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Mitochondrial dysfunction in Down syndrome B-cell acute lymphoblastic leukemia can be targeted using venetoclax

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Author Contribution:

DJ and EW designed the research, performed experiments, performed data analysis, and prepared the manuscript; **ZL** performed experiments, performed data analysis, and prepared the manuscript; **KLS, BK, JF, TW, DT, SCH, JM, BS, and AI** performed experiments; **EH, GT, and JAR** performed data analysis; **DS** performed experiments and data analysis; **AD** performed data analysis and contributed to writing the manuscript; **KWM, HP, and KS** provided critical materials; **JE** provided critical materials and reviewed the manuscript; **CTJ** contributed to writing the manuscript; **JJY** provided genetic samples and contributed to writing the manuscript; and **JRR** contributed to writing the manuscript, **MRV** designed the research and supervised the study.

Data sharing Statement

The datasets generated during the current study will be available in the Gene Expression Omnibus (GEO) repository and INCLUDE DCC. These data are currently private for peer review and will be made publicly available upon publication of this manuscript. The novel DS Cell lines generated here will also be made available to academic community.

Conflict of Interest: JME has provided consulting services for Eli Lilly Co. and Gilead Sciences Inc. and serves on the advisory board of Perha Pharmaceuticals.

Word Count

Down syndrome (DS) is caused by trisomy of chromosome 21 and is associated with a 20-fold increased risk of B-cell acute lymphoblastic leukemia (B-ALL)(1). DS B-ALL patients experience greater treatment related toxicity and relapse, leading to inferior survival when compared to non-DS (NDS) B-ALL patients(1). The causes for higher B-ALL prevalence and chemoresistance remain unclear, which is compounded by a lack of DS B-ALL cell lines (CLs) for study. To address this, we established three DS B-ALL CLs to explore potential therapeutic interventions.

We generated three DS B-ALL CLs (DS-1, DS-76, and DS-81) and performed comprehensive transcriptomic and genomic profiling using RNA-seq and whole genome sequencing (WGS) as we previously described(2). The three CLs demonstrated distinct gene expression profiles, as visualized using uniform manifold approximation and projection tool (UMAP) in the context of our previously published DS B-ALL dataset(2) (Figure 1A). DS-1 harbored an *ERG::CEBPD* rearrangement, that juxtaposes the downstream of *CEBPD* to exon 3 of *ERG* (Figure 1B), that presumably drives *CEBPD* overexpression. This CL has a *FLT3* mutation (Figure 1C), frequently seen in C/EBPα DS B-ALL(2). DS-76 contained the *IGH::CRLF2* rearrangement, a *JAK2* mutation (R683G, Supplemental Figure 1A), and a *BCR::ABL1*-like gene expression profile (Figure 1A). No known gene rearrangements were identified in DS-81, and it was classified as B-other. Notably, biallelic *TP53* mutations are rare in diagnostic DS B-ALL(2), but identified in DS-1 and DS-81, which were both isolated post relapse. DS-1 harbored two *TP53* mutations, located in close proximity to one another and were compound heterozygous, as they are supported by different reads (Figure 1D). For DS-81, the *TP53* mutation affected one allele with the other allele lost due to 17p deletion (Figure 1D, Supplemental Figure 1B). Supplemental Figure 1A contains a full mutational analyses. The data has been deposited (GSE349532). The DS-1 CL lacked *CD19* expression because it was isolated post relapse following CD19- and CD19/CD22-CAR T cells (Figure 1E). All DS B-ALL CLs expressed B lineage genes *CD79A* and *PAX5*, while high levels of *CEBPD* and *ERG* were confirmed in DS-1 (Figure 1E).

De-identified DS B-ALL samples were obtained from patients and families who consented to participate in the University of Colorado study (COMIRB #22-1599). Cryopreserved B-ALL patient samples and patient derived xenografts (PDXs) were thawed and cultured at 1.0×10^6 cells/ml in IMDM (ThermoFisher) supplemented with 20% FBS (Sigma), 1X antibiotic/antimitotic (ThermoFisher), 1X glutamax (ThermoFisher), 55-μM beta-mercaptoethanol (ThermoFisher), 1X BIT (StemCell), IL-3 (10-ng/ml; RnD Systems), and IL-7 (10-ng/ml; Biolegend). DS CLs were cultured the same without BIT or cytokines using 10% FBS. DS-76 and DS-81 CLs were cultured at 1.0×10^6 cells/ml with media doubled after 3 days of culture, then resuspended in fresh media on day 5-6. DS-1 were cultured similarly but seeded at 2.0×10^6 cells/ml. DS B-ALL CLs were generated by culturing cells (1.0×10^6 cells/ml) with 50% media changes every 24-48h for 4-6 weeks without BIT or cytokines. All experiments were conducted on tissue culture treated 6-well or 10cm² plates. RNA was extracted from $1-10 \times 10^6$ cells (RNeasy mini kit, Qiagen). Samples with RIN >7 were used for library prep (3 prime kits, 10X-Genomics) and sequencing (Illumina HiSeq 2000 System, on Illumina NovaSeq-6000 UCCC Genomics Core) as we previously described(2).

To assess targetable pathways for novel therapies in DS B-ALL, we utilized RNA-seq to interrogate the transcriptome of our previously reported DS B-ALL patient cohort (n=249)(2) which was compared to NDS patient samples (n=1988)(3). Ingenuity pathway analyses (IPA) showed downregulation of mitochondrial translation as the second canonical pathway altered in DS (Figure 2A). Gene ontology comparisons showed mitochondrial matrix and mitochondrial protein containing complex as the top cellular components altered and downregulated in DS diagnostic patient samples (Figure 2B). Ribosome pathways were also downregulated and amongst the top ten cellular pathways altered in DS, as was oxidoreductase-complex (Figure 2B). Altogether, this

data indicates that downregulation of mitochondrial and protein translation is a characteristic of the DS BALL transcriptome.

In acute myeloid leukemia (AML), venetoclax resistance can be overcome by inhibition of mitochondrial translation(4), therefore, we hypothesized that venetoclax would also be effective in DS BALL. We found low IC₅₀ values for venetoclax and vincristine in DS CLs (<0.04 μ M), but greater IC₅₀ doses of trametinib were required for all CLs (Figure 3A, Supplemental Figure 2A) which was recently identified as a novel therapy option for DS B-ALL(5). To visualize leukemia growth kinetics and recapitulate the *in vitro* findings, DS B-ALL CLs were lentivirus transduced with pCDH-EF1a-eFFly-eGFP as previously described(6), followed by flow cytometry sorting of GFP⁺ cells. *In vivo* studies using bioluminescence imaging (BLI) showed diminished leukemia growth kinetics for all three DS CLs after 4-weeks of venetoclax (Figure 3B, Supplemental Figure 2B), leading to improved survival (Figure 3C) (IACUC-00251, IBC-1238).

Given our *in vitro* findings with VEN, VCR, and TRAM and the synergy reported between vincristine and trametinib in DS PDXs(5), we tested these combinations with venetoclax *in vitro* using DS B-ALL CLs and found synergy with each combination tested (Figure 3D). Venetoclax was also effective in short term *in vitro* assays using diagnostic DS B-ALL patient samples which displayed similar synergy at 24 hours (Figure 3E). Supplemental Figure 2C displays the cytogenetics of the patient samples used in these assays. Next, we tested the *in vivo* efficacy of venetoclax(7) and vincristine(5) alone and in combination using 2 DS B-ALL PDXs (both *CRLF2-rearranged*). We observed significant reductions in B-ALL after 4-weeks of treatment with all drugs compared to vehicle (Figure 3F). The combination of venetoclax and vincristine resulted in a >10⁵-fold reduction in splenic B-ALL cells compared to vehicle. Overall, venetoclax is active against DS B-ALL and synergistic with both vincristine and trametinib.

The elevated risk of B-ALL in children with DS combined with their increased incidence of chemotherapy intolerance, treatment-related mortality, and relapse(1) warrants the investigation of novel therapies. CLs have been foundational for drug testing in human cancers but have not been available for DS B-ALL to date. Given this, we generated three DS B-ALL CLs that represent distinct subtypes with differing mutations, including a novel *ERG::CEBPD FLT3*-mutant (DS-1). *ERG* is an ETS transcription factor on chromosome 21 frequently deleted or truncated in the *DUX4* B-ALL subset(8). Wildtype *ERG* induced erythromegakaryoblastic leukemia(9), whereas truncated *ERG* induced B-ALL in mice(8). The role of *ERG* as a fusion partner with *CEBPD* warrants further investigation in DS B-ALL. Surprisingly, we found *TP53* mutations in DS-1 and DS-81 CLs, which might be explained by their relapse status(10) and we acknowledge that while DS-1 and DS-76 represent common genetic drivers of DS B-ALL, DS-1 and DS-81 may also represent an extreme sub-population of DS B-ALL, and warrants future studies in relapsed/refractory DS patients.

The efficacy of venetoclax in NDS B-ALL subtypes is controversial and generally ineffective as a single agent(11, 12), whereas we found a consistent sensitivity to venetoclax in DS regardless of mutational subsets, which included efficacy against the DS-76 CL *CRLF2-rearranged* (*BCR::ABL1-like*). Others recently found venetoclax to be effective using DS B-ALL PDXs *in vitro*(13) corroborating our results. Whether venetoclax will be effective in DS patients is unclear, but it is noteworthy that our transcriptome analyses of DS B-ALL patients (n=249)(2) and NDS (n=1988)(3) found downregulation of mitochondrial translation and ribosome function in DS, suggesting this is an intrinsic characteristic of DS-BALL. Toxicity is a critical consideration when evaluating new therapies in DS; in this context, pediatric real-world data (n=41), including 4 with

DS-associated myeloid leukemia, suggests venetoclax is generally well tolerated, with expected cytopenias and infections, although DS-specific toxicity was not reported separately(14).

A weakness of this study was that we could have gained greater power using a larger sample size of DS B-ALL CLs, but we were only successful in generating an n=3 from an n=10 cryopreserved patient samples. Similarly, we had a small sample size of DS B-ALL patient samples for testing chemotherapy *in vitro* (n=5); along with for PDXs (n=2).

In summary, we have generated novel tools to study DS B-ALL by creating and characterizing three distinct CLs. Utilizing the largest DS B-ALL patient RNA-seq dataset, we identified defects in mitochondrial translation and ribosome function in DS. We found that venetoclax targets mitochondrial dysfunction in DS B-ALL and its efficacy at low nanomolar concentrations and synergy with vincristine supports clinical testing of venetoclax for relapsed/refractory DS patients.

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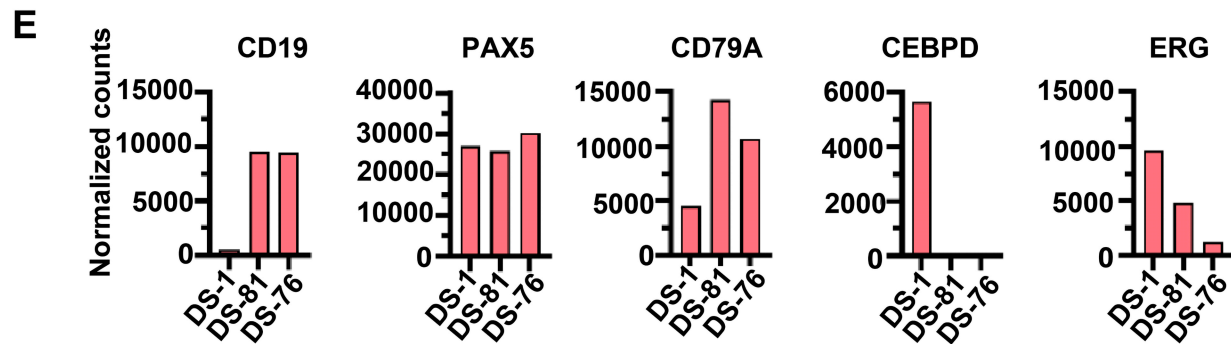
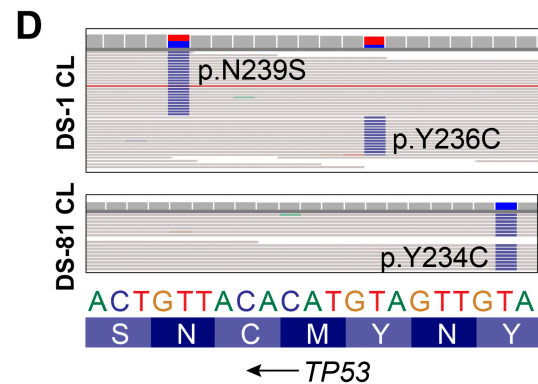
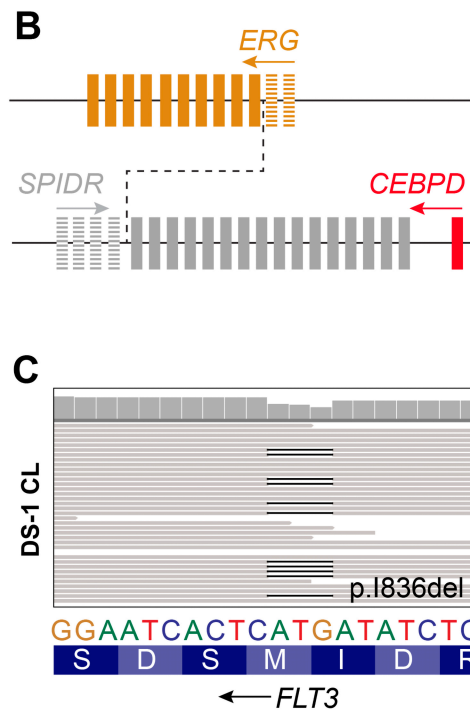
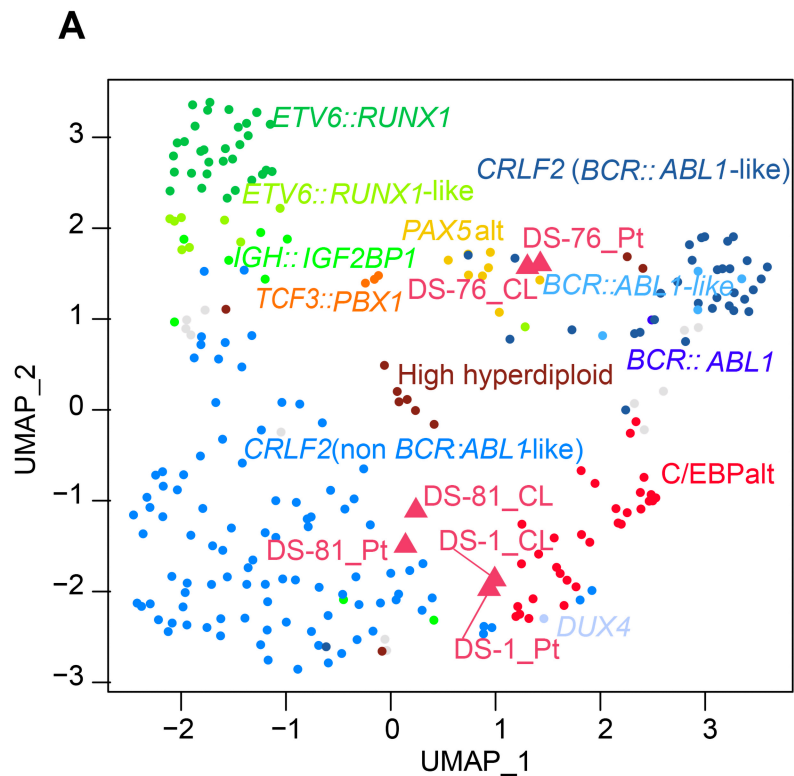
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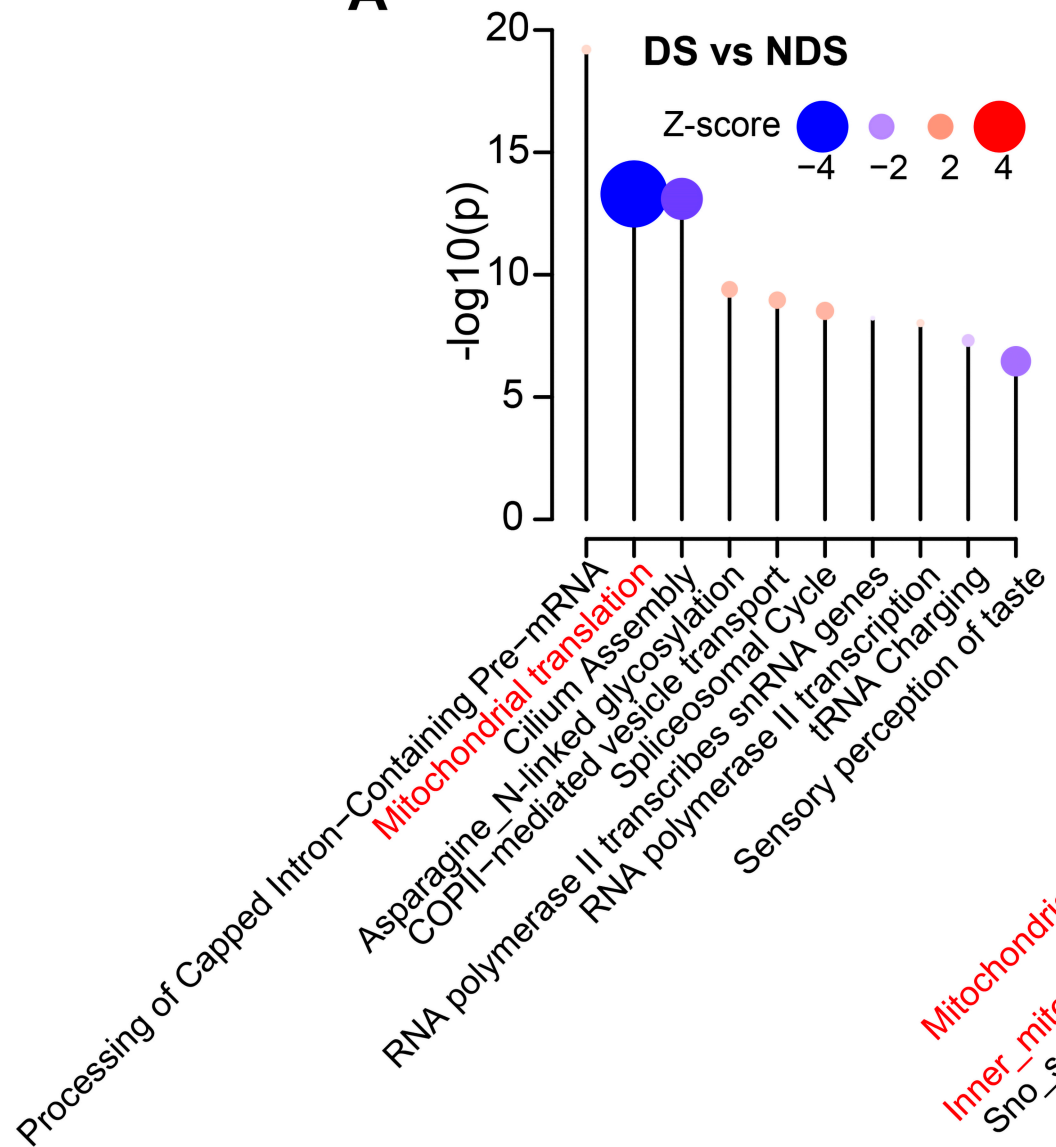
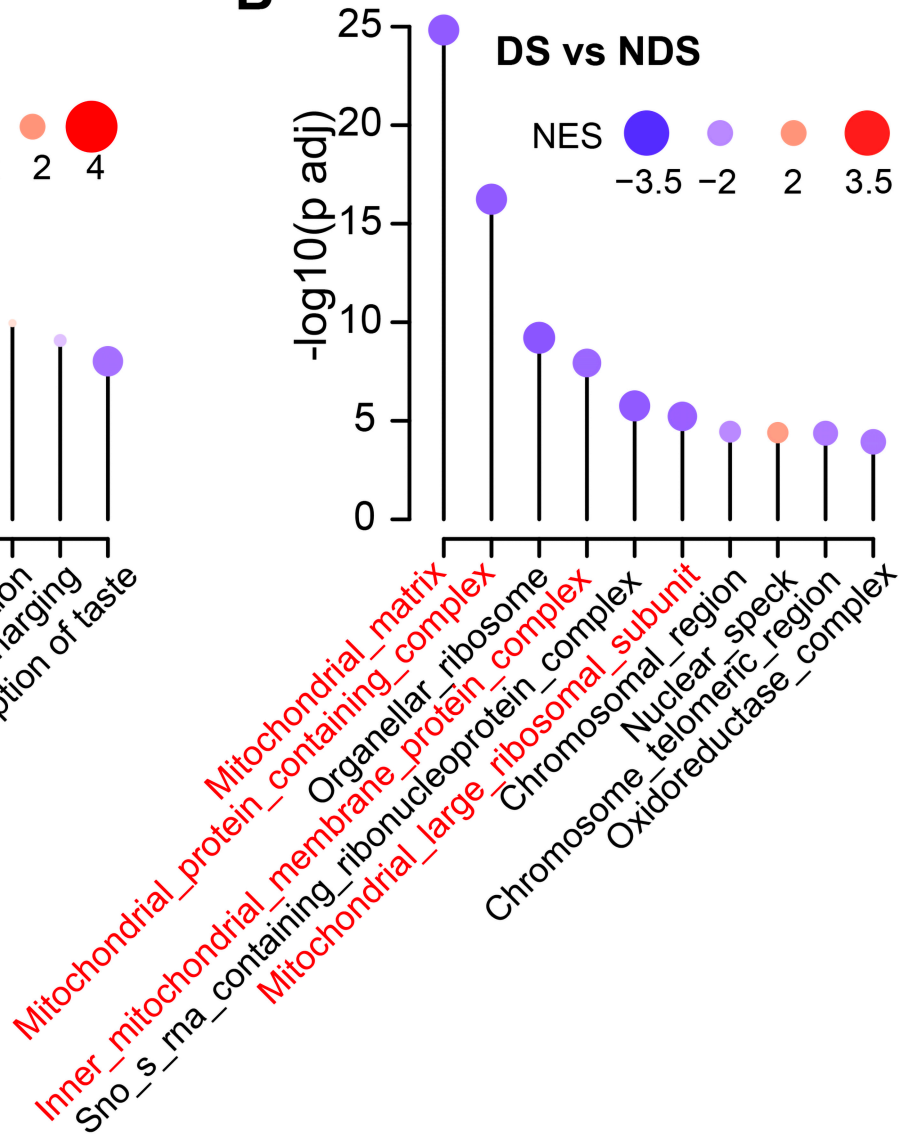
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Figure 1. Generation of three DS B-ALL cell lines and their DS subtype alignment. (A) Visualization of gene expression profiles of the DS cell lines (CLs) and their corresponding patient samples (Pt) using uniform manifold approximation and projection (UMAP) together with previously published 249 primary DS B-ALL samples. **(B)** *ERG::CEBPD* rearrangement identified in DS-1. Each box represents an exon, and solid boxes indicate exons involved in the rearrangement. **(C)** *FLT3* mutation in DS-1. **(D)** *TP53* mutations in DS-1 and DS-81. **(E)** RNA-seq analyses of *CD19*, *PAX5*, *CD79A*, *CEBPD*, *ERG* normalized expression levels in DS B-ALL CLs.

Figure 2. Mitochondrial dysfunction is a hallmark feature in DS B-ALL. (A) Canonical pathway analyses using IPA of RNA-seq from DS (n=249) and NDS (n=1988) patient samples. **(B)** GSEA of RNA-seq from A. Statistical significance was determined using the Mann-Whitney U test.

Figure 3. Metabolic vulnerabilities in DS B-ALL can be therapeutically exploited using venetoclax. (A) IC_{50} values of DS B-ALL cell lines (CLs) treated with venetoclax (VEN, vincristine (VCR), or trametinib (TRAM) for 72h. **(B-C)** NSG mice were tail-vein injected with 1.0×10^6 DS B-ALL CLs from DS-1, DS-76, or DS-81 expressing GFP-Luciferase lentivirus. Then, 2-weeks after injection, treatment with vehicle control or venetoclax (100 mg/kg/day, oral gavage, 5 days/week) was administered for 4-weeks. **(B)** Representative BLI after 4-weeks of Vehicle or VEN treatment in DS-1, DS-76, or DS-81 mice. **(C)** Kaplan-Meier survival curve from B comparing Vehicle (black) and VEN (red) treatment in DS-1, DS-76, or DS-81 NSG mice. 6-8 mice were used for all CLs and treatment groups. **(D)** Summary of synergy scores for combinatorial drug treatments using VEN, VCR, and TRAM from synergyfinder.fimm.fi in DS B-ALL CLs treated for 72h. All CLs were treated with their IC_{50} values and a 4-fold increase/decrease in these values. All treatments were conducted in triplicate. **(E)** DS B-ALL patient samples were thawed and rested over night before treatment with single dose or combinatorial dose of VEN (0.2 μ M), VCR (0.2 μ M), or TRAM (10 μ M) for 24- or 72h. All cells in the FSCa/SSCa gate were analyzed for singlet, $CD34^{+/-}$, $CD45^{+/-}$, $CD3^{-}$, $CD66B^{-}$, $CD14^{-}$, $CD19^{+}$. $CRLF2^{+}$ cells were defined as > 80%-positive. DS-1 patient sample was gated on $CD45^{+/-}$, $CD3^{-}$, $CD66B^{-}$, $CD14^{-}$, $CD19^{-}$, $CD34^{HI}$ as this represented >80% cells. Cells were stained with annexin-V and DAPI to monitor cell death. Live cells=annexin-V/DAPI $^{-}$. Red lines represent average % survival $\pm$ SEM. **(F)** DS-2 and DS-3 PDXs were tail-vein injected into NSG mice as in B-C. Flow cytometry was used to assess B-ALL burden in peripheral blood, when levels of human $CD19^{+}$ reached >1% treatment with VEN as in B-C, VCR 0.5 mg/kg/week, VEN+VCR combo, or Vehicle treatment began (4-weeks). On day-31 of treatment, mice were sacrificed and B-ALL burden in spleens determined using flow cytometry and cell count beads (absolute # of B-ALL cells/spleen). Statistical significance was determined using a Log rank test in C and one-way ANOVA with Tukey posttest in E-F. * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$. ns=not significant. FlowJo was used for flow cytometry analysis that was conducted on a Cytelk Aurora. All drugs were purchased from MedChem Express and resuspended in DMSO.

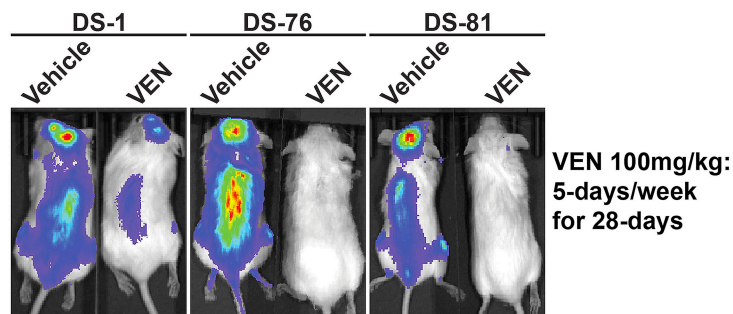


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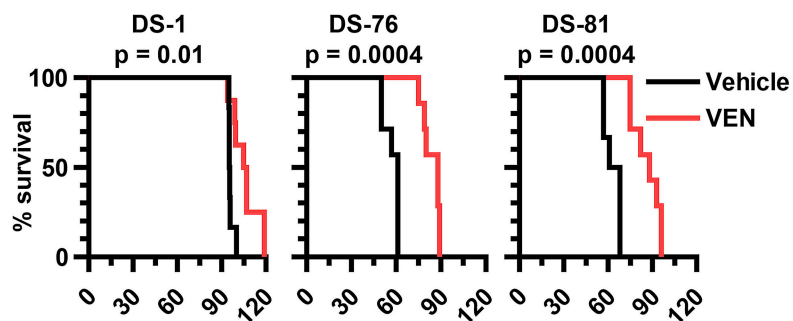
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IC ₅₀ (μM)	DS-1	DS-76	DS-81
Venetoclax (VEN)	0.04	0.015	0.02
Vincristine (VCR)	0.03	0.005	0.005
Trametinib (TRAM)	58.5	1.5	4.6

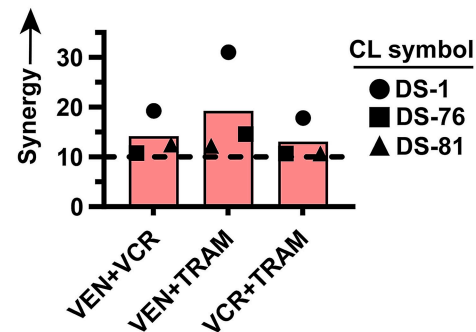
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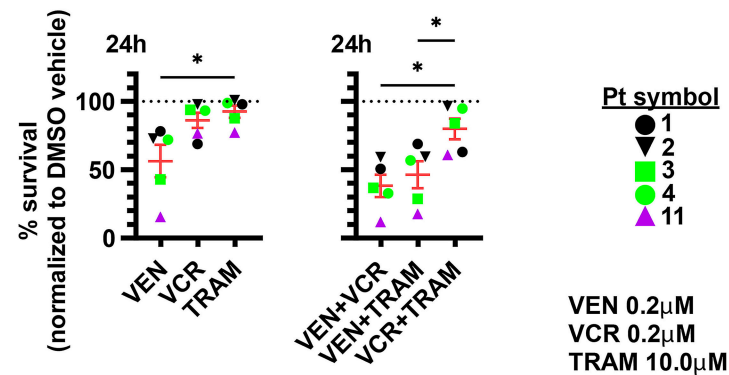
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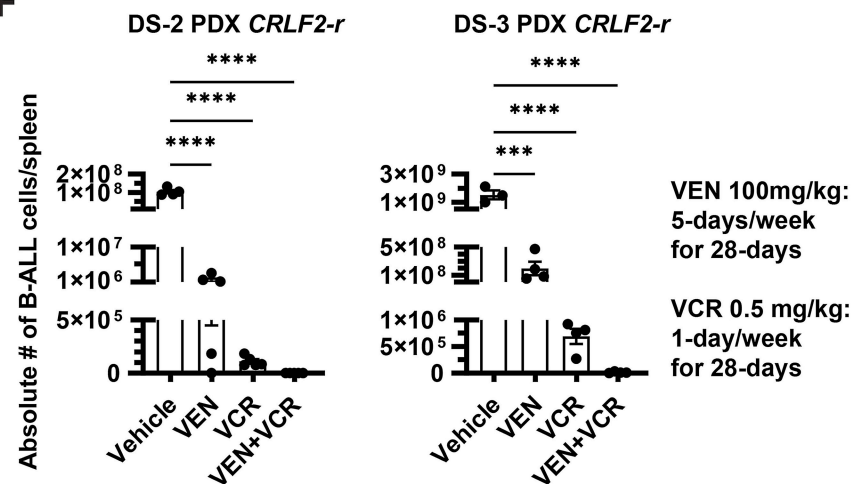
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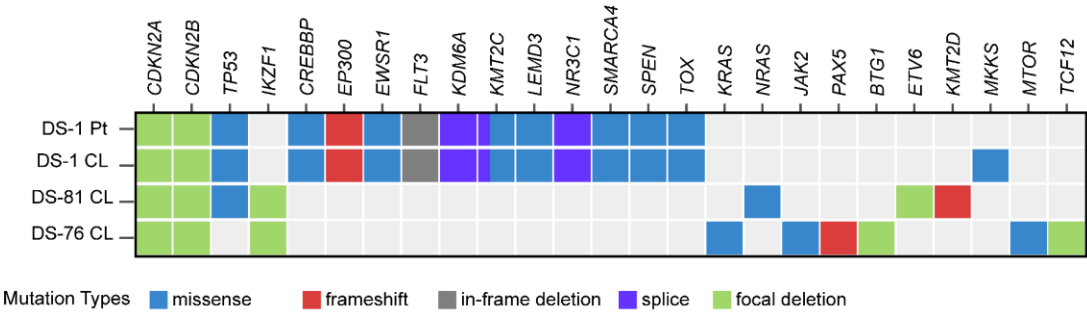
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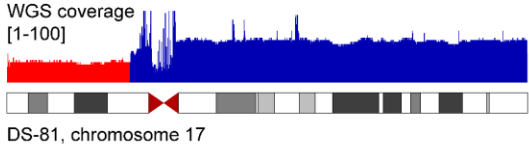
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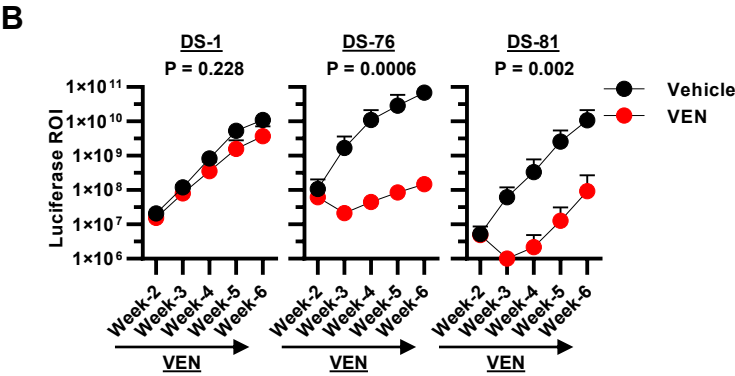
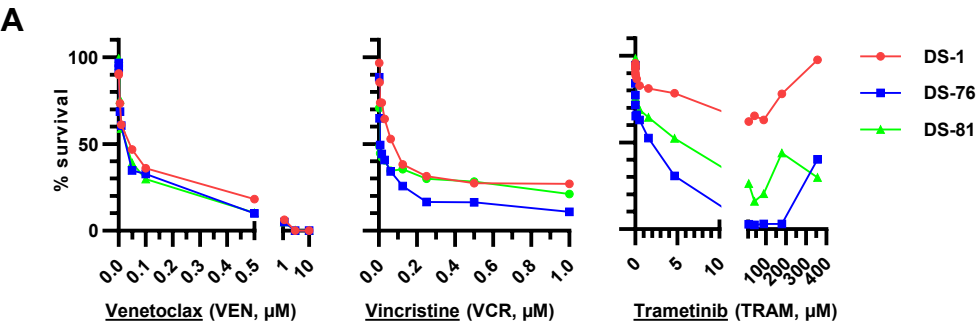
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B



Supplemental Figure 1. Supporting information for Figure 1. (A) Genomic alterations of DS-1 patient sample (Pt) and the three cell lines (CLs). **(B)** Whole-genome sequencing of DS-81 CL and deletion of chromosome 17.



C

B-ALL	Diagnosis	Cytogenetics	CRLF2-r	Age/Gender	BM/PB
DS-1	Relapse, CAR-T 2X	47	No	?/M	BM
DS-2	Primary	47	Yes	unknown	BM
DS-3	Primary	47	Yes	7/M	BM
DS-4	Relapse	47	No	16/M	BM
DS-76	Primary	47, CDKN2A-del	Yes	19/M	PB
DS-81	Relapse	48 (T8 & T21) CDKN2A-del	No	11/M	BM
DS-11	Primary	47	Yes	4/M	BM

Supplemental Figure 2. Supporting chemotherapy and DS B-ALL patient characteristics for Figure 3. (A) Dose response curves for % survival after 72h treatment with Venetoclax (VEN), Vincristine (VCR), and Trametinib (TRAM) in DS-1, DS-76, and DS-81 B-ALL cell lines (CLs) that corresponds to IC_{50} values displayed in Figure 3A. CLs were stained with AO/PI dye (acridine orange/propidium iodide) and viability was determined using a Cellaca automated cell counter. % survival=viable drug/viable vehicle. All doses were tested in triplicate and average % survival shown. All drug doses are given in μM . **(B)** Summary of weekly luciferase ROI values from BLI in Vehicle or VEN treated DS-1, DS-76, or DS-81 NSG mice from Figure 3B-C. **(C)** Table of DS B-ALL patient samples used in this study from Figure 3E-F. CAR-T=chimeric antigen receptor T cell therapy (CD19- and CD19/CD22-targeted); M=male; BM=bone marrow; PB=peripheral blood. Age is given in years. Statistical significance was determined using the Wilcoxon rank sum test in **B**. ROI=region of interest.