LETTER

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# *BCL6* enables Ph$^+$ acute lymphoblastic leukaemia cells to survive *BCR–ABL1* kinase inhibition

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**Tyrosine kinase inhibitors (TKIs) are widely used to treat patients with leukaemia driven by *BCR–ABL1* (ref. 1) and other oncogenic tyrosine kinases[2,3]. Recent efforts have focused on developing more potent TKIs that also inhibit mutant tyrosine kinases[4,5]. However, even effective TKIs typically fail to eradicate leukaemia-initiating cells (LICs)[6–8], which often cause recurrence of leukaemia after initially successful treatment. Here we report the discovery of a novel mechanism of drug resistance, which is based on protective feedback signalling of leukaemia cells in response to treatment with TKI. We identify BCL6 as a central component of this drug-resistance pathway and demonstrate that targeted inhibition of BCL6 leads to eradication of drug-resistant and leukaemia-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 leukaemia (ALL) cells upregulate BCL6 protein levels by approximately 90-fold: that is, to similar levels as in DLBCL (Fig. 1a). Upregulation of BCL6 in response to TKI treatment represents a novel defence mechanism, which enables leukaemia 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 leukaemia cells fail to inactivate p53 and are particularly sensitive to TKI treatment. BCL6$^{-/-}$ leukaemia cells are poised to undergo cellular senescence and fail to initiate leukaemia 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 leukaemia-initiating subclones in tyrosine-kinase-driven leukaemia.

To elucidate mechanisms of TKI resistance in tyrosine-kinase-driven leukaemia, we performed a gene expression analysis including our and published data of TKI-treated leukaemia. We identified *BCL6* as a top-ranking gene in a set of recurrent gene expression changes, some of which are shared with mitogen-activated protein-kinase kinase (MEK) inhibition in BRAF$^{V600E}$ mutant solid tumour cells[10] (Supplementary Figs 2 and 3). TKI-induced upregulation of BCL6 messenger RNA (mRNA) levels was confirmed in multiple leukaemia 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 (ref. 11) and PI3K/AKT[12] pathways downstream of BCR–ABL1. We and others have shown that STAT5 suppresses BCL6 in B cells[13–15]. 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[15], overexpression of FoxO4 induced BCL6 (Fig. 1d). In Ph$^+$ ALL cells, FoxO factors are inactivated by PI3K/AKT[12] signalling, which is reversed by Pten (Supplementary Fig. 4). Deletion of *Pten*, hence, abrogated the ability of the leukaemia cells to upregulate BCL6 in response to TKI treatment (Fig. 1e).

In DLBCL, BCL6 is frequently translocated and suppresses p53-mediated apoptosis[9,16]. Although TKI treatment is less effective in p53$^{-/-}$ Ph$^+$ ALL[17], recent studies showed that TKI paradoxically prevents the upregulation of p53 in response to DNA damage in Ph$^+$ ALL and chronic myelogenous leukaemia[18,19]. 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, whereas 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 (ChIP) in Ph$^+$ ALL (Supplementary Figs 8–11), p53, p21 and p27 were among the genes with the strongest recruitment of BCL6 in TKI-treated ALL (Fig. 2b and Supplementary Figs 9–11). Given that cell-cycle arrest and senescence-associated genes were among the BCL6 targets, we studied the cell-cycle profile of leukaemia cells. BCL6$^{-/-}$ ALL cells divided at a slightly reduced rate compared with BCL6$^{+/+}$ ALL cells (Fig. 2c). Treatment with adriamycin (0.05 μg ml$^{-1}$) had no significant effect on BCL6$^{+/+}$ ALL cells in a senescence-associated β-galactosidase assay[20,21] but revealed that most BCL6$^{-/-}$ leukaemia cells were 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 leukaemia involves acquisition of genetic lesions through DNA damage[22]. Interestingly, a comparative genomic hybridization 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. Because Arf and p53 are critical negative regulators of self-renewal[23], we performed colony-forming assays. The colony frequencies of

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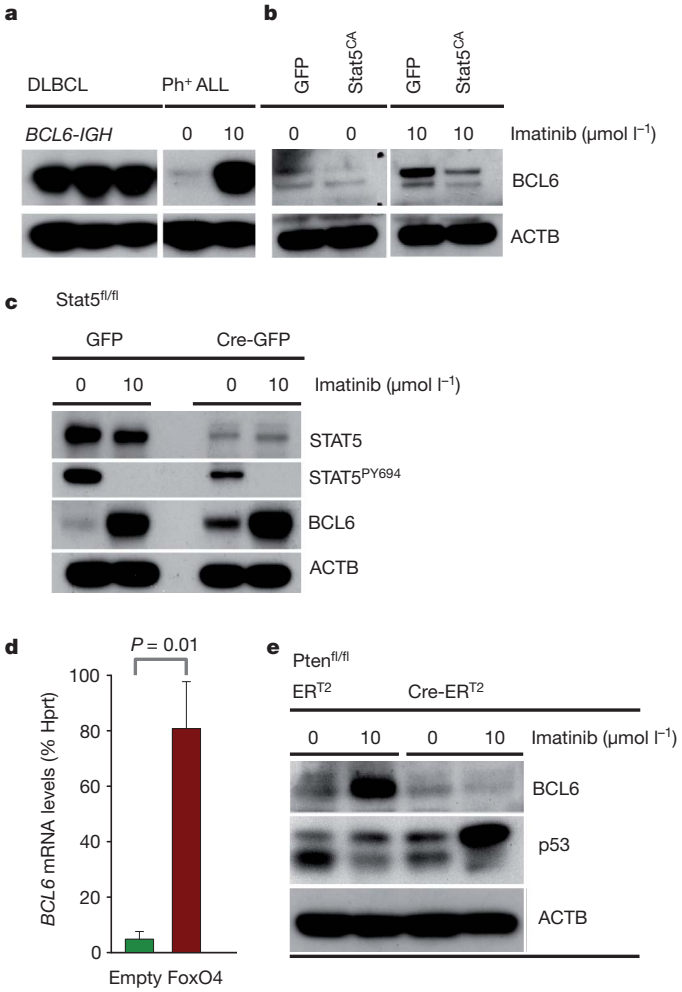


**Figure 1 | Regulation of BCL6 expression in BCR–ABL1 ALL cells.** **a**, $Ph^+$ ALL cells were treated with and without imatinib (10 μmol $l^{-1}$) for 24 h. Upregulation of BCL6 was compared with expression levels in DLBCL by western blot. **b**, *BCR–ABL1*-transformed mouse ALL cells were transduced with a constitutively active Stat5 mutant ($STAT5^{CA}$) or a control vector (green fluorescent protein, 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 collected 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.

$BCL6^{-/-}$ ALL cells were reduced by approximately 20-fold compared with $BCL6^{+/+}$ ALL (Fig. 3a). To study self-renewal *in vivo*, we measured the ability of $BCL6^{+/+}$ and $BCL6^{-/-}$ ALL cells to initiate leukaemia in transplant recipients (Fig. 3b). Using luciferase bioimaging, leukaemia engraftment was observed in both groups after 8 days. $BCL6^{+/+}$ ALL cells rapidly expanded and initiated fatal leukaemia, whereas $BCL6^{-/-}$ ALL cells failed to expand from the initial engraftment foci (Fig. 3c). Some mice that received $BCL6^{-/-}$ ALL cells ultimately succumbed to leukaemia (Fig. 3b). Flow cytometry, however, revealed that the leukaemias 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 and asterisks in Fig. 3b).

Defective leukaemia 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* LICs to the bone marrow microenvironment[24]. Retroviral reconstitution of CD44 markedly increased homing of $BCL6^{-/-}$ ALL cells to the bone marrow niche, but failed to rescue defective leukaemia 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 with only $10^3$ $BCL6^{+/+}$ ALL cells were needed to initiate fatal leukaemia. These findings suggest that the frequency of LIC in $BCL6^{-/-}$ ALL (fewer than 1 in 100,000) is reduced by more than 100-fold compared with $BCL6^{+/+}$ ALL (at least 1 in 1,000). An alternative interpretation would be that LICs occur at a similar frequency in $BCL6^{-/-}$ ALL but with reduced self-renewal activity. To address potential 'exhaustion' of LICs, 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. $BCL6^{-/-}$ leukaemia was not transplantable in secondary recipients (Supplementary Fig. 15). Although these findings do not exclude 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 (*retro-inverso* BCL6 peptide-inhibitor (RI-BPI)), which blocks the repressor activity of BCL6 (ref. 25). 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 leukaemia in a mouse xenograft model. Treatment with RI-BPI resulted in a reduction of colony formation and delayed progression of leukaemia. 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 and Supplementary Fig. 18). Dose–response studies in $BCL6^{+/+}$, $BCL6^{+/-}$ and $BCL6^{-/-}$ ALL (Fig. 4a) showed that sensitivity to imatinib was significantly increased in $BCL6^{-/-}$ (half maximal effective concentration ($EC_{50}$) 0.17 μmol $l^{-1}$) and even in $BCL6^{+/-}$ ALL cells ($EC_{50}$ 0.67 μmol $l^{-1}$) compared with $BCL6^{+/+}$ ALL cells ($EC_{50}$ 1.10 μmol $l^{-1}$; 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[26] 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 partly p53 dependent (Supplementary Fig. 19). In $p53^{-/-}$ ALL cells, the effect of RI-BPI was significantly diminished compared with $p53^{+/+}$ 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-$ER^{T2}$)[26], 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 demonstrated that BCL6 promotes survival of TKI-treated $Ph^+$ ALL (Supplementary Fig. 20).

To test the effect of BCL6 inhibition on TKI resistance, we cultured four primary $Ph^+$ ALL in the presence or absence of imatinib, RI-BPI or a combination of both (Supplementary Fig. 21). Initially, all four $Ph^+$ ALL cases responded to imatinib treatment, but subsequently rebounded and were no longer sensitive to imatinib (10 μmol $l^{-1}$). RI-BPI alone showed only slight effects, whereas the combination of RI-BPI and imatinib rapidly induced cell death and effectively prevented a rebound in all four cases (Supplementary Fig. 21). These

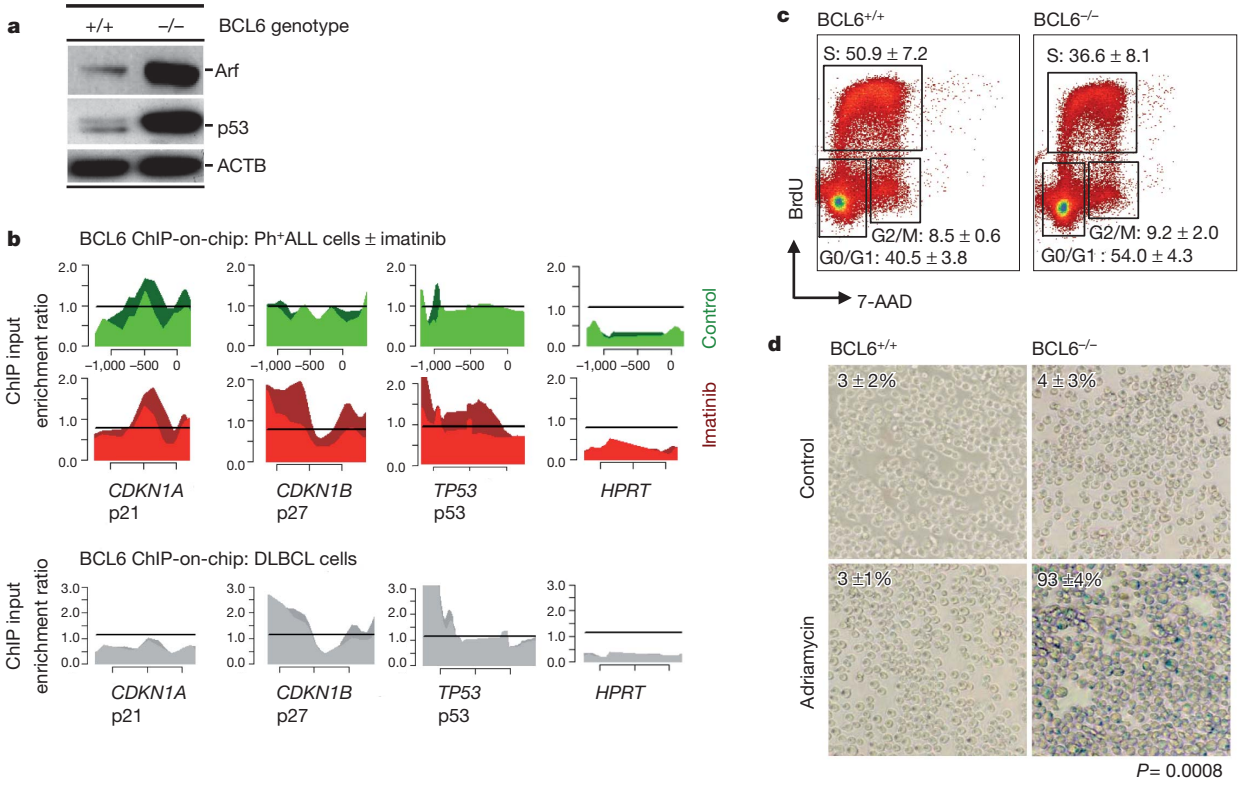


**Figure 2 | BCL6 is required for transcriptional inactivation of the Arf/p53 pathway in BCR–ABL1 ALL.** **a**, Western blot analysis of CDKN2A (Arf) and p53 expression in BCL6$^{-/-}$ and BCL6$^{+/+}$ *BCR–ABL1* ALL cells. **b**, Human Ph$^{+}$ ALL cells (Tom1) were treated with and without imatinib (10 μmol l$^{-1}$) for 24 h and were subjected to ChIP-on-chip analysis using a BCL6-specific antibody. The *y* axis indicates enrichment versus input, 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$^{-1}$ adriamycin for 48 h to induce a low level of DNA damage. Percentages of SA-β-gal$^{+}$ cells are indicated (means ± SD; $n = 3$).

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, four human Ph$^{+}$ ALL cell lines that lacked *BCR–ABL1* kinase mutations (Supplementary Table 1) but which were highly refractory to imatinib (10 μmol l$^{-1}$) 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).

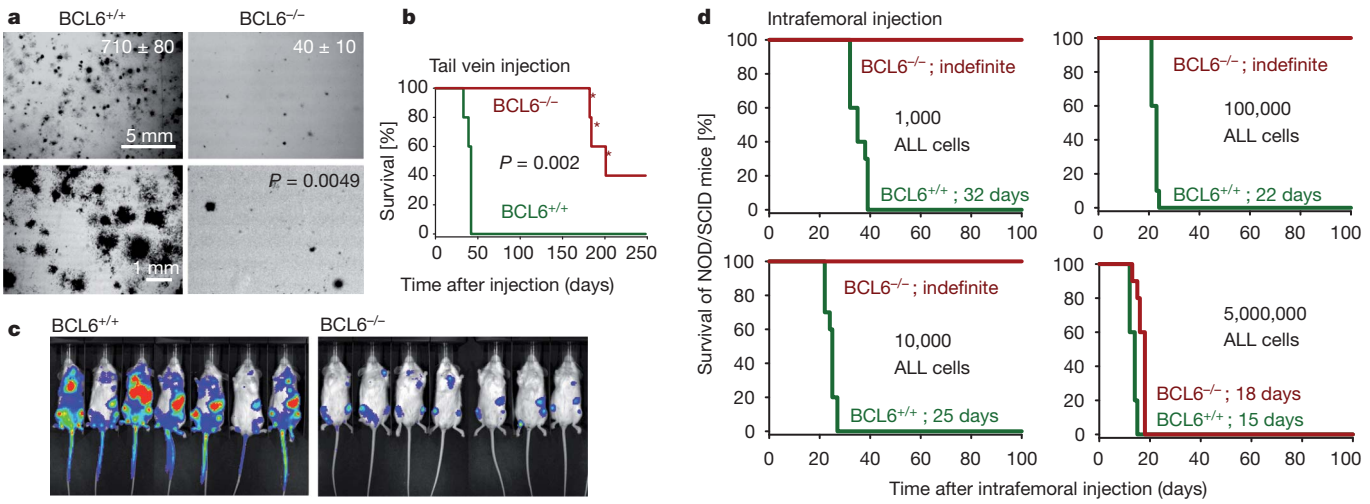


**Figure 3 | BCL6 is required for leukaemia initiation in BCR–ABL1 ALL.** **a**, Ten thousand BCL6$^{-/-}$ or BCL6$^{+/+}$ *BCR–ABL1* ALL cells were plated in semisolid agar, and colonies were counted after 10 days (numbers denote means ± SD, $n = 3$). **b**, Overall survival of mice injected with 100,000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

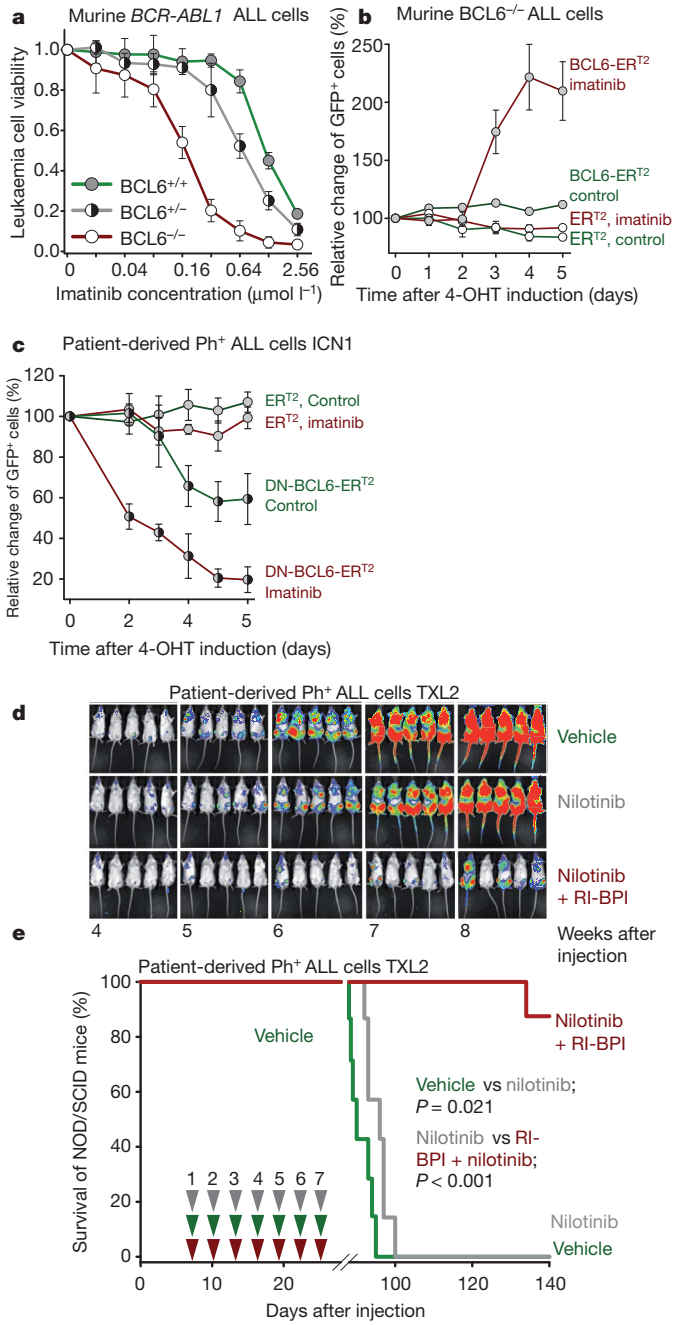


**Figure 4 | BCL6 promotes survival of TKI-treated BCR–ABL1 ALL cells.** **a**, Imatinib sensitivity of BCL6$^{-/-}$, BCL6$^{+/-}$ and BCL6$^{+/+}$ ALL cells was measured in a resazurin viability assay. **b**, BCL6$^{-/-}$ ALL cells were transduced with BCL6-ER$^{T2}$ or ER$^{T2}$ vectors (tagged with GFP). ALL cells were treated with or without 1 μmol l$^{-1}$ imatinib, and BCL6-ER$^{T2}$ or ER$^{T2}$ were induced by 4-hydroxytamoxifen. 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$^{-1}$ imatinib and DN-BCL6-ER$^{T2}$ or ER$^{T2}$ were induced by 4-hydroxytamoxifen. Relative changes of GFP$^{+}$ cells after induction are indicated. **d**, Patient-derived Ph$^{+}$ ALL cells (TXL2) were labelled with luciferase and 100,000 cells were injected. Mice were treated seven times with either vehicle (green), nilotinib (25 mg kg$^{-1}$; grey) or a combination of nilotinib and RI-BPI (25 mg kg$^{-1}$; red). Treated mice are shown in **e**, a Kaplan–Meier survival analysis. Treatment days are indicated by arrowheads.

To study the efficacy of combined tyrosine kinase and BCL6 inhibition *in vivo*, primary Ph$^{+}$ ALL cells were labelled 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[27,28]. Bioimaging demonstrated that seven to ten injections of RI-BPI significantly enhanced the effect of nilotinib (Fig. 4d, e and Supplementary Fig. 23). Whereas all mice treated with nilotinib alone succumbed to leukaemia within 99 days after injection, seven of eight mice treated with RI-BPI/nilotinib combination were still alive after 140 days (Fig. 4d, e). Also, in a model for full-blown mouse leukaemia, 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 Figs 25 and 26 and Supplementary Table 2).

Although transcription factors have been considered intractable therapeutic targets, the recent development of a small molecule inhibitor against BCL6 (ref. 29) holds promise for effectively targeting TKI-resistance in patients with Ph$^{+}$ ALL. Because 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 leukaemia cells (Supplementary Table 1) were cultured on OP9 stroma cells in alpha minimum essential medium without ribonucleotides and deoxyribonucleotides, supplemented with 20% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin. Human ALL cell lines were maintained in RPMI with GlutaMAX containing 20% FBS, 100 IU ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin. Mouse *BCR–ABL1*-transformed ALL cells were maintained in IMDM with GlutaMAX containing 20% FBS, 100 IU ml$^{-1}$ penicillin, 100 μg ml$^{-1}$ 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 murine stem cell virus (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 was collected, filtered through a 0.45 μm filter and loaded by centrifugation (2,000*g*, 90 min at 32 °C) on 50 μg ml$^{-1}$ RetroNectin-coated non-tissue well plates. Extracted bone marrow cells from mice were transduced by *BCR–ABL1* in the presence of 10 ng ml$^{-1}$ recombinant murine interleukin-7 in RetroNectin-coated Petri dishes.

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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**Author 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 samples from patients. H.G. and T.G.G. analysed data. S.H., H.J., J.J.Y., H.W. and B.H.Y. provided important reagents and mouse samples.

**Author Information** The gene expression and ChIP data are deposited in NCBI's Gene Expression Omnibus under accession numbers GSE23743, GSE24426, GSE15179, GSE11794, GSE10086, GSE20987 and GSE24400. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to M.M. (markus.muschen@ucsf.edu).

## METHODS

**Patient samples, human cells and cell lines.** Patient samples (Supplementary Table 1) were provided from the departments of Hematology and Oncology, University Hospital Benjamin Franklin, Berlin, Germany (W.-K.H.) and the USC Norris Comprehensive Cancer Center in compliance with Institutional Review Board regulations (approval from the Ethik-Kommission of the Charité, Campus Benjamin Franklin and the IRB of the University of Southern California Health Sciences Campus). Leukaemia cells from bone marrow biopsy of patients with $Ph^+$ ALL were xenografted into sublethally irradiated NOD/SCID mice by tail vein injection. After passaging, leukaemia cells were collected and cultured on OP9 stroma cells in alpha minimum essential medium (Alpha-MEM, Invitrogen) without ribonucleotides and deoxyribonucleotides, supplemented with 20% fetal bovine serum, 2 mmol $l^{-1}$ L-glutamine, 1 mmol $l^{-1}$ sodium pyruvate, 100 IU $ml^{-1}$ penicillin and 100 μg $ml^{-1}$ streptomycin. The human ALL cell lines BV173, NALM-1, SUP-B15 and TOM1 (obtained from Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ)) were maintained in Roswell Park Memorial Institute medium (RPMI-1640, Invitrogen) with GlutaMAX containing 20% fetal bovine serum, 100 IU $ml^{-1}$ penicillin and 100 μg $ml^{-1}$ streptomycin.

**Retroviral constructs and transduction.** Transfection of retroviral constructs encoding BCR–ABL1-IRES-GFP[30], BCR–ABL1-IRES-Neo, STAT5-CA[31], CD44S-Puro[32], FoxO4-Puro, BCL6-$ER^{T2}$-GFP[26], $ER^{T2}$-GFP, DN-BCL6-$ER^{T2}$-GFP, Cre-$ER^{T2}$-Puro[33], Cre-IRES-GFP, Puro-, Neo- and GFP-empty vector controls were performed using Lipofectamine 2000 (Invitrogen) with Opti-MEM media (Invitrogen). Retroviral supernatant was produced by co-transfecting HEK 293FT cells with the plasmids pHIT60 (ref. 34) (gag-pol) and pHIT123 (ecotropic env) or pHIT456 (amphotropic env). 293FT cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with GlutaMAX containing 10% fetal bovine serum, 100 IU $ml^{-1}$ penicillin, 100 μg $ml^{-1}$ streptomycin, 25 mmol $l^{-1}$ HEPES, 1 mmol $l^{-1}$ sodium pyruvate and 0.1 mmol $l^{-1}$ non-essential amino acids. Regular media were replaced after 16 h by growth media containing 10 mmol $l^{-1}$ sodium butyrate. After incubation for 8 h, the media were changed back to regular growth media. Twenty-four hours later, the virus supernatant was collected, filtered through a 0.45 μm filter and loaded by centrifugation (2,000*g*, 90 min at 32 °C) two times on 50 μg $ml^{-1}$ RetroNectin- (Takara) coated non-tissue six-well plates. Two million to three million cells were transduced per well by centrifugation at 500*g* for 30 min and maintained for 48 h at 37 °C with 5% $CO_2$ before transferring into culture flasks. Transduced cells with oestrogen receptor fusion proteins were induced with 4-hydroxytamoxifen (500 nM).

***In vivo* model for BCR–ABL1-transformed ALL and bioluminescence imaging.** After cytokine-independent proliferation, BCR–ABL1-transformed ALL cells were labelled with a lentiviral vector encoding firefly luciferase with a neomycin selection marker. After selection with 0.5–2 mg $ml^{-1}$ G418 for 10 days, luciferase-labelled ALL cells were injected into sublethally irradiated (250 cGy) NOD/SCID mice. Human primary leukaemia cells were transduced with a lentiviral firefly luciferase carrying a GFP marker. After expansion of sorted $GFP^+$ cells, $1 \times 10^5$ cells were injected through the tail vein into sublethally irradiated NOD/SCID mice. Bioimaging of leukaemia progression in mice was performed at different time points using an *in vivo* IVIS 100 bioluminescence/optical imaging system (Xenogen). D-Luciferin (Promega) dissolved in PBS was injected intraperitoneally at a dose of 2.5 mg per mouse 15 min before measuring the luminescence signal. General anaesthesia was induced with 5% isoflurane and continued during the procedure with 2% isoflurane introduced through a nose cone. All mouse experiments were subject to institutional approval by the Children's Hospital Los Angeles Institutional Animal Care and Use Committee.

**Extraction of bone marrow cells from mice.** To avoid inflammation-related effects in $BCL6^{-/-}$ mice[35], bone marrow cells were extracted from young age-matched $BCL6^{+/+}$ and $BCL6^{-/-}$ mice (younger than 6 weeks of age) without signs of inflammation. Bone marrow cells were obtained by flushing cavities of femur and tibia with PBS. After filtration through a 70 μm filter and depletion of erythrocytes using a lysis buffer (BD PharmLyse, BD Biosciences), washed cells were either frozen for storage or subjected to further experiments.

**$BCL6^{-/-}$, $Stat5^{fl/fl}$, $Pten^{fl/fl}$ and $p53^{-/-}$ mice.** A summary of mouse strains used in this study is provided in Supplementary Table 3. Bone marrow cells from *$BCL6^{-/-}$* (generated in R. Dalla-Favera's laboratory)[36], *$Stat5^{fl/fl}$* (generated in L. Henninghausen's laboratory)[37], *$Pten^{fl/fl}$* (generated in H. Wu's laboratory)[38] and *$p53^{-/-}$* (obtained from Jackson Laboratory) mice were collected and retrovirally transformed by *BCR–ABL1* (ref. 30) in the presence of 10 ng interleukin-7 per milliliter (Peprotech) in RetroNectin- (Takara) coated Petri dishes as described below. All *BCR–ABL1*-transformed ALL cells derived from bone marrow of mice were maintained in Iscove's modified Dulbecco's medium (IMDM, Invitrogen) with GlutaMAX containing 20% fetal bovine serum, 100 IU $ml^{-1}$ penicillin, 100 μg $ml^{-1}$ streptomycin and 50 μM 2-mercaptoethanol. *BCR–ABL1*-transformed ALL cells were propagated only for short periods of time and usually not longer than for 2 months to avoid acquisition of additional genetic lesions during long-term cell culture.

**RI-BPI.** Homo-dimerization of the amino (N)-terminal Broad Complex, Tramtrack, Bric à brac (BTB) domain of BCL6 forms a lateral groove motif, which is required to recruit co-repressor proteins such as BCL6 co-repressor (BCoR), nuclear receptor co-repressor (N-CoR) and silencing mediator of retinoid and thyroid receptors (SMRT). BCoR, NCoR and SMRT interact in a mutually exclusive manner with an 18-amino-acid motif in the lateral groove of the BCL6 BTB domain to form a BCL6 repression complex[39,40]. A recombinant peptide containing the SMRT BBD (BCL6-binding domain) along with a cell-penetrating TAT domain was able to inhibit the transcriptional repressor activity of BCL6[41]. Based on this initial work, the peptidomimetic molecule RI-BPI with superior potency and stability was developed[25] and used for BCL6-inhibition. RI-BPI represents a retro-inverso TAT-BBD-Fu (fusogenic) peptide[25] that was synthesized by Biosynthesis Inc. (Lewisville, TX) and stored lyophilized at −20 °C until reconstituted with sterile, distilled, degassed water immediately before use. The purity determined by high-performance liquid chromatography–mass spectrometry was 95% or higher. RI-BPI was injected intraperitoneally into mice.

**BCR–ABL1 TKI.** Imatinib (STI571) and nilotinib (AMN107) were obtained from Novartis Pharmaceuticals or from LC Laboratories. Stock solutions of imatinib were prepared in sterile, distilled water at 10 mmol $l^{-1}$ and stored at −20 °C. Nilotinib was either dissolved in DMSO (dimethyl sulphoxide) or NMP (N-Methyl-2-pyrrolidone) just before administration. Nilotinib dissolved in DMSO was vortexed with four volumes of peanut butter until a homogeneous mixture was formed. Nilotinib (free base) solubilized in NMP was diluted with PEG 300 (polyethylene glycol 300) in a 10/90 (vol/vol) ratio. Cohorts of mice were treated with oral administration of vehicle or nilotinib (25 mg $kg^{-1}$ $day^{-1}$ or 50 mg $kg^{-1}$ $day^{-1}$) once daily at indicated time points.

**Clonality analysis and spectratyping of B-cell populations.** Immunoglobulin $V_H$–$DJ_H$ gene rearrangements were amplified using PCR primers specific for the J558 $V_H$ region gene with a primer specific for the Cμ constant region gene. Using a FAM-conjugated Cμ constant region or a $J_H$ gene-specific primer in a run-off reaction, PCR products were labelled and subsequently analysed on a capillary sequencer (ABI3100, Applied Biosystems) by fragment-length analysis. Sequences of primers used are given in Supplementary Table 4.

**Affymetrix GeneChip analysis.** Total RNA from cells used for microarray or RT–PCR analysis was isolated by RNeasy (Qiagen) purification. RNA quality was first checked by using an Agilent Bioanalyser (Agilent Technologies). Complementary DNA (cDNA) was generated from 5 μg of total RNA using a poly(dT) oligonucleotide containing a T7 RNA polymerase initiation site and the SuperScript III Reverse Transcriptase (Invitrogen). Biotinylated cRNA was generated and fragmented according to the Affymetrix protocol and hybridized to U133A 2.0 human or 430 mouse microarrays (Affymetrix). After scanning (GeneChip Scanner 3000 7G, Affymetrix) of the GeneChip arrays, the generated CEL files were imported to BRB Array Tool (http://linus.nci.nih.gov/BRB-ArrayTools.html) and processed using the RMA algorithm (Robust Multi-array Average) for normalization and summarization. Relative signal intensities of probe sets were determined by comparing the signal intensity from TKI-treated and untreated cells to the average signal value of the respective cell line or a group of cell lines. The calculated signal ratios of probe sets were visualized as a heatmap with Java Treeview.

**Target validation of RI-BPI in human $Ph^+$ ALL cells.** $Ph^+$ ALL cell lines (BV173, NALM1 and TOM1) were treated with vehicle (control), 10 μmol $l^{-1}$ imatinib or imatinib + 20 μmol $l^{-1}$ RI-BPI for 24 h and maintained in Allprotect (Qiagen) at −80 °C until RNA isolation using an RNeasy Plus kit (Qiagen). RNA integrity was determined using the RNA 6000 Nano LabChip kit on Agilent 2100 Bioanalyser (Agilent Technologies). Two independent samples were analysed for each condition. RNA (1 μg) was hybridized to Agilent 60-mer Whole Human Genome Microarrays (part number G4112A) according to the manufacturer's recommendations. After hybridization, the processed microarrays were scanned with the Agilent DNA microarray scanner (part number G2505C) and extracted with Agilent Feature Extraction software version 8.5 (GE1-v5_10_Apr08). For computational analysis of signal, we used the dye-normalized signal after surrogate algorithm (gProcessedSignal) extracted from the .txt files and process for each array and for all the probes. This value was subjected to $\log_2$ transformation and median array normalization. The fold changes of imatinib compared with control and (imatinib + RI-BPI) compared with imatinib were calculated for each cell line and for each gene. A data set containing previously identified BCL6 target genes (obtained from Nimblegen arrays) was mapped into the Agilent probe sets using the Agilent and NimbleGen array annotation files. To determine if two data sets differed significantly, we compared the fold change in BCL6 target genes with the fold change in BCL6 non-target genes for each data set (imatinib compared with control, and imatinib + RI-BPI compared with imatinib) by the Kolmogorov–Smirnov test[42]. The Kolmogorov–Smirnov test deter-

mines if two data sets (gene expression values for BCL6 target genes and non-target genes) differ significantly. Heat maps and other analysis were obtained using the R statistical software (http://www.r-project.org).

**ChIP-on-chip analysis.** ChIPs were performed with modifications as described[43]. Briefly, $2.5 \times 10^7$ $Ph^+$ ALL cell lines (BV173, NALM1 and TOM1) were treated with or without 10 μmol $l^{-1}$ imatinib for 24 h. Then the cells were double cross-linked with 2 mmol $l^{-1}$ EGS cross linker and 1% formaldehyde. After sonication, immunoprecipitations were performed using 5 μg BCL6 (N3, Santa Cruz Biotechnology) or control IgG antibody (Sigma-Aldrich) from the chromatin fragments of $2.5 \times 10^7$ human $Ph^+$ ALL cells. After validation of enrichment by Q-ChIP, BCL6 or control IgG, ChIP products and their respective input genomic fragments were amplified by ligation-mediated PCR. The products were co-hybridized with the respective input samples to NimbleGen promoter arrays (human genome version 35). Quantitative ChIP was performed again at this stage for selected positive control loci to verify that the enrichment ratios were retained. The genomic products of two biological ChIP replicates were labelled with Cy5 (for ChIP products) and Cy3 (for input) and co-hybridized on custom-designed genomic tiling arrays generated by NimbleGen Systems. These high-density tiling arrays contained 50-residue oligonucleotides with an average overlap of 25 bases, omitting repetitive elements. After hybridization, the relative enrichment for each probe was calculated as the signal ratio of ChIP to input. Peaks of enrichment for BCL6 relative to input were captured with a five-probe sliding window, and the results were uploaded as custom tracks into the University of California Santa Cruz genome browser and graphically represented as histograms. Two replicates were performed with each condition.

**Data analysis of ChIP-on-chip experiments.** To identify target genes of BCL6 in these experiments, we computed the log-ratio between the probe intensities of the ChIP product and input and took moving averages of log-ratio of three neighbouring probes and determined the maximum value for each gene promoter and the random permutation probes as background control[44]. The cut-off for each array was established as higher than the 99th percentile of the 24,175 log-ratio values generated from random permutation probes. A locus with maximum moving average above cut-offs in two replicates was considered a potential binding site. Because this high stringent-overlapping approach can produce a high false-negative rate, we also computed the correlations among peaks between the replicates to rescue promoters that did not pass cut-off in one replicate. We calculated the Pearson correlation coefficient of the probe's signal of the promoter between replicates, and promoters with a correlation higher that 0.8 were rescued and included in our final set of BCL6 targets. In addition, all peaks were mapped back to the genome using BLAT (the BLAST-like Alignment Tool, http://genome.ucsc.edu) to identify genes on opposite strands that could be regulated from the same bidirectional promoter. Two genes were considered to be bidirectional partners when they were located on the opposite strands in a 'head-to-head' orientation and their transcription start sites were separated by less than 0.5 kilobases.

**Comparative genomic hybridization.** To analyse genetic instability and acquisition of genetic lesions during long-term cell culture, genomic DNA of BCR–ABL1-transformed $BCL6^{+/+}$ and $BCL6^{-/-}$ ALL cells was extracted after culturing for 4 months. Genomic DNA was isolated using the PureLink genomic DNA kit (Invitrogen). Three samples of each ALL type were co-hybridized with genomic DNA extracted from normal untransformed mouse cells to NimbleGen mouse 720k Whole-Genome Tiling arrays (NimbleGen Systems) in accordance with the manufacturer's recommendations. Copy number variations were analysed using the FASST-segmentation algorithm in Nexus software (BioDiscovery). Copy-number analysis was performed using a significance threshold of $1 \times 10^{-7}$ and a $\log_2$ ratio cut-off at $\pm 0.2$ for regions sized 1,000 kilobase pairs.

**Senescence-associated β-galactosidase assay.** Senescence-associated β-galactosidase activity was performed on cytospin preparations as described[21]. Briefly, a fixative solution (0.25% glutaraldehyde, 2% paraformaldehyde in PBS pH 5.5 for mouse cells and pH 6 for human cells) was freshly generated. To this end, 1 g paraformaldehyde was dissolved in 50 ml PBS at pH 5.5 by heating followed by addition of 250 μl of a 50% stock glutaraldehyde solution. 1× X-gal staining solution was prepared as follows (10 ml): 9.3 ml PBS/$MgCl_2$, 0.5 ml 20× KC solution (that is, 820 mg $K_3Fe(CN)_6$ and 1,050 mg $K_4Fe(CN)_6 \times 3H_2O$ in 25 ml PBS) and 0.25 ml 40× X-gal (that is, 40 mg 5-bromo-4-chloro-3-indolyl β-D-galactoside per milliliter of *N,N*-dimethylformamide) solution were mixed. For *BCR–ABL1*-transformed ALL cells, 100,000 cells per cytospin were used (700 r.p.m., 8 min). The fixative solution was pipetted on the cytospins and incubated for 10 min at room temperature, then washed twice for 5 min in PBS/$MgCl_2$. Cytospin preparations were submerged in 1× X-gal solution, incubated overnight at 37 °C in a humidified chamber and washed twice in PBS. Slides were mounted before they dried.

**Western blotting.** Cells were lysed in CelLytic buffer (Sigma) supplemented with 1% protease inhibitor cocktail (Pierce). Ten micrograms of protein mixture per sample were separated on NuPAGE (Invitrogen) 4–12% Bis-Tris gradient gels and transferred on PVDF membranes (Immobilion, Millipore). To detect mouse and human proteins by western blot, primary antibodies were used with the WesternBreeze immunodetection system (Invitrogen). The following antibodies were used: human BCL6 (clones D8 and N3, Santa Cruz Biotechnology), mouse BCL6 (rabbit polyclonal, Cell Signaling Technology), Arf (4C6/4, Cell Signaling Technology), p53 (1C12, Cell Signaling Technology), PTEN (A2B1, Santa Cruz), global Stat5 (3H7, Cell Signaling Technology) and phospho-Y694 Stat5 (14H2, Cell Signaling Technology). Antibodies against β-actin were used as a loading control (C4, Santa Cruz).

**Flow cytometry.** Antibodies against mouse CD19 (1D3), B220 (RA3-6B2), CD3 (17A2), CD43 (S7), CD45.1 (A20), CD45.2 (104), CD44 (IM7 and G44-26) and c-Kit (2B8) as well as respective isotype controls were purchased from BD Biosciences. For apoptosis analyses, Annexin V, propidium iodide and 7-AAD were used (BD Biosciences).

**Cell viability assay.** Fifty thousand *BCR–ABL1*-transformed ALL cells per well were seeded in a volume of 100 μl B-cell medium on Optilux 96-well plate (BD Biosciences). Imatinib was diluted in medium and added at the indicated concentration in a total culture volume of 150 μl. After culturing for 3 days, 15 μl of Resazurin (R&D) was added on each well and incubated for 4 h at 37 °C. The fluorescence was read at 535 nm and the reference wavelength was 590 nm. Fold changes were calculated using baseline values of untreated cells as a reference (set to 100%).

**Colony-forming assay.** The methylcellulose colony-forming assays were performed with 10,000 *BCR–ABL1*-transformed mouse $BCL6^{-/-}$ or $BCL6^{+/+}$ or 10,000 human *BCR–ABL1* ALL cells. Cells were re-suspended in MethoCult medium (StemCell Technologies) and cultured on dishes (3 cm diameter) with an extra water supply dish to prevent evaporation. After 7–14 days, colonies were counted.

**Cell-cycle analysis.** For cell-cycle analysis in *BCR–ABL1* ALL cells, the BrdU flow cytometry kit for cell-cycle analysis (BD Biosciences) was used according to manufacturer's instructions. BrdU incorporation (APC-labelled anti-BrdU antibodies) was measured with DNA content (7-amino-actinomycin-D) in fixed and permeabilized cells. The analysis was gated on viable cells that were identified based on scatter morphology[45].

***In vivo* toxicology studies of RI-BPI/nilotinib combinations.** Fifteen adult male C57BL/6 mice were purchased from the National Cancer Institute and randomized in three groups of five. One group was exposed to intraperitoneal administration of RI-BPI 20 mg $kg^{-1}$ of body weight three times a week; the second group was treated with RI-BPI 20 mg $kg^{-1}$ of body weight three times a week plus nilotinib 25 mg $kg^{-1}$ of body weight three times a week by oral gavage. A third group of five mice was treated with vehicle and used as controls. The mice were observed, examined and weighed every other day during the treatment period. Blood was collected at the end of the treatment by retro-orbital bleeding under anaesthesia. All mice were euthanized by $CO_2$ aspiration and the organs were harvested, weighed and macroscopically examined. Histology sections were prepared with haematoxylin and eosin staining. Pictures were taken using a digital camera (Olympus DP72) attached to a light microscope (Axioskop, Carl Zeiss) with ×4 and ×20 Plan Neofluar objectives (Carl Zeiss).


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