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ORIGINAL ARTICLE

Targeting survivin and p53 in pediatric acute lymphoblastic leukemia

JW Tyner[1], AM Jemal[2], M Thayer[3], BJ Druker[1,4] and BH Chang[2]

[1]Division of Hematology and Medical Oncology, and OHSU Knight Cancer Institute, OHSU, Portland, OR, USA; [2]Division of Hematology and Oncology, Department of Pediatrics and OHSU Knight Cancer Institute, OHSU, Portland, OR, USA; [3]Department of Biochemistry and Molecular Biology and OHSU Knight Cancer Institute, OHSU, Portland, OR, USA and [4]Howard Hughes Medical Institute

Despite advances in treatment and outcomes for patients with pediatric acute lymphoblastic leukemia (ALL), there continue to be subsets of patients who are refractory to standard chemotherapy and hematopoietic stem cell transplant. Therefore, novel gene targets for therapy are needed to further advance treatment for this disease. RNA interference technology has identified survivin as a potential therapeutic target. Survivin, a member of the inhibitor of apoptosis (IAP) proteins and chromosome passenger complex, is expressed in hematologic malignancies and overexpressed in relapsed pediatric ALL. Our studies show that survivin is uniformly expressed at high levels in multiple pediatric ALL cell lines. Furthermore, silencing of survivin expression in pediatric ALL cell lines as well as primary leukemic blasts reduces viability of these cells. This includes cell lines derived from patients with relapsed disease featuring cytogenetic anomalies such as t(12;21), Philadelphia chromosome t(9;22), t(1;19) as well as a cell line carrying t(17;19) from a patient with *de novo* ALL. Furthermore, inhibition of survivin increases p53-dependent apoptosis that can be rescued by inhibition of p53. Finally, a screen of randomly selected primary patient samples confirms that survivin-specific small interfering RNA and survivin-targeted drug, YM155, effectively reduce viability of leukemic blasts.
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Introduction

Over the past 50 years, the prognosis for pediatric acute lymphoblastic leukemia (ALL) has changed from a terminal diagnosis to a treatable disease.[1] The success of current therapeutic regimens has given rise to the identification of a subset of patients who will have recurrent or refractory disease. Unfortunately, patients within these subsets have highly resistant disease that may not be overcome even with myeloablative therapy and hematopoietic stem cell transplant. These diseases may have approached the limit of treatment by standard chemotherapeutic regimens. Therefore, new targets for therapy are imperative in the hope of improving outcomes.

A report from the Children's Oncology Group has shown a differential expression profile of relapsed ALL compared with initial diagnosis.[2] One of the genes showing a marked increase in expression in patients with recurrent disease is survivin (aka *BIRC5*). Survivin is an attractive target for therapy because it is expressed mainly during development and in the setting of

malignancy with little to no expression in normal terminally differentiated tissue. Furthermore, survivin overexpression has correlated with resistant and refractory disease in many different malignancies including ALL.[3]

Survivin is a small 16 kD protein that belongs to the inhibitor of apoptosis (IAP) family and also functions as a member of the chromosome passenger complex. Survivin is a unique member of the IAPs in that it is both the smallest member and may not directly interact with caspases. Instead, it may interact with another IAP, smac/Diablo, to regulate apoptosis within the mitochondria.[4] Transcription of survivin shows several splice variants with conserved N-terminal domains, with most of the divergence occurring within or after the baculovirus IAP repeat domain.[5–7] Recent published reports would suggest that these splice variants may have different subcellular localization including the mitochondria and different apoptotic activities.[8]

The role of survivin as a member of the chromosome passenger complex, in which it plays a critical role during mitosis, is better defined.[9] As such, survivin expression is cell-cycle dependent, with the highest expression during G2/M through canonical cell cycle-dependent and cell-cycle homology regions within the proximal promoter.[10] During mitosis, survivin specifically localizes with INCENP and Aurora B kinase within the mitotic apparatus from the centromeres in prophase, kinetochores in metaphase, the mid-plate during anaphase and the mid-body during cytokinesis. Furthermore, it is the direct interaction of survivin with both Aurora B kinase and INCENP that is essential for cell division.[11–13] Survivin phosphorylation at Ser 20 by PLK1 (polo-like kinase) is required for the priming of Aurora B kinase activity to undergo cell division.[14]

In this study we verified the cell-cycle dependence of survivin expression within pediatric ALL cells. We further tested whether several pediatric leukemic cell lines as well as primary patient samples were sensitive to manipulation of survivin expression and activity. Pediatric ALL cell lines have similar expression levels of total survivin. In addition, the expression patterns of survivin among cell lines were similar with an increase in survivin expression during G_2/M. Virtually all of the ALL cell lines tested were sensitive to silencing of survivin including REH (*ETV6-RUNX1*), SUPB15 (*BCR-ABL*) RCH-ACV (*E2A-PBX1*) and HAL01 (*E2A-HLF*). We also identified that targeting survivin either by small interfering RNA (siRNA) or by the survivin-suppressor YM155 in these cell lines increased cell death through the p53-dependent apoptosis pathway. This increase in cell death could then be rescued by silencing p53. Finally, early screening of patient samples with survivin siRNA or YM155 showed sample-specific variation of sensitivity to survivin silencing. The heterogeneity of YM155 responses would suggest that other factors may have importance for primary lymphoblasts to respond to survivin inhibition beyond p53. As such, early disease selection through *in vitro* screening may become

Correspondence: Dr BH Chang, Division of Hematology and Oncology, Department of Pediatrics, OHSU Knight Cancer Institute, Mailcode CDRCP, 3181 SW Sam Jackson Park Road, Portland, OR, USA.
E-mail: changb@ohsu.edu

important for future clinical strategies that would employ survivin as a therapeutic gene target.

Materials and methods

Reagents
Fetal bovine serum (FBS) was obtained from Hyclone Laboratories Inc. (South Logan, UT, USA). All other tissue culture reagents were obtained from Invitrogen Corporation (Carlsbad, CA, USA). The siRNAs (Supplementary Table 1) were from the siGenome SMARTpool designed by Dharmacon (ThermoFisher Scientific, Waltham, MA, USA). Viability assays were performed with CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay from Promega Corporation (Madison, WI, USA). Apoptosis assays were performed using the Guava Nexin Assay (Millipore, Billerica, MA, USA). YM155 was purchased from Selleck (Houston, TX, USA) and solubilized in dimethylsulfoxide at 100 mM stock. Graphical and statistical data were generated using either Microsoft Excel or GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

Cell lines and tissue culture
RCH-ACV (RCH) (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany) is a pediatric ALL cell line from a patient with recurrent disease carrying the *E2A-PBX1* t(1;19) chimeric protein. REH (ATCC) is a pediatric ALL cell line from a patient with recurrent disease carrying the *ETV6-RUNX1* t(12;21) chimeric protein. SUPB15 (American Type Culture Collection (ATCC), Manassas, VA, USA) is a pediatric ALL cell line also from a patient with recurrent disease carrying the *BCR-ABL* t(9;22) translocation. HAL01 cells (DSMZ) are from a pediatric patient with *de novo* ALL with the *E2A-HLF* t(17;19). RCH, REH and HAL01 cells were maintained in RPMI with 10% FBS, 4 mM glutamine and 1% penicillin and streptomycin. SUPB15 cells were maintained in RPMI with 20% FBS, 4 mM glutamine, 50 nM 2-mercaptoethanol and 1% penicillin and streptomycin. All patient samples were obtained with informed consent approved by the institutional review board of Oregon Health and Science University.

Small interfering RNA knockdown, proliferation and induction of apoptosis
Standard electroporation was modified from a previously described protocol.[15] Briefly, 1.5×10^5 cells per condition were resuspended in 75 µl siPORT buffer (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). To the samples, 1–2 µM of siRNA was added. Cells were electroporated at 200 V, 250 µs, 2 pulses, and 20 000 cells per well were plated in triplicate containing 100 µl of culture media. The remaining 60 000 cells were plated into a well containing 500 µl of culture media. For determination of cell viability, the triplicate plates containing 20 000 cells were subjected to the CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay (MTS). For subsequent immunoblot analysis, the plate containing 60 000 cells were harvested and lysed in 20 µl of $1 \times$ sodium dodecyl sulfate (SDS) loading buffer. Identification of induction of apoptosis was performed using the Guava Nexin assay (Millipore). Briefly, triplicate samples containing 20 000 cells were incubated with 60 µl of the Guava Nexin reagent and then analyzed through the microcapillary flow cytometer at varying time points up to 96 h.

Cells were also treated with transductin (Integrated DNA Technologies (IDT), Inc., Coralville, IA, USA) for introduction of siRNA into the cells. A total of 500 nM of siRNA was incubated in phosphate buffered saline (PBS) with 5 µM transductin and added to 2.5×10^5 cells in 0.5 ml of RPMI with 1% bovine serum albumin for

2–4 h at 37 °C. The cell media was then supplemented with 0.5 ml of RPMI containing FBS such that the final concentration of FBS was 10–20% (depending on cell line). A total of 50 000 cells per well were plated in triplicate and grown for 4 days for either MTS or for Gauva Nexin assay. The remaining cells were harvested 48 h after treatment for immunoblot.

Immunoblot analysis
Cells were washed with PBS and lysed with $1 \times$ SDS loading buffer (75 mM Tris, pH 6.8, 3% SDS, 15% glycerol, 8% β-mercaptoethanol, 0.1% bromophenol blue). For mitochondrial fractionation, cells were processed using ApoALert Cell Fractionation Kit (Clontech, Mountain View, CA, USA). All samples were separated by standard SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane (Immobilon-FL, Millipore). Membranes were blocked with Aquablock tm/EIA/WB (EastCoast Bio, Inc., North Berwick, ME, USA) for 1 h, and then incubated with primary antibodies to survivin (Cell Signaling Technology, Inc., Danvers, MA, USA), (Ser 10) phospho-histone H3 (Upstate Biotechnology, Lake Placid, NY, USA), tubulin (Sigma-Aldrich Corp., St Louis, MO, USA), (Ser 15) phospho-p53 (Cell Signaling), p53 (Santa Cruz, Biotechnology, Inc., Santa Cruz, CA, USA) and ABL (BD Biosciences, San Jose, CA, USA) in Aquablock/0.1% Tween-20 overnight at 4 °C. Secondary fluorescent antibodies (Molecular Probes) were used and detected and quantified with Odyssey (LI-COR, Corporate Offices, Lincoln, NE, USA).

Immunofluorescence
ALL cells were grown in complete media and then $\sim 5 \times 10^5$ cells were harvested and resuspended in 0.5 ml of PBS. 2×10^4 cells were spotted onto a cover slip for 10 min. Cells were fixed with 4% formaldehyde at 37 °C for 10 min. Samples were then permeabilized with 0.25% Triton X-100 and washed with PBS. Cells were then stained overnight in 4 °C with primary antibodies and subsequently stained for 1 h with secondary antibody at room temperature. All images were captured with an Olympus (San Jose, CA, USA) BX Fluorescent Microscope using a $\times 50$–100 objective, automatic filter-wheel and Cytovision workstation.

Cell sorting
ALL cell lines were grown in RPMI/10% FBS to a concentration of 5–10×10^5 cells/ml. Cells were then washed with PBS/1% FBS and fixed in 70% ethanol and stored at −20 °C. The samples were then washed with PBS and stained with buffer containing 3 mM EDTA, pH 8.0, 0.05% NP-40, 50 µg/ml propidium iodide and 1 mg/ml RNAse A in PBS.[16] Cells were sorted by DNA content with BD FACS/Aria (BD Biosciences) and harvested with SDS loading buffer.

YM155 dose response
ALL cells lines (5000 cells per well) and primary patient samples (50 000 cells per well) were incubated with graded concentrations of YM155 (0–10 µM) in RPMI with 10% FBS. After 3 days, cells were subjected to MTS for assessment of cell viability. All values were normalized to the no drug control from each respective cell line.

Results

Pediatric ALL cell lines express high levels of survivin mostly at G$_2$/M
Prior studies have suggested that there are varying amounts of survivin expression in primary pediatric ALL samples and in
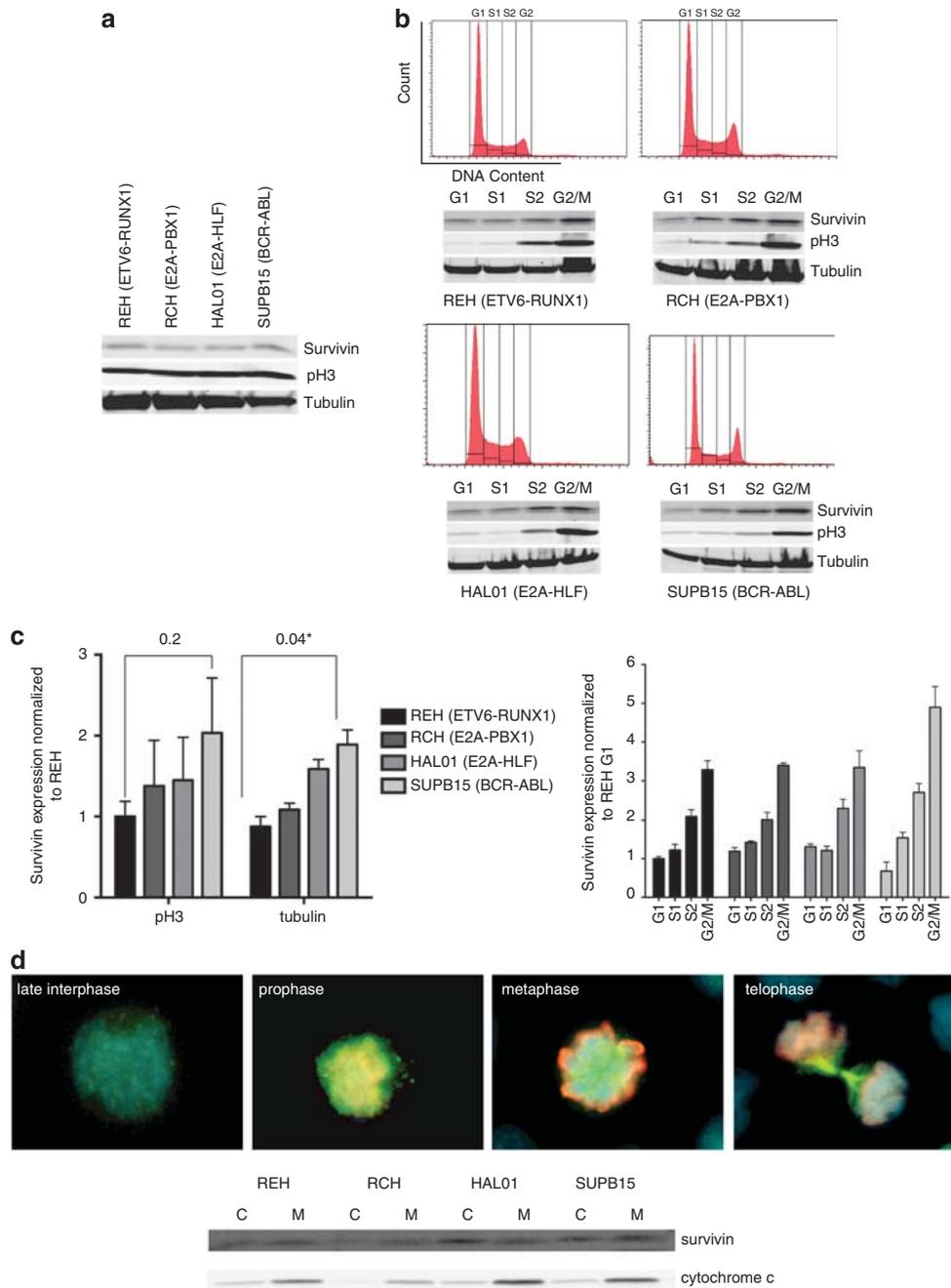
Figure 1 Relative expression of survivin in ALL cell lines and cell-cycle dependence. (**a**) Immunoblot of asynchronous populations ALL cell lines for survivin, (Ser 10) phospho-histone H3 (pH3) and tubulin. The cell lines tested include REH (ETV6-RUNX1), RCH-ACV (E2A-PBX1), HAL01 (E2A-HLF) and SUPB15 (BCR-ABL). The immunoblot shown is representative of three independent experiments. (**b**) Survivin level is dependent on the cell cycle in ALL cell lines. An asynchronous population of ALL cell lines was fixed in 70% ethanol and stained with propidium iodide. Cells were flow sorted by DNA content for G1, early S (S1), late S (S2) and G2/M. Top panels represent the histograms of the cells that were sorted. Bottom panels represent the immunoblots for survivin, pH3 and tubulin. (**c**) Quantification of survivin expression in relation to tubulin and pH3. (Left panel) Each band from immunoblots was quantified by Odyssey for fluorescence intensity and normalized either to tubulin for pH3. Ratios were then compared with REH as control. (Right panel) Graphical representation of the relative quantity of survivin normalized to tubulin from (**b**). Quantity is expressed in comparison with REH in G1. *Denotes $P<0.05$ by student's t-test. (**d**) Subcellular localization of survivin in lymphoblasts. (Upper panel) Each cell line showed similar staining patterns. Representative images of later interphase, prophase, metaphase and telophase are shown from each cell line. 5×10^5 cells of an asynchronous population were dropped onto coverslips and fixed with formaldehyde, and processed for dual immunofluorescence labeling; survivin (green), pH3 (red), DAPI (blue). (Lower panel). Immunoblot of survivin and cytochrome c after fractionation with Apoalert Fractionation. C, cytosolic fraction; M, mitochondrial fraction.

pediatric ALL cell lines.[2,17] To validate this finding, we assessed survivin expression level in a variety of cell lines derived from divergent subsets of pediatric ALL patients, including HAL01 cells that have been used to describe the dependence of survivin overexpression through the chimeric transcription factor E2A-HLF found in t(17;19).[17] Immunoblot of a population of asynchronous cells showed varying amounts of survivin when normalized to tubulin expression, with SUPB15 cells showing a statistically significant difference to REH (t-test 0.04; Figures 1a and c).

Survivin expression is regulated by the cell cycle and plays a role within the chromosome passenger complex.[11–13] These chromosome passenger complex proteins show relatively lower expression in G_1/S phase with the highest expression in G_2/M. To determine whether the small variations of survivin expression observed in cell lines is dependent on the proportion of cells in G_2/M phase at the time of cell lysis, we also examined cell lysates for abundance of Ser 10 phosphorylation on histone H3 (pH3), a marker for G_2/M phase. After normalization of survivin expression to pH3, there appeared to be no significant difference in the amount of survivin expression between cell lines (Figures 1a and c). Furthermore, most of the expressed survivin appears to be in G2/M as shown by flow sorting cells from different stages of the cell cycle followed by immunoblot for survivin, pH3 and tubulin (Figures 1b and c).

The cell cycle-dependent expression of survivin would suggest that in ALL cells lines the major role of survivin would be as a member of the chromosome passenger complex. However, survivin has been shown to exhibit divergent subcellular localizations with certain splice variants exhibiting cytosolic and mitochondrial distribution, suggesting that survivin may also play a role as an IAP.[18] As such, we next wanted to assess whether there are different subcellular pools of survivin within ALL cell lines. We examined survivin expression patterns in RCH, REH, SUPB15 and HAL01 cells by immunofluorescence. In each cell line, the majority of survivin staining was evident in cells undergoing mitosis (Figure 1d and data not shown). There is an increase in survivin staining in early prophase with evident colocalization with chromosomal material, and there is a subsequent increase in the intensity of survivin staining through metaphase. As the chromosomes segregate, survivin stays at the mid-body through cytokinesis. These results are consistent with prior published data on the subcellular localization of survivin in monolayer cells.[8,11] Interestingly, we do observe some variation in punctate cytosolic staining for survivin in interphase cells. The antibody to survivin, which is generated against the N-terminus of the protein (Cell Signaling), is predicted to react to all splice variants. Furthermore, cell fractionation does show that there is a small population of survivin in both the cytosolic and mitochondrial fractions (Figure 1d).

Inhibition of survivin expression reduces cell viability in ALL cell lines

As ALL cell lines appear to express similar amounts of survivin, we next tested whether survivin knockdown would have deleterious effects on viability of these cells. Leukemia cell lines treated with survivin-specific siRNA consistently showed a significant reduction in viability, with RCH, REH, SUPB15 and HAL01 cells each exhibiting at least 40% decrease in cell viability (Figure 2a). Immunoblots performed 48 h after siRNA treatment confirmed siRNA-mediated silencing of survivin of ~50% reduction in levels (Figure 2b and data not shown).

Recently, a novel small-molecule inhibitor, YM155, was developed by Astellas Pharma US, Inc. (Deerfield, IL, USA) as a potent inhibitor of survivin expression.[19] This compound was developed using a screen that identified small molecules that would only inhibit survivin expression at the promoter. As this reagent offered a different mode of survivin suppression, we also tested the impact of this compound on viability of ALL cell lines. Consistent with siRNA results, each of the cell lines tested showed a dose-dependent sensitivity to YM155 as measured by cell viability 72 h after exposure (Figure 2c). Furthermore, inhibition of survivin expression and increase in apoptosis can

be seen in a dose-dependent manner even at 24 h after exposure to YM155 (Supplementary Figure 1). To test whether YM155 sensitivity was specific to inhibition of survivin expression, RCH cells were transfected with pMIG-Survivin. Cells were treated with 1 µM YM155 for 48 h, and then assayed for apoptosis by Annexin V staining. Ectopic expression of survivin in RCH cells partially rescues apoptosis when treated with 1 µM YM155, further validating the selective inhibition of survivin (Supplementary Figure 1d). Interestingly, there was some variation of sensitivity to this compound with REH cells being the most sensitive (half-maximal inhibitory concentration (IC_{50}) 17 nM) and HAL01 showing the least sensitivity (IC_{50} 560 nM). RCH and SUPB15 cells had IC_{50}'s that ranged between 10 and 50 nM. These results would suggest a possible heterogeneity of response among ALL lymphoblasts to inhibition of survivin.

Survivin inhibits the p53-dependent apoptotic pathway in ALL cell lines

Previous studies have suggested that overexpression of survivin inhibits the p53-dependent apoptosis pathway.[20] Therefore, inhibition of survivin may allow for re-activation of this p53-mediated apoptotic program. Prior data suggest that most pediatric ALL cell lines are wild type for *TP53* by gene expression patterns.[21,22] To test whether the cells lines evaluated in this study had an intact p53-dependent cell-cycle arrest, the cells were treated with 0.1 µg/ml of doxorubicin. Doxorubicin is an anthracycline that is known to inhibit resealing of DNA breaks, thereby activating a p53-dependent cell-cycle arrest and apoptosis through the intrinsic pathway. RCH, REH, SUP B15 and HAL01 cells all showed activation of p53 by phosphorylation at Ser 15 (pTP53) within 4 h of treatment (Figure 3a). Furthermore, these cells showed cell-cycle arrest by decrease in pH3.

To test whether the increase in cell death observed after silencing of survivin in ALL cell lines was due to p53 activity, RCH and REH cells were treated with a combination of survivin and p53 siRNA. As previously shown, knockdown of survivin significantly decreased the viability and increased annexin V staining of both RCH and REH cells. Interestingly, treatment with a combination of siRNA to p53 and survivin partially rescued cell viability and decreased annexin V staining (Figure 3b). Immunoblots performed showed similar amounts of protein silencing (Figure 3c), whether alone or in combination. Interestingly, treatment of cells with siRNA to survivin increases the quantity of p53 within the cell (Figure 3c). This increase in p53 is also abrogated by siRNA to p53, suggesting that this phenomenon is from increased transcription or stability of the p53 transcript rather than enhanced stability of the p53 protein. These results are consistent with prior findings that inhibition of survivin expression increases p53-dependent apoptosis and may begin to shed new light as to the mechanism of this inhibition.[23] Prior studies have shown conflicting data as to whether survivin interacts with or is independent of caspases.[17,24,25] For example, Nakahara *et al.*[19] have shown that caspase 3 is activated by YM155 in HRPC cells whereas Okuya *et al.*[17] describe caspase-independent cell death in t(17;19) ALL. Our studies would further agree with Okuya *et al.*[17] that neither caspase 3 nor caspase 9 is activated when treated with either siRNA or YM155, yet they still activate apoptosis in ALL cells (Supplementary Figures 1 and 2).

Ph + ALL cells are sensitive to knockdown of survivin independent of imatinib

As previously discussed, Ph + ALL (BCR-ABL) is a model of resistant disease in which the primary oncogene is well
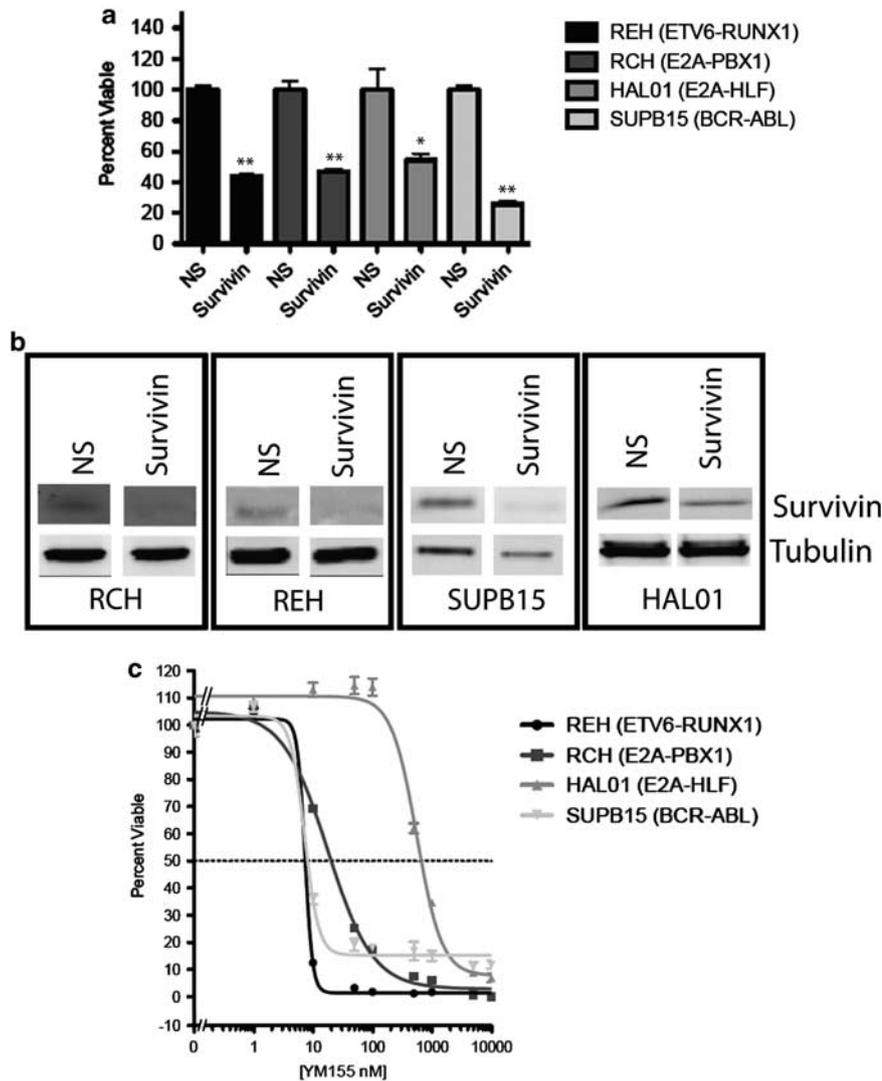
Figure 2 Inhibition of survivin expression inhibits cell viability for REH, RCH, SUPB15 and HAL01. (**a**) Cell viability as measured by MTS 96 h after treatment with siRNA for survivin. All samples were treated with nonspecific (NS) siRNA as a control and normalized to 100%. s.e.m. bars are inserted. $*P<0.05$, $**P<0.01$. (**b**) Representative immunoblots for the expression level of survivin treated with NS siRNA or survivin siRNA. Tubulin expression was used as a control for loading. (**c**) Dose response of ALL cell lines to YM155. Asynchronous populations of cells were treated with increasing doses of YM155 for 72 h. Then, viability was measured by MTS and normalized to no drug control. S.e.m. bars are inserted. Dotted line represents 50% viability.

characterized. Although the addition of imatinib as targeted therapy has improved outcome, there remain patients who continue to relapse. To test whether $Ph+ALL$ would also be sensitive to targeting of survivin, SUPB15 cells were treated with siRNA to survivin, BCR-ABL (ABL),and p53 (Figure 4). Knockdown of survivin increases Annexin V staining that is then rescued by siRNA to p53 (Figures 4a and b). Silencing of survivin or BCR-ABL decreased cell viability by $\sim50\%$ (Figure 4c). Furthermore, simultaneous knockdown of BCR-ABL and p53 had a minimal effect on the cells, suggesting that cell death after silencing of BCR-ABL occurs mainly through a p53-independent mechanism.

Interestingly, the combination of survivin and BCR-ABL silencing did not show an additive effect. It has previously been shown that in certain CML cell lines, the BCR-ABL/mitogen-activated protein kinase pathway increases expression of survivin and that silencing of survivin enhances imatinib-induced cell death.[26] To test whether survivin knockdown could also enhance imatinib-induced cell death, SUPB15 cells were treated with

siRNA for survivin and p53 in the presence of 500 nM imatinib (Figure 4d). In contrast to cells treated with ABL siRNA where no additivity with survivin was observed, knockdown of survivin did show an additive increase in cell death in the presence of imatinib. One possibility for this difference could involve minor variations in signaling that occur after elimination of BCR-ABL protein from cells (siRNA) versus inhibition of BCR-ABL activity without eliminating the protein (imatinib). Another possibility could include inhibition of other non-BCR-ABL pathways by imatinib that could synergize with silencing of survivin. Knockdown of p53 did not rescue the effects on cell viability of either imatinib or ABL siRNA. In contrast, silencing of p53 did rescue the effects of both survivin siRNA and YM155 (Figure 4d, lower panel), and YM155 appears to activate p53 (Supplementary Figure 1a). Cumulatively, these results support the role of survivin as an inhibitor of p53-dependent apoptosis in $Ph+ALL$, which is a pathway independent of BCR-ABL function, suggesting that targeting of survivin could enhance therapy by inhibiting independent pathways to imatinib.
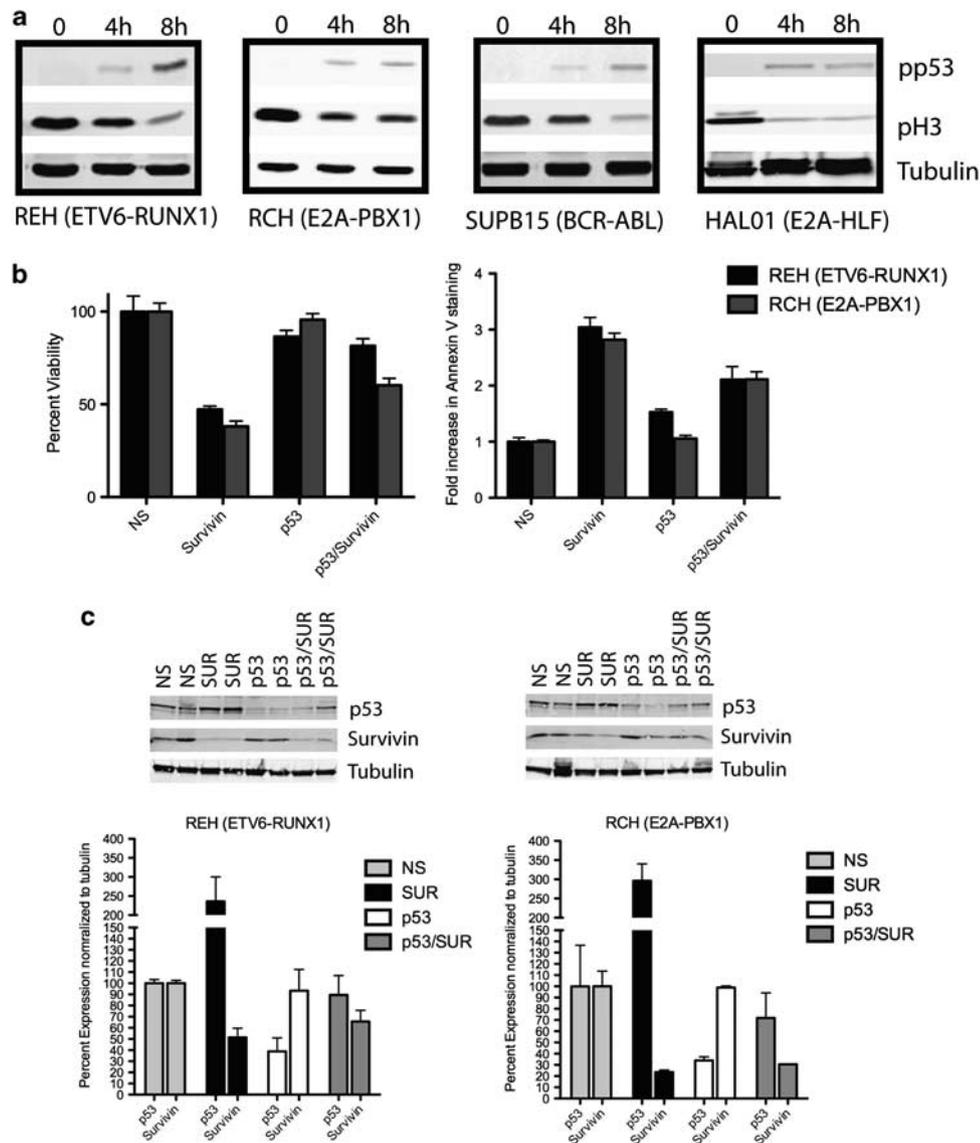
Figure 3 Knockdown of p53 rescues cell death because of silencing of survivin. (**a**) Activation of p53 by treatment with doxorubicin. 1×10^6 cells of RCH, REH, SUPB15 and HAL01 were treated with 0.1 μg/ml of doxorubicin (Sigma) for 0, 4 and 8 h. The cells were then harvested, separated and immunoblotted for (Ser 15)-phospho-p53 (pp53), pH3 and tubulin. S15P p53 evaluated the phosphorylation state of p53 at Ser 15 by activation of the replication checkpoint. pH3 was again used as a marker for cell-cycle arrest reflecting a decrease in the number of cells entering mitosis. (**b**) Partial rescue of cell viability by knockdown of p53. RCH and REH cells were treated with siRNA to NS, survivin, p53 and a combination of p53/survivin. Viability was tested by MTS and normalized to NS (left panel). Apoptosis was then evaluated by Annexin V binding (right panel). (**c**) Immunoblots of representative duplicate experiments to quantify the expression of survivin, and p53 after treatment with siRNA. Lower panels show the relative expression normalized to tubulin quantified by fluorescence intensity.

Primary patient samples are sensitive to inhibition of survivin

Our results would suggest that patients with pediatric ALL could benefit from inhibition of survivin. For initial, preclinical validation of this strategy, we isolated leukemic blasts from fresh primary ALL samples and treated the cells with siRNA to survivin. Treatment of two, randomly selected primary patient samples with survivin siRNA showed response with a 30–50% decrease in cell viability (Figure 5a). To further validate survivin as a *bona fide* therapeutic target, we treated fresh primary patient samples with YM155. Treatment of four patient samples revealed a range of sensitivity to this drug from IC_{50} values ⩽10 nM to IC_{50}'s exceeding 1 μM consistent with the cell lines (Figure 5b). Interestingly, the samples that showed the highest IC_{50}'s were the HAL01 cells and the patient sample with

E2A-HLF. Immunoblots were also performed to identify the expression levels of survivin as compared with tubulin and pH3 (Figures 5c and d). There was a distribution of variability of expression that does not appear to correlate with sensitivity to YM155. For example, patient 5 (relapsed with *ETV6-RUNX1*) was most sensitive to YM155, yet had one of the lowest levels of survivin expression when normalized to tubulin. However, this patient had relatively high expression when normalized to pH3. This would suggest that the cell cycle-independent expression of survivin is a more important correlate for YM155 sensitivity than overall survivin expression. In contrast, samples with E2A-HLF (patient 3, Figure 5, and HAL01, Figure 2) exhibited less sensitivity to YM155 despite high expression of survivin, suggesting that other factors may play an important role in YM155 sensitivity. E2A-HLF cell lines have previously been
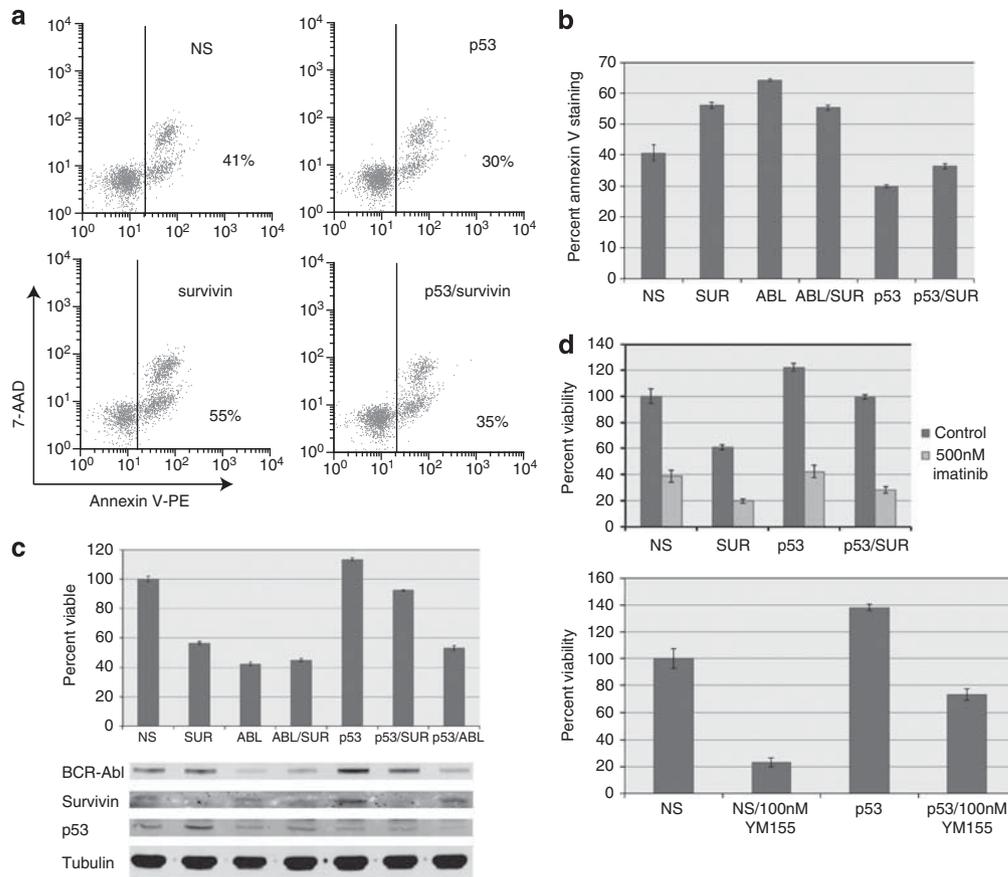
Figure 4 Sensitivity of inhibition of survivin in SUPB15 cells is dependent on intact p53. (**a**) Flow cytometric representation of apoptosis by staining for Annexin V and 7-aminoactinomycin D (7-AAD). SUPB15 cells (BCR-ABL) were treated with siRNA to NS, SUR, p53 and p53/SUR. (**b**) A graphical representation of Annexin V staining in SUPB15 treated with NS, survivin, ABL, ABL/survivin, p53 and p53/survivin. (**c**) Graphical representation of the percent viability of SUPB15 cells treated with NS, survivin, p53 and p53/survivin. The lower panel represents the immunoblots performed on the samples treated with the siRNA and stained for BCR-ABL, survivin, p53 and tubulin. Viability was tested by MTS and normalized to nonspecific siRNA (NS). (**d**) Treatment of SUPB15 cells with imatinib or YM155. Top panel shows cells treated with siRNA in the presence of 500 nM imatinib. Cells were treated with siRNA, and then subsequently incubated in 500 nM imatinib for 96 h. Bottom panel shows the partial rescue of SUPB15 cells treated with 100 nM YM155 for 96 h. S.e.m. bars are inserted into each graph.

shown to overexpress the drug efflux protein ABCB1,[27] which may reduce the amount of YM155 within the cell, thereby increasing the IC_{50}. These studies would suggest that preselection of patients by *in vitro* screening for sensitivity to YM155 would be important in future studies using this compound for clinical trials.

Discussion

In contrast to prior reports, our data have shown that ALL cell lines express similar amounts of survivin and that most of the protein is expressed during G_2/M. Meanwhile, primary patient samples do show significant variability in survivin expression. This study also demonstrates that knockdown of survivin in ALL increases p53-dependent apoptosis. Furthermore, in the BCR-ABL line SUPB15, reduction of BCR-ABL activity or expression does not increase the p53-dependent apoptotic pathway. Finally, we show that screening of primary samples with either siRNA to survivin or the selective suppressor YM155 has the potential to identify samples that are more sensitive to survivin inhibition.

There is evidence to support the interaction of survivin with the p53-dependent apoptotic pathway. In BaF3 cells, it has been

shown that overexpression of survivin has the ability to inhibit degradation of Mdm2 (murine double minute 2), thereby promoting degradation of p53.[28] Other studies have shown that p53 has the ability to decrease survivin expression.[20] In human lung and ovarian cancer cell lines, activation of p53 decreases expression of survivin by changing acetylation of the survivin promoter rather than direct binding. In our studies, silencing of survivin did show a qualitative increase in p53 expression. These findings would suggest that there is a critical relationship with survivin and p53 whereby higher levels of survivin inhibit p53 either by degradation or inhibition of expression, whereas inhibition of survivin activates p53 and increases p53 expression. Taken together, our studies support the critical role for the interaction of survivin with the p53-dependent apoptotic pathway as an important regulator of growth in pediatric lymphoblasts.

Molecular targeting of survivin continues to be an intriguing concept for therapy. It is a protein whose expression is almost exclusively confined to dividing cells such as malignant tissue and normal hematopoietic stem cells and colonic epithelium.[29,30] Although survivin is expressed differentially within the cell cycle with the highest expression at G_2/M, some studies have suggested a baseline increase in expression in malignant cells.[31] Also, higher expression of survivin has been shown in
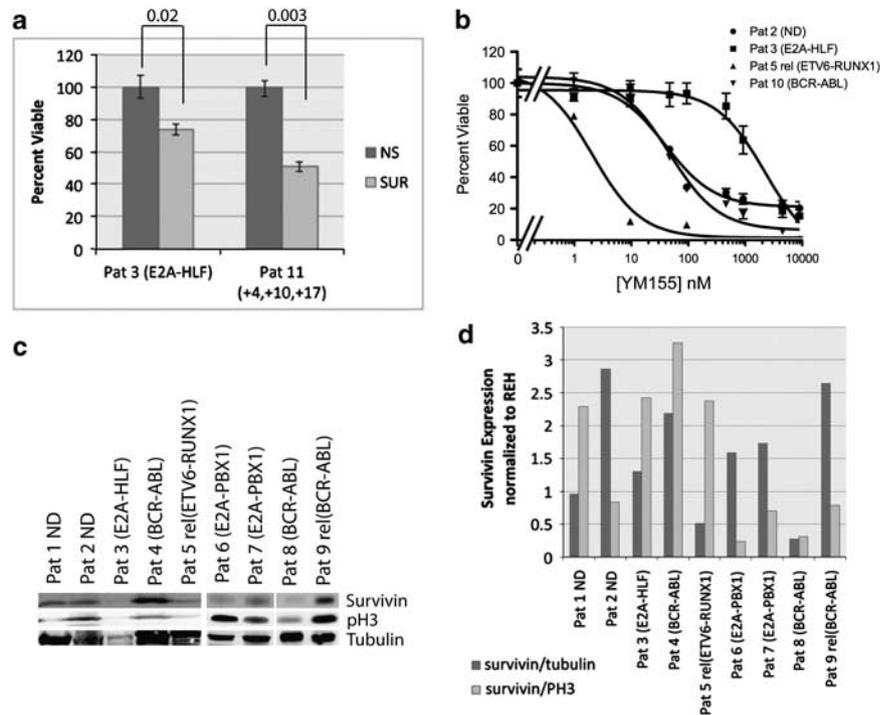
Figure 5 Primary patient (Pat.) samples show sensitivity to inhibition of survivin. See Supplementary Table 2 for patient details. (**a**) Two random fresh primary patient samples were treated with siRNA to NS and survivin. Viability was then assayed 4 days later by MTS, and normalized to viability to NS. (**b**) Dose response of patient samples to YM155. Fresh mononuclear cells were separated by Ficoll and ∼50 000 cells were plated into each well containing increasing concentrations of YM155 from 1 nM to 1 μM. Viability was assayed 3 days later by MTS and normalized to no drug control. Viability was then assayed 3 days later by MTS. All plots contain s.e.m. bars. (**c**) Immunoblots of patient samples for survivin pH3 and tubulin. Approximately 1×10^7 cells were lysed in 100 μl of SDS loading buffer and 20 μl was run on polyacrylamide gel electrophoresis (PAGE). (**d**) Graphical representation of survivin expression normalized to either tubulin or pH3 intensity. These ratios were then compared with REH ratios as a control.

recurrent and relapsed disease.[3] Furthermore, in cell lines there does appear to be a weak but direct correlation with the quantity of survivin expression and sensitivity to YM155.[32] Our studies would suggest that there is a distribution of expression of survivin in primary patient samples and the expression level may not correlate with sensitivity to targeting of survivin.

There are many ways of targeting survivin such as antisense oligonucleotides, ribozymes, siRNA, dominant-negative mutants, small-molecule antagonists and immunotherapy.[33] Many of these compounds are in early clinical phase I and II trials. Interestingly, most of these compounds are well tolerated with minimal toxicities and appear to have modest effects on heavily pretreated patients when administered as monotherapy. Our studies would suggest that pediatric ALL patients will also benefit from inhibition of survivin. Future studies are currently under development for combination of these survivin-targeted agents with other therapeutics.

In our studies we chose to utilize YM155 because this drug has the advantage over siRNA or antisense oligos in that it does not require a specific delivery system. When the drug is given as a continuous intravenous infusion for 7 days, it was well tolerated and also had modest effects on the diseases, including diffuse large B-cell lymphomas.[32] Our studies would suggest that specific patient samples or disease subtypes may respond differently to these drugs. Therefore, selection of patients based on *in vitro* sensitivity screens may improve response rates to this and other survivin-targeted compounds.

Clearly, there are a variety of potential mechanisms that may explain the heterogeneous responses observed to inhibition of survivin. As knockout studies have confirmed survivin as an essential protein and it is also expressed in all cells undergoing

mitosis (including normal hematopoietic cells),[29] one could argue that complete inhibition of survivin as a therapy may have increased side effects because of its essential role within the cell. Instead, perhaps selective inhibition of survivin as an IAP may provide a better therapy. Our data, which were largely collected using siRNA in which only partial silencing of survivin was achieved, may suggest that a therapeutic strategy that also achieved only partial inhibition of survivin could increase p53-dependent apoptosis in a select group of pediatric ALL including Ph + ALL. Therefore, partial inhibition of survivin may have a selective role in adjuvant therapy for these diseases. In contrast, cell line studies using YM155 have not shown a correlation with sensitivity to survivin inhibition and p53 status.[19,32] These observations would suggest that other mechanisms must be involved regarding inhibition of survivin and cell death.

Future studies must be performed to address several unanswered questions. The first is the mechanism by which survivin inhibits the p53-dependent apoptotic pathway in leukemic cells. Previous data in t(17;19) cell lines would suggest that survivin sensitivity is mediated through a mechanism independent of caspases 3 and 9. Yet, we have observed that both the cell line HAL01 and the primary t(17;19) patient sample were relatively insensitive to YM155. This may suggest that there are other mechanisms involved in this disease as opposed to the other samples such as drug efflux. Another concern would be the relative utility of targeting survivin in pediatric patients with ALL. Our data would suggest that there may be a selective population that is more sensitive to inhibition of survivin. Currently, there are clinical trials using antisense oligonucleotides (EZN-3042) to survivin either under development or in phase I trials for relapsed leukemias. Our prediction would be a

heterogeneous response in these trials as only a subset of patients within each disease subtype would be predicted to exhibit survivin sensitivity, based on our findings. Furthermore, our studies show the importance of preselection of subjects by *in vitro* inhibitory screens to identify subjects more likely to respond to survivin inhibition. Therefore, we would propose to perform *in vitro* screening to assist with subject selection.

In conclusion, our data support the concept that targeting survivin may be of clinical benefit in pediatric ALL. Furthermore, this targeting would be selective to the role of survivin as an IAP as opposed to its fundamental role in the chromosome passenger complex. Future studies will be needed to verify these results in primary patient samples and to identify patients who will respond to therapies targeting survivin.

Conflict of Interest

Acknowledgements

Author contributions

BHC and JWT designed the experiments; BHC and AMJ performed the experiments; and BHC, JWT, AMJ, MT and BJD wrote the manuscript.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)