A transgenic mouse model of Down syndrome acute lymphoblastic leukemia identifies targetable vulnerabilities

Children with Down syndrome (DS) have a 20-fold increased risk of B-acute lymphoblastic leukemia (B-ALL) compared to children without DS.[1] The mechanism underlying the increased risk is not well understood. Outcomes in DS-ALL are poorer than in children without DS, with increased risk of relapse and treatment-related mortality,[2] making it imperative to identify new therapeutic targets to improve outcomes. DS-ALL is notable for harboring *CRLF2/JAK2* alterations in 50% of cases,[3,4] demonstrating hyperactive RAS signaling in up to 80% of cases,[5] and exhibiting *PAX5* and *KRAS* mutations in 25% and 15% of cases, respectively.[6] Experimental systems are needed to better understand the pathogenesis of DS-ALL and test targeted therapies. Here, we report the generation of a *de novo* mouse model and cell lines recapitulating key features of DS-ALL, and demonstrate cytotoxicity of agents which may have efficacy in DS-ALL and other leukemias, including the NAMPT inhibitor FK866 as well as agents targeting DNA damage responses, HSP90, autophagy, and JAK signaling. We created novel mouse models of DS and non-DS ALL by introducing *Kras*^{G12D} and *Pax5* heterozygosity, both driven by *CD19-Cre*, in the Dp16 mouse model of DS[7] and in littermate control wild-type (WT) mice. *Kras*^{G12D}*.Pax5*$^{+/-}$ mice on both Dp16 and WT backgrounds developed highly penetrant B-ALL, with 8 out of 9 Dp16 and 23 out of 23 WT moribund leukemic mice demonstrating expansion of a B-progenitor population (typically B220^{+}CD43^{+}CD24^{+}CD25^{+}) (Figure 1A) in the bone marrow, spleen, and lymph nodes (Figure 1B). Notably, the Dp16 *Kras*^{G12D}*.Pax5*$^{+/-}$ B-ALL mice had a significantly shorter

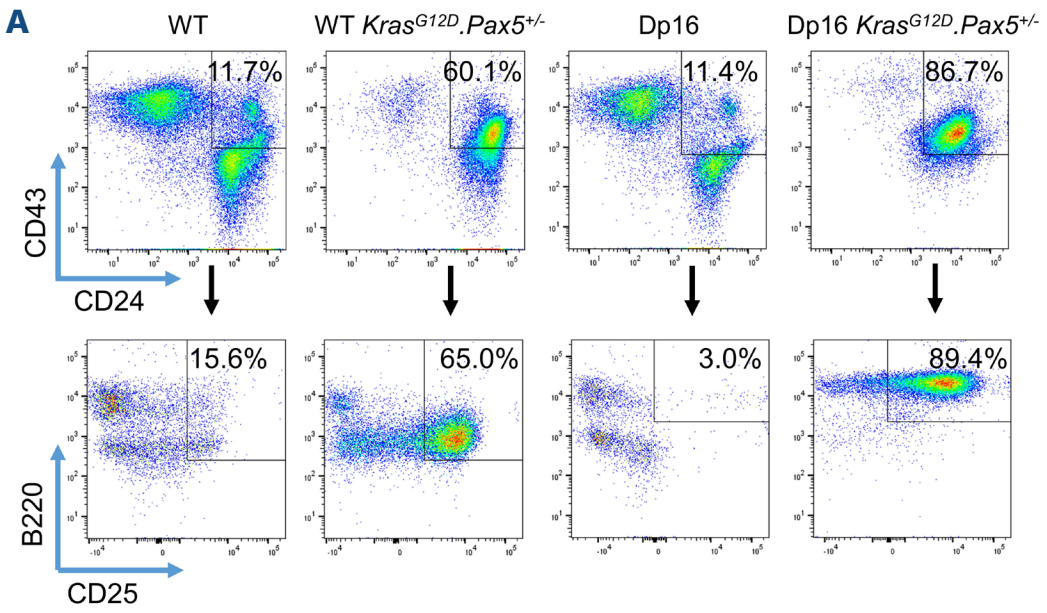


Figure 1. *Kras*^{G12D}*.Pax5*$^{+/-}$ mice develop B-cell acute lymphoblastic leukemia, with a shorter latency in the Dp16 *versus* wild-type background. (A) Nearly all moribund *Kras*^{G12D}*.Pax5*$^{+/-}$ mice (23 out of 23 wild-type [WT], 8 out of 9 Dp16) assayed by flow cytometry demonstrated expansion of a B-cell acute lymphoblastic leukemia (B-ALL), typically B220^{+}CD43^{+}CD24^{+}CD25^{+}, in the blood, bone marrow, and spleen relative to age-matched healthy control Dp16 and WT mice. Representative dot plots from splenic cells are shown. (B) Dp16 *Kras*^{G12D}*.Pax5*$^{+/-}$ and WT *Kras*^{G12D}*.Pax5*$^{+/-}$ mice demonstrated enlarged spleens and lymph nodes (LN) compared to non-leukemic control Dp16 and WT mice: *P<0.05, **P<0.001, ***P<0.0001, Mann-Whitney U test. (C) Dp16 *Kras*^{G12D}*.Pax5*$^{+/-}$ and WT *Kras*^{G12D}*.Pax5*$^{+/-}$ mice developed B-ALL with high penetrance, with a significantly shorter median latency in Dp16 *Kras*^{G12D}*.Pax5*$^{+/-}$ compared to WT *Kras*^{G12D}*.Pax5*$^{+/-}$ mice (80 *vs.* 114 days, P<0.0001, log-rank test). Latency to disease was also shorter in Dp16 *Kras*^{G12D} compared to WT *Kras*^{G12D} mice (P=0.01), and Dp16 *Pax5*$^{+/-}$ compared to WT *Pax5*$^{+/-}$ mice (P<0.01). Mice were monitored for at least 500 days.

latency to disease than WT *Kras*^{G12D}.*Pax5*$^{+/-}$ B-ALL mice (80 *vs*. 114 days, *P*<0.0001). Dp16 mice with either *Kras*^{G12D} or *Pax5* heterozygosity alone also developed leukemias significantly earlier and at greater penetrance than WT mice with these alterations (*P*<0.05), but with a longer latency and less complete penetrance than mice bearing both alterations (Figure 1C). While Dp16 *Pax5*$^{+/-}$ mice developed B-ALL, Dp16 or WT *Kras*^{G12D} mice typically developed myeloid or T-cell disease (*Online Supplementary Figure S1A-C*). We confirmed disease transplantability by injecting splenic blasts from leukemic Dp16 and WT *Kras*^{G12D}.*Pax5*$^{+/-}$ mice into NSG mice. Most primary samples (7 out of 8 WT, 7 out of 8 Dp16) generated leukemia in one or more recipient mice, with marrow and splenic infiltration with blasts of the same B-progenitor immunophenotype as the primary cells. There was no difference in survival in NSG recipient mice of WT *versus* Dp16 B-ALL blasts (*Online Supplementary Figure S1D, E*). All animal experiments were performed with the approval of the Baylor College of Medicine Institutional Animal Care and Use Committee. Next, we generated immortal B-ALL cell lines from 5 Dp16 and 2 WT *Kras*^{G12D}.*Pax5*$^{+/-}$ mice by incubating splenic blasts isolated from secondary NSG mouse xenografts with IL-7-supplemented medium for at least one month. These B-ALL lines displayed a B220^{+}CD43^{+}CD24^{+}CD25^{+} B-progenitor immunophenotype, similar to that of primary mouse samples (*Online Supplementary Figure S1F*) and maintained the presence of the Dp16 transgene (*data not shown*).

We performed whole transcriptome sequencing to characterize NSG-expanded Dp16 and WT *Kras*^{G12D}.*Pax5*$^{+/-}$ B-ALL blasts compared to control early B-lineage (B220^{+}) bone marrow (BM) cells from age-matched healthy Dp16 and WT mice. Gene set enrichment analysis (GSEA) using Hallmark gene sets demonstrated increased expression of oncogenic Myc targets and oxidative phosphorylation associated genes in both B-ALL models (Figure 2A), as well as upregulation of common human B-ALL oncogenes (including *Kras* and *Flt3*) and downregulation of genes frequently inactivated in human B-ALL (including *Ikzf1* and *Pax5*)[6,8] (*Online Supplementary Figure S2A*). The Dp16 *Kras*^{G12D}.*Pax5*$^{+/-}$ B-ALL model also displayed significant upregulation of DNA repair signaling compared to healthy Dp16 control B cells (Figure 2A). We also compared gene expression in Dp16 and WT control B cells. Genes upregulated in human DS *versus* non-DS lymphoid cells,[9] including *Dyrk1a, Runx1, Hmgn1, Rcan1, Ifnar1, Ifnar2*, and *Ifngr2*, were among the top upregulated genes in Dp16 *versus* WT B cells (*Online Supplementary Figure S2B*).

We conducted whole exome sequencing to determine if Dp16 and WT *Kras*^{G12D}.*Pax5*$^{+/-}$ B-ALL have secondary alterations common in human leukemia. We observed that 8 out of 8 leukemias arising independently in mice had an additional mutation in *Pax5* and/or *Ikzf1*, and 6 out of 8 had alterations in epigenetic-associated genes, alterations also commonly observed in both DS and non-DS ALL.[6,8] Interestingly, 5 out of 6 mice with additional *Pax5* alterations had a *Tox* mutation, alterations also observed to co-occur in human B-ALL (Figure 2B).[10]

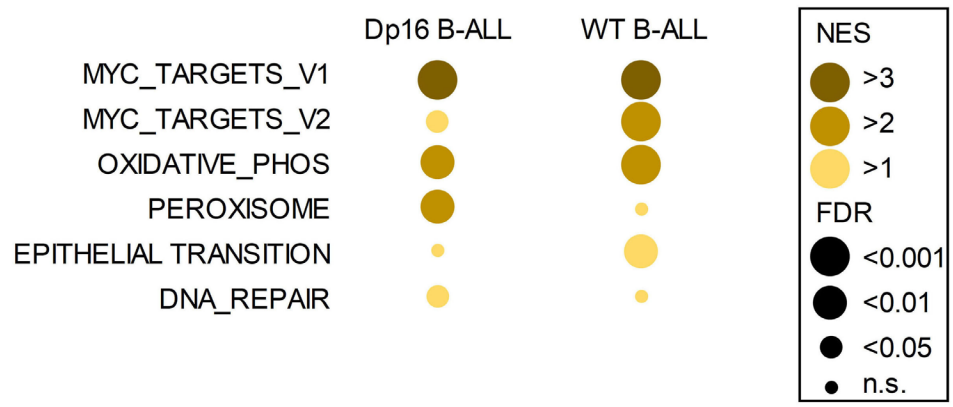
Next, we performed drug screens in the Dp16 and WT *Kras*^{G12D}.*Pax5*$^{+/-}$ B-ALL cell lines and in 4 DS-ALL patient samples to evaluate novel therapeutic agents. We performed a high-throughput screen with the Broad Institute Informer Set in 2 Dp16 and 2 WT *Kras*^{G12D}.*Pax5*$^{+/-}$ B-ALL cell lines. Inhibitors of mitosis, DNA damage response, and kinases were effective in all 4 cell lines. Only the HSP90 inhibitor AT13387 was significantly more effective in the Dp16 *versus* WT *Kras*^{G12D}.*Pax5*$^{+/-}$ B-ALL cell lines (34.01 *vs*. 47.5 nM, *P*=0.04) (*Online Supplementary Figure S3A*). We also performed cytotoxicity testing using a custom leukemia panel of 35 agents. We found that luminespib, bortezomib, inotuzumab, JQ1, palbociclib, vorinostat, and most kinase inhibitors reduced viability of at least 3 out of 4 B-ALL cell lines and 2 out of 4 DS-ALL patient samples at nanomolar-range concentrations. Many other compounds tested were effective in all mouse B-ALL cell lines, but only in one DS-ALL patient sample, potentially due to heterogeneity of these cases (*Online Supplementary Figure S3B*).

We chose the most effective drugs from the custom leukemia panel and the Broad Institute Informer Set high-throughput screen, and from among those identified from prior work,[11] for testing in PDX-expanded DS and non-DS B-ALL patient samples. All patient samples were collected with informed consent under a protocol approved by the Baylor College of Medicine Institutional Review Board. We selected agents which had demonstrated cytotoxicity in the Dp16 *Kras*^{G12D}.*Pax5*$^{+/-}$ B-ALL cell lines and DS-ALL patient samples, and have shown good bioavailability and safety profiles in prior mouse xenograft studies and/or potential clinical utility in phase I trials. Most tested agents significantly reduced the viability of both DS-ALL and non-DS ALL patient samples (Figure 3A). There were no significant differences in sensitivity based on DS *versus* non-DS or *CRLF2*-mutated *versus* *CRLF2*-wild-type status. DS-ALL and non-DS ALL samples were most sensitive to therapies targeting the DNA damage response (dinaciclib), autophagy (bafilomycin A1), JAK signaling (cucurbitacin I), and glycolysis (FK866). We confirmed the on-target mechanism of FK866 cytotoxicity via inhibition of the glycolytic enzyme NAMPT, by demonstrating that NAD+ supplementation in K187 cells prevented cytotoxicity of FK866 but not doxorubicin (Figure 3B). Finally, we tested 2 effective agents, FK866 and cucurbitacin I, *in vivo* in mice xenografted with *CRLF2/JAK2*-mutated DS-ALL patient samples 839 and K187. Cucurbitacin I modestly reduced peripheral blood (PB) disease burden of 839-xenografted mice and had no effect on PB or BM burden or spleen weights of K187-xenografted mice (*Online Supplementary Figure S3C-E*). FK866 significantly reduced PB burden of both 839 and K187-xenografted mice throughout treatment, and reduced spleen weight and BM burden of K187 mice (Figure 3C-E).

Other mouse models and cell lines recapitulating DS-ALL have been developed, but these require viral transduction and/or transplantation into WT mice.[12,13] To generate our novel syngeneic DS-ALL mouse model, we chose the Dp16 mouse

model because it contains a large complement of Hsa21 orthologues, including the Down syndrome critical region, and we induced *Kras*^{G12D}, *Pax5* heterozygosity, or both specifically in B cells via CD19-mediated Cre expression. The latency to disease for each genotype (*Kras*^{G12D}*.Pax5*$^{+/-}$, *Kras*^{G12D}, and *Pax5*$^{+/-}$) was significantly shorter for Dp16 than for WT mice,

A



B

Pathway	Gene	Dp16 B-ALL 599	Dp16 B-ALL 631	Dp16 B-ALL 707	Dp16 B-ALL 713	WT B-ALL 625	WT B-ALL 626	WT B-ALL 677	WT B-ALL 683
RAS	*Kras*	G12D	G12D	G12D	G12D	G12D	G12D	G12D	G12D
B-cell pathway/ lymphoid	*Bcl11a*		L147P						
	Ebf1							Q32R	
	Ikzf1				G205Rfs*2	S251_S387del		C332_E333inG	R75L
	Pax5	P80R	S86P	G85R			P316T	V26Gfs*49	R38C
	Tox	E140D	E140D	E140D				E140D	E140D
Epigenetic/ remodeller	*Arid1a*								W1546R
	Arid5b							N480S	
	Asxl1	A1467T							
	Chd4					L75P			
	Ezh2						E116K		
	Kdm6b	K979Rfs*104							
	Kmt2a			E806D, E821P					E806D, E821P
	Kmt2b								P1120L
	Kmt2d					Q2786R			
JAK-STAT	*Tyk2*	H261R							
Kinase	*Abl1*						S857G		
	Pdgfrb	R703Q							
Transcription	*Elf1*				E103K				
	Gata3	L102Q							
	Zfhx3						L1726S		
Membrane	*Gnas*		Q510R						
	Muc4			start SNV					
DNA repair	*Atm*			R32C, Q1078H, A2119T					R32C, Q1078H, A2119T
Others	*Cux1*			A1423V					
	Serp2				A34T				
	Syne1	V1597L							E1291Q

Missense
Frameshift
Nonsense
In-frame insertion
Start-loss

Figure 2. Dp16 and WT *Kras*^{G12D}*.Pax5*$^{+/-}$ B-cell acute lymphoblastic leukemia recapitulate gene expression signatures and co-occurring mutations commonly seen in human B-cell acute lymphoblastic leukemia. (A) Gene set enrichment analysis revealed significant upregulation of Myc targets and oxidative phosphorylation in both Nod scid gamma (NSG)-expanded Dp16 *Kras*^{G12D}*.Pax5*$^{+/-}$ and wild-type (WT) *Kras*^{G12D}*.Pax5*$^{+/-}$ B-cell acute lymphoblastic leukemia (B-ALL) compared to healthy Dp16 and WT bone marrow (BM) B cells. DNA repair genes were also significantly upregulated in Dp16 *Kras*^{G12D}*.Pax5*$^{+/-}$ B-ALL. Approximate Normalized Enrichment Score (NES) and False Discovery Rate (FDR) q-values are indicated by colors and size of circles. (Legend on right) Significant FDR q-value<0.05, N=4 for B-ALL groups, N=5 for healthy control BM B cells. n.s.: not significant. (B) Whole exome sequencing revealed both Dp16 *Kras*^{G12D}*.Pax*$^{5+/-}$ and WT *Kras*^{G12D}*.Pax5*$^{+/-}$ B-ALL have mutations in genes and pathways also frequently mutated in human B-ALL, including those involved in B-cell development and epigenetic regulation. N=4; mouse ID indicated at top of each column; only non-synonymous mutations with a variant allele frequency ≥20% are shown.

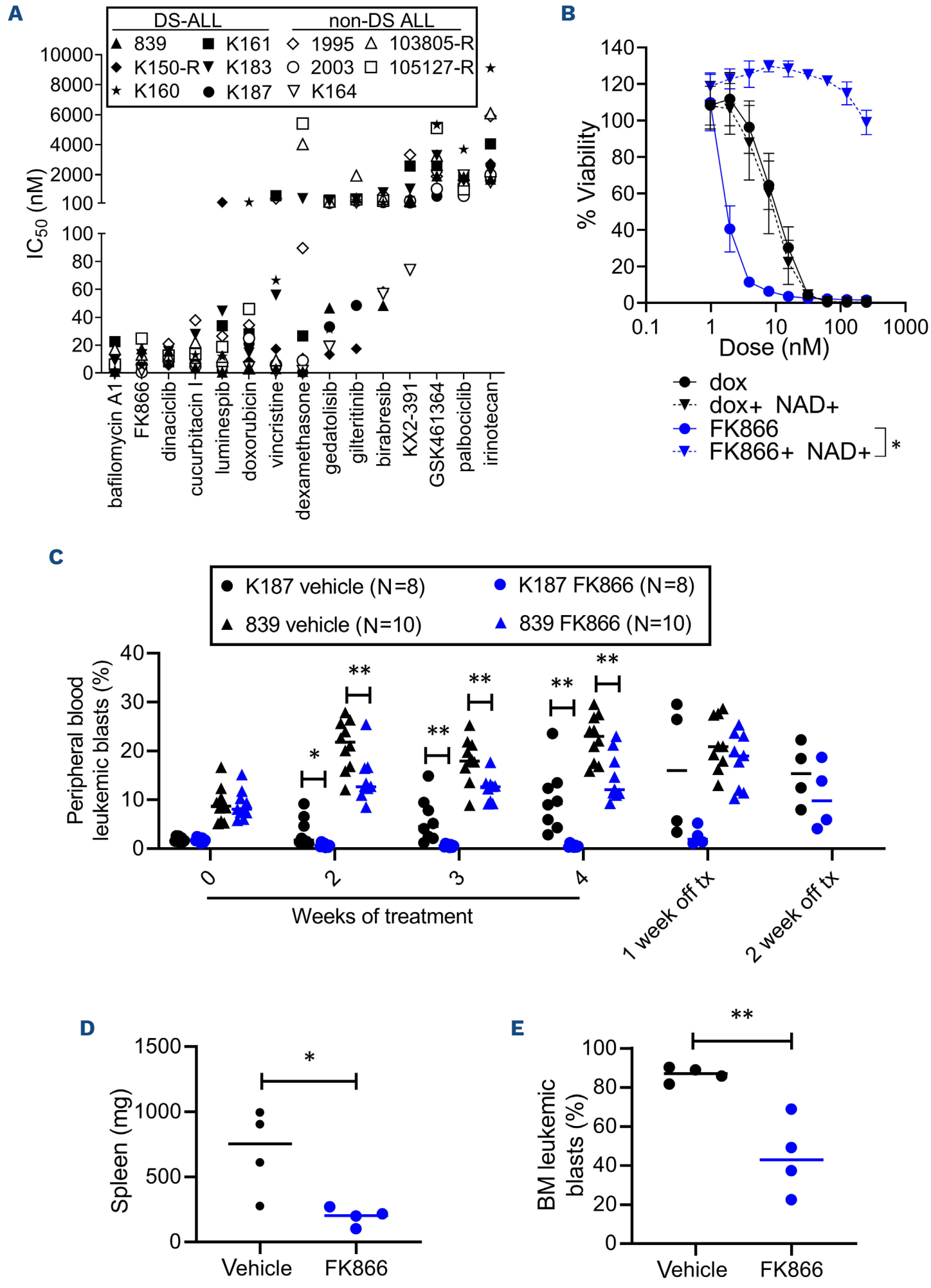


Figure 3. Targeted agents are effective against Down syndrome acute lymphoblastic leukemia and non-Down syndrome acute lymphoblastic leukemia patient samples *in vitro*, and FK866 reduces Down syndrome acute lymphoblastic leukemia disease burden *in vivo*. (A) Down syndrome acute lymphoblastic leukemia (DS-ALL) and non-DS ALL patient samples were incubated with 12 targeted therapies selected from the 2 screening studies, and 3 standard chemotherapies (doxorubicin, vincristine, and dexamethasone). Cells were incubated with each drug at 11 doses from ~1 nM to 4 μM for 72 hours (hr), before viability was measured with CellTiter-Glo. Bafilomycin A1, FK866, dinaciclib, and cucurbitacin I were effective in all patient samples at low nanomolar concentrations. Samples with IC_{50} values >10 μM are not displayed (N=6 for DS-ALL, N=5 for non-DS-ALL). (B) Blasts isolated from a mouse xenografted with DS-ALL patient sample K187 were pretreat-

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ed with NAD+ (triangles) or vehicle (circles), before 72-hr incubation with up to 250 nM doxorubicin (black) or FK866 (blue). The effect of FK866 was selectively reversed by NAD+ supplementation, indicating FK866 functions by inhibiting NAMPT. *P<0.0001, Student t test. (C) NSG mice xenografted with DS-ALL patient samples K187 (circles) or 839 (triangles) were treated with vehicle (black) or 20 mg/kg FK866 (blue) via intraperitoneal injection five days per week for four weeks. FK866 significantly reduced peripheral blood (PB) disease burden at all tested timepoints during treatment (tx). By one week post treatment, there was no difference in PB blast burden between groups: *P<0.05, **P<0.01, Student t test. Investigators were blinded to PB sample identity. FK866 also significantly reduced (D) spleen weight and (E) BM leukemic disease burden after four weeks of treatment in K187 mice (N=4): *P<0.05, **P<0.01, Student t test.

demonstrating the leukemogenic effect of the Dp16 genetic background. Importantly, this DS-ALL mouse model contains a fully native marrow microenvironment and immune system, providing a valuable opportunity to study interactions between native DS BM cell populations and developing B-ALL.

Triplication of specific Hsa21 genes likely directly contributes to the increased risk of DS-ALL, and Hsa21 genes *DYRK1A* and *HMGN1* may be therapeutic targets in DS-ALL.[12,13] We observed *Dyrk1a*, *Hmgn1*, and *Runx1* were significantly upregulated in Dp16 BM B cells compared to WT, supporting utility of our model for studies of novel therapies. We also observed upregulation of *Ifnar1*, *Ifnar2*, and *Ifngr2*, paralleling a key feature of immune dysregulation observed in DS,[14] which may contribute to features associated with DS-ALL.

Despite differences in latency to disease between the Dp16 and WT B-ALL mouse models, the GSEA analyses and mouse cell line drug screening revealed limited biological differences between the blasts in each model. Both the Dp16 and WT B-ALL cells demonstrated upregulation of pathways involving Myc signaling and oxidative phosphorylation, and both mouse and human DS and non-DS B-ALL cells were similarly sensitive to novel therapeutic agents. These results parallel another chemosensitivity study, which showed equal sensitivity of DS and non-DS ALL samples to several chemotherapies.[15] Finally, NSG recipients of established Dp16 and WT *Kras*^{G12D}.*Pax5*$^{+/-}$ leukemic blasts demonstrated the same latency to disease. Overall, our findings suggest the main difference between the Dp16 and WT *Kras*^{G12D}.*Pax5*$^{+/-}$ models is in the timing of leukemia initiation, rather than features of the resulting leukemic blasts.

The Dp16 *Kras*^{G12D}.*Pax5*$^{+/-}$ B-ALL mouse model presented here is a powerful tool for studies of DS-ALL. It constitutes a *de novo* ALL in a DS model organism with a native stromal microenvironment and immune system, and demonstrates a transcriptome profile and secondary alterations that recapitulate key features of DS-ALL. Our *in vitro* screen identified several new agents with potential utility in DS and non-DS ALL, and our *in vivo* DS-ALL PDX studies confirmed cytotoxicity of the NAMPT inhibitor FK866.

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Disclosures

No conflicts of interest to disclose.

Contributions

JJJ designed and conducted experiments, analyzed and interpreted data, and wrote the manuscript. MR, MA, RR, MT, LR and GD conducted experiments. BZ and PS analyzed and interpreted data. RTP, CCS, PJD, MMM and JJY designed experiments, and analyzed and interpreted data. KRR designed experiments, analyzed and interpreted data, and wrote the manuscript. All authors reviewed and approved the manuscript.

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Data-sharing statement

The corresponding authors are available to share any requested original data and protocols with other investigators. RNA-Seq data were deposited in the European Nucleotide Archive (ENA), with the accession number PRJEB68384.

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