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 caspase-3 and caspase-9 inactivation. *BIRC5* is also involved in stabilizing the microtubule–kinetochore dynamics. Based on the Affymetrix mRNA expression data, we here show that *BIRC5* expression is strongly upregulated in neuroblastoma compared with 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 P53 and CASP2. We show that *BIRC5* silencing indeed resulted in activation of P53 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.

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Introduction

Neuroblastomas 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 20–35% overall survival (van Noesel & Versteeg 2004, Henry *et al.* 2005, Maris *et al.* 2007).

Chromosome 17q is gained in majority of neuroblastoma, and *BIRC5* (survivin) is one of the genes located in the 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

(Adida *et al.* 1998, Islam *et al.* 2000). 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 caspase-9 (CASP9), thus preventing apoptosis formation and caspase-3 (CASP3) 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 (Marusawa *et al.* 2003, Song *et al.* 2003, Dohi *et al.* 2004, Zangemeister-Wittke & Simon 2004).

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), Aurora kinase B (AURKB), and INCENP by which it regulates microtubule dynamics at the kinetochores. Independent of the chromosomal passenger complex, BIRC5 can stabilize the microtubules by directly binding to them (Altieri 2006, Lens *et al.* 2006, Ruchaud *et al.* 2007). If the microtubule–kinetochore dynamics are disturbed, mitotic catastrophe occurs. This results in P53 and caspase-2 (CASP2) activation after which the mitochondrial apoptotic pathway is activated (Castedo *et al.* 2004a,b). BIRC5 has five isoforms, generated by alternative splicing. Three variants are anti-apoptotic (BIRC5, BIRC5 Δ Ex3, and BIRC5 3B) and two variants may be pro-apoptotic (BIRC5 2B and BIRC5 2 α ; Caldas *et al.* 2005, Fangusaro *et al.* 2006).

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 with various normal tissues, adult tumors, and its tissue of origin (fetal adrenal medulla). High expression of BIRC5 in neuroblastoma correlated to a bad prognosis independent 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 P53 was activated after BIRC5 silencing. These data strongly suggest that apoptosis after BIRC5 knock down is caused by mitotic catastrophe.

Materials and 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 Nano-Drop ND-1000 and quality was determined using the RNA 6000 Nanoassay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). For Affymetrix Microarray analysis, fragmentation of RNA, labeling, hybridization to HG-U133 Plus 2.0 microarrays, and scanning were carried out according to the manufacturer's protocol (Affymetrix, Inc., Santa Clara, CA, USA). The expression data were normalized with the MAS5.0 algorithm within the GCOS program of Affymetrix. Target intensity was set to 100 ($\alpha_1=0.04$ and $\alpha_2=0.06$). If more than one probe set was available for one gene, the probe set with the highest expression was selected, considering that the probe set was correctly located on the gene of interest. The commonly available neuroblastoma data sets we used were of Delattre (Fix *et al.* 2008) and Lastowska (geo ID: gse13136). The commonly available data sets 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 commonly available data set of 18 neuroblastoma (13 high-stage neuroblastoma and five low-stage neuroblastoma), three samples of the adrenal cortex, and three samples of adrenal neuroblasts were used (De Preter *et al.* 2006). The correlation between BIRC5 expression and prognosis and the difference in expression between neuroblastoma and other tumors or normal tissue were analyzed using the bioinformatic platform R2.

Tissue array

Paraffin-embedded tumors were cut into 4 μ m 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 0.3% H₂O₂ solution in 100% methanol. Subsequently, the slides were rinsed thoroughly in

distilled water and pretreated with a boiling procedure for 10 min 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, Cambridge, UK). Slides were incubated for 1 h 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, Duiven, The Netherlands) 1:1 diluted in PBS for 15 min, followed by a 30 min incubation with poly-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 using 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% FCS, 4 mM L-glutamine, 100 μ g/ml streptomycin, and 100 U/ml penicillin. The other cell lines were grown in DMEM, supplemented with 10% FCS, 10 mM L-glutamine, 10 U/ml penicillin, non-essential amino acids (1 $\times$), and 10 μ g/ml streptomycin. Cells were maintained at 37 °C under 5% CO₂. For primary references of these cell lines, see Molenaar *et al.* (2008).

EZN-3042 and transfection procedures

Cells were transfected 24 h after plating in 10–30% confluence. The locked nucleic acid-antisense oligo-nucleotide (LNA-ASO; provided by Santaris Pharma, Hørsholm, Denmark) was dissolved in PBS and was transfected with Lipofectamine 2000 (Invitrogen) following the 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 nucleotides are depicted in capitals; the small letters represent DNA nucleotides. Dose efficacy curves were made to calculate the IC₅₀ levels (concentration drug needed for 50% cell survival).

Lentiviral short-hairpin RNA 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 h after transfection, which was purified by filtration and ultracentrifuging. The concentration was determined by a p24 ELISA.

Cells were plated in 10% confluency. After 24 h, cells were transduced with lentiviral *BIRC5* shRNA (Sigma, TRCN0000073720) in various concentrations (multiplicity of infection: 0.5–3). SHC-002 shRNA (non-targeting shRNA: CAACAAGATGAAGAG-CACCAA) was used as a negative control. Twenty-four hours after transduction, medium was refreshed and puromycin was added to determine the efficacy of transduction. Protein was harvested 72 h after transduction and analyzed by western blot. Cells were harvested 48 and 72 h 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 to 6250 nM.

CASP2 inhibition

Twenty-four 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, Minneapolis, MN, USA) was added to the cells following the manufacturer's protocol in concentrations between 10 and 50 μ M. The cells were transduced with *BIRC5* shRNA at the same time. Both the immunofluorescence and the MTT assay were performed 48 h after treatment.

MTT assay

Cells were plated in a 10–30% confluence in a 96-well plate and transfected after 24 h with EZN-3042 and EZN-3046 as described earlier. Forty-eight hours after transfection, 10 μ l thiazolyl blue tetrazolium bromide (MTT, Sigma M2128) was added. After 4–6 h of incubation, 100 μ l of 10% SDS and 0.01 M HCl were added to stop the reaction. The absorbance was measured at 570 and 720 nm using a plate reader (BioTek, Winooski, VT, USA). 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 (Chou & Talalay 1984) using the CalcuSyn Software (bisoft, Cambridge, UK).

RT-PCR

SKNBE cells were harvested 24 h 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 min. A mix was added with the final concentrations of 2 mM MgCl₂, 0.5 mM dNTP, 1×Fs-buffer, and superscript III (Invitrogen, 100 U) in mQ. The reaction was performed at 50 °C for 60 min and 70 °C for 15 min. The primers (Biolegio, Nijmegen, The Netherlands) used for PCR of *BIRC5* were as follows: forward: 5'-GCATGGGTGCCCC-GACGTTG-3', reverse: 5'-GCTCCGGCCAGAGGC-CTCAA-3'. RT-PCRs were performed in a final concentration of 312.5× diluted cDNA, 1 ng/µl forward primer and reverse primer, and 2× diluted reddy mix (ABgene, Epsom, UK) in mQ. After activation of Taq at 94 °C, PCR followed with 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min with a final extension at 72 °C for 5 min. Equal volumes of PCR products were electrophoresed through a 1% agarose gel in TBE buffer.

Western blotting

Twenty-four hours after transfection with EZN-3042, 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 and electroblotted on a transfer membrane (Millipore, Gloucestershire, UK). 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, Franklin Lakes, NJ, USA), P53 (Neomarkers, BP53-12, Newmarket, Suffolk, UK), and β-actin mouse monoclonal (Abcam, ab6276). The secondary antibodies used were a secondary sheep anti-mouse or anti-rabbit HRP linked antibody (Amersham, Diegem, Belgium) or secondary antibodies provided by LI-COR. Proteins were visualized using an ECL detection kit (Amersham), or with the Odyssey bioanalyzer (LI-COR, Lincoln, NE, USA).

FACS analysis

Twenty-four and 48 h 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 h 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, North York, ON, Canada) 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 and 1 mM Na₂VO₃). Antibodies used for IP were *BIRC5* rabbit monoclonal (Cell Signaling, 2808, Boston, MA, USA) 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. Immuno-complexes were washed, heated at 95 °C for 10 min, 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 1 h 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 h after treatment. We used mouse anti-α tubulin (1:1000, Sigma) as a primary antibody and goat anti-mouse (Alexa, Invitrogen A11029, Paisley, UK) 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, Burlingame, CA, USA). At least five pictures were made per slide and quantified for number of aberrant cells. Significance between groups was calculated using Student's *t*-test.

Results

BIRC5 expression in neuroblastoma tumors

BIRC5 is upregulated in several kinds of tumors and is widely investigated as a drug target (Altieri 2003). 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 Materials and methods). First, we analyzed the expression of *BIRC5* in three 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.

Neuroblastomas have a significantly increased expression of *BIRC5* compared with normal tissues and also compared with various adult tumor types that are known to have increased *BIRC5* expression (Fig. 1a). Many neuroblastomas 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 with 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 study 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 6.8×10^{-7} was observed at a cutoff expression value of 400; Fig. 1d). The poor prognosis was also significant independent of chromosome 17q gain ($P = 4.0 \times 10^{-3}$ in tumors with 17q gain, data not shown). To verify whether the *BIRC5* RNA expression is representative for *BIRC5* protein expression, we performed immunohistochemistry on 39 tumor samples and divided them into three groups based on the *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* knock down

To assess the role of *BIRC5* in neuroblastoma, we silenced *BIRC5* expression in neuroblastoma cell lines by various methods. We first used a *BIRC5* LNA-ASO

Figure 1 *BIRC5* expression in neuroblastoma tumors. (a) Average *BIRC5* expression data in neuroblastoma (black), other tumors (gray), and normal tissue (white) based on Affymetrix profiling. The error bars indicate the s.e.m.; 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 based on Affymetrix expression data. 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 based on Affymetrix expression data. Every dot represents one tissue sample and the horizontal line represents the average RNA expression. (d) Kaplan–Meier curve based on Affymetrix expression data and 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 log rank *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.

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 (Beane *et al.* 2007, Mook *et al.* 2007). LNA-ASO binds to RNA, prevents transcription, and activates RNase H resulting in cleavage of the target RNA molecule (Petersen & Wengel 2003, Vester & Wengel 2004). We used a panel of ten neuroblastoma cell lines, all of which expressed relatively high BIRC5 expression as established by Affymetrix profiling (Table 1). 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 and 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 knockdown 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 the 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 in the PARP cleavage product of 80 kDa, 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 (Rubinson *et al.* 2003). The lentivirally delivered shRNA (which targeted another sequence in BIRC5 than in EZN-3042) also downregulated BIRC5 expression and induced PARP cleavage in the IMR32 cell line, while a control lentivirus (SHC002) did not affect BIRC5 levels (Fig. 2c right).

To validate the apoptotic response of BIRC5 knock down 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 fivefold 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

Table 1 IC₅₀ of EZN-3042 in neuroblastoma cell lines. 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

	MYCN ampl	LOH 1p	17q gain	Survivin expression	IC ₅₀ EZN-3042 (nM)	IC ₅₀ EZN-3046 (nM)
SHEP 21N	Y	N	Y	1142	3	> 50
IMR32	Y	Y	Y	2052	3	> 50
SK-N-BE	Y	Y	Y	6765	9	> 50
TR14	Y	Y	Y	858	22	> 50
SJNB10	Y	Y		1210	40	> 50
SJNB-8	Y	Y	Y	1208	45	> 50
SK-N-AS	N	N	Y	1044	46	> 50
NGP-c4	Y	Y		1423	128	> 250
SK-N-FI	N	N	Y	730	164	> 250
SJNB-6	Y	Y	Y	1943	166	> 250

Figure 2 Apoptosis of neuroblastoma cell lines after *BIRC5* knock down. (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 and EZN-3046 (control). Three bands are shown, which represent different *BIRC5* isoforms. (c) Western blot of *BIRC5* expression after treatment with EZN-3042 or *BIRC5* shRNA. 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.

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. After this process, apoptosis is mediated by activation of CASP2 and P53 (Castedo *et al.* 2004a,b). We reasoned that inhibition of CASP2 could rescue cells from mitotic

catastrophe-induced apoptosis, 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 (Nutt *et al.* 2005, Basu *et al.* 2008, Shen *et al.* 2008, Tinnikov *et al.* 2009). 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 with 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* knock down can guide compound combination strategies. Simultaneous inhibition of other genes in the same signal transduction

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. Forty-eight 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. Forty-eight 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.

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 CI between 0.9 and 1.1 for all concentration combinations as calculated with the Chou Talalay method (Chou & Talalay 1984). The dose–effect curves of BIRC5 LNA-ASO with ZM447439 in SKNBE are shown in [Supplementary Figure 1](#), see section on [supplementary data](#) given at the end of this article.

Discussion

In this paper, we show that BIRC5 is strongly over-expressed in human neuroblastoma tumors compared with 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 earlier (Adida *et al.* 1998, Islam *et al.* 2000). 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 (Lens *et al.* 2006) 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 (data not shown). Also, BIRC5 is established as an E2F target (Jiang *et al.* 2004). In neuroblastoma, a high E2F activity is related to a bad prognosis (Molenaar *et al.* 2011).

An important role for BIRC5 in mitosis has extensively been shown in other tumors (Lens *et al.* 2003, Marusawa *et al.* 2003, Zangemeister-Wittke & Simon 2004, Wheatley & McNeish 2005, Altieri 2006). This role has also been described before in a neuroblastoma cell line; however, unlike our data, BIRC5 knock down induced caspase-independent cell death (Shankar *et al.* 2001). In addition, apoptosis after BIRC5 inhibition has been shown in neuroblastoma cell lines by others using compounds that are less specific and therefore less suitable for functional analysis of BIRC5 in neuroblastoma compared with BIRC5 antisense techniques (Pyrko *et al.* 2006, Muhlethaler-Mottet *et al.* 2008). 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 (Marusawa *et al.* 2003, Song *et al.* 2003, Dohi *et al.* 2004). 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 knock down has often resulted in an 8N peak on FACS analysis in cell lines of several tumor types (Lens *et al.* 2003, 2006). 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 (Castedo *et al.* 2004a).

We validated BIRC5 as a drug target in neuroblastoma by showing an apoptotic response after BIRC5 knock down using two independent antisense techniques. RNA interference functions via the RISC, which can bind and cleave the target mRNA (Rubinson *et al.* 2003) while LNA-ASO such as EZN-3042 bind mRNA, and activates RNase H-dependent mRNA cleavage (Petersen & Wengel 2003, Vester & Wengel 2004, Hansen *et al.* 2008). 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 (Petersen & Wengel 2003, Vester & Wengel 2004, Hansen *et al.* 2008). 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 (Hansen *et al.* 2008,

Sapra et al. 2010). 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 (Fest et al. 2009, Ryan et al. 2009). 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 (Pyrko et al. 2006). Promising results have been reached with YM155, a small molecule triggering transcriptional repression of BIRC5 (Nakahara et al. 2007, Altieri 2008, Tolcher et al. 2008, Giaccone et al. 2009, Satoh et al. 2009). 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 (Tolcher et al. 2008, Giaccone et al. 2009, Satoh et al. 2009).

BIRC5 LNA-ASO combined with an AURKB inhibitor showed an additive effect for both cell lines tested, which suggests rationale for combining BIRC5 inhibitors with one of these compounds in clinical trial. However, BIRC5 inhibition should be first 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.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-11-0207>.

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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