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**Short title**

Proteomics identify therapeutic vulnerabilities

**Disclosures**

The authors declare no conflicts of interest.

**Data availability statement**

The data set generated and analyzed during the current study will be made available at PRIDE.¹

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Author contributions
L.B., J.B., M.S., J.-P.B., A.B., M.R., O.A. and U.F. planned and directed the study. Patient-derived xenograft models were provided by B.B., B.M. and J.-P.B. F.B., J.T.D., V.M., F.D., D.L. and O.A. conducted proteomic profiling and analyzed proteomic data. D.P. provided bioinformatic analyses. L.B. designed and performed the in vitro experiments, supported by V.J., J.S.-D. and R.H.. N.Q. generated dose-response curves. S.B. provided intellectual contributions to the project and to the interpretation of the results. L.B., V.J. and U.F. wrote the manuscript. Figures were designed and drafted by L.B., D.P., N.Q., V.J., J.S.-D. and U.F. All authors edited and contributed to the final manuscript.
Although therapy escalation has led to improved 5-year overall survival rates for patients with B-cell acute lymphoblastic leukemia (B-ALL), few effective treatment options are available for relapsed and treatment-resistant disease. This applies particularly to specific subtypes of B-ALL, such as patients harboring TCF3 (formerly E2A) fusions. TCF3, encoding members of the E protein (class I) family of helix-loop-helix transcription factors, is a master regulator of B-cell development and is involved in several chromosomal translocations associated with lymphoid malignancies, such as the translocation t(1;19)(q23;p13.3), resulting in the TCF3::PBX1 fusion (5% of pediatric B-ALL) or the translocation t(17;19)(q22;p13) generating the TCF3::HLF fusion (~0.5% of pediatric B-ALL).\(^2\) Omics research for the discovery of novel treatment strategies in hematological cancer is still based largely on transcriptomics, although it is increasingly recognized that this does not translate well into the expression of proteins, which are the main targets of drugs and functional entities of biological processes. In this study, we comprehensively analyzed the proteomic landscapes of TCF3::HLF+ (n=6) and TCF3::PBX1+ (n=5) B-ALL employing primary patient-derived xenografts (PDX), liquid chromatography tandem mass spectrometry and data-dependent acquisition. Approval for the study reported here was granted by the Ethics Committee of the Medical Faculty of the Christian-Albrechts-University, Kiel, Germany (vote DS08/13). We detected 6863 proteins (6123 without ≥2 missing values, Supplementary Table 1), which allowed a clear distinction between TCF3::HLF+ and TCF3::PBX1+ leukemia by unsupervised hierarchical clustering and principal component analysis (Figure 1A,B). Proteomic profiling proved a useful tool for prioritizing drug targets, as only 8.45% of the significantly differentially expressed genes (n=119 of 1409, p<0.05 and minimal log2 fold change of ±1) previously detected by RNA sequencing\(^2\) showed differential expression on protein level confirmed by our proteomic analysis (Supplementary Figure 1A). In contrast, 34.8% (n=119 of 342) of differentially regulated proteins detected by proteomics were also differentially expressed on RNA level. As a proof-of-concept, we examined overlap of differentially expressed genes (cut-off: \(p<0.05\) and minimal log2 fold change of ±1) from RNA sequencing and proteomic analysis obtained from a previously published dataset of ETV6::RUNX1+ (n=9) and high hyperdiploid (n=18) primary ALL
patient samples.\textsuperscript{3} While only 3.63\% (n=82 of 2262) of differentially expressed genes detected via RNA sequencing showed differential expression on protein level, 92.13\% (n=82 of 89) of differentially regulated proteins were also differentially expressed on RNA level (Supplementary Figure 1B).

In order to identify protein classes presenting specific therapeutic vulnerabilities, we performed gene set enrichment analysis (GSEA). We identified several gene sets enriched in either of the two subgroups (Figure 1C). RNA biology, mitochondrial translation and cellular respiration were the most prominent enriched gene sets for TCF3::HLF+ leukemia. In addition, strongly increased MYC expression and enrichment in MYC targets (Figure 1D,E) were detected, consistent with TCF3::HLF-driven activation of a MYC enhancer cluster previously shown using extensive functional genomics.\textsuperscript{4}

For TCF3::PBX1+ leukemia, immune response/cell cycle, actin cytoskeleton, cell morphogenesis and RTK signaling were among the most prominent enriched gene sets (Figure 1C). We validated therapeutic vulnerabilities indicated by GSEA using high-throughput drug screening. To this end, we tested the sensitivity of leukemic cell lines (TCF3::HLF+: HAL-01; TCF3::PBX1+: 697 and RCH-ACV) and mononuclear cells from peripheral blood of three healthy donors against a drug library of over 600 FDA-approved or clinical trial phase I-IV anti-cancer drugs. TCF3::HLF+ and TCF3::PBX1+ leukemic cells showed a differential response towards 109 drugs based on the area under the curve (AUC) as response parameter (Figure 2A, Supplementary Table 2; AUC < 0.8 and > 1.2 as a cut-off). Compared to our previous screening of bioactive compounds (n=98) employing the PDX samples,\textsuperscript{2} the cell lines showed similarly increased sensitivity towards compounds, such as BCL2 and mTOR inhibitors for TCF3::HLF+, and aurora kinase and polo-like kinase inhibitors for TCF3::PBX1+ B-ALL (Figure 2A). In addition, we identified novel potential drug targets. These included MDM2 and DNA/RNA synthesis for TCF3::HLF+ and microtubule/tubulin and cyclin-dependent kinases (CDK) for TCF3::PBX1+ leukemic cells (Figure 2A). To confirm these findings, we chose drugs from those groups, which did not affect normal peripheral blood cells (Figure 2B-E), and treated TCF3::PBX1+ (RCH-ACV) and TCF3::HLF+ (HAL-01) cells with half IC\textsubscript{50}, IC\textsubscript{50} and double IC\textsubscript{50} concentrations to investigate apoptosis.
induction. We demonstrated increased caspase 3/7 activity and apoptotic subG1 cells in TCF3::PBX1+ B-ALL in response to the microtubule/tubulin inhibitor ixabepilone and the CDK inhibitor SNS-032. For TCF3::HLF+ leukemic cells, we verified increased apoptotic cell death upon idasanutlin (MDM2 inhibitor) and bleomycin sulfate (DNA/RNA synthesis inhibitor) treatment (Figure 2F-I).

Besides the detection of vulnerabilities to specific drug classes, we aimed to identify novel targets for drug development. In our proteomic analyses, the B-lymphoid tyrosine kinase (BLK) was the most significantly upregulated protein for the TCF3::PBX1+ subtype (Figure 3A; Supplementary Figure 1C; Supplementary Table 1; minimal log2 fold change of ±1 and significance level of p<0.05 as cut-offs). BLK encodes a non-receptor tyrosine kinase of the src family of proto-oncogenes that plays an important role in precursor (pre) B-cell receptor (BCR) signaling and early B-cell development.5 RNA sequencing and expression microarray data by us and others supported this finding (Figure 3B-D). We examined human gene expression data derived from four independent data sets of >3000 leukemia cases6-9 available at the R2: Genomics analysis and visualization platform (http://r2.amc.nl). These data indicated a subpopulation of leukemia samples that highly co-expresses BLK and PBX1 (Figure 3B; Supplementary Figure 1D-F). In two of the data sets, information on chromosomal translocations was available. There, the BLK and PBX1 co-expressing subpopulation was specifically associated with the TCF3::PBX1 fusion (Figure 3B; Supplementary Figure 1D).8,9 In the Microarray Innovations in LEukemia (MILE) study8 all 36 cytogenetically identified TCF3::PBX1+ cases were BLKhph expressing (n=1897 other leukemia or myelodysplastic syndrome (MDS), n=71 normal controls). Similarly, in another study9 all six TCF3::PBX1+ cases and none of the other samples (n=185 other B-ALL, n=3 normal controls) were both PBX1 and BLK high expressing. RNA sequencing data of our cohort showed high RNA expression of BLK in all TCF3::PBX1+ leukemia cases (n=5 at diagnosis, n=8 after transplantation into NSG mice)2 compared to TCF3::HLF+ cases (n=5 at diagnosis, n=22 after transplantation) (Figure 3C,D).
Thus, we hypothesized that interference with BLK signaling might present a potential treatment strategy for TCF3::PBX1-rearranged B-ALL in particular. To test this, we treated TCF3::PBX1+ BLK^{high} (RCH-ACV) and TCF3::HLF+ BLK^{low} (HAL-01) cells with a first selective irreversible BLK inhibitor (BLK-IN-2)\(^{10}\). BLK^{high} cells responded in a dose-dependent manner starting at nanomolar concentrations (IC\(_{50}\)=0.2169 µM), whereas BLK^{low} cells showed little or no response (≥167-fold less, IC\(_{50}\)=36.20 µM) (Figure 3E; Supplementary Figure 1G-I). We further tested the impact of BLK-IN-2 on other genetic subtypes of B-ALL and noticed preferential sensitivity of only the TCF3::PBX1+ subtype to BLK-IN-2 (Supplementary Figure 1I). To test if BLK inhibition synergizes with the specific vulnerabilities identified in our proteomic screen, we performed combined treatment with ixabepilone (microtubule/tubulin inhibitor). Indeed, both drugs synergized in TCF3::PBX1+ cell lines, but not in the TCF3::HLF+ cell line HAL-01 (Figure 3F-H). We further tested, if interference with pre-BCR signaling including BTK inhibitors would have the same impact. To this end, we tested the response of TCF3::PBX1+ B-ALL cell lines to ibrutinib and other BTK-targeting drugs (acalabrutinib, spebrutinib, LFM-A13). The response, however, was low and did not differ from cells lacking pre-BCR expression.

As previously reported by Geng et al., BLK is a signature gene of adult TCF3::PBX1+ B-ALL.\(^9\) Combining ChIP sequencing, DNA methylation and expression profiling, the authors identified hypomethylation and overexpression of BLK in adult TCF3::PBX1+ B-ALL. In this study, upregulated genes targeted by TCF3::PBX1 included pre-BCR components and pre-BCR downstream signaling molecules.\(^{11}\) Ligand-independent autonomous tonic pre-BCR activation via self-aggregation is a main mechanism for pre-BCR activation and leads to constitutive activation of BLK\(^{11}\) (indicated by phosphorylation of the activating tyrosine Y388) observed in several TCF3::PBX1+ cell lines and primary B-ALL.\(^{11}\) Pre-BCR function induces activation of the transcription factor BCL6, which further increases pre-BCR signaling in a self-enforcing positive feedback loop and directly activates BLK transcription. More than 90% of TCF3::PBX1+ leukemia cases are pre-BCR+ and critically rely on pre-BCR-dependent
signaling for proliferation.\textsuperscript{5} Thus, targeting BLK to abrogate pre-BCR downstream signaling presents an attractive approach for therapeutic intervention in \textit{TCF3::PBX1+ B-ALL}.

Previously, interference with pre-BCR signaling has been suggested as a therapeutic option for \textit{TCF3-rearranged ALL}\textsuperscript{12} and the inhibitors ibrutinib, dasatinib and idelalisib to be effective against \textit{TCF3::PBX1+ B-ALL}\textsuperscript{5,12,13} Inhibition of BTK, a downstream signaling kinase of the BCR, by ibrutinib is clinically beneficial in BCR+ B-cell malignancies such as non-Hodgkin lymphomas and multiple myeloma. However, in our analyses, the response of \textit{TCF3::PBX1+ B-ALL} cell lines to BTK-targeting drugs was low and not corresponding to pre-BCR expression. This is in line with the observation that ibrutinib exerts a cytostatic rather than a cytotoxic effect on pre-BCR+ B-ALL cells\textsuperscript{5} and is further supported by the lack of \textit{in vivo} effectivity against \textit{TCF3::PBX1+ primografts}\textsuperscript{14}. However, \textit{TCF3::PBX1+ PDX} samples responded well to the tyrosine kinase inhibitor dasatinib\textsuperscript{2}. Still, high doses might be required for targets other than \textit{BCR::ABL1} and might be limited in the relapse setting due to toxicity\textsuperscript{13}.

Taken together, proteomic-based profiling is a powerful tool to discover highly specific and sensitive cancer biomarkers and oncogenic pathway activation.\textsuperscript{15} Here, we uncovered proteomic alterations associated with \textit{TCF3::HLF+} or \textit{TCF3::PBX1+ B-ALL} and revealed potential therapeutic options for these subtypes. These include previously known sensitivities for \textit{TCF3::HLF+} (e.g. BCL2 and mTOR) and \textit{TCF3::PBX1+ B-ALL} (e.g. aurora kinase and polo-like kinase), as well as potential novel drug targets, such as MDM2 and DNA/RNA synthesis for \textit{TCF3::HLF+} or microtubule/tubulin and CDK for \textit{TCF3::PBX1+ leukemic cells}. Our data suggest that \textit{TCF3::PBX1+ B-ALL} might be sensitive to treatment with selective BLK inhibitors, especially in combination with microtubule/tubulin targeting drugs, such as ixabepilone. Due to high BLK expression in \textit{TCF3::PBX1+ B-ALL} cells, such inhibitors could selectively eradicate leukemic cells at doses eliciting less side-effects on normal tissue. As a limitation of our study, this was not tested in mouse models. In future studies, it would be interesting to apply BLK inhibition to suitable mouse models of \textit{TCF3::PBX1+ leukemia} and to test synergism with other
Larger numbers of patient samples need to be tested to show the applicability for TCF3:PBX1+ leukemia.

References

Figure legends

Figure 1: Proteomic profiling distinguishes TCF3::HLF+ and TCF3::PBX1+ leukemia and uncovers therapeutic vulnerabilities for both subtypes. Unsupervised hierarchical clustering (A), principal component analysis (PCA, B) and gene set enrichment analysis (GSEA, C) was performed on the proteomic data of six TCF3::HLF+ (in red) and five TCF3::PBX1+ (in orange) B-ALL patient-derived xenograft samples. For unsupervised hierarchical clustering, the 10% most variable proteins were used based on the standard deviation. PCA was performed on all proteins. Both subtypes clearly segregate into distinct clusters suggesting highly distinct proteomic landscapes. (C) GSEA is based on all proteins and identifies several gene sets enriched in either of the two subgroups. (D) Proteomic data shows enrichment of MYC protein expression in TCF3::HLF+ versus TCF3::PBX1+ leukemic samples in this study. To determine differential expression, non-parametric Mann-Whitney t-test (two-tailed) was used. (E) Gene set enrichment plot of MYC targets showing a positive correlation with TCF3::HLF+ leukemia.

Figure 2: High-throughput drug screening and functional analysis confirm therapeutic vulnerabilities for TCF3::HLF+ and TCF3::PBX1+ leukemic cells. (A) HAL-01 (TCF3::HLF+), 697 and RCH-ACV (both TCF3::PBX1+) were exposed to a drug library, consisting of over 600 compounds. The supervised heatmap is based on the area under the curve (AUC, minimal fold change of <0.8 or >1.2 as a cut-off) as response parameter. Presented are groups of drugs with a differential response between TCF3::PBX1+ and TCF3::HLF+ leukemic cells. High sensitivity is shown in blue and low sensitivity is shown in white. (B-E) Presented are the dose-response curves for ixabepilone (B), SNS-032 (C), bleomycin sulfate (D) and idasanutlin (E), showing a differential response of TCF3::HLF+ versus TCF3::PBX1+ leukemic cells. Human peripheral blood cells from three healthy donors (in black) served as control cells (B-E). (F-I) RCH-ACV and HAL-01 were treated with ixabepilone (10 nM, 20 nM, 40 nM), SNS-032 (133 nM, 266 nM, 532 nM), idasanutlin (131 nM, 262 nM, 524 nM) or bleomycin
sulfate (235 nM, 470 nM, 940 nM) dissolved in DMSO or DMSO as negative control. (F,G) Bar graphs representing the fold change in caspase 3/7 activity 24 h after treatment. Caspase 3/7 activity was determined using the Caspase-Glo 3/7 Assay (Promega). (H,I) Bar graphs represent the proportion of apoptotic cells (subG₁) 48 h after treatment. Cell cycle profiles were generated by flow cytometric measurement of propidium iodide intercalation into DNA after partial cell lysis in hypotonic buffer (0.1% sodium citrate, 0.1% Triton X-100, 0.5 mg/ml RNase A containing 40 µg/ml propidium iodide). Values shown in (F-I) represent mean ±SEM of three biologically independent replicates. ns: not significant; *p<0.05; **p<0.01; ***p<0.001 (t-test).

Figure 3: Proteogenomic profiling detects BLK as a marker of TCF3::PBX1+ B-ALL targetable by the selective BLK inhibitor BLK-IN-2. (A) The volcano plot presents significantly (red) or non-significantly (grey) dysregulated proteins in TCF3::PBX1+ (left) or TCF3::HLF+ (right) leukemias. BLK is the most significantly dysregulated protein for the TCF3::PBX1+ subtype (minimal log2 fold change of ±1 and significance level of p<0.05 as cut-offs). (B) Gene expression data of human patient samples is derived from data sets available at the R2: Genomics analysis and visualization platform (http://r2.amc.nl). Dot plot presents PBX1 and BLK expression in healthy controls (black), TCF3::PBX1+ (orange) and other unstratified leukemic samples (grey). Data of the Mixed Leukemia-MILE - 2004 - MAS5.0 - u133p2 study⁸ is shown. Three further studies are presented in Supplementary Figure 1D-F. (C,D) BLK RNA expression in TCF3::HLF+ (n=5) and TCF3::PBX1+ (n=5) B-ALL at the time of diagnosis (C) and after transplantation into NSG mice (TCF3::HLF+: n=22; TCF3::PBX1+: n=8) (D). Data is derived from our previous study². To determine differential expression, non-parametric Mann-Whitney t-test (two-tailed) was used. (E) Dose-response curves for BLK-IN-2 show a differential response of TCF3::HLF+ (red) versus TCF3::PBX1+ (orange) leukemic cells. (F-H) Synergy drug screening of BLK-IN-2 and ixapepilone reveals a strong synergistic effect in TCF3::PBX1+ cell lines, while no such effect is detected in the TCF3::HLF+ cell line HAL-01.
Representative synergy plots of 3 independent experiments are shown. Drug concentration ranges were chosen according to the pre-determined IC\textsubscript{50}-values of each cell line (RCH-ACV and 697: 5 nM-500 nM BLK-IN-2, 0.25 nM-25 nM ixabepilone; HAL-01: 0.25 µM-25 µM BLK-IN-2, 5 nM-500 nM ixabepilone). DMSO was used as a negative control. ZIP synergy score analysis was conducted using the synergyfinder package version 3.0.14 with additional baseline correction.
Supplement

Supplementary Tables (see excel files)

Supplementary Table 1: List of proteins detected upon proteomic profiling of TCF3::HLF and TCF3::PBX1 positive leukemias.

Supplementary Table 2: Result of the high-throughput drug screening performed on TCF3::PBX1 and TCF3::HLF positive leukemic cells. The drugs listed were purchased from MedChemExpress (Monmouth Junction, NJ, USA) as a customized library and were provided as 10 mM dimethylsulfoxide (DMSO) stock solutions. The area under the curve (AUC) serves as response parameter.
Supplementary Figure 1: (A) Proteomic analysis for target prioritization in TCF3-rearranged ALL.

Patient derived B cell acute lymphoblastic leukemia (B-ALL) xenografts (PDX) (n=5 TCF3::PBX1+ and n=6 TCF3::HLF+) were analyzed by RNA sequencing as previously reported by us. 1 1409 significantly differentially expressed genes were detected within the two subtypes of B-ALL (9.5% of n=14,830 in total, cutoffs: p<0.05 and minimal log2 fold change of ±1) via RNA sequencing. Proteomic analysis carried out in the present study detected 342 differentially abundant proteins in the same PDX samples.
using the same cutoffs (5.6% of n=6123 in total). 34.8% of differentially regulated proteins could be confirmed to be also differentially expressed on RNA level (n=119 of 342). In contrast, less than 10% of differentially regulated genes detected by RNA sequencing could be proven to have a significant impact on protein expression as detected by proteomics (n=119 of 1409, 8.45%) in our analyses. Therefore, 91.55% of the detected differentially expressed genes could be excluded as immediate targets for B-ALL subtype specific drug development by prioritization using proteomics. For proteomic analysis, liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed. All peptide samples were separated and analyzed by coupling a RSLCnano system (Ultimate 3000, Thermo Scientific, Bremen, Germany) online to an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific). Briefly, after LC-MS/MS and data Dependent Acquisition (DDA), data were searched using Sequest HT through proteome discoverer (version 2.4) and label free quantification (LFQ) was performed by using myProMS pre-processing to quantitatively profile changes in the proteome of TCF3-translocated samples. For data analysis, unsupervised consensus clustering approaches (e.g. NMF and SOM clustering) and supervised strategies (e.g. LIMMA and SAM) were used. Downstream pathway analyses were conducted using bioinformatics algorithms including Ingenuity Pathway Analysis (IPA, QIAGEN) and Gene Set Enrichment Analysis (GSEA). (B) Overlap of significantly dysregulated genes detected by RNA sequencing and proteomics in primary samples of ETV6::RUNX1+ and high hyperdiploid ALL. Previously published RNA sequencing and proteomic data of primary ALL samples (n=9 ETV6::RUNX1+ and n=18 high hyperdiploid)² were filtered for significance (cutoffs: p<0.05 and minimal log2 fold change of ±1). 2262 significantly differentially expressed genes were detected in the two subtypes of B-ALL using RNA sequencing, while proteomics detected 89 differentially expressed proteins in these samples using the same cutoffs. The majority of differentially regulated proteins was also found to be differentially expressed on RNA level (n=82 of 89, 92.13%). In contrast, only 3.63% of differentially regulated genes detected by RNA sequencing were identified as differentially expressed proteins by proteomics (n=82 of 2262). (C) High BLK expression in
**TCF3::PBX1+ PDX samples.** BLK protein expression data of TCF3::HLF+ and TCF3::PBX1+ samples is derived from the proteomic data generated in this study. To determine differential expression, non-parametric Mann-Whitney t-test (two-tailed) was used. (D-F) **BLK is highly coexpressed with PBX1.** mRNA expression data of human patient samples is derived from data sets available at the R2: Genomics analysis and visualization platform (http://r2.amc.nl). Dot plots present PBX1 and BLK expression in healthy controls (black), TCF3::PBX1+ (orange) and other unstratified leukemic samples (grey). Data sets shown are the B-ALL - Melnick - 194 - custom - gpl15088 study (D), the Tumor ALL (BCP) Pediatric (combat) - Boer - 654 - MAS5.0 - u133p2 study (E) and Tumor ALL (TARGET) - Roberts - 283 - MAS5.0 - u133p2 data set (F). (G,H) **Validation of BLK expression on mRNA and protein level in TCF3::PBX1+ and TCF3::HLF+ leukemia cell lines.** (G) BLK mRNA expression is increased in TCF3::PBX1+ (orange) versus TCF3::HLF+ cells (red). mRNA expression was normalized to the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB). (H) Shown is the corresponding BLK protein expression analyzed by specific Western blot. Pan-actin was used as a loading control. (I) **BLK-IN-2 specifically targets TCF3::PBX1+ cells.** Dose-response curves for BLK-IN-2 showing differential responses of TCF3::HLF+ HAL-01 cells (red, IC_{50}=no response), TCF3::PBX1+ RCH-ACV cells (orange, IC_{50}=0.18 µM), IGH::DUX4+ NALM-6 cells (green, IC_{50}=3.4 µM), KMT2A::AFF1+ SEM cells (grey, IC_{50}=4.5 µM), ETV6::RUNX1+ REH cells (black, IC_{50}=10.3 µM) and the B lymphoblastoid cell line NC-NC (rose, IC_{50}=11.3 µM). Cell lines were treated with BLK-IN-2 (0.005 µM-25 µM) for 72 h. Representative drug response curves of two independent experiments are shown. Treatment was conducted in 384-well plates with technical triplicates. Data is depicted as the mean ±SEM. DMSO was used as a negative control. Cell viability was determined via CellTiter-Glo (Promega) measurement using a Tecan SPARK 10M reader.
Supplementary References