Identifying therapeutic targets by elucidating signaling pathways in pediatric lymphoid leukemias
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Effect of *IKZF1* deletions on signal transduction pathways in Philadelphia chromosome negative pediatric B-cell precursor Acute Lymphoblastic Leukemia (BCP-ALL)

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ABSTRACT

Background: IKZF1 deletions are an unfavorable prognostic factor in children with Philadelphia chromosome positive (Ph+) as well as negative (Ph-) acute lymphoblastic leukemia (ALL). Although IKZF1 deletions occur in 10-15% of Ph- ALL cases, effects of IKZF1 deletions on signaling pathways in this group have not been extensively studied. Therefore, in this study we aimed to study the effect of IKZF1 deletions on active signal transduction pathways.

Methods: Multiplex ligation-dependent probe amplification (MLPA) was used to determine IKZF1 deletions and other copy number alterations in 109 pediatric B-Cell Precursor ALL (BCP-ALL) patients. Kinase activity profiling of 45 primary Ph- BCP-ALL patients (31 IKZF1 wild type patients and 14 patients harboring an IKZF1 alteration) and western blot analysis of 14 pediatric BCP-ALL samples was performed to determine active signal transduction pathways.

Results: Unsupervised hierarchical cluster analysis of kinome profiles of 45 pediatric Ph- ALL cases showed no clustering based on IKZF1 status. Comparing the phosphorylation intensities of peptides associated with signaling pathways known to be involved in BCP-ALL maintenance, we did not observe differences between the two groups. Western blot analysis of 14 pediatric BCP-ALL samples showed large variations in phosphorylation levels between the different ALL samples, independent of IKZF1 status.

Conclusions: Based on these results we conclude that, although IKZF1 deletions appear to be an important clinical prognostic factor, we were unable to identify a unique IKZF1 dependent protein expression signature in pediatric Ph- ALL and consequently no specific targets for future therapy of Ph- IKZF1 deleted BCP-ALL could be identified.
INTRODUCTION

Overall survival rates for children with Acute Lymphoblastic Leukemia (ALL), the most common type of cancer in children, are approaching 90%.1 Historically, risk stratification of newly diagnosed children was based on age and white blood cell count (WBC), but nowadays includes extensive cytogenetic and molecular analyses. In the past 5 years, genome wide approaches, studying DNA copy number alterations in ALL, have identified novel molecular markers that can be used for further risk stratification, including IKZF1 deletions as a predictor of poor outcome. IKZF1 deletions can be identified in approximately 70% of the children with Philadelphia chromosome positive (Ph+) ALL and in 10-15% of the children with Philadelphia chromosome negative (Ph-) ALL and are associated with an increased relapse risk and decreased overall survival in both groups.2-4 More recent studies indicate that the genomic context in which IKZF1 deletions occur is more important for prognosis as for example CRLF2 and JAK2 mutations are more common in IKZF1 deleted BCP-ALL.5-7 In pediatric B-cell progenitor ALL (BCP-ALL), 80% of the IKZF1 deletions are found in a Philadelphia chromosome negative background.

IKZF1, which encodes the transcription factor Ikaros, is essential for normal lymphoid development, whereas for erythroid and myeloid lineage differentiation IKZF1 is less critical.8 Mice deficient for IKZF1 show a complete arrest in B-lymphocyte development while mice heterozygous for a dominant-negative mutation of IKZF1 develop T cell leukemia and lymphoma with a 100% penetrance.9,10 During normal development, Ikaros restricts the G1-S transition of the cell cycle when it binds to the DNA, by regulating transcription of cell cycle regulator genes e.g. a positive effect on cell cycle inhibitors CDKN1A (p21Cip1) and CDKN1B (p27Kip1).11 Phosphorylation of Ikaros by casein kinase II (CK2) temporarily reduces Ikaros binding to DNA and thereby facilitates progression through the S phase of the cell cycle.11 Furthermore, Ikaros can be phosphorylated by spleen tyrosine kinase (SYK) and bruton’s tyrosine kinase (BTK).12,13 These phosphorylation events are essential for nuclear localization, regulation of DNA binding activity, and an optimal transcriptional function of Ikaros.12,13

IKZF1 deletions observed in BCP-ALL are typically mono-allelic, either resulting in a loss of function or the expression of a dominant-negative isoform.14 The dominant-negative isoforms lack the DNA binding N-terminal zinc fingers, preventing DNA binding after dimerization with Ikaros.15 As a result, the control of Ikaros on the G1-S transition is abolished leading to hyperproliferation and the development of leukemia.11

Although the cure rates for children with BCP-ALL have improved substantially, the outcome after ALL relapse remains poor. Since IKZF1 deletions increase the risk of relapse, new therapeutic options aiming to improve cure rates for this specific subtype of ALL are needed. We have previously shown that insight into active signal transduction pathways allows identification of interesting targets for future therapy.16-19 At the level of signal transduction, Iacobucci et al. showed on western blot analysis a higher STAT5 phosphorylation in IKZF1 deleted compared to IKZF1 wild type adult BCP-ALL patients with unknown cytogenetic background.20 However,
this observation might also be associated with BCR-ABL1 activity as in adult BCP-ALL patients.

IKZF1 deletions are more common in Ph+ ALL.\textsuperscript{21} Additionally, Ikaros-reconstitution in two IKZF1 deleted Philadelphia positive ALL patients resulted in an upregulation of the B-cell receptor (BCR) signaling pathway and a concomitant cell cycle arrest; showing that in Ph+ ALL pre-B cell receptor signaling suppresses proliferation through an Ikaros-mediated cell cycle arrest.\textsuperscript{22}

Although IKZF1 deletions in children are most commonly found in a Philadelphia negative background, the effect of IKZF1 deletions on signaling pathways in Philadelphia negative ALL have not been extensively studied. Therefore, in this study we aimed to study the effect of IKZF1 deletions on active signal transduction pathways in Philadelphia negative pediatric BCP-ALL using kinome profiling.

MATERIAL AND METHODS

Patients

Primary blood and bone marrow samples from newly diagnosed ALL patients were collected after getting written informed consent in accordance with the regulations and protocols of the medical ethics committee of the University Medical Center Groningen. Overall, we collected material of 109 Philadelphia negative BCP-ALL patients. Mononuclear cells were isolated by Lympho-prep (Nycomed, Zürich, Switzerland) density gradients and cryopreserved in liquid nitrogen until use. The cryopreserved leukemia cells were thawed rapidly at 37°C and diluted in a 6 ml volume of newborn calf serum, as described earlier.\textsuperscript{23}

DNA isolation

Genomic DNA was extracted from mononuclear cells using the QIAamp DNA easy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. All isolated DNA was quantified by NanoDrop spectrophotometry (NanoDrop, Wilmington, DE, USA).

Multiplex Ligation-Dependent Probe Amplification (MLPA)

Targeted copy number screening of eight selected loci was performed in the cohort by multiplex ligation-dependent probe amplification (MLPA) using the P335-B2 SALSA MLPA kit (MRC-Holland, Amsterdam, The Netherlands). The assay includes probes for each of the eight exons of the IKZF1 gene and is able to detect deletions of the whole gene as well as all types of focal intragenic deletions. Selected exons of the genes BTG1, CDKN2A/B, EBF1, ETV6, PAX5, RB1 and the PAR1 region (approx. 230 kbp downstream of SHOX, CRLF2, CSF2RA and IL3RA) are also covered. Probe mix and hybridization buffer (MRC-Holland) were added in equal amounts to 50 ng of genomic DNA followed by heat denaturation and overnight hybridization of the probes at 60°C. Subsequently, ligation was performed and the ligation products were amplified by PCR.
using a 6-FAM fluorophore-labeled primer set (MRC-Holland). The amplification products were quantified and identified by capillary electrophoresis on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

**Data analysis:** Data were analyzed using Gene Mapper v.4.0 software (Applied Biosystems). Normalization of the data was carried out by dividing the peak area of each probe by the average peak area of the control probes. This normalized peak pattern was divided by the average peak pattern of all the samples in the same experiment. The resulting values were 1 for every wild-type peak, 0.5 for heterozygous deletions and 1.5 for heterozygous duplications.

**PepChip**

Kinase activity profiles of 45 primary Ph- BCP-ALL patients were determined using the PepChip™ Kinomics microarray system (Pepscan, Lelystad, the Netherlands) and performed as described previously.\(^\text{18,19}\) The microarray contain 1,024 peptides in triplicate (1,008 unique target peptides and 16 peptides used for production) derived from known phosphorylation sites from human protein sequences that can be phosphorylated by the kinases in the sample lysate. Per sample, 0.5 x 10^6 cells were lysed in 100 μl of M-PER Mammalian Protein Extraction Buffer containing 1 μl Phosphatase Inhibitor and 1 μl Protease Inhibitor (Pierce, Rockford, IL, United States). Peptide array incubation mix was produced by adding 10 μl of filter-cleared activation mix onto 90 μl cell lysate. Peptide array incubation mix was loaded on the chip and incubated for 2 hours at 37°C in a closed humid box at saturated humidity. Subsequently, the peptide array was washed and blow dried with compressed air or N\(_2\) and the chips were exposed to a phospho-storage screen for 24-96h. The amount of bound \(^{33}\)P-labelled ATP to the peptides specifies the amount of peptide phosphorylation and was analyzed with array software (ScanAlyze, Eisen Lab, University of California at Berkely, Berkely, CA, United States).

**Data analysis:** Data were analyzed as described previously.\(^\text{18,19}\) In short, background was subtracted and the spot intensities were quantile normalized. A Pearson’s correlation coefficient was determined over the triplicates (Excel 2003, Microsoft Office, Redmond, WA, United States). Slides with a correlation <0.6 over the triplicates were excluded from further analysis. The correlation over the triplicates was <0.6 in none of the samples. Cluster, statistical and heatmap analysis were performed using Qlucore Omics Explorer 3.0 (Qlucor AB, Lund, Sweden). The file containing the processed raw data can be found in the supplementary information (Supplementary Table 1).
Western blot analysis

Primary ALL cells were solved in laemmli sample buffer (Bio-Rad laboratories, Veenendaal, the Netherlands). Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transported to nitrocellulose membranes. Membranes were blocked in 7.5% skimmed milk and incubated overnight with primary antibodies for phospho-Src_Y416, phospho-Syk_Y323, phospho-ERK1/2_T202/Y204, phospho-CREB_S133, phospho-p38_T180/Y182, phospho-Akt_S473, phospho-mTOR_S2448, phospho-GSK3α/β_S21/S9, phospho-MDM2_S166, phospho-Chk2_T68, phospho-RB1_S807/811, phospho-STAT3_Y705, phospho-STAT5_Y694 (Cell Signaling, Danvers, MA, USA), or phospho-p27_T187 (Abgent, San Diego, CA, United States) and for 1 hour with HRP conjugated secondary antibodies (Dako, Glosturp, Denmark). Protein bands were visualized using the ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, United States) and ImageLab software (version 5.0, Bio-Rad Laboratories). Loading control was visualized using β-actin (Santa Cruz Biotechnology, Dallas, TX, United States).

RESULTS

Generation of kinase activity profiles in IKZF1 deleted versus IKZF1 wild type Philadelphia negative pediatric BCP-ALL

MLPA analysis revealed that among the 109 Philadelphia negative pediatric BCP-ALL patients tested, 17 (15.6%) patients harbor an IKZF1 deletion whereas one patient (0.9%) showed a gain of IKZF1. We generated kinase activity profiles of 31 IKZF1 wild type patients and 14 patients with an IKZF1 alteration (13 patients with an IKZF1 deletion and 1 patient with a gain of IKZF1). No material was available for kinome profiling of the other three patients with an IKZF1 deletion. Among the 14 patients harboring an IKZF1 alteration, deletions in exons 1 through 8 (4 patients, 28.6%), 4 through 7 (3 patients, 21.4%), and 4 through 8 (3 patients, 21.4%) were most frequent (Figure 1). Patients’ characteristics are shown in Figure 1. Patients harboring an IKZF1 alteration appeared to be older compared to IKZF1 wild type patients (8.4 versus 5.7 years, respectively, \( P = 0.038 \)). Furthermore, MLPA analysis showed more CSF2RA alterations in the group with IKZF1 alterations compared to the IKZF1 wild type group (\( P = 0.003 \)).

Kinase activity profiles were generated to reveal potential differences in signaling differences between IKZF1 deleted and non-deleted Ph− pediatric BCP-ALL cases. Compared to previously performed kinome arrays, we observed a good variability in peptide phosphorylation intensities among the 1,008 unique target peptides and within the 45 individual patients, indicating that the experimental conditions were optimal. Unsupervised hierarchical cluster analysis showed no clustering based upon IKZF1 status (Figure 2A). Moreover, no differential clustering could be observed based on karyotype and copy number alterations of PAX5, ETV6, CDKN2A, CDKN2B, and CRLF2 (Figure 2A). The clustering in peptide activity was not correlated to patients’
Clinical and genetic characteristics as well as copy number alterations identified using Multiplex Ligation-Dependent Probe Amplification (MLPA) analysis are shown for 45 patients who were included in the kinome analysis.

characteristics as age, gender, and white blood cell count (WBC) at diagnosis (Supplementary Figure 1). To evaluate whether \textit{IKZF1} deletions display a unique kinase signature we studied differences in peptide phosphorylation intensities by \textit{t}-test analysis. Thirty-eight peptides were differentially phosphorylated between \textit{IKZF1} deleted (\textit{N} = 13) and \textit{IKZF1} wild type (\textit{N} = 31) pediatric Ph - BCP-ALL patients: phosphorylation of 14 peptides was higher in the \textit{IKZF1} deleted group and 24 peptides showed reduced phosphorylation intensities (\textit{P} \leq 0.05, Figure 2B, Supplementary Table 2). In Figure 2B, normalized peptide intensities are shown with each variable normalized to mean 0 and variance 1. Although we observed thirty-eight peptides to be differentially phosphorylated, absolute differences of normalized peptide phosphorylation intensities between \textit{IKZF1} deleted and wild type samples were small (Supplementary Figure 2 and Supplementary Table 2).
Chapter 5

Focusing on the normalized peptide phosphorylation intensities, high phosphorylation levels of peptides derived from Cytohesin-1_S394 and Cytohesin-2_S392 were shown. The list of top 100 most highly phosphorylated peptides also showed only subtle variations between *IKZF1* deleted and wild type cases (Supplementary Table 3). Although no gross differences in peptide phosphorylation levels were observed, the list of top 100 highest phosphorylated peptides included a number of potentially druggable targets for the treatment of BCP-ALL. Among the top 100 most highly phosphorylated peptides, we identified 86 peptides previously defined as activated peptides in pediatric ALL including cAMP responsive element binding protein 1 (CREB1), peptides derived from protein kinases related to the PI3K/Akt-signaling pathway including peptides related to phosphatidylinositol 3 kinases and ribosomal S6 kinases, and peptides derived from regulators of the cell cycle including checkpoint kinase 2 and retinoblastoma 1.19

**Signal transduction pathway activation in response to *IKZF1* status**

To focus more closely on active signal transduction pathways, we determined peptide phosphorylation of proteins involved in important signaling pathways for BCP-ALL cell proliferation and survival (e.g. the BCR signaling pathway, the MAPK, PI3K/Akt/mTOR, JAK/STAT5 signaling pathways), adhesion pathways, and regulators of the cell cycle (including p21Cip1 and p27Kip1, Figure 3). Activity of these signaling pathways could be observed in both *IKZF1* wild type and *IKZF1* deleted pediatric Ph<sup>-</sup> ALL patients. Comparing phosphorylation intensities of peptides associated with these main signaling pathways showed no differences in pathway activation between *IKZF1* wild type and *IKZF1* deleted Ph<sup>-</sup> ALL (Supplementary Table 4).

**Figure 2** Kinome profile in Philadelphia chromosome negative pediatric BCP-ALL patients

**(A)** Unsupervised hierarchical clustering of 45 pediatric BCP-ALL cases; 13 *IKZF1* deleted, 1 *IKZF1* gain, and 31 *IKZF1* wild type Philadelphia chromosome negative patients based on 1,008 unique target peptides. No distinct clustering could be observed based on *IKZF1* status, neither on genetic background or other copy number alterations.

**(B)** Unsupervised hierarchical clustering of 44 pediatric BCP-ALL cases; 13 *IKZF1* deleted and 31 *IKZF1* wild type patients based on 38 differentially phosphorylated peptides identified by t-test. Each row represents a peptide, each column represents a single ALL sample. The magnitude of deviation from the median is represented by the color saturation with each variable normalized to mean 0 and variance 1. Red and green spots display the phosphorylation intensity above and below the median, respectively.
Effect of IKZF1 deletions on signal transduction pathways in pediatric Ph- BCP-ALL

A

B
Figure 3 Signal transduction in *IKZF1* deleted versus wild type cases in Philadelphia chromosome negative pediatric BCP-ALL patients

Shown are peptide phosphorylation intensities of proteins involved in important signal transduction pathways for BCP-ALL cell proliferation and survival. Results are expressed in gray scale representing the mean peptide phosphorylation intensity of all category peptides.

Phosphorylation levels of key signaling proteins in *IKZF1* deleted and wild type BCP-ALL

Previously, upregulation of the B-cell receptor (BCR) signaling pathway was reported in adult Ph⁺ ALL in response to Ikaros-reconstitution, as well as an increased STAT5 phosphorylation in *IKZF1* deleted versus wild type adult ALL cases with unknown cytogenetic background.²⁰,²² Although various peptides involved in the BCR signaling pathway and peptides derived from STAT5 were present on the array, we did not observe these differences in our Ph⁻ cases. Therefore, in addition to the kinome profiles, we performed western blot analysis of 14 pediatric BCP-ALL samples; seven Philadelphia negative *IKZF1* wild type patients, six Philadelphia
negative IKZF1 deleted patients, and one Philadelphia positive IKZF1 deleted patient (Figure 4). The western blot results showed a large variation in phosphorylation levels between the different ALL samples. STAT5_Y694 phosphorylation could be detected in the Philadelphia positive patient (patient no 14) and in two Philadelphia negative patients with an IKZF1 deletion (patients no 6 and 8), but was not detectable in the other IKZF1 deleted Philadelphia negative patients (patients no 7, 9, 10, and 13, Figure 4). The western blot results showed that protein phosphorylation of Src_Y416, Syk_Y323, ERK1/2_T202/Y204, CREB_S133, p38_T180/Y182, Akt_S473, mTOR_S2448, GSK3α/β_S21/S9, MDM2_S166, p27_T187, Chk2_T68, Rb1_S807/811, and STAT3_Y705 was clearly independent of IKZF1 status. Neither did we observe a clear relation between the western blot results and known copy number alterations of PAX5, ETV6, Rb1, CDKN2A, CDKN2B, or CRLF2 (Figure 4). In conclusion, our kinome and western blot results suggest that IKZF1 deletions do not predict a unique protein expression signature in pediatric Ph- ALL.

DISCUSSION

IKZF1 deletions are found in approximately 70% of the children with Philadelphia chromosome positive (Ph+) ALL and in 10-15% of the children with Philadelphia chromosome negative (Ph-) ALL. In both groups, IKZF1 deletions are associated with an increased risk on relapse and decreased overall survival.2-4 In Ph- ALL, the effect of IKZF1 deletions on outcome is most pronounced in children with an intermediate treatment response based on the minimal residual disease at days 42 and 84.24 Although multiple studies have established IKZF1 as a prognostic factor in pediatric BCP-ALL, the effect of IKZF1 deletions on signaling pathways in Philadelphia negative B-cell precursor ALL is poorly understood. In previous studies, we have shown that kinome profiling can be used successfully to describe active signal transduction pathways in pediatric malignancies.16-19 In this study we used kinome profiling to elucidate the effect of IKZF1 deletions on active signal transduction pathways.

Unexpectedly, unsupervised hierarchical cluster analysis revealed no clustering between IKZF1 deleted and wild type patients. Furthermore, peptide phosphorylation intensities between IKZF1 deleted and wild type Ph- BCP-ALL patients were very comparable as shown by the phosphorylation intensities of the thirty-eight differentially phosphorylated peptides and by the list of top 100 most highly phosphorylated peptides. While focusing on important signaling pathways involved in cell proliferation and survival of BCP-ALL cells we showed activity of all these pathways, however, no differences between the IKZF1 deleted versus the IKZF1 wild type group could be observed. Moreover, western blots of several key proteins involved in ALL signaling showed a variety of phosphorylation events, clearly unrelated to IKZF1 status.
Figure 4 Protein phosphorylation profiles in Philadelphia chromosome negative pediatric BCP-ALL patients

Western blot analysis of 14 pediatric BCP-ALL samples; seven Philadelphia negative \(IKZF1\) wild type, six Philadelphia negative \(IKZF1\) deleted patients, and one Philadelphia positive \(IKZF1\) deleted patient showing phosphorylation expression levels of a selection of proteins. The results show a great diversity in phosphorylation levels between the different ALL samples independently of \(IKZF1\) status and other copy number alterations.
Although no clear differences in peptide phosphorylation intensities could be observed, kinome profiles showed a remarkable high phosphorylation of peptides derived from Cytohesin-1_S394 and Cytohesin-2_S392. Cytohesins have been described as ErbB receptor activators.\textsuperscript{25} It has been described that Cytohesin overexpression enhances epidermal growth factor receptor (EGFR) signaling in human cancers including lung cancer and colorectal cancer.\textsuperscript{25,26} Although kinase domain mutations of ErbB receptors are uncommon in acute leukemias, in vitro inhibition of ErbB2 reduces cell proliferation especially when combined with BCR-ABL tyrosine kinase inhibitors in Ph\textsuperscript{+} ALL, suggesting a role for ErbB signaling pathway activation in leukemia.\textsuperscript{27,28} Therefore, it will be interesting to further explore the role of Cytohesin in BCP-ALL.

Although we did not identify a unique kinome signature as a result of \textit{IKZF1} status, effects of \textit{IKZF1} deletions on gene expression were described previously. Iacobucci \textit{et al.} showed that \textit{IKZF1} deletions display a unique gene expression signature in a cohort of adult B-ALL patients, including patients with a Philadelphia translocation and B-ALL patients negative for known molecular rearrangements.\textsuperscript{20} The gene expression signature was characterized by the downregulation of genes regulating B-cell lineage development and DNA repair upon DNA damage response genes and upregulation of cell cycle/apoptosis genes, JAK/STAT signaling and stem cell self-renewal.\textsuperscript{20} More recently, besides an upregulation of genes associated with B-cell proliferation, an upregulation of genes involved in cell adhesion and communication was also observed in pediatric Ph\textsuperscript{+} ALL.\textsuperscript{29}

In addition to a unique \textit{IKZF1} dependent gene expression profile, a subtype of precursor BCP-ALL with a similar gene expression profile compared to Philadelphia-positive ALL was identified several years ago (Ph-like ALL).\textsuperscript{2,30} Importantly, 68% of the patients with Ph-like ALL showed deletions in \textit{IKZF1}.\textsuperscript{31} Within the Ph-like ALL subtype, 5-year event-free survival rates in patients with \textit{IKZF1} alterations were inferior compared to Ph-like \textit{IKZF1} wild type ALL patients.\textsuperscript{31} Recently, Roberts \textit{et al.} defined the genomic landscape of Ph-like ALL in a large cohort of children and adolescents to elucidate kinase-activating genetic alterations which might include potential leads for targeted therapy.\textsuperscript{31} Genomic alterations activating kinase signaling were identified in 91% of the Ph-like ALL patients (\(N = 156\)) including ABL-class fusions, rearrangements of JAK2 or CRLF2, genetic alterations including IL7R and FLT3, and Ras pathway mutations.\textsuperscript{31} Patients harboring \textit{IKZF1} alterations (\(N = 96, 61.5\%\)) were distributed over all groups of different kinase alterations, indicating a high degree of heterogeneity within the group of \textit{IKZF1} deleted patients.\textsuperscript{31} Our results and the results of Roberts \textit{et al.} suggest that patients harboring \textit{IKZF1} alterations represent a heterogeneous subgroup when evaluated at the level of active signal transduction pathways. The identification of potential targets for tyrosine kinase inhibitors therefore appears to be dictated by upstream genomic alterations that activate kinase signaling or cytokine receptor pathways rather than \textit{IKZF1} status per se.
CONCLUSIONS

The aim of this study was to elucidate the effect of *IKZF1* deletions on active signal transduction pathways using kinome profiling and western blot analysis in children with Ph⁻ BCP-ALL. Although *IKZF1* deletions are an important clinical prognostic factor we were unable to identify a unique *IKZF1* associated protein expression signature in pediatric Ph⁻ BCP-ALL and consequently no specific targets for future therapy of Ph⁻ *IKZF1* deleted BCP-ALL were identified.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

**Supplementary Table 1** Processed raw data values of the PepChip

*This table can be found in the online supplementary information of the journal.*

**Supplementary Table 3** Top 100 most highly phosphorylated peptides

Shown is the list of top 100 highest phosphorylated peptides in *IKZF1* deleted and *IKZF1* wild type Philadelphia negative pediatric BCP-ALL patients. Highlighted peptides are overlapping peptides between *IKZF1* deleted and *IKZF1* wild type.

*This table can be found in the online supplementary information of the journal.*

**Supplementary Table 4** List of proteins involved in important signaling pathways for BCP-ALL

Shown are the mean normalized phosphorylation intensities of multiple peptides derived from indicated proteins as well as *P*-values are shown for *IKZF1* deleted (*N* = 13) and *IKZF1* wild type (*N* = 31) pediatric patients.

*This table can be found in the online supplementary information of the journal.*
### Supplementary Table 2 List of 38 differentially phosphorylated peptides

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<th>Peptide</th>
<th>Mean normalized phosphorylation intensity IKZF1 wild type</th>
<th>Mean normalized phosphorylation intensity IKZF1 deleted</th>
<th>P-value</th>
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<td>TRKA_Y490</td>
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<td>-89.56</td>
<td>0.002</td>
</tr>
</tbody>
</table>
### Supplementary Table 2 List of 38 differentially phosphorylated peptides (Continued)

<table>
<thead>
<tr>
<th>Peptide (Gene)</th>
<th>Mean normalized phosphorylation intensity IKZF1 wild type</th>
<th>Mean normalized phosphorylation intensity IKZF1 deleted</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin dependent kinase 5_Y15</td>
<td>117.92</td>
<td>-0.13</td>
<td>0.021</td>
</tr>
<tr>
<td>Mnk2_T379</td>
<td>45.11</td>
<td>-56.85</td>
<td>0.009</td>
</tr>
<tr>
<td>Sperm associated antigen 1_S317</td>
<td>147.22</td>
<td>55.48</td>
<td>0.039</td>
</tr>
<tr>
<td>WT1_S365</td>
<td>-6.55</td>
<td>-83.01</td>
<td>0.049</td>
</tr>
<tr>
<td>Grb2_Y209</td>
<td>11.25</td>
<td>-88.94</td>
<td>0.020</td>
</tr>
<tr>
<td>E1A binding protein p300_S1834</td>
<td>-11.51</td>
<td>-116.11</td>
<td>0.007</td>
</tr>
<tr>
<td>Serum response factor_S83</td>
<td>92.38</td>
<td>4.36</td>
<td>0.021</td>
</tr>
<tr>
<td>Ezrin_T353</td>
<td>44.67</td>
<td>-55.24</td>
<td>0.019</td>
</tr>
<tr>
<td>NIK_T559</td>
<td>18.84</td>
<td>-58.15</td>
<td>0.034</td>
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<tr>
<td>Crystallin, alpha A_T140</td>
<td>3.64</td>
<td>-82.50</td>
<td>0.048</td>
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<tr>
<td>Sepiapterin reductase_S213</td>
<td>59.56</td>
<td>-37.92</td>
<td>0.012</td>
</tr>
<tr>
<td>Colony-stimulating factor 3 receptor_ Y814</td>
<td>-3.18</td>
<td>-87.53</td>
<td>0.006</td>
</tr>
</tbody>
</table>

### Supplementary Figure 1 Unsupervised hierarchical clustering on patients’ characteristics

Unsupervised hierarchical clustering of 1,008 unique target peptides using Qlucore Omics Explorer 3.0 showed no distinct clustering based on the patients’ characteristics age, gender and white blood cell count (WBC).
Supplementary Figure 2 Absolute phosphorylation intensities of 38 differentially phosphorylated peptides

Supervised hierarchical clustering of 44 pediatric BCP-ALL cases; 13 IKZF1 deleted and 31 IKZF1 wild type Philadelphia chromosome negative patients based on 38 peptides identified by t-test. Each row represents a peptide, each column represents a single ALL sample. Absolute phosphorylation intensities are shown by the color saturation, red and green spots display high and low phosphorylation intensities, respectively.
REFERENCES


