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BCL6 enables Ph+ acute lymphoblastic leukemia cells to survive *BCR-ABL1* kinase inhibition

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Abstract

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Contributions

C.D. and M.M. conceived the study and wrote the paper. M.M. and A.M. designed experiments and interpreted data. C.D., C.H., S. Shojaee, L.C., S. Swaminathan, L.K., S.-M.K, R.N., M.B., E.P. and Y.-M.K. designed and performed experiments and interpreted data. W.-K.H., H.P.K. and N.H. provided and characterized patient samples. H.G. and T.G.G. analyzed data. S.H., H.J., J.J.Y., H.W., B.H.Y. provided important reagents and mouse samples.

Competing financial interests

The authors declare no competing financial interests.

Tyrosine kinase inhibitors (TKI) are widely used to treat patients with leukemia driven by *BCR-ABL1*[1] and other oncogenic tyrosine kinases[2,3]. Recent efforts focused on the development of more potent TKI that also inhibit mutant tyrosine kinases[4,5]. However, even effective TKI typically fail to eradicate leukemia-initiating cells[6–8], which often cause recurrence of leukemia after initially successful treatment. Here we report on the discovery of a novel mechanism of drug-resistance, which is based on protective feedback signaling of leukemia cells in response to TKI-treatment. We identified BCL6 as a central component of this drug-resistance pathway and demonstrate that targeted inhibition of BCL6 leads to eradication of drug-resistant and leukemia-initiating subclones.

BCL6 is a known proto-oncogene that is often translocated in diffuse large B cell lymphoma (DLBCL)[9]. In response to TKI-treatment, *BCR-ABL1* acute lymphoblastic leukemia (ALL) cells upregulate BCL6 protein levels by ~90-fold, i.e. to similar levels as in DLBCL (Fig. 1a). Upregulation of BCL6 in response to TKI-treatment represents a novel defense mechanism, which enables leukemia cells to survive TKI-treatment: Previous work suggested that TKI-mediated cell death is largely p53-independent. Here we demonstrate that BCL6 upregulation upon TKI-treatment leads to transcriptional inactivation of the p53 pathway. BCL6-deficient leukemia cells fail to inactivate p53 and are particularly sensitive to TKI-treatment. $BCL6^{-/-}$ leukemia cells are poised to undergo cellular senescence and fail to initiate leukemia in serial transplant recipients. A combination of TKI-treatment and a novel BCL6 peptide inhibitor markedly increased survival of NOD/SCID mice xenografted with patient-derived *BCR-ABL1* ALL cells. We propose that dual targeting of oncogenic tyrosine kinases and BCL6-dependent feedback (Supplementary Fig. 1) represents a novel strategy to eradicate drug-resistant and leukemia-initiating subclones in tyrosine kinase-driven leukemia.

To elucidate mechanisms of TKI-resistance in tyrosine kinase-driven leukemia, we performed a gene expression analysis including our and published data of TKI-treated leukemia. We identified *BCL6* as a top-ranking gene in a set of recurrent gene expression changes[10], some of which are shared with MEK inhibition in $BRAF^{V600E}$ mutant solid tumor cells[11] (Supplementary Fig. 2–3). TKI-induced upregulation of BCL6 mRNA levels was confirmed in multiple leukemia subtypes carrying oncogenic tyrosine kinases (Supplementary Fig. 2). The BCR-ABL1 kinase, encoded by the Philadelphia chromosome (Ph), represents the most frequent genetic lesion in adult ALL, defines the subtype with a particularly poor prognosis[1,4,5] and was therefore chosen as focus for this study.

To elucidate the regulation of BCL6 in Ph^+ ALL, we investigated the JAK2/STAT5[12] and PI3K/AKT[13] pathways downstream of BCR-ABL1. We and others have shown that STAT5 suppresses BCL6 in B cells[14–16]. TKI-mediated upregulation of BCL6 was diminished by constitutively active STAT5(Fig. 1b) and deletion of *STAT5* was sufficient to upregulate BCL6, even in the absence of TKI-treatment (Fig. 1c). In agreement with previous work[16], overexpression of FoxO4 induced BCL6 (Fig. 1d). In Ph^+ ALL cells, FoxO factors are inactivated by PI3K/AKT[13] signaling, which is reversed by Pten (Supplementary Fig. 4). Deletion of *Pten*, hence, abrogated the ability of the leukemia cells to upregulate BCL6 in response to TKI-treatment (Fig. 1e).

In DLBCL, BCL6 is frequently translocated and suppresses p53-mediated apoptosis[9,17]. While TKI-treatment is less effective in $p53^{-/-}$ Ph^+ ALL[18], recent studies showed that TKI paradoxically prevent the upregulation of p53 in response to DNA damage in Ph^+ ALL and CML[19,20]. A comparative gene expression analysis of $BCL6^{-/-}$ and $BCL6^{+/+}$ ALL cells (Supplementary Fig. 5) identified Cdkn2a (Arf), Cdkn1a (p21), p53 and p53bp1 as potential BCL6 target genes (Supplementary Fig. 6). Arf and p53 protein levels were indeed unrestrained in $BCL6^{-/-}$ ALL (Fig. 2a). TKI-treatment of $BCL6^{+/+}$ ALL resulted in strong upregulation of BCL6 with low levels of p53, while $BCL6^{-/-}$ALL cells failed to curb p53 expression levels (Supplementary Fig. 7). Likewise, TKI-treatment increased excessively p53 levels when *Pten*-deficient ALL cells failed to upregulate BCL6 (Fig. 1e).

Identifying direct targets of BCL6 by chromatin immunoprecipitation in Ph^+ ALL (Supplementary Fig. 8–11), p53, p21 and p27 were among the genes with the strongest recruitment of BCL6 in TKI-treated ALL (Fig. 2b; Supplementary Fig. 9–11). Given that cell cycle-arrest and senescence-associated genes were among the BCL6 targets, we studied the cell cycle profile of leukemia cells. $BCL6^{-/-}$ ALL cells divided at a slightly reduced rate compared to $BCL6^{+/+}$ ALL cells (Fig. 2c). Treatment with adriamycin (0.05 μg/ml) had no significant effect on $BCL6^{+/+}$ ALL cells in a senescence-associated β-galactosidase assay[21–22] but revealed that the vast majority of $BCL6^{-/-}$ leukemia cells was poised to undergo cellular senescence (Fig. 2d). These findings demonstrate that even low levels of BCL6 in the absence of TKI-treatment are critical to downregulate Arf/p53.

Clonal evolution of leukemia involves acquisition of genetic lesions through DNA damage[23]. Interestingly, a CGH analysis revealed that genetic lesions were less frequent in BCL6 deficient ALL (Supplementary Fig. 12), suggesting that increased sensitivity to DNA damage limits clonal evolution in the absence of BCL6. Since Arf and p53 are critical negative regulators of self-renewal[24], we performed colony-forming assays. The colony frequencies of $BCL6^{-/-}$ ALL cells were reduced by ~20-fold compared to $BCL6^{+/+}$ ALL (Fig. 3a). To study self-renewal *in vivo*, we measured the ability of $BCL6^{+/+}$ and $BCL6^{-/-}$ ALL cells to initiate leukemia in transplant recipients (Fig. 3b). Using luciferase bioimaging, leukemia engraftment was observed in both groups after 8 days. $BCL6^{+/+}$ ALL cells rapidly expanded and initiated fatal leukemia, whereas $BCL6^{-/-}$ ALL cells failed to expand from the initial engraftment foci (Fig. 3c). Some mice that received $BCL6^{-/-}$ ALL cells ultimately succumbed to leukemia (Fig. 3b). Flow cytometry, however, revealed that the leukemias in the $BCL6^{-/-}$ group were in fact derived from endogenous $CD45.1^+$ cells of the irradiated recipients and not from the injected $CD45.2^+$ donor ALL cells (Supplementary Fig. 13; asterisks in Fig. 3b).

Defective leukemia-initiation may be a consequence of impaired homing to the bone marrow niche. Indeed, $BCL6^{-/-}$ ALL cells lack expression of CD44 (Supplementary Fig. 14), which is critical for homing of *BCR-ABL1* leukemia-initiating cells (LIC) to the bone marrow microenvironment[25]. Retroviral reconstitution of CD44 markedly increased homing of $BCL6^{-/-}$ ALL cells to the bone marrow niche, but failed to rescue defective leukemia-initiation (Supplementary Fig. 14).

Using intrafemoral injection to circumvent homing defects, a limiting dilution experiment (Fig. 3d) showed that 5 million $BCL6^{-/-}$ ALL cells compared to only 10^3 $BCL6^{+/+}$ ALL cells were needed to initiate fatal leukemia. These findings suggest that the frequency of LIC in $BCL6^{-/-}$ ALL (<1 in 100,000) is reduced by >100-fold compared to $BCL6^{+/+}$ ALL (≥1 in 1,000). An alternative interpretation would be that LIC occur at a similar frequency in $BCL6^{-/-}$ ALL but with reduced self-renewal activity. To address potential "exhaustion" of LIC, we performed a serial transplantation with ALL cells that gave rise to disease in primary recipients after injection of 5 million ALL cells. From the bone marrow, we isolated $CD19^+$ ALL cells for secondary intrafemoral injection. $BCL6^{-/-}$ leukemia was not transplantable in secondary recipients (Supplementary Fig. 15). While these findings do not rule out the possibility that the LIC frequencies are reduced in $BCL6^{-/-}$ ALL, they support the notion of LIC "exhaustion" after secondary transplantation.

To explore the therapeutic usefulness of pharmacological inhibition of BCL6, we tested a BCL6 inhibitor (RI-BPI), which blocks the repressor activity of BCL6[26]. Gene expression analysis confirmed that RI-BPI is a selective and potent inhibitor of BCL6 (Supplementary Fig. 16). We investigated the effect of RI-BPI on the self-renewal capacity of primary Ph^+ ALL and the initiation of leukemia in a mouse xenograft model. Treatment with RI-BPI resulted in a reduction of colony formation and delayed progression of leukemia. Likewise, treatment of Ph^+ ALL with RI-BPI induced cellular senescence (Supplementary Fig. 17).

We next examined how gene dosage of BCL6 affects responses to TKI. For instance, *Pten*$^{-/-}$ ALL cells lack the ability to upregulate the p53-repressor BCL6 and are more sensitive to Imatinib (Fig. 1e; Supplementary Fig. 18). Dose-response studies in $BCL6^{+/+}$, $BCL6^{+/-}$ and $BCL6^{-/-}$ ALL (Fig. 4a) showed that Imatinib-sensitivity was significantly increased in $BCL6^{-/-}$ (EC_{50}: 0.17 μmol/l) and even in $BCL6^{+/-}$ ALL cells (EC_{50}: 0.67 μmol/l) compared to $BCL6^{+/+}$ ALL cells (EC_{50}: 1.10 μmol/l; Fig. 4a). These findings indicate that maximum levels of BCL6 expression are required to prevent TKI-induced cell death. Indeed, inducible activation of BCL6-ER^{T2} constructs[27] in $BCL6^{-/-}$ ALL cells conferred a strong survival advantage in the presence of Imatinib (Fig. 4b). Activation of BCL6 in $BCL6^{+/+}$ ALL cells induced cell cycle exit (not shown) and no additional survival advantage, because these cells already achieved maximal upregulation of endogenous BCL6 (Fig. 1a).

To address the role of BCL6-mediated repression of p53 in TKI-resistance, $p53^{-/-}$ and $p53^{+/+}$ ALL cells were treated with RI-BPI. The synergistic effect between TKI-treatment and RI-BPI is indeed partially p53-dependent (Supplementary Fig. 19). In $p53^{-/-}$ ALL cells, the effect of RI-BPI was significantly diminished compared to $p53^{+/+}$ ALL.

To confirm that BCL6 has a similar function in patient-derived Ph^+ ALL, primary ALL cells were transduced with a dominant-negative BCL6 mutant (DN-BCL6-ER^{T2})[27], which resulted in a marked competitive disadvantage of Ph^+ ALL cells, that was further enhanced by Imatinib treatment (Fig. 4c). Similar observations in mouse ALL and in an established Ph^+ ALL cell line demonstrate that BCL6 promotes survival of TKI-treated Ph^+ ALL (Supplementary Fig. 20).

To test the effect of BCL6 inhibition on TKI-resistance, we cultured 4 primary Ph^+ ALL in the presence or absence of Imatinib, RI-BPI or a combination of both (Supplementary Fig. 21). Initially, all 4 Ph^+ ALL cases responded to Imatinib treatment, but subsequently rebounded and were no longer sensitive to Imatinib (10 μmol/l). RI-BPI alone showed only slight effects, while the combination of RI-BPI and Imatinib rapidly induced cell death and effectively prevented a rebound in all 4 cases (Supplementary Fig. 21). These findings suggest that prolonged treatment with a combination of Imatinib/RI-BPI prevents acquisition of TKI-resistance. We next examined the effect of Imatinib/RI-BPI combinations on primary TKI-resistance in Ph^+ ALL. To this end, 4 human Ph^+ ALL cell lines that lack *BCR-ABL1* kinase mutations (Supplementary Table 1) but are highly refractory to Imatinib (10 μmol/l) were treated with or without Imatinib, RI-BPI or a combination of both. Imatinib alone did not achieve a therapeutic response, whereas the combination with RI-BPI potentiated the effect of Imatinib on the refractory ALL cells (Supplementary Fig. 22).

To study the efficacy of combined tyrosine kinase and BCL6 inhibition *in vivo*, primary Ph^+ ALL cells were labeled with luciferase and xenografted into mice. Recipient mice were treated with either vehicle, Nilotinib or a combination of Nilotinib and RI-BPI. Nilotinib is more potent than Imatinib, which only has marginal effects in mice[28],[29]. Bioimaging demonstrated that 7–10 injections of RI-BPI significantly enhanced the effect of Nilotinib (Fig. 4d–e and Supplementary Fig. 23). While all mice treated with Nilotinib alone succumbed to leukemia within 99 days after injection, 7 of 8 mice treated with RI-BPI/Nilotinib combination were still alive after 140 days (Fig. 4d–e). Also in a model for full-blown mouse leukemia, TKI/RI-BPI combinations proved effective and significantly prolonged survival (Supplementary Fig. 24). Establishing a potential therapeutic window of Nilotinib/RI-BPI combinations, we found no evidence of relevant toxicity (Supplementary Fig. 25–26; Supplementary Table 2).

Although transcription factors have been considered intractable therapeutic targets, the recent development of a small molecule inhibitor against BCL6[30] holds promise to effectively target TKI-resistance in patients with Ph^+ ALL. Since TKI-resistance develops in virtually all cases of Ph^+ ALL, it appears particularly important to target this novel pathway of TKI-resistance.

Methods summary

Cell culture

Primary leukemia cells (Supplementary Table 1) were cultured on OP9 stroma cells in Alpha MEM without ribonucleotides and deoxyribonucleotides, supplemented with 20% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Human ALL cell lines were maintained in RPMI with GlutaMAX containing 20% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Mouse *BCR-ABL1*-transformed ALL cells were maintained in IMDM with GlutaMAX containing 20% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol. Cell cultures were kept at 37°C in a humidified incubator under a 5% CO_2 atmosphere.

BCR-ABL1 transformation

Transfection of a MSCV-based retroviral vector encoding BCR-ABL1 was performed using Lipofectamine 2000. Retroviral supernatant was produced by co-transfecting 293FT cells with the plasmids pHIT60 and pHIT123. Virus supernatant were harvested, filtered through a 0.45 μm filter and loaded by centrifugation (2,000 × g, 90 min at 32°C) on 50μg/ml RetroNectin coated non-tissue well plates. Extracted bone marrow cells from mice were transduced by *BCR-ABL1* in the presence of 10 ng/ml recombinant murine Interleukin-7 (IL-7) in RetroNectin-coated petri dishes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

4-OHT	4-hydroxytamoxifen
ALL	acute lymphoblastic leukemia
ChIP	chromatin immunoprecipitation
CML	chronic myelogenous leukemia
DLBCL	diffuse large B cell lymphoma
GC	germinal center
Ig	immunoglobulin
IL	Interleukin
LIC	leukemia-initiating cells
n	denotes the number of independent experiments
Ph	Philadelphia chromosome
RI-BPI	*retro-inverso* BCL6 peptide-inhibitor

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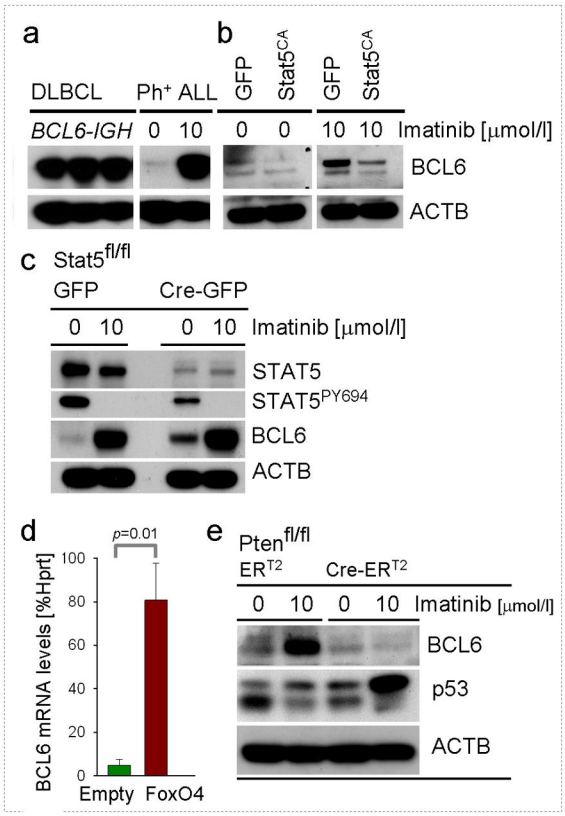

Figure 1. Regulation of BCL6 expression in BCR-ABL1 ALL cells

a, Ph^+ ALL cells were treated with and without Imatinib (10 μmol/l) for 24 hours. Upregulation of BCL6 was compared to expression levels in DLBCL with *BCL6-IGH* translocation by Western blot. **b**, *BCR-ABL1*-transformed mouse ALL cells were transduced with a constitutively active Stat5 mutant (STAT5-CA) or a control vector (GFP) and treated either with or without Imatinib. BCL6 Western blot was performed using β-actin as loading control. **c**, BCL6 expression upon Imatinib-treatment was studied by Western blot in the presence or absence of Cre-mediated deletion of *Stat5* in *BCR-ABL1*-transformed $Stat5^{fl/fl}$ mouse ALL. **d**, Mouse *BCR-ABL1* ALL cells were transduced with FoxO4-Puromycin or a Puromycin control vector and subjected to antibiotic selection. Cells were harvested and BCL6 mRNA levels were measured by qRT-PCR relative to Hprt. **e**, Imatinib-induced BCL6 expression was studied by Western blot in the presence or absence of Cre-mediated deletion of *Pten* in *BCR-ABL1*-transformed $Pten^{fl/fl}$ mouse ALL cells.

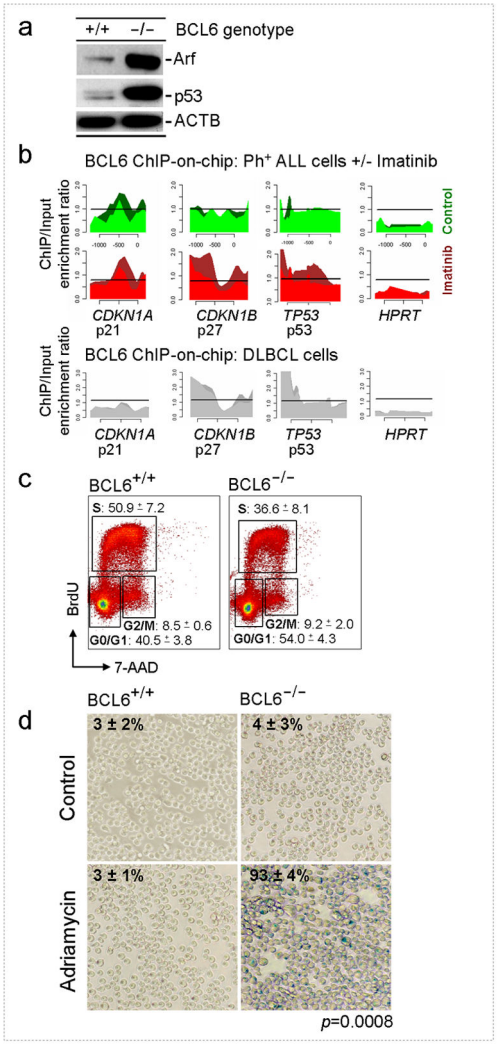

Figure 2. BCL6 is required for transcriptional inactivation of the Arf/p53 pathway in BCR-ABL1 ALL

a, Western blot analysis of CDKN2A (Arf) and p53 expression in $BCL6^{-/-}$ and $BCL6^{+/+}$ *BCR-ABL1* ALL cells. **b,** Human Ph^+ ALL cells (Tom1) were treated with and without Imatinib (10 μmol/l) for 24 hours and were subjected to ChIP-on-chip analysis using a BCL6-specific antibody. The y-axis indicates enrichment versus input and the x-axis the location of probes within the respective loci relative to the transcriptional start site. The dark and light green (Control) or red (Imatinib) tracings depict two replicates. Recruitment to *CDKN1A*, *CDKN1B*, *TP53* and *HPRT* (negative control) is shown in Ph^+ ALL cells and one DLBCL cell line (OCI-Ly7). **c**, Cell cycle analysis (BrdU/7-AAD staining). **d**, Staining for senescence-associated β-galactosidase (SA-β-gal). ALL cells were treated with or without 0.05 μg/ml adriamycin for 48 hours to induce a low level of DNA damage. Percentages of SA-β-gal^+ cells are indicated (means ± SD; n=3).

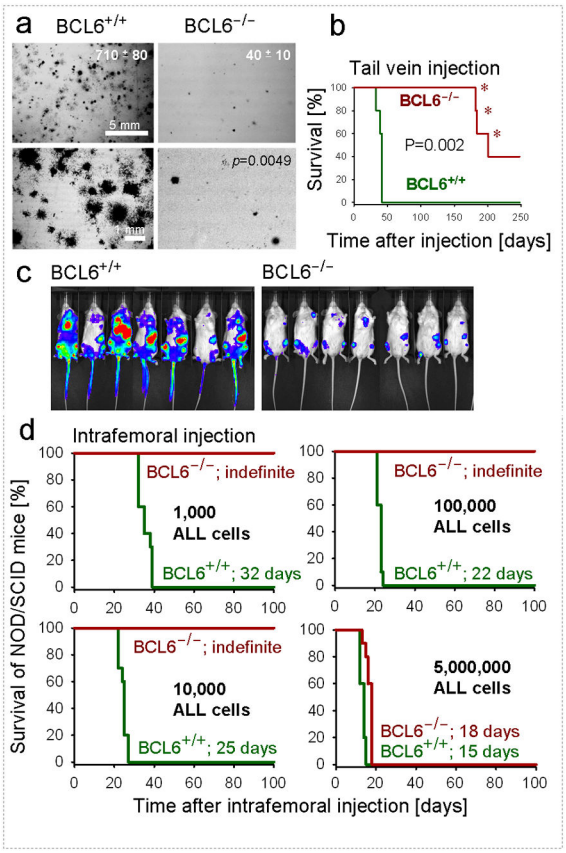

Figure 3. BCL6 is required for leukemia-initiation in BCR-ABL1 ALL

a, 10,000 BCL6$^{-/-}$ or BCL6$^{+/+}$ *BCR-ABL1* ALL cells were plated in semisolid agar, and colonies were counted after ten days (numbers denote means ± SD, n=3). **b**, Overall survival of mice injected with 100,000 BCL6$^{-/-}$ and BCL6$^{+/+}$ *BCR-ABL1* ALL cells was compared by Kaplan-Meier analysis. **c**, For a SCID leukemia-initiating cell (SL-IC) experiment, BCL6$^{-/-}$ and BCL6$^{+/+}$ *BCR-ABL1* ALL cells were labeled with firefly luciferase and were intravenously injected into sublethally irradiated NOD/SCID mice. Mice that developed CD45.1^{+} endogenous leukemia instead of leukemia from injected CD45.2^{+} cells are indicated by asterisks (see Supplementary Fig. 13). **d**, The SL-IC assay was repeated as a limiting dilution experiment (10^3, 10^4, 10^5, 5 million cells) and leukemia cells were directly injected into the femoral bone marrow to circumvent potential engraftment defects.

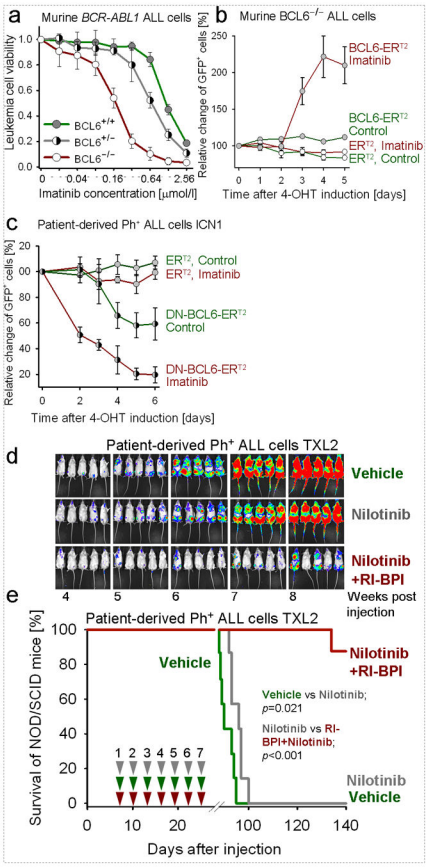

Figure 4. BCL6 promotes survival of TKI-treated BCR-ABL1 ALL cells

a, Imatinib-sensitivity of BCL6$^{-/-}$, BCL6$^{+/-}$ and BCL6$^{+/+}$ ALL cells was measured in a Resazurin viability assay. **b**, BCL6$^{-/-}$ ALL cells were transduced with BCL6-ER^{T2} or ER^{T2} vectors (tagged with GFP). ALL cells were treated with or without 1 μmol/l Imatinib, and BCL6-ER^{T2} or ER^{T2} were induced by 4-OHT. Relative changes of GFP^{+} cells after induction are indicated. **c**, Patient-derived Ph^{+} ALL cells (ICN1) were transduced with inducible dominant-negative BCL6 (DN-BCL6-ER^{T2}) or ER^{T2} control vectors. ALL cells were treated with or without 10 μmol/l Imatinib and DN-BCL6-ER^{T2} or ER^{T2} were induced by 4-OHT. Relative changes of GFP^{+} cells after induction are indicated. **d**, Patient-derived Ph^{+} ALL cells (TXL2) were labeled with luciferase and 100,000 cells were injected. Mice were treated 7 times with either vehicle (gray), Nilotinib (25 mg/kg; green) or a combination of Nilotinib and RI-BPI (25 mg/kg; red). Treated mice are shown in **e**, a Kaplan-Meier survival analysis. Treatment days are indicated by arrow heads.