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PTEN opposes negative selection and enables oncogenic transformation of pre-B cells

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Phosphatase and tensin homolog (PTEN) is a negative regulator of the phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT) signaling pathway and a potent tumor suppressor in many types of cancer. To test a tumor suppressive role for PTEN in pre-B acute lymphoblastic leukemia (ALL), we induced Cre-mediated deletion of Pten in mouse models of pre-B ALL. In contrast to its role as a tumor suppressor in other cancers, loss of one or both alleles of Pten caused rapid cell death of pre-B ALL cells and was sufficient to clear transplant recipient mice of leukemia. Small-molecule inhibition of PTEN in human pre-B ALL cells resulted in hyperactivation of AKT, activation of the p53 tumor suppressor cell cycle checkpoint and cell death. Loss of PTEN function in pre-B ALL cells was functionally equivalent to acute activation of autoreactive pre-B cell receptor signaling, which engaged a deletional checkpoint for the removal of autoreactive B cells. We propose that targeted inhibition of PTEN and hyperactivation of AKT triggers a checkpoint for the elimination of autoreactive B cells and represents a new strategy to overcome drug resistance in human ALL.

The majority of newly generated pre-B cells in the bone marrow are eliminated at the pre–B cell receptor (pre-BCR) checkpoint1. Critical survival and proliferation signals come from the pre-BCR; if pre-B cell clones fail to express a functional pre-BCR, then signaling output is too weak. If the pre-BCR binds to ubiquitous self-antigen (auto-reactive immunoglobulin μ heavy chain; μ-HC), then pre-BCR signals are strong. Both attenuation below a minimum (such as a nonfunctional pre-BCR) and hyperactivation above a maximum (such as an autoreactive pre-BCR) threshold of signaling strength trigger negative selection and cell death. Approximately 75% of newly generated pre-B cells express an autoreactive μ-HC2,3, highlighting the importance of stringent negative selection of autoreactive clones at the pre-BCR checkpoint. Although autoreactive pre-B cell clones are eliminated owing to the toxicity of strong pre-BCR signaling1,3, sustained activation of PI3K-AKT signaling is sufficient to rescue B cell survival in the absence of a functional BCR4 and is required for pre-B cell survival3. Likewise, germline mutations in humans that result in either loss or hyperactivation of PI3K-AKT signaling have equally deleterious effects on human early B cell development6, suggesting that early B cells undergo selection for an intermediate level of PI3K signaling.

PTEN is a key negative regulator of the PI3K-AKT pathway and functions as a dual protein and lipid phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-triphosphate (PIP3). PTEN counteracts the activity of PI3K, which phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) to generate PIP3, the membrane anchor and ligand of AKT’s pleckstrin homology (PH) domain7. Deletions or inactivating mutations of PTEN are frequently observed in all main types of human cancer (on average 8.3% among 37,898 samples studied)8. The common outcome of these lesions is increased membrane levels of PIP3 and AKT hyperactivation. Genetic lesions of PTEN mutations also have a major role in hematopoietic malignancies. For instance, lesions in PTEN and in genes encoding components of the PI3K-AKT pathway are present in up to 50% of T cell lineage ALL cases5.

RESULTS
Pten is required for initiation and maintenance of pre-B ALL in vivo
To study a potential role of PTEN and negative regulation of PI3K-AKT signaling, we developed BCR-ABL1- and NRASG12D-, driven mouse models of pre-B ALL (Fig. 1). To this end, we transformed interleukin (IL)-7–dependent pre-B cells from the bone marrow of mice carrying loxP-flanked (floxed) Pten (Ptenflo/flo and Ptenfr/fr mice)10 with oncogenic BCR-ABL1 or NRASG12D. BCR-ABL1 represents the driver oncogene in Philadelphia chromosome–positive (Ph+) ALL, the most common subtype of pre-B ALL in adults (~30%). RAS pathway lesions affect components encoded by NRAS, KRAS, PTPN11 and NF1, and these occur in ~50% of both adult and pediatric ALL11. Together, BCR-ABL1- and NRASG12D-driven pre-B ALL reflect two major nonoverlapping types of human ALL (~80%). For inducible deletion of Pten, pre-B ALL cells were transduced with a construct expressing

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a tamoxifen (Tam)-inducible estrogen receptor empty vector control (ERT2) or one expressing Cre (Cre-ERT2). After selection of the transduced cells with puromycin, Cre was activated by Tam treatment (1 μmol/liter), which induced excision of the loxp-flanked Pten alleles and depletion of PTEN protein within 2 d (Fig. 1a). Notably, inducible Cre-mediadated deletion of Pten in pre-B ALL cells resulted in the rapid cell death of leukemia cells (Fig. 1b and Supplementary Fig. 1a). To address whether loss of PTEN affected not only survival of established leukemia but also initiation of leukemia, we reversed the order and first induced deletion of Pten in IL7-dependent Ptenfl/fl pre-B cells and subsequently induced BCR-ABL1–mediated transformation. Two days after induction of Cre, Pten+/− and Pten−/− pre-B cells were transduced with a construct encoding green fluorescent protein (GFP)-tagged BCR-ABL1 (BCR-ABL1GFP). Pten+/− pre-B cells showed rapid outgrowth of BCR-ABL1GFP-expressing clones, indicating leukemic transformation. In contrast, Pten−/− pre-B cells carrying BCR-ABL1GFP were not susceptible to malignant transformation by BCR-ABL1 (Fig. 1c and Supplementary Fig. 1b). These findings were recapitulated in an in vivo transplant setting. BCR-ABL1–transformed Ptenfl/fl pre-B ALL cells caused a fatal leukemia in transplant–recipient mice within 18 d. Luciferase bioimaging revealed that Cre-mediadated deletion of Pten did not interfere with engraftment of pre-B ALL cells. However, pre-B ALL cells failed to initiate fatal disease in the absence of PTEN, and the transplant recipients survived for indefinite periods of time (Fig. 1d). Minimal residual disease (MRD) analysis, using genomic PCR, revealed no trace of covert leukemia clones (Supplementary Fig. 1c).

Pten deletion also induced G0-G1 cell cycle arrest and senescence in pre-B ALL cells (Fig. 2c,d). To assess the effect of acute ablation of Pten in fully established BCR-ABL1 and NRASG12D–driven pre-B ALL cells in vivo, 100,000 Ptenfl/fl pre-B ALL cells carrying tamoxifen-inducible Cre were injected into sublethally irradiated NOD.CB17-Prkdcscid/l (NOD-SCID) recipient female mice for engraftment. Five days after transplantation of Ptenfl/fl pre-B ALL cells, leukemia initiation and engraftment was confirmed by using bioluminescence imaging, and Cre was induced by ten consecutive daily injections of tamoxifen. Consistent with in vitro results, Pten deletion caused leukemia regression in vivo (Fig. 2e) and prolonged overall survival in the recipient mice. We conclude that Pten is required for both initiation and maintenance of pre-B ALL in vivo.

Pre-B ALL cells are exempt from genetic lesions of PTEN

Given the unexpected sensitivity of pre-B ALL cells to even a moderate dose reduction of Pten, we reassessed the concept of Pten as a tumor suppressor in human cancer and in leukemias and lymphomas. A reanalysis of genetic lesions of Pten in human cancer revealed a high frequency of mutations in solid cancer (8.3% in 37,898 samples) and hematological malignancies (8.4% in 2,548 samples). In addition to point mutations, deletions of Pten at chromosome 10q23 are frequent in cancer (5.3% in 8,071 samples studied; Fig. 3a). These findings are in agreement with previous mechanistic studies demonstrating a tumor suppressor role of Pten in acute myeloid leukemia16, chronic myeloid leukemia (CML)17, T cell lineage acute lymphoblastic leukemia18,19, and mature B cell lymphoma20–22. However, point mutations in Pten were not detected in any of the 694 pre-B ALL patient samples evaluated. Likewise, no Pten deletions were found in the 231 pre-B ALL cases studied (Fig. 3a). Similarly, although oncogenic activation of the PI3K–AKT pathway in leukemia and lymphoma also occurs through activating mutations in genes encoding agonists of the PI3K–AKT pathway, pre-B ALL cases do not harbor such mutations (Supplementary Fig. 3). Together, these genetic data suggest that Pten lesions and other mutations that lead to oncogenic activation of the PI3K–AKT pathway are not favorable in pre-B ALL and that Pten may have a fundamentally different role in pre-B ALL than in other hematopoietic malignancies.

High expression levels of PTEN in patient-derived pre-B ALL cells

Consistent with these findings, our analysis of patient-derived samples showed that Pten promoter regions are hypermethylated in B cell lymphomas (n = 68) but not in pre-B ALL samples (n = 83; Fig. 3b). Reverse-phase protein array (RPPA) measurements for 155 newly diagnosed cases of adult ALL, 22 cases of T cell lineage ALL and 11 cases of mature B cell lymphoma (the M.D. Anderson Cancer Center (MDACC) (1983–2007) cohort)23 revealed higher PTEN protein levels in samples from individuals with pre-B ALL than in those from individuals with T cell lineage ALL or mature B cell lymphoma (Fig. 3e). We confirmed these findings by western blot analyses, using a panel of sorted normal human CD19+ pre-B cells (n = 3), patient-derived pre-B ALL cells (n = 8) and B cell non-Hodgkin’s lymphoma samples (n = 4) (Fig. 3d). The MDACC (1983–2007) cohort RPPA data set replicated results from previous work24 that identified high expression levels of PTEN as a predictor of favorable outcome in individuals with T cell lineage ALL (Supplementary Fig. 4). In contrast, RPPA data for diagnostic samples from individuals with pre-B ALL showed the opposite trend, with greater-than-median expression levels of PTEN in diagnostic pre-B ALL samples being predictive of shorter relapse-free survival times (Supplementary Fig. 4).
Figure 1 Pten is required for leukemic transformation of pre-B cells. (a) Representative PCR (left; n = 3) and western blot (right; n = 3) analysis for Pten deletion (Ptenfl/fl) after induction of Cre expression by Tam treatment of BCR-ABL1–transformed Ptenfl/fl pre-B cells. β-actin was used as loading control. (b) Viability of BCR-ABL1–transformed pre-B ALL cells, as measured by flow cytometry, after tamoxifen-dependent induction of Pten deletion (n = 3). Error bars represent mean ± s.d. P < 0.0001; as calculated by contingency table. (c) The fraction of BCR-ABL1–GFP+ pre-B cells, measured over time using flow cytometry. Error bars represent mean ± s.d. P < 0.0001; as calculated by contingency table. (d) Luciferase bioimaging of NOD-SCID recipient mice 9 d (left) and 14 d (right) after injection of Ptenfl/fl BCR-ABL1–transformed pre-B ALL cells that were transduced with constructs expressing either Tam-inducible Cre (Cre) (bottom) or an empty vector (EV) control (top) and in which Pten deletion was induced 24 h before injection. Graph (bottom) shows survival analysis of the mice (n = 7 mice per group). Color bar for luminescent signal intensity shows photons/s/cm²/steradian. Scale bars, 2 cm. P = 0.0002 was calculated by Mantel-Cox log-rank test. (e) Microarray gene expression analysis after 48 h of induction of Pten deletion in BCR-ABL1–transformed pre-B ALL cells. (f) Representative flow cytometry analysis (of n = 3) for cell surface expression of the indicated molecules before (Ptenfl/fl) + EV and after (+ Cre) deletion of Pten. FSC, forward scatter. (g) Representative flow cytometry analysis (of n = 3) for the expression of the B cell markers CD19 and B220 in cells after the deletion of Pten in vitro (+ Cre; n = 3) (left) or in vivo (Ptenfl/fl × Cd79a-Cre; n = 4) (right). (h) Representative western blot analysis (of n = 4) of Ptenfl/fl BCR-ABL1–transformed pre-B ALL cells before (EV) and after (Cre) Ptenfl/fl deletion. β-actin was used as a loading control.

Pten regulates AKT activity downstream of the pre-B cell receptor

To elucidate the mechanistic basis of Pten dependency in pre-B ALL, we first tested the hypothesis that loss of Pten results in hyperactivation of PI3K-AKT signaling and subsequent cell death. In agreement with this hypothesis, we found that inducible deletion of Pten in both BCR-ABL1– and NRASG12D–driven pre-B ALL cells resulted in increased phosphorylation of AKT at both Ser473 and Thr308 (Fig. 3e). Consistent with Pten-dependent feedback regulation, deletion of Pten caused hyperactivation of PI3K-AKT signaling and downregulation of the pre-BCR, IL-7R and CD19 (Figs. 1f–h and 3f), all of which mediate PI3K-AKT activation in pre-B cells. SYK is a B cell–specific tyrosine kinase that links pre-BCR signaling to PI3K activation12–15. To measure the contribution of individual components of the PI3K-AKT pathway in inducing toxicity, we used selective small-molecule inhibitors of the SYK (PRT06207), PI3K (BKMI120) and AKT (AZD5363) kinases (Fig. 3f). Consistent with the toxicity induced by hyperactivation of AKT in pre-B ALL cells, pharmacological inhibition of AKT by treatment with AZD5363 greatly reduced the toxicity mediated by Pten deletion (Fig. 3g and Supplementary Fig. 5a). Also inhibition of SYK (using PRT06207) and PI3K (using BKMI120) mitigated the toxic effects of the Pten deletion. Given that BCR-ABL1 and NRASG12D can activate AKT independently of SYK and PI3K, treatment of cells with the AKT inhibitor AZD5363 had the strongest protective effect in reducing cell death induced by the Pten deletion (Fig. 3h and Supplementary Fig. 5b).
Both alleles of Pten are required for survival and proliferation of pre-B ALL cells

Deletion of PTEN in human cancer typically affects one allele, whereas the other copy is retained\(^2\), suggesting that cancer cells are selected for the deletion of one but not both alleles of PTEN. Indeed, deletion of both Pten alleles induces p53-dependent senescence in a model of prostate cancer, unless Trp53 is also deleted\(^3\). For this reason, we studied the consequences of both heterozygous and homozygous deletion of Pten in mouse pre-B ALL cells (Supplementary Fig. 6). By comparing the cells in which either one (Pten\(^{+/\beta}\)) or both (Pten\(^{−/\beta}\)) alleles of Pten were deleted, we found that, in contrast to solid tumor cells, both heterozygous and homozygous deletion of Pten affected colony formation and proliferation of pre-B ALL cells and induced cellular senescence. The deleterious effects of mono- and bi-allelic Pten ablation in pre-B ALL cells were indistinguishable (Supplementary Fig. 6a–c). Likewise, deletion of either both or only one allele of Pten compromised leukemogenesis in an in vivo transplant experiment, and no difference was noted between pre-B ALL cells carrying a deletion of one or both alleles of Pten (Supplementary Fig. 6d). Western blot analysis revealed that deletion of one allele of Pten resulted in increased phosphorylation of AKT (Supplementary Fig. 6e); however, this increase was less than the AKT phosphorylation observed in response to the bi-allelic deletion of Pten.
AKT hyperactivation eliminates autoreactive B cells

Unlike other cell types, pre-B cells are selected for on the basis of an inducible activation (CA) forms of SYK (SYKCA) and AKT (AKTCa) and measured pre-B ALL cell viability in the presence or absence of the Syk, PI3K and AKT inhibitors. As predicted, SYKCA rapidly induced cell death in pre-B ALL cells, which could be almost completely rescued by inhibition of SYK, PI3K and AKT kinase activity (Fig. 4d and Supplementary Fig. 7b). Likewise, hyperactivation of AKT (AKTCa) was toxic in pre-B ALL cells, and toxicity was reduced by treatment with AKT kinase inhibitor AZD5363 (Fig. 4e and Supplementary Fig. 7c). Collectively, these findings suggest that hyperactivation of PI3K-AKT signaling engages a deletional checkpoint for the removal of autoreactive B cell clones.

To demonstrate the toxic effects of SYK and AKT hyperactivation in a genetic experiment, we transduced pre-B ALL cells with constitutively active (CA) forms of SYK (SYKCa) and AKT (AKTCa) and measured pre-B ALL cell viability in the presence or absence of the Syk, PI3K and AKT inhibitors. As predicted, SYKCa rapidly induced cell death in pre-B ALL cells, which could be almost completely rescued by inhibition of SYK, PI3K and AKT kinase activity (Fig. 4d and Supplementary Fig. 7b). Likewise, hyperactivation of AKT (AKTCa) was toxic in pre-B ALL cells, and toxicity was reduced by treatment with AKT kinase inhibitor AZD5363 (Fig. 4e and Supplementary Fig. 7c). Collectively, these findings suggest that hyperactivation of PI3K-AKT signaling engages a deletional checkpoint for the removal of autoreactive B cell clones.

B cell–specific expression and activity of PTEN

To test the basic premise of a checkpoint for the elimination of autoreactive pre-B cells, we reprogrammed pre-B ALL cells into myeloid lineage cells using an inducible CCAAT–enhancer-binding

Figure 3 Pre-B ALL cells do not harbor genetic lesions in PTEN and do not tolerate hyperactivation of PI3K-AKT signaling. (a) Frequencies of genetic lesions in PTEN, including mutations and deletions, from the Catalog of Somatic Mutations in Cancer (COSMIC) database for 16 types of human cancer. (b) Comparison of the level of CpG methylation in the PTEN promoter region in pre-B cells from healthy donors (n = 12), bone marrow biopsies from patients with pre-B ALL (n = 83) and patient-derived B cell non-Hodgkin's lymphoma cells (n = 68). (c) Comparison of PTEN expression, using the RPPA assay, for subjects with newly diagnosed adult ALL (n = 155), T cell lineage ALL (n = 22) or mature B cell lymphoma (n = 11). P values in b,c were calculated by two-sided Mann-Whitney-Wilcoxon test. (d) Western blot analysis (top) and quantification (bottom) of PTEN expression in a panel of sorted normal human CD19+ pre-B cells (n = 3), patient-derived pre-B ALL cells (n = 8) and samples from individuals with B cell non-Hodgkin's lymphoma (n = 4). β-actin was used as loading control. P values were calculated by Student’s t-test. (e) Representative western blot analysis (n = 3) for AKT activation after deletion of Pten in BCR-ABL1− or NRASQ61D1-transformed pre-B ALL cells. β-actin was used as loading control. AKT-pS473, phospho-Ser473 AKT; AKT-pThr308, phospho-Thr308 AKT. (f) Schematic of pre-BCR signaling and its interaction with the PI3K-AKT pathway. AZD, AZD5363; PRT, PRT06207; BKM, BKM120. (g) Fraction of GFP+ cells, as measured by flow cytometry, after Pten deletion in BCR-ABL1−transformed (left) or NRASQ61D1-transformed (right) Pten−/− pre-B cells and treatment with the AKT inhibitor AZD5363 (3 μmol/liter). (h) Fraction of GFP+ cells after Pten-deleted BCR-ABL1−transformed pre-B ALL cells in the presence or absence of AZD5363 (3 μmol/liter), the PI3K inhibitor BKM120 (1 μmol/liter) or the SYK inhibitor PRT06207 (3 μmol/liter). Throughout, for cells transduced with EV or a Cre-expressing vector, P values were calculated by contingency table, by comparing the treatment group with the untreated control group (Ctrl). All P values for EV-transduced cells are not significant (P > 0.5, not shown). Error bars represent s.d. Results are representative of three independent experiments.
Figure 4  Hyperactivation of AKT is a defining feature of autoreactive pre-BCR signaling and triggers a checkpoint for the removal of autoreactive pre-B cells.

(a) Schematic of the reconstitution system for inducible activation of pre-BCR signaling using a Tam-inducible (Blnk-ER) form of the pre-BCR linker molecule BLNK (also known as SLP-65). Addition of Tam releases BLNK from its complex with heat-shock protein 90 (HSP90) and allows for assembly of the pre-BCR signaling complex within minutes. LYN, LYN proto-oncogene, Src family tyrosine kinase. (b) Quantification of GFP+ cells, relative to that in EV-transduced cells, after activation of pre-B cell signaling in BCR-ABL1–transformed pre-B ALL cells with 2 d of pre-treatment with inhibitors of SYK (PRT, 3 µmol/liter) or AKT (AZD, 3 µmol/liter). For each curve, the P value was calculated by contingency table, by comparing treatment groups with untreated control group (Ctrl). (c) Representative western blot analysis of phosphorylated SYK, AKT and the ribosomal protein RPS6 5 min after addition of tamoxifen. (d) Relative changes in the percentages of GFP+ cells after transduction of pre-B ALL cells with a construct that expresses a GFP-tagged constitutively active SYK (SYKCA, SykY348E,Y352E) or empty vector (EV) control in the presence of vehicle or inhibitors of AKT (AZD, 3 µmol/liter), PI3K (BKM, 1 µmol/liter) and SYK (PRT, 3 µmol/liter). (e) Relative changes in the percentages of GFP+ cells after transduction of pre-B ALL cells with a GFP-tagged retroviral vector encoding myristoylated AKT (AKTCA) or GFP (EV) control in the presence or absence of the AKT inhibitor (AZD, 3 µmol/liter). (f) Representative flow cytometry analysis of (n = 3) for B–myeloid reprogramming of BCR-ABL1–transformed Ptenfl/fl pre-B ALL (CD19+Mac1+) to myeloid lineage (CD19+Mac1+) cells after induction of C/EBP-α by doxycycline (Dox) treatment. (g) Representative western blot analysis of (n = 2) for the expression of C/EBP-α, the B cell lineage–specific transcription factor PAX5, total and phospho-Ser473 AKT, and PTEN in B–lineage and B–myeloid reprogrammed cells. (h) Changes in the frequency of GFP+ cells in B–myeloid (CD19+Mac1+) and pre-B cells (CD19+Mac1+) after induction of Cre-GFP by Tam treatment. P < 0.0001 was calculated using contingency table. All results presented in this figure are representative of two independent experiments with triplicates. Error bars (b,d,e,h) represent mean ± s.d. For curves in b,d,e, P values were calculated by contingency table comparing treatment groups with the respective untreated control group (Ctrl).

Protein alpha (C/EBP-α) vector system. To this end, we engineered Ptenfl/fl pre-B ALL cells with a doxycycline-inducible vector system for expression of the myeloid transcription factor C/EBP-α, which results in B–myeloid lineage conversion (Fig. 4f–h). Inducible expression of C/EBP-α in pre-B ALL cells in pre-B ALL is not only downregulated PAX5, a transcription factor that determines B cell lineage identity, but also resulted in downregulation of PTEN levels, demonstrating that PTEN expression levels depend on B cell lineage identity (Fig. 4g). C/EBP-α–mediated myeloid lineage conversion also resulted in a 45-fold increase in phosphorylation of Ser473 in AKT, as compared to that in the EV-transduced cells, which mirrors transcriptional repression of PTEN in myeloid cells (Fig. 4g). These findings are consistent with the notion that myeloid cells, unlike B lineage cells, are permissive to the loss of PTEN function and hyperactivation of AKT signaling.

To test whether B–myeloid lineage conversion erases dependency on PTEN-mediated negative regulation of AKT activity, Ptenfl/fl pre-B ALL cells carrying inducible C/EBP-α or empty-vector controls (EV) were also induced to express Cre (Fig. 4h). Inducible deletion of Pten resulted in a rapid elimination of B cell–lineage ALL cells (CD19+Mac1+) as in previous experiments (Fig. 1 and Supplementary Fig. 1). By contrast, deletion of Pten in B–myeloid–reprogrammed cells (CD19+Mac1+) resulted in a slight increase in the frequency of Cre-GFP+ cells, reflecting a relative survival advantage for Pten deletion in myeloid cells (Fig. 4h). These findings are consistent with a previous study that identified PTEN as a tumor suppressor in BCR-ABL1–transformed myeloid leukemia and reinforce the notion that normal pre-B and pre-B ALL cells are uniquely dependent on negative regulation of AKT by PTEN.

The deleterious effects of Pten deletion in pre-B ALL are linked to B cell lineage identity

Pre-B (Ph+ ALL) and CML are both driven by the oncogenic BCR-ABL tyrosine kinase but markedly differ with respect to PTEN expression levels (Fig. 5a,b). PTEN protein levels were high in BCR-ABL1–driven mouse pre-B ALL cells and in patient-derived Ph+ ALL cells (n = 5) but barely detectable in mouse CML-like and in patient-derived CML cells (n = 5) (Fig. 5a,b). This difference is consistent with regulation of Pten levels during B–myeloid reprogramming (Fig. 4g). Although pre-B cells are subject to a deletional checkpoint for the removal of autoreactive clones, this is not the case for other cell types (including myeloid cells). Similarly, deletion of Pten in normal myeloid progenitor cells showed no effects but caused toxicity in normal pre-B cells (Fig. 5c). Moreover, Cre-mediated Pten deletion had no deleterious effects in BCR-ABL1 myeloid lineage CML but caused cell death in BCR-ABL1–transformed pre-B ALL cells (Fig. 5d). Induction of cell death after Pten deletion in pre-B ALL cells was paralleled by accumulation of the ARF, p53 and p21 cell cycle checkpoint molecules. In contrast, Pten deletion in myeloid leukemia cells induced neither activation of checkpoint molecules nor cell death.
in B lymphoid cells, which resulted in depletion of glucose reserves and in near-exhaustion of cellular ATP levels (Fig. 5g). Taken together, these findings indicate that deletion of Pten in pre-B ALL cells caused profound metabolic distress in conjunction with strong activation of p53 checkpoint activation in pre-B ALL cells. It should be noted that we tested alternative mechanisms of toxicity related to Pten deletion, including dephosphorylation of signal transducer and activator of transcription 5 (STAT5) and accumulation of cellular reactive-oxygen species (ROS). Unlike metabolic stress and p53-mediated cell death, loss of STAT5 phosphorylation and increased ROS levels were only observed in BCR-ABL1–transformed ALL cells and could not be mechanistically validated in other ALL subtypes (data not shown).

Validation of PTEN as a therapeutic target in patient-derived pre-B ALL cells

Despite the well-characterized function of PTEN as a tumor suppressor, to test whether PTEN represents a therapeutic target in human pre-B ALL, we used patient-derived pre-B ALL cells in which either of two distinct shRNAs specific for Pten or a scrambled control shRNA were delivered using a lentivirus. Expression of the Pten-specific shRNA hairpins reduced PTEN protein levels by twofold to fourfold as compared to expression of the nontargeting (scrambled) control, as determined by western blot analysis (Fig. 6a). shRNA-mediated knockdown of PTEN in three human myeloid lineage CML cell lines did not affect cell viability, as compared to cells expressing the control shRNA (Fig. 6b and Supplementary Fig. 9). In contrast, PTEN knockdown induced cell death in cells from four individuals with pre-B ALL who harbored the BCR-ABL1 (subjects LA2X and ICN1), TCF3-PBX1 (subject ICN12) or KRASG12V (subject LA7X) oncogenes (Fig. 6b and Supplementary Fig. 9). We conclude that PTEN is essential for the survival of human pre-B ALL cells, thus validating PTEN as a potential therapeutic target in human pre-B ALL.

**Figure 5** Pre-B cell–specific functions of PTEN in normal progenitor cells and in leukemia. (a) Representative western blot analysis of Pten protein levels in mouse cytokine-dependent myeloid progenitor cells (Sca1+ c-Ki67 Lin-), IL-7–dependent pre-B cells, BCR-ABL1–transformed myeloid and pre-B leukemia cells. PTEN/β-actin densitometry ratios were normalized to ratios calculated for myeloid progenitor cells. P values were calculated by Student’s t-test. (b) Top, western blot analysis for PTEN expression in patient-derived CML (n = 5) and patient-derived B-lineage ALL (n = 5) cells. Bottom, PTEN/β-actin densitometry ratios were calculated, and P = 0.0001 was calculated by Student’s t-test. (c) The percentages of viable cells were measured after Cre-induced Pten deletion in myeloid progenitor cells (c, left) and pre-B cells (c, right) and their BCR-ABL1–transformed counterpart CML-like (d, left) and pre-B ALL (d, right) cells (n = 2). P values were calculated using contingency tables. (e) Representative western blot analysis (of n = 2) for expression of cell cycle–checkpoint molecules ARF, p53 and p21 after deletion of Pten in BCR-ABL1–transformed myeloid leukemia and pre-B ALL cells. (f) Representative western blot analysis (of n = 3) for total p53 and p53 (phospho-Ser15) after 48 h of Cre–induced Pten deletion in BCR-ABL1–transformed pre-B ALL in the presence of the AKT inhibitor (AZD, 3 μmol/liter) or vehicle. (g) Glucose consumption, lactate production and ATP levels on day 2 following induction of Cre in Ptenfl/fl BCR-ABL1–transformed pre-B ALL or myeloid reprogrammed cells. Values obtained were normalized to the number of viable cells and are shown as average relative levels. P values were calculated by Student’s t-test. β-actin was used as loading control (a,b,e,f). Error bars (a,c,d,g) represent mean ± s.d.
Figure 6 A small-molecule inhibitor of PTEN is specifically toxic in pre-B ALL. (a) Representative western blots (of n = 3) (top) and quantification (bottom) after PTEN knockdown with either of two PTEN-specific shRNAs (shPTEN-1 and shPTEN-2) in pre-B ALL xenografts and CML cell lines. β-actin was used as a loading control. P values were calculated by Student’s t-test comparing PTEN levels in cells expressing each PTEN-specific shRNA to that from cells expressing the scrambled shRNA. (b) Viability of three CML cell lines (KYO-1, solid line; KUB12, short-dashed line; JURL-MK1, long-dashed line) (left) and three patient-derived pre-B ALL xenografts (LAX7R, solid line; LAX2, short-dashed line; ICN12, long-dashed line) (right) after transduction with either of two shRNAs specific for PTEN or a scrambled (Scram) sequence. P values were calculated using contingency tables comparing average viability of cells for each shRNA versus scrambled control. (c) Chemical structure of the PTEN inhibitor SF1670. (d) Representative western blot analysis (of n = 4) for induction of the AKT signaling pathway after SF1670 (10 µmol/liter) treatment of cells from two individuals with pre-B ALL. β-actin was used as loading control. (e) Viability of human cells from individuals with pre-B ALL (n = 5, red) or CML (n = 3, green) after SF1670 treatment. Error bars represent mean ± s.d. P < 0.0001 was calculated using contingency table comparing viability of cells from individuals with pre-B ALL versus those with CML. (f) Representative western blot analysis (of n = 3) for p21, p53 and p53 (phospho-Ser15) on pre-B ALL cells treated with SF1670 (10 µmol/liter). β-actin was used as loading control. (g) Viability of BCR-ABL1–transformed pre-B ALL cells with or without Trp53 deletion after SF1670 treatment. Error bars represent mean ± s.d. P < 0.001 was calculated by contingency table. (h) Viability of cells from two individuals with pre-B ALL after treatment for 1 h or 3 h with SF1670 (10 µmol/liter) for three consecutive days. P values were calculated by contingency table comparing average cell viability after each treatment to those of vehicle-treated cells. The results are representative of three independent experiments. (i) Glucose consumption, lactate production and ATP levels in a CML cell line (KYO-1) and in a patient-derived pre-B ALL cells (LAX7R) after a 24-h treatment with SF1670 (2.5 µmol/liter). Values obtained were normalized to the number of viable cells and are shown as average relative values. P values were calculated by Student’s t-test. Error bars represent mean ± s.d.

Preclinical evaluation of PTEN inhibitor in pre-B ALL treatment

To test potential therapeutic usefulness of the pharmacological inhibition of PTEN, we studied the effects of a small-molecule inhibitor of PTEN, SF1670 (refs. 30, 31), in human pre-B ALL cells (Fig. 6c). Biochemical validation of SF1670 activity revealed that treatment with this PTEN inhibitor induced activation of AKT signaling in cells from individuals with pre-B ALL who carried the BCR-ABL1 or KRASG12V oncogenes within 1 h of treatment (Fig. 6d). SF1670 treatment strongly and selectively induced cell death in pre-B ALL cells at similar concentrations that were previously used to stimulate neutrophils in vivo31 (concentration required for stimulation of 50% of cells [IC$_{50}$] = 1.2 µmol/liter; Fig. 6c and Supplementary Fig. 10a). In addition, treatment with SF1670 shows a similar B cell lineage–specific effect to that observed using genetic approaches (i.e., deletion of Pten and shRNA-mediated knockdown of PTEN), as myeloid CML cells were either not affected by SF1670 treatment or affected only at higher concentrations (Fig. 6e). In addition, treatment of human pre-B ALL cells with SF1670 phenocopied the effects of a genetic deletion of Pten in Pten$^{fl/fl}$ in pre-B ALL cells. Of note, the acute deletion of Pten in Pten$^{fl/fl}$ pre-B ALL cells resulted in phosphorylation (Ser15) and activation of p53 (Fig. 5f). Similarly, small-molecule inhibition of PTEN resulted in rapid activation of the p53 and p21 checkpoint molecules and in p53 phosphorylation at Ser15 (Fig. 6f). To test whether p53 checkpoint activation represents an important aspect of toxicity in pre-B ALL cells that results from acute PTEN inhibition, we transduced Trp53fl/+ BCR-ABL1–transformed pre-B cells with a Cre-expressing or an empty-vector (EV) construct. Notably, Trp53 deletion caused a substantial shift in the dose-response curves of cells treated with SF1670. (Fig. 6g). Although Cre-mediated deletion of Pten resulted in the rapid loss of cell surface expression of CD19 and the pre-BCR (VpreB) (Fig. 1f and Supplementary Fig. 10b), we observed the same effects in human pre-B ALL cells that were treated for 2 d with SF1670 (Supplementary Fig. 10b). Because prolonged systemic inhibition of PTEN would presumably raise safety concerns owing to its role as a tumor suppressor in a wide range of cell types8,18, we tested the effects of short, transient inhibition of PTEN using SF1670 treatment. A single exposure to SF1670 for 3 h (and subsequent washout of SF1670) induced AKT hyperactivation and caused significant cell death in pre-B ALL cells (P < 0.001; Fig. 6h).

Small-molecule inhibition of PTEN also recapitulated metabolic features of the genetic deletion of Pten (Fig. 6i). Although SF1670 treatment resulted in a moderate stimulation of glycolysis (glucose consumption and lactate production) in CML cells, stimulation of glycolytic responses was much stronger in B lymphoid ALL cells (Fig. 5g). However, SF1670 treatment resulted in a net increase in cellular ATP in myeloid CML cells but a near-complete depletion of energy reserves in patient-derived pre-B ALL cells. Thus, SF1670 treatment induced pre-B cell–specific depletion of ATP and energy crisis, which recapitulated the measurements we observed in response to Cre-mediated deletion of Pten in myeloid leukemia and pre-B ALL cells (Fig. 5g).
DISCUSSION
Unlike other cell types, pre-B cells are under intense selective pressure and have to pass a central B cell tolerance checkpoint that removes autoreactive clones. The vast majority of newly generated pre-B cells express an autoreactive immunoglobulin μ-heavy chain, which results in strong pre-B cell receptor signaling, and are eliminated at this checkpoint. Humans carrying germline mutations that result in hyperactive PI3K-AKT signaling have profound B cell lymphopenia, presumably because almost all newly generated pre-B cells are subject to negative selection owing to hyperactive PI3K-AKT signaling. Recent work from our group demonstrated that central B cell tolerance checkpoints are fully functional in human pre-B ALL cells, despite their malignant transformation. Here we demonstrated that PTEN represents a critical gatekeeper of this checkpoint. Despite its tumor suppressor function in all major subtypes of cancer, PTEN is required to prevent indiscriminate checkpoint activation. Reflecting the unique function of PTEN in central B cell tolerance, pre-B ALL was the only subtype of human cancer that we found to be exempt from PTEN lesions (Fig. 3a). Previous work demonstrated that pre-B cells need a minimum level of PI3K-AKT signaling to survive and that targeting of this pathway is potentially useful in the treatment of human pre-B ALL. However, here we propose that acute inhibition of PTEN or direct pharmacological hyperactivation of PI3K-AKT signaling may represent a strategy to trigger central B cell tolerance checkpoints and thereby overcome conventional drug resistance in human pre-B ALL.

METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. Gene Expression Omnibus: the array data has been deposited with accession code GSE34829.

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AUTHOR CONTRIBUTIONS
S.S. and M.M. designed experiments and interpreted data; M.M. conceived the study, obtained funding, coordinated collaborations and wrote the paper; S.S., L.N.C., M.B., V.C., K.N.C. and H.G. performed experiments and analyzed data; Y.H.Q., A.M. and S.M.K. provided and characterized patient samples or cell lines and clinical outcome data; H.W. provided important reagents and mouse samples; M.D.v.M., T.E., A.H., G.C., S.M.K., T.G.G. and H.J. provided conceptual input to the design of the study.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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AUTHOR CONTRIBUTIONS
S.S. and M.M. designed experiments and interpreted data; M.M. conceived the study, obtained funding, coordinated collaborations and wrote the paper; S.S., L.N.C., M.B., V.C., K.N.C. and H.G. performed experiments and analyzed data; Y.H.Q., A.M. and S.M.K. provided and characterized patient samples or cell lines and clinical outcome data; H.W. provided important reagents and mouse samples; M.D.v.M., T.E., A.H., G.C., S.M.K., T.G.G. and H.J. provided conceptual input to the design of the study.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.
ONLINE METHODS

Patient samples, human cells and cell lines. We obtained primary patient samples (Supplementary Table 1) in compliance with the Institutional Review Board of the University of California, San Francisco. Human leukemia cells were maintained in Minimum Essential Medium alpha (MEM-α, Invitrogen, Carlsbad, CA) supplemented with GluMAX, 20% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin and incubated at 37°C in a humidified incubator with 5% CO₂. We obtained informed consent from all participants. Primary human ALL cells (patient samples) were cultured on irradiated OP9 stromal cells (American Type Culture Collection, Manassas, VA) in the same medium as described above.

Retroviral transduction. A complete list of vectors used in this study is provided in Supplementary Table 2. Transfections of the murine stem cell virus (MSCV)-based retroviral constructs were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with Opti-MEM medium (Invitrogen). Retroviral supernatant was produced by cotransfecting 293FT cells (DSMZ, Germany) with the plasmids pHT60 (gag-pol) and pHIT123 (ecotropic env). Cultivation was performed in high-glucose Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) with GluMAX containing 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 mM HepES, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. Regular medium were replaced after 16 h by growth medium containing 10 mM sodium butyrate. After an 8-h incubation, the medium was changed back to regular growth medium. 24 h later, the virus supernatant was harvested, filtered through a 0.45-μm filter and loaded by centrifugation at 600 g for 30 min and were maintained for 72 h at 37°C with 5% CO₂ before transfer into culture flasks.

Preparation of bone marrow cells from mice. A complete list of mice used in this study is provided in Supplementary Table 3. All mouse experiments were subject to institutional approval by the University of California San Francisco Institutional Animal Care and Use Committee. Bone marrow cells were extracted from mice younger than 6 weeks of age. Bone marrow cells were obtained by flushing the 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), the washed cells were either frozen for storage (in 10% DMSO, 70% PBS, 30% methylcellulose) or transduced per well by centrifugation at 600 g for 30 min and were maintained for 72 h at 37°C with 5% CO₂ before transfer into culture flasks.

BCR-ABL1–driven mouse model of pre-B ALL and myeloid leukemia. Bone marrow cells from Ptenfl/fl (n = 3, female) and Ptenfl/+ (n = 2, female) mice (Supplementary Table 3) were collected and retrovirally transformed with a vector expressing BCR-ABL1. For B cell lineage leukemias, transduction was performed after culturing bone marrow cells with IL-7 (10 ng/ml) for 1 week. To generate myeloid lineage leukemias, bone marrow cells were cultured with 10 ng/ml recombinant mouse IL-3, 25 ng/ml recombinant mouse IL-6, and 50 ng/ml recombinant mouse stem cell factor (SCF) for 1 week (PeproTech). This treatment leads to selection of hematopoietic progenitor cells (Lin−Sca−1−c-Kit+) (LSK) cells. Transformation of precursor B cells and LSK cells with the BCR-ABL1 construct generates pre-B ALL and CML-like models, respectively. After BCR-ABL1 transduction, all cytokines were removed from the culture to selectively select the nontransduced cells. Both ALL and CML cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM, Invitrogen) supplemented with GluMAX, 20% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol. BCR-ABL1–transformed ALL cells were propagated only for short periods of time and usually not longer than 2 months to avoid acquisition of additional genetic lesions during long-term cell culture.

In vivo transplantation of leukemia cells. For in vivo experiments, mouse ALL cells were labeled with retroviral firefly luciferase and selected based on antibiotic resistance (blasticidin). The mouse ALL cells were then injected via the tail vein into sublethally irradiated (300 Cgy) NOD-SCID recipient mice. Engraftment was monitored using luciferase bioimaging (VIS 100 bioluminescence/optical imaging system; Xenogen). α-luciferin (Xenogen), dissolved in PBS, was injected intraperitoneally at a dose of 2.5 mg/mouse 15 min before measuring light emission. 6- to 8-week-old female NOD-SCID mice (Jackson Laboratories, ME) were randomly allocated into each treatment group. The minimal number of mice in each group was calculated by using the ‘cowper’ function in the R/Hmisc package. No blinding was used.

C/EBPα–mediated reprogramming of pre-B cells into myeloid cells. The tetracycline operon–CCAAAT/enhancer binding protein alpha–green fluorescent protein (Teto/C/EBPα-GFP) construct was generated as a modification of the previously published Teto–C/EBPα–tdTomato lentivirus27. Ptenfl/+ pre-B ALL cells were first transduced with a construct expressing the tetracycline-regulated transcriptional transactivator (rtTA), selected for puromycin resistance and subsequently infected with the Teto–C/EBPα construct. C/EBPα was induced in the Ptenfl/+ pre-B ALL cells by addition of 2 μg/ml doxycycline (Sigma-Aldrich). Staining for cell surface antigens was done with directly conjugated antibodies against CD11b (phycocerythrin (PE)-conjugated; Mac1) and CD19 (PerCP-Cy5.5–conjugated) (EBioscience). To measure the effects of Pten deletion in C/EBPα–reprogrammed pre-B ALL cells, deletion was induced with a tamoxifen-inducible Cre or an empty vector control (0.5 μmol/L). The nuclear stain DAPI and 7-aminocinoinolactone D (7-AAD) at 1 μg/ml were used as viability markers.

Array-based promoter methylation analysis using HELP. The HELP (HpaII tiny-fragment enrichment by ligation-mediated PCR) assay was performed as previously described35. One microgram of high-molecular-weight DNA was digested overnight with isoschizomer enzymes HpaII andMspI, respectively (New England BioLabs (NEB), Ipswich, MA). DNA fragments were purified using phenol-chloroform, resuspended in 10 mM Tris-HCl pH 8.0, and used immediately to set up the ligation reaction with MspI-HpaII–compatible adapters and T4 DNA ligase. Ligation-mediated PCR was performed with enrichment for the 200- to 2,000-bp products, and the products were then submitted for labeling and hybridization onto a human HG_17 promoter custom-designed oligonucleotide array covering 25,626 HpaII-amplifiable fragments within the promoters of the genes. For analysis, raw data (.pair) files were generated using NimbleScan software. Signal intensities at each HpaII-amplifiable fragment were calculated as a robust (25% trimmed) mean of their component probe-level signal intensities. Any fragments found within the level of the background Msp signal intensity, measured as 2.5 mean absolute deviation (MAD) above the median of random probe signals, were considered to be ‘failed’ probes and were removed. A median normalization was performed on each array by subtracting the median log-ratio (HpaII/MspI) of that array (resulting in a median log-ratio of 0 for each array).

Western blotting. Cells were lysed in Celllytic buffer (Sigma, St. Louis, MO) supplemented with phenylmethylsulfonyl fluoride (PMSF), phosphatase and protease inhibitor cocktail (Pierce, Rockford, IL). 15 μg of protein mixture per sample was separated on NuPAGE (Invitrogen, Carlsbad, CA) 4–12% Bis-Tris gradient gels and transferred to PVDF membranes (Immobilon, Millipore, Temecula, CA). For the detection of mouse and human proteins by western blotting, primary antibodies were used together with the WesternBreeze immunodetection system (Invitrogen). Details of the antibodies used are in Supplementary Table 4.

Flow cytometry. Antibodies used for flow cytometry experiments are listed in Supplementary Table 4. For apoptosis analyses, annexin V, propidium iodide and 7-AAD (BD Biosciences) were used. A Fortessa scanner was used for flow cytometry, and FlowJo v.10 was used for data analysis.

Senescence-associated β-galactosidase assay. Senescence-associated β-galactosidase activity was performed on cytosin preparations. Briefly, a fixation solution (0.25% glutaraldehyde, 2% paraformaldehyde in PBS (pH 5.5 for mouse cells) was prepared fresh. 1 g paraformaldehyde was dissolved in 50 ml PBS at pH 5.5 by heating followed by addition of 250 μl of a 50% glutaraldehyde stock solution. 1× X-gal staining solution (10 ml) was prepared as follows: 9.3 ml PBS-MgCl₂, 0.5 ml 20× KC solution (820 mg K₂Fe(CN)₃ and 1,050 mg K₃Fe(CN)₆·3H₂O in 25 ml PBS) and 0.25 ml 40× X-gal (40 mg 5-bromo-4-chloro-3-indolyl β-D-galactoside per ml of NaN₃-dimethyformamide) solutions

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were mixed. 200,000 cells per cytospin were used (700 r.p.m., 8 min). The fixative solution was pipetted onto cytospins and incubated for 10 min at room temperature, then washed twice for 5 min in PBS-MgCl2. Cytospin preparations were submerged in 1× X-gal solution, incubated overnight at 37 °C in a humidified chamber and washed twice in PBS. Images were acquired using regular microscopy, and the percentage of stained cells was calculated by counting.

**Colony-forming assay.** Methylcellulose colony-forming assays were performed with 10,000 cells after transformation with Cre-ER<sup>12</sup> or ER<sup>2</sup> and 2 d of treatment with 0.5 μmol/liter tamoxifen. Cells were resuspended in murine MethoCult medium (StemCell Technologies, Vancouver, BC, Canada) and cultured on dishes (3 cm in diameter) with an extra water supply dish to prevent evaporation. After 7–14 d, the total number of colonies per plate was counted by microscopy.

**Cell cycle analysis.** For cell cycle analysis, the BrdU flow cytometry kit for cell cycle analysis (BD Biosciences) was used according to manufacturer's instructions. 5-bromo-2′-deoxyuridine (BrdU) incorporation (APC-labeled anti-BrdU antibodies) was measured along with DNA content (using 7-AAD) in fixed and permeabilized cells. The analysis was gated on viable cells that were identified based on scatter morphology.

**Cell viability assay.** 100,000 cells per well were seeded in a volume of 100 μl of medium in OptiX 96-well plates (BD Biosciences, San Jose, CA). Inhibitors were diluted in medium and added at the indicated concentration in a total culture volume of 120 μl. After 3 d in culture, 12 μl of Resazurin (R&D, Minneapolis, MN) was added to each well, and the plates were incubated for 4 h at 37 °C. The fluorescent signal was monitored using 535-nm excitation wavelength and 590-nm emission wavelength. Fold changes were calculated using baseline values of untreated cells as a reference (set to 100%). Each sample was measured in triplicate.

**RNA purification and expression analysis.** Total RNA was purified using the RNeasy kit (Qiagen, Valencia, CA). RNA quality was checked by using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). cDNA was generated from 5 μg of total RNA using a poly(dT) oligonucleotide and the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Biotinylated cRNA was generated and fragmented according to the Affymetrix protocol and hybridized to mouse Gene 1.0 ST (Affymetrix, High Wycombe, UK). 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. The GEO accession number for the arrays is GSE43482.

**Proteomic profiling.** Proteomic profiling was performed using reverse-phase protein array (RPPA) analysis<sup>23</sup> on peripheral blood and bone marrow specimens from 192 patients with acute lymphocytic leukemia (ALL), including 192 samples obtained at diagnosis and 12 paired diagnosis-relapse samples, that were evaluated at the University of Texas M.D. Anderson Cancer Center (MDACC) between 1983 and 2007 (two from the 1980s, 45 from the 1990s). Samples were acquired during routine diagnostic assessments in accordance with the regulations and protocols (Lab 01-473 after September 2001, prior protocols for samples before then) approved by the Investigational Review Board (IRB) of MDACC. Informed consent was obtained in accordance with Declaration of Helsinki. Samples were analyzed under an IRB-approved laboratory protocol (Lab 05-0654). Samples were selected for inclusion based on availability in the MDACC Leukemia Sample Bank. Patients aged 15 or over were included; another eight pediatric ALL cases were on the array but are not included in this analysis. Samples underwent Ficoll separation to yield a mononuclear fraction. The samples were normalized to a concentration of 1 × 10<sup>4</sup> cells/μl and a whole-cell lysate was prepared as previously described.<sup>24</sup> All protein samples were prepared from fresh cells on the day of collection. Of the 192 newly diagnosed ALL cases, most were treated with the hyper CVAD (cyclophosphamide, vincristine, adriamycin and dexamethasone) regimen either alone (n = 91) or in combination with rituximab (n = 31), a tyrosine kinase inhibitor (n = 28) or nelarabine (n = 3). A modification of the Berlin-Frankfurt-Mannheim regimen was used in 23 cases. The remaining 16 cases received miscellaneous combination regimens that were in use over this time period.

**Glucose, lactate and ATP measurements.** Glucose and lactate levels were measured using the Amplex Red Glucose/Glucose Oxidase Assay Kit (Invitrogen) and the t-Lactate Assay Kit (Cayman Chemical), respectively, according to the manufacturers’ protocols. Glucose and lactate concentrations were measured in fresh and spent medium. Total ATP levels were measured using the ATP Bioluminescence Assay Kit CLS II (Roche) according to the manufacturer’s protocol. 10<sup>6</sup> cells/ml were seeded in fresh medium and treated as indicated in the figure legends. The relative levels of glucose consumed and lactate produced, and the total amount of ATP, are shown, and all values were normalized to the number of viable cells. Inhibitors used in this study are listed in Supplementary Table 5.

**Reconstitution model for pre-BCR signaling.** Pre-B cells from Blnk<sup>−/−</sup>Rag2<sup>−/−</sup> Igll1<sup>−/−</sup> triple-knockout transgenic mice<sup>25</sup> were engineered to express a non-autoreactive pre-BCR (μ-HC<sup>WA</sup>), an autoreactive pre-BCR (μ-HC<sup>AM</sup>) or an empty-vector control (EV). The cells were transduced with a tamoxifen-inducible (Blnk-ER) form of the pre-BCR linker molecule Blnk (also known as SLP-65). Addition of tamoxifen (Tam) releases Blnk from its complex with heat-shock protein (HSP) 90 and allows for assembly of the pre-BCR signaling complex within minutes.

**Statistical analysis.** Unpaired, two-tailed Student’s t-tests were used to compare colony number, S phase percentage and frequency of cellular senescence between different groups. A two-sided Mann-Whitney-Wilcoxon test was used to compare methylation values of ALL versus normal pre-B or B-cell lymphoma groups and RPPA data of newly diagnosed adult ALL versus T cell lineage ALL or mature B cell lymphoma, using R version 2.14.0. The Mantel-Cox log-rank test (two-sided) was used to compare survival data between different groups. R package ‘survival’ version 2.35-8 was used for the survival analysis.

35. Khulan, B. et al. Comparative isoschizomer profiling of cytosine methylation: the HELP assay. Genome Res. 16, 1046–1055 (2006).