in tumors with 17q gain, data not shown). To verify if the *BIRC5* RNA expression is representative for BIRC5 protein expression we performed immunohistochemistry on 39 tumor samples and divided them in three groups based on BIRC5 protein staining. We compared this to the BIRC5 RNA expression of these tumors and found a significant correlation (fig 1e).

*Apoptosis of neuroblastoma cell lines after BIRC5 knockdown*

To assess the role of *BIRC5* in neuroblastoma, we silenced *BIRC5* expression in neuroblastoma cell lines by various methods. We first used a BIRC5 Locked Nucleic Acid-Antisense Oligonucleotide (LNA-ASO) that targets the coding sequence of exon 4 of *BIRC5* (EZN-3042). LNA bases contain a methylene bridge that connects the 2'-oxygen of the ribose with the 4'-carbon, which makes it more stable than a regular oligonucleotide and suitable for therapeutic application.\(^{22,23}\) LNA-ASO binds to RNA, prevents transcription and activates RNAse H resulting in cleavage of the target RNA molecule.\(^{24,25}\) We used a panel of ten neuroblastoma cell lines, which all expressed relatively high BIRC5 expression as established by Affymetrix profiling
Figure 1: BIRC5 expression in neuroblastoma tumors based on Affymetrix RNA expression data

(A) Average BIRC5 expression data in Neuroblastoma (black), other tumors (grey) and normal tissue (white). The error bars indicate the standard error; the number of tumor samples is given between brackets. (B) BIRC5 expression data in normal adrenal cortex, non malignant fetal adrenal neuroblasts and high stage and low stage neuroblastoma. Every dot represents one tissue sample and the horizontal line represents the average RNA expression. (C) BIRC5 expression data in all neuroblastoma stages according to the INSS. Every dot represents one tissue sample and the horizontal line represents the average RNA expression. (D) Kaplan Meier curve based on the survival data of 88 neuroblastoma tumors. The expression cutoff is 400. The upper line represents the survival curve of patients with low BIRC5 expression and the lower line represents the survival curve of patients with high BIRC5 expression. The logrank P-value is shown in the graph. (E) BIRC5 RNA expression data are represented by the Y-axis. The three groups of protein expression are represented by the X-axis. Every dot is one tumor sample and the horizontal line shows the average RNA expression. The P-values are depicted above the graph.
BIRC5 Knockdown in Neuroblastoma

We determined the sensitivity of neuroblastoma cell lines for EZN-3042 by MTT assays. The IC50 (concentration drug needed for 50% cell viability reduction) of EZN-3042 determined by dose efficacy curves varied from 2 to 170 nM (fig 2a, table 1). No correlation between sensitivity to EZN-3042 and genetic aberrations or BIRC5 expression levels in neuroblastoma cell lines was found (table 1).

For further analysis of EZN-3042 we used for each cell line its own IC50 value as a concentration. We analyzed the specific knock-down of BIRC5 RNA after treatment of the cells with EZN-3042 by RT-PCR. The main transcript of BIRC5 is clearly expressed in SKNBE and is reduced after EZN-3042 treatment. Furthermore, low levels of the alternative splice variants BIRC5 2B and BIRC5 ΔEx3 were detected. All three variants were strongly silenced (fig 2b). We subsequently analyzed the effect of EZN-3042 for all ten neuroblastoma cell lines by Western blot. EZN-3042 treatment strongly reduced BIRC5 expression in all cell lines and showed an increase of the PARP cleavage product of 80 kD, confirming an apoptotic response (fig 2c).

To exclude that the apoptotic response was caused by an off-target effect of EZN-3042, we also used lentivirally mediated shRNA silencing. The mode of action of shRNA differs from LNA-ASO, as shRNAs are cleaved by the cellular machinery into siRNA that can bind to the RNA-induced silencing complex (RISC), which in turn can bind and cleave the target mRNA. The lentivirally delivered shRNA (which targeted another sequence in BIRC5 than EZN-3042) also down-regulated BIRC5 expression and induced PARP cleavage in the IMR32 cell line, while a control lentivirus (SHC002) did not affect BIRC5 levels (fig 2c right).

<table>
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<tr>
<th>Cell Line</th>
<th>MYCN ampl</th>
<th>LOH 1p</th>
<th>17q gain</th>
<th>Survivin expression</th>
<th>IC50 EZN-3042 (nM)</th>
<th>IC50 EZN-3046 (nM)</th>
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*Table 1*: The IC50 (concentration drug needed for 50% less cell viability) of all neuroblastoma cell lines of the panel for EZN-3042 compared and the control EZN-3046 was determined by MTT assays. The occurrence of the most important genetic aberrations in neuroblastoma is also shown in this table.
Figure 2: Apoptosis of neuroblastoma cell lines after BIRC5 knockdown

(A) The curve of cell viability determined by an MTT-assay after transfection of SKNBE with EZN-3042 and EZN-3046 (control). Cell viability is represented on the Y-axis and the EZN-3042 concentration in nM is represented on the X-axis. (B) RT-PCR of BIRC5 expression on RNA level after treatment of SKNBE with EZN-3042 (S) and EZN-3046 (control: C). Three bands are shown, which represent different BIRC5 isoforms. (C) Western blot of BIRC5 expression after treatment with EZN-3042 (S) or BIRC5 shRNA (sh). The Western blot was incubated with BIRC5, PARP and Actin. (D) FACS analysis of IMR32 after treatment with BIRC5 shRNA. Pictures were made of the cells that were harvested for FACS analysis. The Y-axes of the graphs represent the number of events and the X-axes represent the size of the particles detected. Apoptosis is shown by the sub G1-peak.
To validate the apoptotic response of BIRC5 knockdown and to analyze the effect on the cell cycle distribution we performed FACS analysis. After transduction of IMR32 with BIRC5 shRNA, the sub G1 fraction showed a more than a 5 fold increase from 6.3 to 33.6%, while the cell cycle distribution remained the same (fig 2d). Also after treatment of NGP-c4, SKNAS and IMR32 with EZN-3042, the sub G1 fractions increased with 19%, 28% and 22% respectively above the baseline levels (<7%), without a change in cell cycle distribution (data not shown). We conclude that silencing of BIRC5 results in a strong apoptotic response in neuroblastoma cells.

**Anti-apoptotic effect of BIRC5 is mediated by its role in the chromosomal passenger complex**

BIRC5 has been shown to inhibit the intrinsic apoptotic pathway via direct interaction with XIAP and DIABLO, but it can also stabilize the microtubules at the kinetochores. The mode of action of BIRC5 is relevant when it is used as a drug target, as this may predict synergistic effects with other targeted drugs. We therefore analyzed the binding partners of BIRC5 protein in IMR32 cells by co-immunoprecipitation. Immunoprecipitation of protein lysates with a BIRC5 antibody did not reveal co-immunoprecipitation of XIAP or DIABLO. As a positive control, we also performed immunoprecipitation of the same lysates with an antibody to DIABLO, which showed a clear co-immunoprecipitation of XIAP (fig 3a, left). DIABLO is located in the mitochondria in non-apoptotic cells which could explain the negative IP results between DIABLO and BIRC5. To confirm that BIRC5 is not bound to DIABLO in an apoptotic state, we induced apoptosis by addition of ABT263, an established BCL2 inhibitor. No interaction between BIRC5 and DIABLO was seen (fig 3a, right) which confirms our previous conclusion. Although these negative results do not formally exclude interaction of BIRC5 with DIABLO or XIAP, it suggests that alternative mechanisms are involved.

To demonstrate whether the anti-apoptotic function of BIRC5 in neuroblastoma cells is mediated by its role in the chromosomal passenger complex, we further analyzed IMR32 cells after silencing of BIRC5. We first performed immunofluorescence analyses of cells. IMR32 cells became large after shRNA mediated BIRC5 silencing and showed an increase in the number of micronuclei (fig 3b, panel 1, 2 and 4; P = 0.02). This suggested a disturbance of normal DNA segregation during mitosis, which could lead to the apoptotic response by mitotic catastrophe. Apoptosis after this process is mediated by activation of CASP2 and P53.13,14 We reasoned that inhibition of CASP2 could rescue cells from mitotic catastrophe induced apoptosis,
Figure 3: Anti-apoptotic effect of BIRC5 is mediated by its role in the chromosomal passenger complex

(A) Co-immunoprecipitation of untreated IMR32 cells (left) and IMR32 and SKNSH cells with or without ABT263 treatment (right). Co-immunoprecipitation was performed with BIRC5 and DIABLO antibodies. Western blot has been incubated with BIRC5, DIABLO, and XIAP antibodies. (B) IMR32 cells were transduced with BIRC5 shRNA and treated with ZVDVAD at the same time. 48 hours after treatment immunofluorescence was performed. The cells were stained with α-tubulin antibody (green) and DAPI (blue). Of each sample one picture is shown. Examples of cells with aberrant nuclei are indicated with an arrow. In the graph the percentage of cells with aberrant nuclei is represented and the P-values are indicated. (C) IMR32 cells were transduced with BIRC5 shRNA and treated with ZVDVAD at the same time. The phenotype is shown by the pictures. 48 hours after treatment cell proliferation was determined by an MTT-assay. (D) Western blot after transduction of IMR32 with BIRC5 shRNA and after transfection with EZN-3042. The Western blot was incubated with BIRC5, P53, PARP and Actin.
but not from apoptosis resulting from inhibiting BIRC5 in the intrinsic apoptotic pathway. We therefore treated IMR32 cells with ZVDVAD, a widely used CASP2 inhibitor.\textsuperscript{27-30} Silencing of BIRC5 expression with addition of ZVDVAD indeed rescued the cells from BIRC5 shRNA-induced apoptosis (fig 3c). Moreover, blocking of apoptosis by ZVDVAD increased the number of large cells with multiple micronuclei compared to cells treated with BIRC5 shRNA alone (fig 3b, panel 3 and 4; \( P = 0.01 \)). Finally, we analyzed whether BIRC5 silencing results in P53 activation. Western blot analysis indeed showed a strong increase of the P53 levels after BIRC5 silencing with EZN-3042 as well as with lentiviral shRNA (fig 3d). We conclude that the apoptosis mediated by BIRC5 silencing is associated with mitotic catastrophe and P53 activation and can be rescued by CASP2 inhibition. These data strongly suggest that the anti-apoptotic function of BIRC5 in neuroblastoma cells is exerted by its role in the chromosomal passenger complex.

**Combination of BIRC5 LNA-ASO with an AURKB inhibitor**

The insight that mitotic catastrophe is involved in the apoptotic response after BIRC5 knockdown can guide compound combination strategies. Simultaneous inhibition of other genes in the same signal transduction pathway, such as AURKB could lead to additional or synergistic effects. Therefore we performed MTT synergy assays of BIRC5 LNA-ASO combined with ZM447439 (AURKB inhibitor) in SKNBE and IMR32. In both cell lines BIRC5 LNA-ASO showed an additive effect with a combination index between 0.9 and 1.1 for all concentration combinations as calculated with the Chou Talalay method.\textsuperscript{20} The dose-effect curves of BIRC5 LNA-ASO with ZM447439 in SKNBE are shown in Fig. 4.

![Combined dose dependency curves in SKNBE](image)

**Figure 4: Combined dose dependency curves in SKNBE**
The curves of cell viability determined by an MTT-assay after treatment with ZM447439 combined with fixed concentrations of EZN-3042. Cell viability is represented on the Y-axis and the ZM447439 concentration in nM is represented on the X-axis. The legend shows which line represents which BIRC5 LNA-ASO concentration.
Discussion

In this paper we show that BIRC5 is strongly over-expressed in human neuroblastoma tumors compared to all other tissues and that over-expression correlates with a poor prognosis. BIRC5 inhibition with several antisense techniques causes a clear apoptotic response. We were not able to detect protein interactions between BIRC5 and DIABLO or XIAP. However, we could show mitotic catastrophe and P53 activation after BIRC5 inhibition and we could rescue apoptosis by a CASP2 inhibitor. We conclude that the microtubule stabilization and kinetochore functions of BIRC5 play a major role in neuroblastoma maintenance and that BIRC5 inhibition results in mitotic catastrophe and apoptosis.

The BIRC5 over-expression and its correlation to poor prognostic factors in neuroblastoma patients that we found have been described before.\textsuperscript{4,5} The high expression could partly depend on 17q gain but the significant correlation of BIRC5 with prognosis independent of 17q gain suggests regulation of BIRC5 by other mechanisms as well. One possibility is that the high expression is caused by its cell cycle dependent expression pattern\textsuperscript{11} and because neuroblastoma are fast dividing tumors. This is supported by the fact that BIRC5 expression is correlated to the expression of several important cell cycle genes (unpublished data). Also, BIRC5 is established as an E2F target.\textsuperscript{31} In neuroblastoma a high E2F activity is related to a bad prognosis (Molenaar et al., submitted).

An important role for BIRC5 in mitosis has extensively been shown in other tumors.\textsuperscript{7,10,32-34} This role has also been described before in a neuroblastoma cell line, however unlike our data, BIRC5 knockdown induced Caspase independent cell death.\textsuperscript{35} In addition, apoptosis after BIRC5 inhibition has been shown in neuroblastoma cell lines by others using compounds which are less specific and therefore less suitable for functional analysis of BIRC5 in neuroblastoma compared to BIRC5 antisense techniques.\textsuperscript{36,37} The interactions between BIRC5, XIAP and DIABLO have never been shown in neuroblastoma cell lines, but data on these interactions are available from experiments in other tumor types using over-expression constructs of one of the interacting genes.\textsuperscript{6-8} In this paper we investigated the endogenous interactions between BIRC5 and DIABLO or XIAP and found DIABLO and XIAP to only interact with each other but we did not find an interaction with BIRC5, neither after apoptosis induction. Our results do not rule out that BIRC5 in addition has a role in the intrinsic apoptotic pathway. However, such a
function does not seem to be essential in the apoptotic response triggered by BIRC5 silencing.

BIRC5 knockdown has often resulted in an 8N-peak on FACS analysis in cell lines of several tumor types.\textsuperscript{11,32} Cells with 4N DNA cannot divide due to destabilized microtubules after which they start to re-duplicate the DNA. In our FACS analyses of neuroblastoma cell lines after BIRC5 silencing, we never observed an 8N peak (fig 2d). A possible explanation is that the apoptotic pathway is activated before the cells start to re-duplicate their DNA, resulting in a tetraploid G1 phase arrests. This is in accordance with our finding with immunofluorescence (fig 3b). The cells become large with multiple micronuclei, indicating that they are not able to divide properly, while the amount of DNA does not increase. This phenotype is known as mitotic catastrophe.\textsuperscript{13}

We validated BIRC5 as a drug target in neuroblastoma by showing an apoptotic response after BIRC5 knockdown using two independent antisense techniques. RNA interference functions via the RNA-Induced Silencing Complex (RISC), which can bind and cleave the target mRNA\textsuperscript{26} while LNA-ASO such as EZN-3042 bind mRNA, and activate RNAse H dependent mRNA cleavage.\textsuperscript{24,25,38} Since these antisense techniques have a different mechanism and since we chose different target sequences, it is unlikely that the apoptotic response after BIRC5 inhibition is caused by off-target effects. All cell lines tested appeared to undergo apoptosis after BIRC5 inhibition although some cell lines were less sensitive. This could be caused by a difference in transfection efficiency of EZN-3042.

The validation of BIRC5 as viable drug target in neuroblastoma warrants further development of targeted inhibition of BIRC5 in this pediatric malignancy. EZN-3042 is developed as a human treatment modality.\textsuperscript{24,25,38} In general, due to insufficient delivery to solid tumors and low potency of traditional antisense oligonucleotides, pharmacological activity has been difficult to obtain in these tissues. However, it has been shown that solid tumors can be targeted by Locked Nucleic Acids.\textsuperscript{38,39} BIRC5 based vaccines are found to reduce primary tumor growth and spontaneous liver metastasis in a neuroblastoma xenograft model and are currently in Phase I/II clinical trial in adult tumors.\textsuperscript{40,41} Alternatively, small molecules are available that inhibit the BIRC5 activation pathway. One option is to use CDK1 inhibitors to block phosphorylation and activation of BIRC5 by this kinase. Also 2,5-Dimethyl-celecoxib (DMC), was shown to have an antitumor activity, possibly by inhibiting BIRC5.\textsuperscript{37}
Promising results have been reached with YM155, a small molecule triggering transcriptional repression of BIRC5.\textsuperscript{42-46} Phase-I and II clinical studies in various tumor types showed an antitumor effect of YM155 at a dose that does not cause severe toxicities.\textsuperscript{43,45,46}

BIRC5 LNA-ASO combined with an AURKB inhibitor showed an additive effect for both cell lines tested, which suggests rational for combining BIRC5 inhibitors with one of these compounds in clinical trial. However, BIRC5 inhibition should first be tested in combination with the currently used cytostatics and before a clinical trial can be designed BIRC5 inhibitors should be extensively tested in in vivo neuroblastoma models.

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Reference List