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EFFICACY OF RETINOIDS IN IKZF1-MUTATED BCR-ABL1 ACUTE LYMPHOBLASTIC LEUKEMIA

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AUTHOR CONTRIBUTIONS

M.L.C., R.K.G, T.C. and C.G.M. designed experiments. M.L.C., J.L., E.M.P., L.H.K., Y.C., D.P.-T., M.J.A., W.C., L.L., K.G.R., K.M., I.I., J.P., V.E.C., K.K., V.P., and C.G.M. performed experiments. E.M.P., F.N., S.P.D, J.E.D., C.L.W., J.R. and S.L. provided clinical samples and data. Y.-L.Y., J.M, A.M., R.T.W. and R.A.D supplied reagents. M.L.C., J.L., C.Q., S.-C.C., J.M., G.S., M.R., D.M., M.E., P.G., Y.-D.W., B.F., J.C.P., S.B., L.J., J.P., V.P. and C.G.M. analyzed data. M.L.C. and C.G.M. wrote the manuscript.

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SUMMARY

Alterations of *IKZF1*, encoding the lymphoid transcription factor IKAROS, are a hallmark of high risk acute lymphoblastic leukemia (ALL), however the role of *IKZF1* alterations in ALL pathogenesis is poorly understood. Here we show that in mouse models of BCR-ABL1 leukemia, *Ikzf1* and *Arf* alterations synergistically promote the development of an aggressive lymphoid leukemia. *Ikzf1* alterations result in acquisition of stem cell-like features, including self-renewal and increased bone marrow stromal adhesion. Retinoid receptor agonists reversed this phenotype, partly by inducing expression of IKZF1, resulting in abrogation of adhesion and self-renewal, cell cycle arrest and attenuation of proliferation without direct cytotoxicity. Retinoids potentiated the activity of dasatinib in mouse and human *BCR-ABL1* ALL, providing an additional therapeutic option in *IKZF1*-mutated ALL.

INTRODUCTION

ALL is the most common tumor and a leading cause of cancer death in the young (Stock, 2010; Inaba et al., 2013). B-progenitor ALL (B-ALL) is more common than T-lineage ALL, and comprises a number of subtypes characterized by constellations of chromosomal alterations, submicroscopic deletions and sequence mutations (Mullighan, 2013). Loss-of-function or dominant-negative deletions and mutations of lymphoid transcription factor genes including *PAX5* (encoding paired box 5), *IKZF1* (IKAROS), and *EBF1* (early B-cell factor 1) are observed in the majority of B-ALL cases (Mullighan et al., 2007).

IKZF1 alterations are a hallmark of high-risk B-ALL, particularly *BCR-ABL1* positive (Ph^+) ALL (Mullighan et al., 2008) and Ph-like ALL, which is characterized by a range of genetic alterations driving cytokine receptor and kinase signaling (Den Boer et al., 2009; Mullighan et al., 2009; Roberts et al., 2012; Roberts et al., 2014). *IKZF1* alterations include deletions that result in loss of expression of wild-type (WT) IKZF1 (IK1), and focal deletions or sequence mutations that alter IKZF1 function. A common deletion involving exons 4-7 results in expression of the IK6 isoform that lacks the N-terminal DNA-binding zinc fingers, but retains the C-terminal zinc fingers responsible for dimerization (Mullighan et al., 2008).

IK6 has dominant negative effects, in part by mislocalizing WT IKZF1 from the nucleus to the cytoplasm. Sequence mutations commonly involve zinc finger residues that mediate DNA binding (Mullighan et al., 2009). *IKZF1* alterations are also common in chronic myeloid leukemia (CML) at progression to lymphoid blast crisis, but are rare at chronic phase and progression to myeloid blast crisis, suggesting a central role in determining disease lineage and progression to acute leukemia (Mullighan et al., 2008). Additional genetic alterations are also observed in *BCR-ABL1* lymphoid leukemia, most commonly deletion of *CDKN2A/B* (INK4/ARF) in approximately 50% of cases (Mullighan et al., 2008).

IKZF1 alterations are associated with poor outcome in Ph− positive ALL, despite the advent of TKI therapy (Martinelli et al., 2009; van der Veer et al., 2014) and Ph-negative B-ALL (Mullighan et al., 2009; Kuiper et al., 2010). Consequently, new therapeutic approaches to improve the outcome of *IKZF1*-mutated ALL are required. However, a detailed understanding of the relative roles of *IKZF1* alterations and concomitant genomic alterations in lymphoid leukemogenesis and resistance to therapy is lacking. IKZF1 is required for the specification of the lymphoid lineage (Georgopoulos et al., 1994) by activating a lymphoid transcriptional network while repressing stem cell-, myeloid-, and erythroid-specific genes (Yoshida et al., 2010). *Ikzf1* haploinsufficiency accelerates the onset of *BCR-ABL1* lymphoid leukemia (Virely et al., 2010), and deletions of selected N-terminal zinc fingers in B cells results in stromal adhesion and progression to acute leukemia in mice (Schjerven et al., 2013; Joshi et al., 2014). Howevever, these studies do not fully recapitulate the genomic alterations in human *IKZF1*-mutated leukemia (including *BCR-ABL1*, expression of IK6 and *CDKN2A/B* deletion), nor do they directly model the role of *IKZF1* alterations in determining disease lineage and responsiveness to TKI therapy.

Here we describe mouse models of *Ikzf1*-mutated, Ph^+ ALL. We have used these models to examine the effects of different *Ikzf1* alterations, including haploinsufficiency and/or expression of IK6, and *Arf* loss, on disease lineage and responsiveness to TKI therapy. We examine the role of *Ikzf1* alterations on the acquisition of hematopoietic stem-cell like features, and have used these models as a platform of drug discovery to identify agents that enhance responsiveness to TKI therapy.

RESULTS

IKAROS Alterations in Human *BCR-ABL1* B-ALL

We previously reported a prevalence of *IKZF1* alterations in approximately 15% of childhood ALL, and over 80% of Ph^+ ALL (Mullighan et al., 2008; Zhang et al., 2011). However, the prevalence of *IKZF1* alterations has differed between studies. We examined *IKZF1* status in large cohorts of childhood and adult Ph-positive and negative ALL (Figure 1A and Table S1) (Roberts et al., 2014). Twenty-five percent of childhood and 44% of young adult precursor B-cell ALL cases had alterations of *IKZF1*. Exon 4-7 (IK6) deletions were present in 22.9% of B-ALL cases, and biallelic alterations in 13.2% of cases. *IKZF1* sequence mutations were observed in 2.6% of childhood and 3.4% of young adult ALL. Many sequence alterations were missense mutations in the N-terminal zinc fingers at or near

residues known to be critical for DNA binding (Cobb et al., 2000), and are thus likely to be loss-of-function and/or exert dominant negative effects (Figure 1B).

To compare the effects of point mutations to IK6, we expressed six different IKZF1 point mutant alleles, as well as WT IKZF1 and IK6 in *Arf*$^{-/-}$ BCR-ABL1-expressing pre-B cells. These experiments were performed in *Arf*$^{-/-}$ cells as although *Cdkn2a* encodes both p19^{Arf} and p16^{Ink4a}, prior studies have loss of Arf but not Ink4a promotes the development of BCR-ABL1 ALL (Kamijo et al., 1997; Williams et al., 2006; Signer et al., 2010). Enforced expression of WT IKZF1 was not tolerated and resulted in cell death. In contrast, all Ikzf1 point mutant alleles resulted in perturbed subcellular localization of the protein (Figure 1C). Endogenous IKZF1 exhibited punctate nuclear staining, whereas IK6 or IKZF1 point mutant alleles exhibited cytoplasmic or perturbed nuclear localization (Figure 1C). Thus, like IK6 (Nishii et al., 2002), the IKZF1 sequence mutations observed in human ALL result in cellular mislocalization and may also perturb IKZF1 function.

Cooperativity of *Ikzf1* and *Arf* Alterations in *BCR-ABL1* lymphoid leukemogenesis

To examine the relative contributions of *IKZF1* and Arf alterations in leukemogenesis, we used unfractionated, lineage negative (lin-) and pre-B cell retroviral bone marrow (BM) transplant models of BCR-ABL1 leukemia. Consistent with prior data (Virely et al., 2010), heterozygosity for an *Ikzf1* null allele increased the penetrance and reduced latency of pre-B cell ALL in both whole BM and pre-B cell transplant models (Figures 2A-B). The leukemias were of pre-B cell lineage (CD43^{+}, B220^{+}, CD19^{+}, BP-1^{+}, and IgM^{-}; Figure S1A). There was no expansion of lymphoid progenitors in *Ikzf1*$^{+/-}$ marrow (Figure S1B), and *Ikzf1*$^{+/-}$ mice did not develop leukemia in the absence of BCR-ABL1, suggesting that loss of IKZF1 does not promote leukemogenesis by expanding the pool of lymphoid progenitors susceptible to transformation.

Human Ph^{+} ALL tumors commonly harbor recurring DNA copy number alterations in addition to deletion of *IKZF1*, including deletions of *CDKN2A/B*, *PAX5*, *EBF1* and *MEF2C* (Mullighan et al., 2008; Mullighan et al., 2009). Using a comparative genomic hybridization microarray with dense tiling of targets of genetic alteration in human B-ALL (Table S2), we identified deletions also observed in human B-ALL, including deletions of *Ebf1* and *Cdkn2a/b*, but not *Ikzf1* (Figure S1C). These data support the notion that IKZF1 haploinsufficiency promotes tumorigenesis, but this process involves the sequential acquisition of additional genetic alterations, including second hits disrupting transcriptional regulation of lymphoid development.

The lineage of BCR-ABL1 leukemia has been attributed to the cellular target of transformation (McLaughlin et al., 1987; Daley et al., 1990), but the importance of cooperating genetic alterations has not been formally examined. To examine the role of *Ikzf1* and *Arf* alterations in determining disease lineage, we adopted a retroviral BM transplant model of BCRABL1 leukemia, in which expression of BCR-ABL1 in lineage-negative hematopoietic progenitors robustly induces myeloproliferative disease that recapitulates CML (Daley et al., 1990). As expected, WT BM expressing BCR-ABL1 resulted in a fully penetrant myeloid leukemia (Figure 2C; Figure S1D). In combination with IK6, BCR-ABL1 drove either myeloid or B-lymphoid disease (Figure 2C; Figure S1D). On an *Arf*$^{-/-}$

background, BCR-ABL1 resulted in 29% myeloid tumors and 71% B-lymphoid tumors; with IK6, BCR-ABL1 uniformly induced BALL (Figure 2C; Figure S1D). One recipient of $Arf^{-/-}$ BCR-ABL1 cells developed a bilineal tumor with both myeloid and lymphoid (Figures 2D and S1E). All other tumors were either myeloid or lymphoid by immunophenotype and morphology (Figures 2D and S1F), and were retransplantable maintaining immunophenotypic fidelity to the primary tumor (Figure S1E). These results indicate that both IK6 and loss of *Arf* shift differentiating hematopoietic precursors toward a lymphoid fate during BCR-ABL1-driven leukemogenesis.

Ikzf1 Alterations Reduce Responsiveness of BCR-ABL1 ALL to Dasatinib

We next examined the effects of *Ikzf1* haploinsufficiency, IK6 and *Arf* loss on responsiveness to TKI therapy. To establish ALL models of each *Arf/Ikzf1* genotype with equivalent tumor burden at the commencement of therapy, 2×10^5 pre-B cells from WT, $Arf^{-/-}$, or $Ikzf^{+/-}$ mice expressing MSCV-BCR-ABL1-ires-*luc* and either MSCV-IK6-IRES-GFP (MIG-IK6) or empty vector (MIG) were inoculated into sublethally irradiated WT recipients. Dasatinib or vehicle was commenced at equivalent tumor burden as determined by bioluminescent imaging (Figure 2E). ALL lacking *Ikzf1* or *Arf* alterations was not modeled due to incomplete penetrance and highly variable latency.

Ikzf1 haploinsufficiency and IK6 both reduced disease latency in a synergistic fashion ($Ikzf1^{+/+}$ + IK6 vs $Ikzf1^{+/-}$ + IK6 $p<0.0001$; Table 1 and Figures 2F-2H). Expression of IK6 resulted in a reduced response to dasatinib treatment, also in a synergistic manner with *Ikzf1* haploinsufficiency (Figure 2F-2H). The reduced effect of dasatinib in *Ikzf1*-altered leukemias was not due to impaired inhibition of ABL1 (as measured by STAT5 and CRKL phosphorylation, Figure S1G) or the acquisition of ABL1 tyrosine kinase domain mutations (data not shown).

Increased Adhesion of Ikzf1-altered BCR-ABL1 pre-B Cells

We next examined the growth kinetics and phenotypic properties of BCR-ABL1 pre-B cells of various *Ikzf1* and *Arf* genotypes (WT, $Arf^{-/-}$, $Ikzf1^{+/-}$, and $Ikzf1^{+/-}$;$Arf^{+/-}$ with and without IK6). Both *Ikzf1* haploinsufficiency and expression of IK6 reduced doubling times (Figure S2A).

We observed striking effects of all *Ikzf1* alterations on intercellular adhesion (Figure 3A). Regardless of genetic background, BCR-ABL1 pre-B cells harboring *Ikzf1* alterations exhibited marked changes in their morphology with adherence in sphere-like aggregates *in vitro*. This was observed for *Ikzf1* haploinsufficiency, expression of IK6 or zinc finger point mutant alleles, and shRNA-knockdown of *Ikzf1* (Figure S2B). Knockdown of *Ikzf3* (AIOLOS), an IKAROS family member that with IKZF1 is part of the Mi-2/nucleosome remodeling and deacetylase (NuRD) complex (Zhang et al., 2012b), resulted in similar cellular aggregation (Figure S2B, panel XIV). Knockdown of *Ikzf2* (HELIOS), that does not share a role in the NuRD complex and is not expressed in pre-B cells, had no effect.

To assess whether the increased adhesion of *Ikzf1*-altered BCR-ABL1 pre-B cells conferred an advantage in homing and engraftment in the BM niche, we transplanted 2×10^5 $Arf^{-/-}$ pre-

B cells expressing BCR-ABL1 and either IK6-MIG or MIG and harvested calvaria at 24, 48, and 72 hours post-injection to track the infiltration of GFP-labeled cells in the BM cavities utilizing multiphoton microscopy. By 48 hours sufficient cells were visible in each group to enable accurate quantitation of engraftment (Figure S2C). No quantitative differences in homing were detected, however IK6-expressing cells adopted spindle-like morphology, with adherence to and infiltration of surrounding BM stroma (Figure 3B). To assess localization in the BM niche, we repeated this experiment using *Prrx1-Cre;*Ai9 recipient mice, which express tdTomato in mesenchymal stem cells, osteoblasts and CXCL12-abundant reticular cells (Greenbaum et al., 2013), followed by *in vivo* antibody-based marking of the BM vasculature. This showed localization of leukemic cells adjacent to perivascular mesenchymal cells and SCA-1^+ arteriolar endothelial cells (Figure 3C). This stromal adherence *in vivo* was recapitulated *in vitro* by measuring adherence to the fibronectin, which showed increased adherence of IK6-expressing cells, comparable to purified mHSCs, after short-term incubation (Figure 3D).

Characterization of the Adhesive Phenotype of *Ikzf1*-altered Leukemia

We next performed transcriptomic and proteomic profiling of $Arf^{-/-}$ BCR-ABL1 pre-B cells with or without expression of IK6, and immunophenotyping of a large cohort of human Ph^+ ALL cases of known *IKZF1* genotype (Table S5). These analyses showed highly significant correlation of the transcriptomic and proteomic signatures of BCR-ABL1 IK6 *v.* non-IK6 leukemias, with gene set enrichment analysis p and FDR q values <0.0001 for enrichment of the expression signature in the proteomic signature. IK6-expressing leukemic cells showed overexpression of multiple adhesion molecules implicated in leukemic and stem cell adherence, including THY1 (thymocyte differentiation antigen 1, CD90), SELL (L-selectin), and the THY1 ligand ITGA5 (integrin alpha 5) (Saalbach et al., 2005) as well as other genes involved in THY1 integrin signaling, including PTK2 (protein kinase 2; or focal adhesion kinase, FAK) and PTK2B (Figures 3E-F and S2D-E; Tables S3 and S4). THY1 is a GPI-anchored glycoprotein involved in adhesion (Rege and Hagood, 2006) and integrin signaling (Barker and Hagood, 2009). THY1 is expressed by thymocytes, hematopoietic stem cells (Craig et al., 1993; Mayani and Lansdorp, 1994; Notta et al., 2011) and stem cell-enriched populations in non-BCR-ABL1 leukemia (Lamkin et al., 1994; Yamazaki et al., 2009), but not normal B-cells. THY1 and ITGA5 are both expressed at high levels in mouse and human HSCs (Figure S2F-G), and mouse and human HSCs were highly adherent to fibronectin in vitro (Figures 3D and S2H). *Thy1* is a target of IKZF1 transcriptional repression (Zhang et al., 2012b). Analysis of existing chromatin immunoprecipitation (ChIP) sequencing data (Ferreiros-Vidal et al., 2013), and ChIP-PCR of BCR-ABL1 $Arf^{-/-}$ pre-B cells showed that *Thy1*, *Sell*, *Cd28*, and *Itga5* are IKZF1 targets in primary mouse pre-B cells (Figures S2I-J).

All BCR-ABL1 pre-B cell lines harboring an *Ikzf1* alteration displayed increased THY1 expression relative to controls (Figures 3G and S2K). IK6-expressing tumors cells from both lineage negative and pre-B cell experiments, and BCR-ABL1 cells following RNAi-mediated Ikzf1/3 knockdown showed increases of THY1 expression (Figures 3E-G and S2K-M). Immunophenotyping of 98 human ALL samples showed higher THY1 expression in *IKZF1*-altered BCR-ABL1 B-ALL cases compared to those lacking IKZF1 alterations and non-Ph^+ cases (Figure 3H; Table S5).

To examine the role of adhesion molecule upregulation, we incubated *Arf*$^{-/-}$ BCR-ABL1 IK6 pre-B cells with a neutralizing anti-THY1 antibody, which abrogated the clustering phenotype, whereas an antibody directed to B220 and an IgG control had no effect (Figure 3I). shRNA-mediated knockdown of THY1, SELL, or ITGA5 also disrupted cellular aggregation *in vitro* (Figures 3J and S2N). Conversely, overexpression of THY1, SELL, or ITGA5 alone in *Arf*$^{-/-}$ BCR-ABL1-expressing pre-B cells was sufficient to induce clustering (Figure 3K and Figure S2O), demonstrating that THY1, SELL, and ITGA5 mediate adhesion of IKZF1-altered cells.

Ikzf1 Alterations Result in Acquisition of a Stem Cell-like Phenotype

The gene expression profile of IK6-expressing BCR-ABL1 *Arf*$^{-/-}$ pre-B cells was significantly enriched for gene sets upregulated in human Ph^{+} B-ALL, transcriptional targets of IKZF1 and gene sets representative of human embryonic (Wong et al., 2008), hematopoietic (Doulatov et al., 2010; Novershtern et al., 2011) and leukemic (Zhang et al., 2012a) stem cells (Figure 4A). This is consistent with previous observations in human *IKZF1*-altered ALL (Mullighan et al., 2009), and suggests that *IKZF1* alterations impair hematopoietic development and confer stem-cell like features. To investigate this, we examined single cell colony forming potential of WT, *Ikzf1*$^{+/-}$, and *Arf*$^{-/-}$ BCR-ABL1 pre-B cell lines with and without IK6 as a measure of self-renewal. In all genetic backgrounds, *Ikzf1*-alterations conferred increased clonogenicity (Figure 4B). Serial re-plating of the *Arf*$^{-/-}$ groups demonstrated that IK6-expressing cells maintain a consistent level of clonogenicity over eight weeks, while cells expressing empty vector decrease the number of colonies formed over time (Figure 4C). WT cultures could not be maintained more than two serial platings due to loss of ARF. Addition of THY1-neutralizing antibody to the media prior to single cell sorting reduced the ability of IK6-expressing cells to form colonies (Figure 4D).

RXR Agonists Induce IKZF1 Expression and Ameliorate the Effects of *Ikzf1* Alterations

As *IKZF1* alterations induce stem cell and adhesive properties, and are associated with poor response to TKI therapy in human ALL, we performed a drug screen to identify agents that reverse this stem-cell-like phenotype and enhance TKI sensitivity. We used high-throughput microscopy to measure the clustering of IK6-expressing *Arf*$^{-/-}$ BCR-ABL1 pre-B cells with the aim of identifying compounds that abolish their sphere-forming capacity without directly affecting viability, although direct cytotoxicity was also measured (Figure S3A-C). Disruption of clustering suggested that a drug may reverse the stem-cell like characteristics induced by *IKZF1* alteration, and may augment responsiveness to existing therapy.

An initial screen of 356 compounds currently in clinical use or trials (Table S6) identified bexarotene as the agent that most potently inhibited cellular aggregation (Figure 5A). Bexarotene is a synthetic retinoid that activates retinoid X receptors (RXRs). A secondary screen using 127 nuclear hormone receptor effectors (Table S6) identified carbacyclin, all-*trans* retinoic acid (ATRA), 9-*cis* retinoic acid (RA), and 13-*cis* RA as potent inhibitors of cellular aggregation. Carbacyclin is a peroxisome proliferator-activated receptor (PPAR) agonist, whereas ATRA and 9/13-*cis* RA activate retinoic acid receptors (RARs), although all have promiscuous activity for multiple receptors. All compounds identified converge on

the retinoid pathway, whereby RXRs, RARs, and PPARs can interchangeably heterodimerize to transcriptionally activate target genes containing appropriate response elements (Altucci et al., 2007). *Rara, Rarg, Rarres, Rxra, Rxrb, Ppard, and Ppargc1b* were expressed in *Arf*$^{-/-}$ BCR ABL1 pre-B cells, all of which harbor IKZF1 binding sites, with significant upregulation of *Rxra*, encoding retinoid X receptor alpha, in IK6 expressing cells (Figures S3D-E). As these agents exhibit cross reactivity for the different retinoid receptors, we also treated IK6-expressing *Arf*$^{-/-}$ BCR-ABL1 pre-B cells with RXR-specific agonist LG100268 (LG) and the RAR-specific agonist, AGN-195183 (AGN). LG abrogated the clustering phenotype *in vitro*, whereas AGN and the L-selectin inhibitor rivipansel had no effect on adhesion (Figure S3F). In addition, connectivity map analysis (Lamb et al., 2006) identified the gene expression signature of 13-*cis*-retinoic acid as negatively enriched in that of *Arf*$^{-/-}$ BCR-ABL1 MIG-IK6 *v.* MIG leukemia, further supporting the importance of this pathway in *Ikzf1*-altered ALL (P=0.0048, specificity=0.0079, Table S7).

Each non-specific retinoid abrogated sphere formation of *Arf*$^{-/-}$ BCR-ABL1 IK6-expressing pre-B cells (Figure 5A), and arrested cell proliferation (Figure 5B) at the G_0/G_1 phase of the cell cycle (Figure 5C), with minimal apoptosis (Figure S3G). With the exception of carbacyclin, this was associated with partial maturation, with reduced CD43 and increased CD25 expression (Figure 5D), and on transcriptomic analysis, reversal of the hematopoietic stem cell state and induction of a B-cell differentiation program (Figure S3H). THY1 and CD28 expression was reduced after 72 hours of treatment *in vitro*, with drug-dependent effects on ITGA5 and SELL levels (Figure 5E). Expression of the downstream integrin signaling mediator PTK2B was also reduced by each compound (data not shown). All retinoids profoundly decreased the colony-forming potential of IK6-expressing pre-B cells (Figure 5F). In calvarial BM imaging experiments, *in vivo* treatment with bexarotene at 0, 24 and 48 hours post-transplant, restored the spherical, non-adherent phenotype of transplanted *Arf*$^{-/-}$ BCR-ABL1 IK6-expressing pre-B cells (Figure 5G). In addition, leukemic burden was significantly decreased in mice treated with bexarotene (Figure 5H).

IKZF1 has alternate non-coding first exons, and the promoter, 5′ untranslated region, and gene body of *IKZF1* harbors multiple putative retinoic acid response elements (RARE; Figure S4A). *Ikzf1* is a direct target of RXR signaling, as determined by ChIP-PCR in *Arf*$^{-/-}$ BCR ABL1 IK6 pre-B cells. No enrichment was observed for RAR binding at the *Ikzf1* locus (Figure S4A). Retinoid treatment resulted in induction of WT *IKZF1*, but not IK6 expression from the first non-coding exon in both mouse and human leukemic cells as determined by RNA-seq (Figures 6A-B). Western blotting, RT-PCR and immunofluorescence confirmed selective induction of WT IKZF1 but not IK6 by retinoids, and partial relocalization of IKZF1 to the nucleus in IK6-expressing cells (Figures 6C-G). Moreover, 1745 (85%) of 2061 genes differentially expressed in *Arf*$^{-/-}$ BCR-ABL1 IK6 cells following bexarotene treatment were IKZF1 targets (Ferreiros-Vidal et al., 2013), compared to 40% of non-differentially expressed genes ($P<2\times10^{-16}$), supporting the notion that induction of IKZF1 directly contributes to the effects observed upon retinoid treatment. Multiple RAREs within the genomic region of *IKZF1* deleted in the IK6 allele showed significant enrichment for RXR binding, particularly after treatment with the RXR-specific agonist LG100268 (Figure S4A). In addition, the *IKZF1* locus has a putative intragenic

enhancer within the region of IK6 deletion marked by H3K27 acetylation in K562 (BCR-ABL1$^+$ *IKZF1* WT cells, Figure S4B) that has been shown to interact with the *IKZF1* promoter through Chromatin Interaction Analysis Paired-End Tags only in K562 among the 6 ENCODE cell lines (Li et al., 2012), suggesting that this region that is lost in IK6-deleted cases influences regulation of IKZF1 expression.

To test if directly increasing IKZF1 expression levels recapitulates the effects of retinoids, we enforced expression of IKZF1 in *Arf*$^{-/-}$ BCR-ABL1 pre-B cells with or without expression of IK6. Enforced expression of IKZF1 is not tolerated by *Ikzf1* WT cells and results in apoptotic cell death (Figure 1C). In contrast, IKZF1 expression in IK6-expressing cells did not suppress proliferation or cause cell death (Figure S4C), but resulted in attenuation of aggregation accompanied by a decrease in THY1 and ITGA5 expression (Figures 6H and 6I). Therefore in *IKZF1*-altered cells, the induction of WT *Ikzf1* by retinoids leads to abrogation of the adherent phenotype, but cell cycle arrest and differentiation are caused by retinoid-related, non-IKZF1-dependent mechanisms. In contrast, bexarotene did not significantly reduce proliferation in mouse BCR-ABL1 leukemic cells lacking Arf/Ikzf1 alterations (Figure S4D). Reduced proliferation and abrogation of clustering was observed in non-BCR-ABL1 human leukemic cells with *IKZF1* alterations, including Ph-like ALL cells expressing *PAG1-ABL2* or harboring *EPOR* rearrangements (Figure S4D-E), as has been reported for retinoids in non-BCR-ABL1 ALL cells (Zhang et al., 2002; Lin et al., 2007)

Retinoids Increase Responsiveness of Ikzf1-altered Leukemias to TKIs

Using *in vitro* viability assays with drug added at the time of cell plating, bexarotene significantly increased responsiveness to dasatinib in IK6-expressing *Arf*$^{-/-}$ BCR-ABL1 IK6 pre-B cells (data not shown). Response to dasatinib was also improved in *Arf*$^{-/-}$ BCR-ABL1 pre-B cells lacking IK6, consistent with retinoid induction of IKZF1 expression in non-IK6 expressing cells causing cell cycle arrest and subsequent death (Figures 1C, S4C).

We examined the combinatorial and single-agent effects of bexarotene, dasatinib and additional cytotoxic agents used in ALL therapy, L-asparaginase and dexamethasone, in mice transplanted with *Arf*$^{-/-}$ BCR-ABL1 pre-B cells with or without IK6 expression. In contrast to L-asparaginase and dexamethasone (Boulos et al., 2011), bexarotene monotherapy resulted in significant benefit without detectable toxicity (Figure 7A and S5A). Dasatinib monotherapy increased survival with inferior responsiveness in the IK6 group (Figure 7A). The combination of dasatinib and bexarotene resulted in the greatest survival advantage, with a near-doubling of survival time in IK6 tumors compared to dasatinib monotherapy. Thus, bexarotene enhances efficacy of TKI therapy in Ph$^+$ tumors regardless of *Ikzf1* status, but with the greatest potentiation observed in *Ikzf1*-altered tumors.

To test the activity of retinoids in human leukemic cells, we established xenografts of Ph$^+$ ALL that faithfully recapitulate a range of *IKZF1* genotypes. The tumors harbored homozygous deletion of *CDKN2A/B (*i.e. INK4/ARF−null), and had deletions or sequence mutations of *IKZF1* (*IKZF1* Δ4-7 (IK6), Δ2-7 or R502W). Xenografts were treated with dasatinib in combination with bexarotene, ATRA or the FAK inhibitors PF-562271, NVP-

TAE226, and PF-573228 *ex vivo*. This resulted in significant potentiation of cell killing at increasing concentrations of the drugs (Figures 7B and S5B).

DISCUSSION

Here, we have examined large B-ALL cohorts to define the spectrum of *IKZF1* alterations, and used this to inform the development of faithful mouse models of human Ph^+ ALL. These models incorporate modeling of ARF loss, the second most common genetic alteration in Ph^+ ALL, which has been shown to reduce the latency of experimental Ph^+ ALL, but has not previously been co-modeled with *IKZF1* alterations (Williams et al., 2006; Williams et al., 2007). Importantly, we have studied *Ikzf1* haploinsufficiency and expression of IK6. In contrast to previously modeled *Ikzf1* deletions (Joshi et al., 2014), IK6 lacks all 4 N-terminal zinc fingers required for normal DNA binding, and arises from the most common focal *IKZF1* deletion observed in human ALL.

Using these models and complementary genomic and proteomic approaches, we have shown that perturbation of IKZF1 activity is a central event in the pathogenesis of BCR-ABL1 lymphoid leukemogenesis and reduced response to TKI therapy. We show that *Ikzf1* alterations induce a hematopoietic stem cell-like gene expression program accompanied by upregulation of adhesion molecules and signaling pathways that mediate abnormal adherence. We provide evidence that *Ikzf1* alterations confer stem cell like properties, as demonstrated by increased single cell colony formation and induction of adhesion molecules in mouse and human ALL.

Increased expression of THY1 (Yamazaki et al., 2009) and integrins (Hsieh et al., 2013; Miller et al., 2013) have been noted previously in ALL and *Ikzf1*-mutant mouse B-cells (Joshi et al., 2014) but not in Ph^+ ALL. In gain- and loss-of-function studies, we have shown that upregulation of these genes is a consequence of *IKZF1* alterations and directly contributes to enhanced adhesion and self-renewal properties in *Ikzf1*-altered BCR-ABL1 cells. Moreover, we have shown that each adhesion molecule is a direct transcriptional target of IKZF1, providing a mechanism for deregulation of expression in *IKZF1*-mutant leukemia.

The reversal of this phenotype with retinoids was striking and emphasizes the utility of drug screens interrogating phenotypes more complex than killing alone. Administration of retinoid agonists resulted in profound reversal of the stem cell transcriptional program, and abrogation of adherence and self-renewal, without direct cytotoxicity. The *Ikzf1* locus has multiple retinoid receptor binding sites and strikingly, retinoids selectively induced expression of WT IKZF1 resulting in expression of IKZF1 target genes. Using adhesion assays and calvarial imaging, we showed that retinoids completely reversed the stromal adhesion of IKZF1-altered leukemic cells. Together with the observation that enforced IKZF1 expression also abrogated adhesion, these findings suggest that retinoid-mediated induction of IKZF1 directly contributes to the reversal of stem cell features and increased responsiveness to dasatinib in Ph^+ ALL. The observed induction of *IKZF1* expression suggests that retinoids may have less effect in the minority of ALL cases with biallelic alterations resulting in complete loss of IKZF1 expression. However, in contrast to enforced expression of IKZF1, retinoids induced differentiation and cell cycle arrest independent of

IKZF1 induction, indicating that retinoids have both IKZF1 dependent and independent effects in Ph^+ ALL (Figure 7C).

Retinoids are approved for use in several diseases, including all-*trans*-retinoic acid in acute promyelocytic leukemia (Park and Tallman, 2011), and bexarotene in cutaneous T-cell lymphoma (Connolly et al., 2013), but have not been previously systematically investigated in Ph^+ ALL. Our findings provide a rationale for clinical evaluation of these agents in *IKZF1*-altered Ph^+ ALL. Collectively, these results indicate that *IKZF1* alterations have important roles in determining disease lineage, reduced TKI sensitivity, and induction of stem cell features in Ph^+ B-ALL, and that disrupting these phenotypes by targeting the retinoid pathway, or downstream signaling pathways, represent important treatment opportunities that should be pursued in clinical trials.

EXPERIMENTAL PROCEDURES

Mouse modeling of BCR-ABL1 leukemia

For whole BM experiments, unmanipulated BM was transduced with retrovirus for three hours and 1 million unsorted cells were transplanted by tail vein injection into lethally irradiated WT recipients. To derive pre-B cells, BCR-ABL1-transduced BM was cultured in the absence of feeder layers or cytokines, cells were subsequently transduced with retroviruses on retronectin and 2×10^5 sorted cells were injected by tail vein into sublethally irradiated WT recipients. For lin-cell transplantation experiments, unmanipulated whole BM cells lacking lineage markers were isolated and transduced with retroviruses prior to sorting and injection into lethally irradiated WT recipients. Mice were housed in an American Association of Laboratory Animal Care (AALAC)-accredited facility and were treated on Institutional Animal Care and Use Committee (IACUC)-approved protocols in accordance with NIH guidelines.

Analysis of Human Leukemias

Previously reported cohorts (Mullighan et al., 2007; Mullighan et al., 2008; Mullighan et al., 2009; Roberts et al., 2012; Roberts et al., 2014) and ninety-eight adult B-progenitor ALL samples collected on the Eastern Cooperative Oncology Group E2993 (Rowe et al., 2005) and ClinicalTrials.gov identifier NCT00002514 and Alliance for Clinical Oncology C10403 (clinicaltrials.gov NCT00558519) studies were analyzed for *IKZF1* deletions and mutations by SNP 6.0 microarrays (Affymetrix, Santa Clara, CA) and by genomic PCR and Sanger Sequencing as previously described (Mullighan et al., 2007; Mullighan et al., 2009; Mullighan et al., 2011). All samples were obtained with patient or parent/guardian provided informed consent under protocols approved by the Institutional Review Board at each COG and ECOG institution and St. Jude Children's Research Hospital. All samples were de-identified prior to analysis.

Statistical Analyses

Data analyses were performed using GraphPad Prism Version 6.0 (GraphPad, La Jolla, CA). All data are presented as mean ± SD. Significance was determined using Student's t test, ANOVA, or Mantel-Cox log rank test as appropriate. For survival studies, Kaplan-Meier

curves were generated and Mantel-Cox p values were determined for pairwise comparisons of cohorts. A *P* value of less than 0.05 was considered significant.

Other Procedures

All methods are described in detail in the Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- *Ikzf1* alterations confer stem-like properties and increased adhesion in *BCR-ABL1* ALL
- IKZF1-altered BCR-ABL1^{+} leukemias display reduced responsiveness to dasatinib
- Retinoids reverse the effects of *Ikzf1* alterations, partially by induction of IKZF1
- Retinoids potentiate the activity of dasatinb in human and mouse *BCR-ABL1* ALL

SIGNIFICANCE

The prognosis of high-risk acute lymphoblastic leukemia remains suboptimal despite contemporary chemotherapy and the advent of targeted therapeutic approaches. Deletions or mutations of *IKZF1* are a hallmark of high-risk ALL, but an understanding of how *IKZF1* alteration contribute to leukemia development are lacking. Here we show that *IKZF1* alterations drive lymphoid lineage, a stem cell-like phenotype, abnormal bone marrow adhesion, and poor responsiveness to tyrosine kinase inhibitor (TKI) therapy. Using a high-content screen, we show that retinoids reverse this phenotype in part by inducing expression of wild-type IKZF1, and increase responsiveness to TKIs. These findings provide insight into the pathogenesis of high-risk ALL and potential therapeutic approaches.

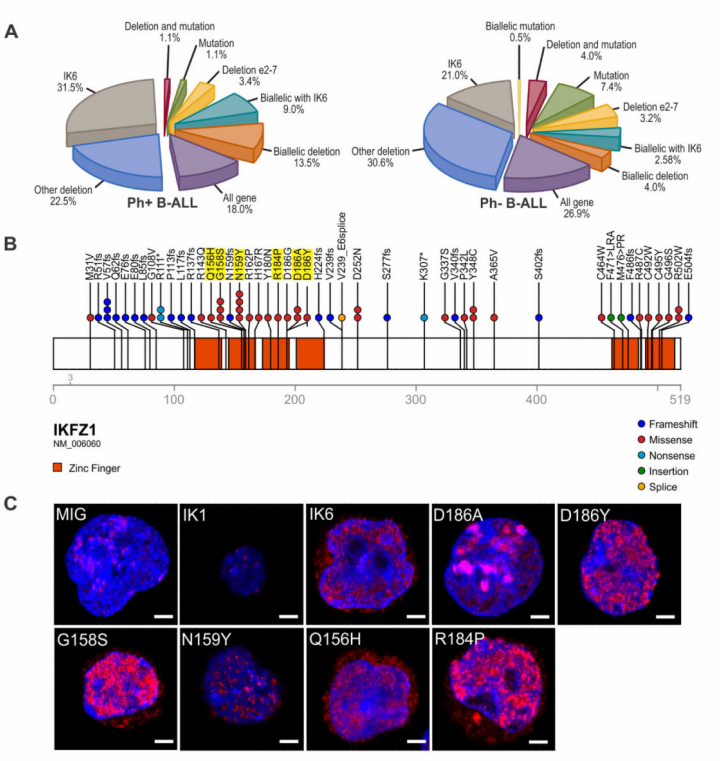

Figure 1. *IKZF1* alterations in human B-ALL

(A) Distribution of *IKZF1* alterations in human Ph$^+$ (left, n=89) and Ph- (right, n=405) B-ALL. (B) Point mutations found in human B-ALL. Highlighted mutations were modeled in *Arf*$^{-/-}$ BCR ABL1-expressing pre-B cells studied in (C). (C) WT IKZF1, IK6, and six IKZF1 point mutants were expressed in *Arf*$^{-/-}$ BCR-ABL1-expressing pre-B cells (scale bars, 2 m).

See also Table S1.

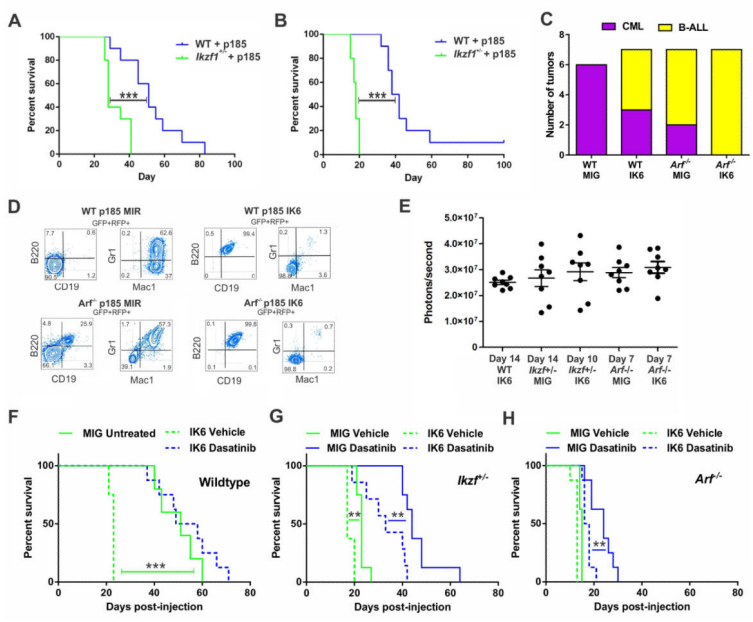

Figure 2. Perturbation of IKZF1 drives a lymphoid leukemia with less responsiveness to TKI therapy

(A) Unmanipulated BM from WT or *Ikzf1*$^{+/-}$ mice transduced with p185 BCR-ABL1 expressing retrovirus and immediately transplanted into lethally irradiated recipients. Statistical significance was assessed by log rank Mantel Cox ***p<0.0005; n=10 mice per group. (B) Transplantation of *in vitro*-derived BCR-ABL1 transformed pre-B cells into sublethally irradiated recipients. Statistical significance was assessed by log rank Mantel Cox ***p<0.0005; n=10 mice per group. (C) Summarized leukemia lineage data from lin- BM transplant experiments (see also Figure S1) (X^2 p=0.0029; n≥6 mice per group). (D) Representative flow cytometric analysis of tumors derived from p185 BCR-ABL1 transduced lin- BM cells to determine disease lineage. (E) *In vivo* quantification of luciferase activity, representing disease burden, in mice transplanted with preB cells of the defined genotypes (WT, *Arf*$^{-/-}$, or *Ikzf*$^{+/-}$) transduced with BCR-ABL1-ires-luciferase and empty vector (MIG) or IK6. Raw data points ± S.D. are plotted. Treatment was started when the average luminescence was greater than 2 × 10^7 photons/second, indicating a substantial and similar disease burden at the start of therapy between genotypic groups shown in (F-H) and Table 1. (F) Kaplan Meier survival curves of vehicle or dasatinib-treated mice inoculated with pre-B cells derived from WT BM described in (E). (G) Kaplan Meier survival curves of vehicle or dasatinib-treated mice inoculated with pre-B cells derived from *Ikzf*$^{+/-}$ BM described in (E). (H) Kaplan Meier survival curves of vehicle or dasatinib-treated mice inoculated with pre-B cells derived from *Arf*$^{-/-}$ BM described in (E). Statistical significance was access by log-rank Mantel-Cox test in F-H. **p < 0.005; n=8 mice per group.

See also Figure S1 and Table S2.

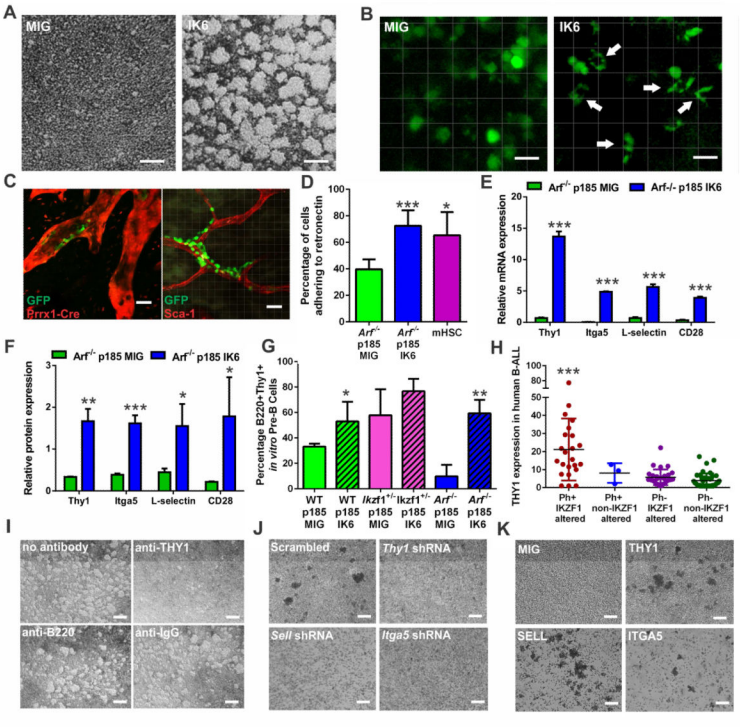

Figure 3. IKZF1 alterations induce aberrant adhesion in BCR-ABL1 leukemic cells

(A) Suspension cultures of *Arf*$^{-/-}$ BCR-ABL1 (p185) pre-B leukemic cells showing adherent growth in IK6 expressing cells *in vitro* (scale bars, 100μM). (B) Calvarial imaging of mice transplanted with *Arf*$^{-/-}$ BCR-ABL1-transduced pre-B cells expressing MIG or IK6-GFP *in vivo* (white arrows indicate cells with marked change in pre-B cell morphology; scale bars, 20μM). (C) Calvarial imaging of *Arf*$^{-/-}$ BCR-ABL1 IK6-GFP leukemic cells in *Prrx1*-Cre;Ai9 tomato mice showing associations with the PRRX1^{+} perivascular stromal cells (left) and *in vivo*-labeled SCA-1^{+} arterioles (right) in the BM niche (scale bars, 20μM). (D) *Arf*$^{-/-}$ BCR-ABL1 IK6-expressing cells are more adherent to fibronectin monolayers *in vitro*, comparable to murine hematopoietic stem cells (mHSC). Data are means ± S.D.; *p<0.05, *** p<0.0005; n=3 biological replicates. (E) The adherent phenotype of IK6-expressing *Arf*$^{-/-}$ BCR-ABL1 pre-B cells is accompanied by increased expression of adhesion markers compared to empty vector controls as determined by mRNA-sequencing. Data are means ± S.D.; *** p<0.0005; n=4 biological replicates per group. (F) Proteomic analysis by tandem mass tag based mass spectrometry confirms increased protein expression of adhesion markers in IK6-expressing pre-B cells. Data are means ± S.D.; ***p<0.0005, **p<0.005, *p<0.05; n=3 biological replicates per group. (G) Flow cytometric analysis for THY1 in cultured WT, *Ikzf1*$^{+/-}$, and *Arf*$^{-/-}$ BCR-ABL1-transduced cells expressing empty vector or IK6. Data are means ± S.D.; **p<0.005, *p<0.05; n=3 biological replicates. (H) Increased expression THY1 (CD90) by immunophenotypic analysis of human BCR-ABL1 (Ph^{+}) and *IKZF1*-mutated leukemias. Data are means ± S.D.; **p<0.005, 98 human cases were analyzed, see also Table S5. (I) Cellular adherence of *Arf*$^{-/-}$ BCR-ABL1 IK6-expressing pre-B cells is abrogated *in vitro* by anti-THY1 antibody, but not B220 and IgG control antibodies (scale bars, 100μM). (J) Cell aggregation is also attenuated by shRNA

knockdown of *Thy1*, *Sell*, or *Itga5* (scale bars, 100μM). (K) Overexpression of THY1, SELL, or ITGA5 resulted in cellular aggregation (scale bars, 100μM).
See also Figure S2 and Tables S3-S5.

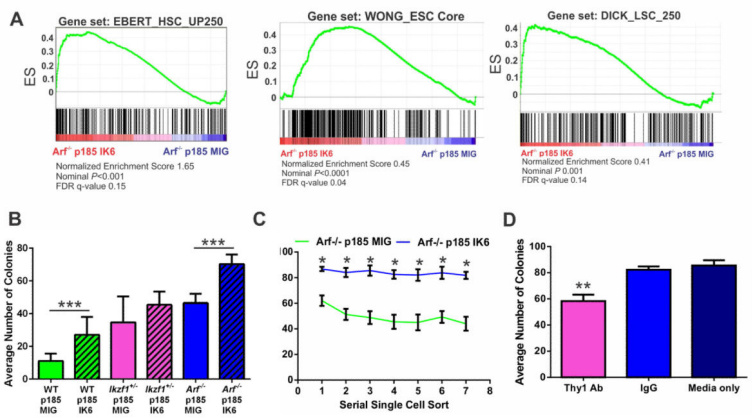

Figure 4. IKZF1 alterations induce stem cell-like features in BCR-ABL1 leukemic cells

(A) GSEA analyses of $Arf^{-/-}$ BCR-ABL1 MIG vs IK6-expressing pre-B cells showing IK6 expression is associated with acquisition of a stem-like gene expression programs. A full list of significantly enriched gene sets is provided in Table S3. (B) IKZF1 perturbation and *Arf* loss synergistically result in enhanced self-renewal of BCR-ABL1-expressing pre-B cells, as shown by the ability of single cell cultures to form colonies. Data are means ± S.D.; ***p<0.0005, n=3 biological replicates performed in triplicate. (C) Single cell replatings were sustained over serial passages in the immortal $Arf^{-/-}$ lines. Data are means ± S.D.; *p<0.05; n=4 technical replicates (96 well plates). (D) A neutralizing THY1 antibody attenuates the colony-forming ability of $Arf^{-/-}$ BCR-ABL1 IK6 pre-B cells. Data are means ± S.D.; **p<0.005; n=3 biological replicates.

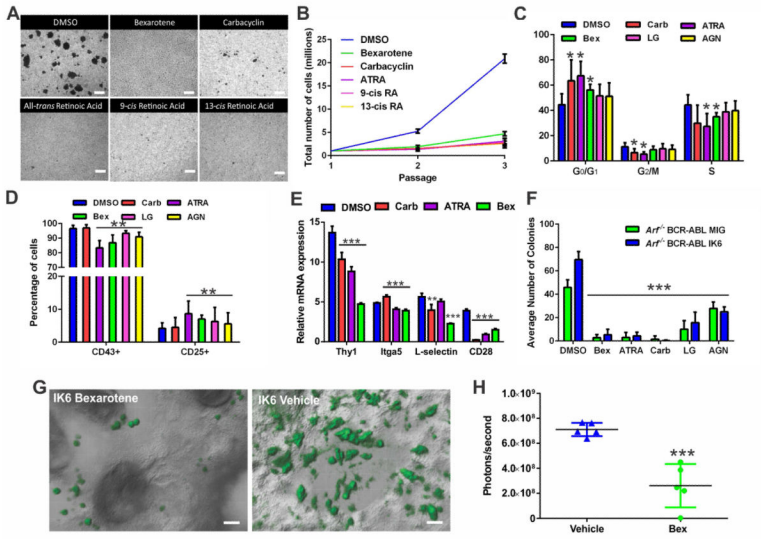

Figure 5. Retinoids disrupt cellular aggregation and reverse the stem-cell like features of BCR-ABL1 leukemic cells

(A) Suspension cultures of *Arf*$^{-/-}$ BCR-ABL1 IK6-expressing cells exposed to DMSO or retinoids at 1μM for 72 hours. Each drug abolishes adherent growth induced by IK6 (scale bars, 100μM). (B) *In vitro* growth kinetics of DMSO or retinoid-treated *Arf*$^{-/-}$ BCR-ABL1 IK6-expressing cells. Data are means ± S.D.; n=3 biological replicates. (C) Retinoid treatment arrests *Arf*$^{-/-}$ BCR-ABL1 IK6-expressing pre-B cells at the G_0/G_1 phase of the cell cycle. Data are means ± S.D.; *p<0.05; n=3 biological replicates performed in triplicate. (D) Flow cytometry of retinoid-treated *Arf*$^{-/-}$ BCR ABL1 IK6-expressing pre-B cells shows decreased CD43 expression and increased CD25 levels. Data are means ± S.D.; **p<0.005; n=3 biological replicates performed in triplicate. (E) Relative expression of aberrantly expressed adhesion molecules in *Arf*$^{-/-}$ BCR-ABL1 IK6-expressing pre-B cells following retinoid treatment, as determined by mRNA-sequencing. Data are means ± S.D.; ***p<0.0005, **p<0.005; n=4 biological replicates. (F) Retinoid treatment attenuates the self-renewal of *Arf*$^{-/-}$ BCR-ABL1 IK6-expressing pre-B cells as demonstrated by single cell colony forming ability. Data are means ± S.D.; ***p<0.0005; n= 3 biological replicates performed in triplicate 96 well plates. (G) *In vivo* bex treatment reversed the adherent phenotype of *Arf*$^{-/-}$ BCR-ABL1 IK6-GFP cells in the calvarial BM niche (scale bars, 20μM). (H) Luciferase quantification of vehicle or bex-treated mice 14 days after inoculation with 2×10^5 *Arf*$^{-/-}$ p185 IK6 leukemic cells. Daily vehicle or 45mg/kg oral bex treatment commenced at the time of transplant. Raw data points ± S.D. are plotted; ***p<0.0005; n=5 mice per group.

See also Figure S3 and Tables S6 and S7.

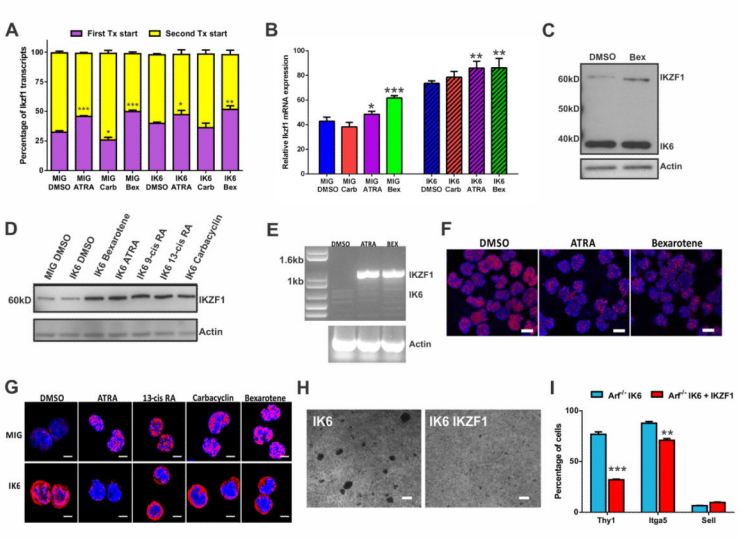

Figure 6. Retinoids induce IKZF1 expression, but not IK6, in BCR-ABL1 leukemic cells

(A) Proportion of *Ikzf1* transcripts from alternate transcriptional start sites as determined by mRNA-seq in $Arf^{-/-}$ p185-expressing pre-B cells. Data are means ± S.D.; ***p<0.0005, **p<0.005, *p<0.05; n=4 biological replicates. (B) *Ikzf1* mRNA levels are increased after retinoid treatment in $Arf^{-/-}$ p185 MIG or IK6 pre-B cells. Data are means ± S.D.; ***p<0.0005, ***P* < 0.005, *p<0.05; n=4 biological replicates. (C) Western blot of retinoid-treated murine $Arf^{-/-}$ BCR-ABL1 IK6-expressing pre-B cells. (D) Western blot of retinoid-treated human *CDKN2A*-deleted (*Arf*-null) BCR-ABL1^{+} IK6-expressing B-ALL cells. (E) RT-PCR analysis of retinoid-treated human BCR-ABL1 IK6 B-ALL cells for IKZF1 and IK6 transcripts. (F) Immunofluorescence for IKZF1 in *CDKN2A*-deleted (*Arf*-null) BCR-ABL1^{+} IK6-expressing B-ALL cells (scale bars, 5 μm). (G) Immunofluorescence for IKZF1 in murine $Arf^{-/-}$ BCR-ABL1 MIG (top) and IK6-exressing (bottom) pre-B cells (scale bars, 2 μm). (H) Overexpression of IKZF1 in $Arf^{-/-}$ BCR-ABL1 IK6 pre-B cells abrogates cellular adhesion *in vitro* (scale bars, 100μm). (I) Overexpression of IKZF1 in $Arf^{-/-}$ BCR-ABL1 IK6 pre-B cells downregulates THY1 and ITGA5 (as determined by flow cytometry). Data are means ± S.D.; ***p<0.0005, **p<0.005; n=3 technical replicates.

See also Figure S4.

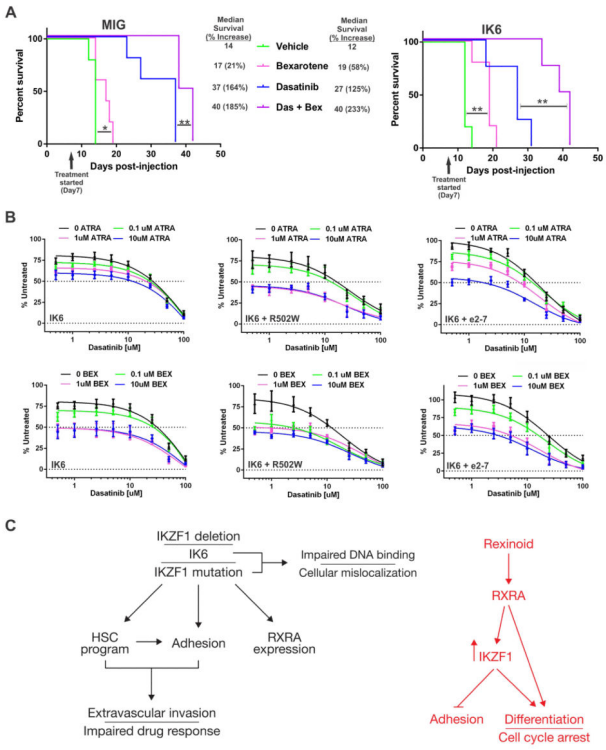

Figure 7. Retinoids potentiate TKI therapy in BCR-ABL1 leukemia

(A) WT C57Bl/6 mice were engrafted with *Arf*$^{-/-}$ BCR-ABL1 MIG-IK6 or MIG cells and randomized to vehicle, bex and/or dasatinib. On Kaplan-Meier analysis, bexarotene significantly increased survival time, with the greatest effect observed in mice treated with both drugs. **p<0.005, *p<0.05; n=5 mice per group. (B) Dose-response curves of human BCR-ABL1 *CDKN2A/B*-deleted (*i.e.*, INK4/ARF$^{-/-}$), *IKZF1* Δ4-7 (IK6), IK6 + R502W mutation, or IK6 + Δ2-7 leukemic cells harvested from NSG mice and immediately treated *ex vivo* with increasing concentrations of bexarotene (Bex), or all-*trans*-retinoic acid (ATRA) at increasing concentrations of dasatinib. Each retinoid inhibited cell proliferation, even at very low concentrations of dasatinib, and reduced the TKI IC_{50}. n=3 biological replicates performed in triplicate. (C) schematic summarizing effects of IKZF1 alterations and their reversal by retinoids.

See also Figure S5.

Table 1

Genetic Combination	Day Treatment Started	Median Survival (post-injection) Untreated	Median Survival (post-injection) Dasatinib
WT MIG	n/a	51	n/a
WT IK6	14	23	51.5
Ikzf$^{+/-}$ MIG	14	23	44
Ikzf$^{+/-}$ IK6	10	17	31.5
Arf$^{-/-}$ MIG	7	15	24
Arf$^{-/-}$ IK6	7	13	17