Pre–B cell receptor–mediated cell cycle arrest in Philadelphia chromosome–positive acute lymphoblastic leukemia requires *IKAROS* function

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B cell lineage acute lymphoblastic leukemia (ALL) arises in virtually all cases from B cell precursors that are arrested at pre–B cell receptor–dependent stages. The Philadelphia chromosome–positive (Ph$^+$) subtype of ALL accounts for 25–30% of cases of adult ALL, has the most unfavorable clinical outcome among all ALL subtypes and is defined by the oncogenic BCR-ABL1 kinase and deletions of the *IKAROS* gene in >80% of cases. Here, we demonstrate that the pre–B cell receptor functions as a tumor suppressor upstream of *IKAROS* through induction of cell cycle arrest in Ph$^+$ ALL cells. Pre–B cell receptor–mediated cell cycle arrest in Ph$^+$ ALL cells critically depends on *IKAROS* function, and is reversed by coexpression of the dominant-negative *IKAROS* splice variant IK6. *IKAROS* also promotes tumor suppression through cooperation with downstream molecules of the pre–B cell receptor signaling pathway, even if expression of the pre–B cell receptor itself is compromised. In this case, *IKAROS* redirects oncogenic BCR-ABL1 tyrosine kinase signaling from SRC kinase-activation to SLP65, which functions as a critical tumor suppressor downstream of the pre–B cell receptor. These findings provide a rationale for the surprisingly high frequency of *IKAROS* deletions in Ph$^+$ ALL and identify *IKAROS*-mediated cell cycle exit as the endpoint of an emerging pathway of pre–B cell receptor–mediated tumor suppression.

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Abbreviations used: ALL, acute lymphoblastic leukemia; DN, dominant-negative; IGHM, Ig μ heavy chain; mRNA, messenger RNA; Ph, Philadelphia chromosome; Tet, tetracycline; tTA, Tet-controlled transactivatore.

The pre–B cell receptor promotes differentiation and proliferation signals in B cell precursor cells within the bone marrow (Rolink et al., 2000). It consists of an *Ig μ heavy chain* (μ chain; *IGHM*) coupled to the surrogate light chain with its two components, VpreB (*VPREB1*) and λ5 (*IGLL1*), which promote constitutive pre–B cell receptor signaling (van Loo et al., 2007; Fig. S1). Productive rearrangement of Ig V_H to DJ_H gene segments is a prerequisite for the expression of a functional μ chain, which is linked to the Igα and Igβ transmembrane signaling chains (Guo et al., 2000). The key components of the proximal pre–B cell receptor signaling cascade are assembled and stabilized by the linker protein

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SLP65 (or BLNK, BASH; Fu et al., 1998; Hayashi et al., 2000; Fig. S1). In the absence of SLP65, the function of the pre–B cell receptor is compromised and SLP65-deficient B cell precursors are arrested at the pre–B cell stage (Jumaa et al., 1999). In virtually all cases, B cell lineage acute lymphoblastic leukemia (ALL) is derived from B cell precursors that are arrested at pre–B cell receptor–dependent stages of development. Among ALL in adults, the Philadelphia chromosome (Ph) encoding the oncogenic BCR-ABL1 tyrosine kinase, represents the most frequent cytogenetic abnormality (~25–30% of cases) and defines the ALL subset with the most unfavorable prognosis (Mancini et al., 2005). Ph^+ ALL is also characterized by deletions of the *IKZF1* (*IKAROS*) gene that are found in >80% of Ph^+ ALL cases (Mullighan et al., 2008). *IKZF1* deletions typically lead to the expression of dominant-negative IKAROS variants (e.g., IK6) that are characterized by loss of N-terminal zinc fingers that mediate DNA binding, whereas the C-terminal dimerization domain is retained (Klein et al., 2006; Iacobucci et al., 2008; Reynaud et al., 2008). Based on a previous study of 12 cases of Ph^+ ALL, our group described inactivation of the pre–B cell receptor in Ph^+ ALL based on nonfunctional *IGHM* gene rearrangements (Klein et al., 2004) and down-regulation of pre–B cell receptor–related signaling molecules (Klein et al., 2004, 2006). Here, we confirm these observations based on 57 cases of human Ph^+ ALL as compared with normal pre–B cells and 54 cases of Ph^- ALL and elucidate the mechanism of pre–B cell receptor–mediated tumor suppression in Ph^+ ALL.

RESULTS

Ph^+ ALL clones are selected against expression of a functional pre–B cell receptor

To investigate the role of the pre–B cell receptor in Ph^+ ALL, we studied the configuration of the *IGHM* locus in sorted normal human B cell precursor cells by single-cell PCR, and in 54 cases of Ph^- and 57 cases of Ph^+ ALL. The frequency of normal human B cell precursors lacking coding capacity for a μ chain decreased from 41% in pro–B ($CD19^+$ $CD34^+$) to 13% in pre–B ($CD19^+$ $VpreB^+$) and to 12% in immature B cells ($CD10^+$ $CD20^+$). Because pre–B cell receptor selection represents an ongoing process, it is possible that some $CD19^+$ $VpreB^+$ and $CD10^+$ $CD20^+$ cells were viably sorted even though these cells lacked coding capacity for a μ chain and were therefore destined to die. In addition, in some cells, a second productively rearranged *IGHM* allele may have been missed in our single-cell PCR analysis. Compared with random distribution of nonfunctional *IGHM* alleles (calculated based on the statistical model described in Table S1), we found evidence for positive selection of functional *IGHM* alleles in pre–B cells ($P = 0.03$) and immature B cells ($P = 0.01$; green asterisks, Fig. 1 A).

In contrast, 47 of 57 (83%) of patient-derived Ph^+ ALL cases carried only nonfunctional *IGHM* V_HDJ_H gene rearrangements (Fig. 1 A and Table S1). Ph^+ ALL cases are selected against expression of a functional *IGHM* gene rearrangement ($P = 0.01$; red asterisk, Fig. 1 A). Negative selection of pre–B cell receptor expression is specific for Ph^+ ALL because in a group of 54 cases of Ph^- ALL, including ALL carrying *E2A-PBX1* ($n = 8$), *TEL-AML1* ($n = 11$), or *MYC* ($n = 4$) gene rearrangements and ALL with hyperdiploid ($n = 18$) and normal karyotype ($n = 13$), no evidence for negative selection against functional *IGHM* alleles was found (Fig. 1 A).

Lack of pre–B cell receptor function in Ph^+ ALL cells

We next tested whether the pre–B cell receptor is functional in the few cases of Ph^+ ALL that harbor at least one productively rearranged *IGHM* allele. The function of the pre–B cell receptor was studied in 7 Ph^+ and 10 Ph^- ALL cell lines. As a control, we used bone marrow B cell precursor cells from four healthy donors. Engagement of the pre–B cell receptor using μ chain–specific antibodies resulted in a strong Ca^{2+} signal in normal pre–B cells, but none of the seven Ph^+ ALL cases (Fig. 1 B). Because normal bone marrow B cell precursors were only gated on the pan–B cell antigen CD19, we cannot exclude that IgM^+ immature B cells rather than μ chain$^+$ pre–B cells responded to μ chain/IgM engagement. For this reason, we also tested 10 Ph^- ALL cases, 7 of which showed a strong Ca^{2+} signal in response to pre–B cell receptor engagement (Fig. 1 B). We conclude that even in the few cases in which a μ chain can be expressed, the Ph^+ ALL cells lack pre–B cell receptor function, indicating that pre–B cell receptor function is compromised in most, if not all, cases of Ph^+ ALL.

Down-regulation of pre–B cell receptor–related signaling molecules in Ph^+ ALL

To investigate expression levels of pre–B cell receptor–related signaling molecules in human Ph^+ ALL cells, we performed a metaanalysis of published gene expression data of 15 cases of Ph^+ ALL (Ross et al., 2003) and normal human B cell precursors (van Zelm et al., 2005), as described in Fig. S2. Interestingly, a group of pre–B cell receptor–related genes including *SYK*, *SLP65*, *IGLL1*, *VPREB1* (surrogate light chain), *IGHM* (μ chain), *CD79B* (Igβ signaling chain), and *IRF4* are consistently down-regulated in Ph^+ ALL as compared with normal human B cell precursors (Fig. 1 C, left). Analysis of four Ph^+ ALL cell lines (BV173, Nalm1, SUP-B15, and TOM1; Fig. 1 C, middle) that were cultured in the presence or absence of STI571 (Imatinib) showed that inhibition of the BCR-ABL1 kinase restored normal messenger RNA (mRNA) levels of *SYK*, *SLP65*, *IGLL1*, *VPREB1*, *IGHM*, and *IRF4*.

Genomic deletions of pre–B cell receptor–related genes in Ph^+ ALL

We next studied genomic deletions in the same set of pre–B cell receptor–related genes using the 250 *Nsp*I SNP-chip platform and found deletions in at least one of these genes in all 22 Ph^+ ALL cases analyzed. We detected *VPREB1* and *IGLL1* deletions in 5 of 22 cases and deletions in the *SYK*, *SLP65*, *CD79B*, *IRF4*, and *BTLA* gene at least once (Fig. 1 C, right). With the exception of *VPREB1* and *IGLL1*, all these deletions were heterozygous, so their functional significance

remains to be investigated. Previous studies showed that heterozygous deletions of *PAX5* lead to haploinsufficiency (Mullighan et al., 2007; Kawamata et al., 2008) and to the expression of dominant-negative mutants as in the case of *IKAROS* (Mullighan et al., 2008). Deletions of the *VPREB1* gene encoding part of the pre–B cell receptor surrogate light chain were subsequently verified by FISH analysis and quantitative genomic PCR (Fig. S3). 3 monoallelic and 2 biallelic

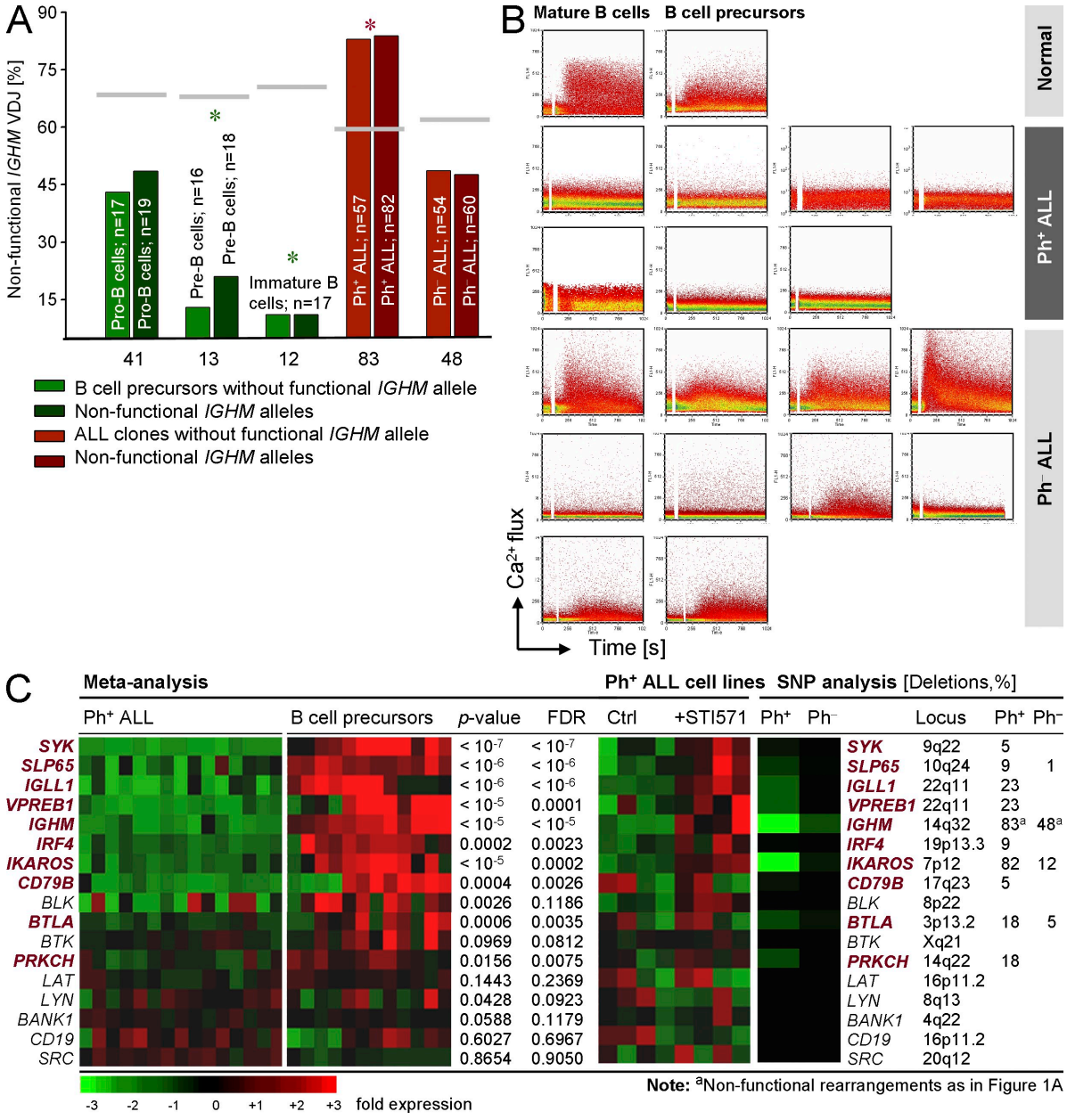

Figure 1. Pre–B cell receptor function in normal human B cell precursors and Ph+ ALL. The configuration of the Ig heavy chain (*IGHM*) locus was studied in bone marrow pro–B cells, pre–B cells, immature B cells, 57 cases of Ph+ ALL, and 54 cases of Ph-negative ALL (A). The frequencies of cells/ALL clones lacking a functional *IGHM* gene rearrangement (light bars) and the total frequency of nonfunctional *IGHM* alleles in these populations (dark bars) are shown. The expected frequencies of cells/ ALL clones lacking coding capacity for a pre–B cell receptor based on random distribution (Table S1) are indicated as horizontal gray lines. Asterisks denote significant differences from random distribution ($P < 0.05$). Ca^{2+} mobilization in response to pre–B cell receptor engagement was studied in normal B and pre–B cells, 7 cases of Ph+ ALL and 10 Ph-negative ALL (B). A metaanalysis of published gene expression data for pre–B cell receptor–related genes in 15 cases of Ph+ ALL and normal human B cell precursors was performed (C, left). P values and false discovery rates (FDR) are indicated. Ph+ ALL cell lines (BV173, Nalm1, SUP-B15, and TOM1) were cultured in the presence or absence of 10 μmol/STI571 (Imatinib) for 16 h and analyzed by Affymetrix U133A2.0 microarrays (C, middle). 22 cases of Ph+ ALL were analyzed by a SNP mapping assay (C, right). The frequency of deletions is given in percent. The frequencies of deletions in our dataset of 22 cases of Ph+ ALL are plotted against the frequencies of deletions found in Ph− ALL (Mullighan et al., 2007).

deletions of the *VPREB1* locus were identified in 22 cases of Ph^+ ALL, but not in any of 22 Ph^- ALL cases ($P < 0.05$). We conclude that deletions in pre–B cell receptor–related genes represent a novel and frequent feature of Ph^+ ALL.

Pre–B cell receptor function during leukemic transformation in *BCR-ABL1* transgenic mice

To investigate the function of the pre–B cell receptor during progressive leukemic transformation in vivo, we studied B cell precursors in *BCR-ABL1* transgenic mice (Heisterkamp et al., 1990) at different stages of malignant transformation. B cell precursors of (a) wild-type littermates and (b) *BCR-ABL1* transgenic mice at <60 d of age in the absence of any indication of leukemia (preleukemic), (c) mice with full-blown leukemia (>90 d of age) before and (d) after 7 d of treatment with the BCR-ABL1 kinase inhibitor AMN107 (Nilotinib; Table S2). Interestingly, *BCR-ABL1* transgenic pre–B cells represent a normal polyclonal population before the onset of leukemia, but mainly belong to one single clone in full-blown leukemia (Fig. 2 A). Treatment with AMN107 eradicates the dominant leukemia clone and restores a normal polyclonal pre–B cell repertoire. B cell development in *BCR-ABL1* transgenic mice with overt leukemia was arrested at a pre–B cell receptor–negative stage of development (Fig. 2 B). As opposed to wild-type and preleukemic B cells, B lymphoid cells in full-blown leukemia did not express Ig μ-heavy chains (Fig. 2 B). Treatment of the leukemic mice with AMN107, however, restored phenotypically normal B cell differentiation (Fig. 2 B). Ca^{2+} release in response to pre–B cell receptor engagement was compromised in mice with full-blown leukemia, yet restored by treatment of leukemic mice with AMN107 (Fig. 2 C).

mRNA levels of pre–B cell receptor–related signaling molecules were studied in sorted $CD19^+$ $AA4.1^+$ bone marrow pre–B cell populations from wild-type mice and *BCR-ABL1* transgenic mice before and after leukemic transformation and after 7 d of treatment with AMN107. Although mRNA levels for most pre–B cell receptor–related molecules were similar in wild-type and "preleukemic" *BCR-ABL1* transgenic mice, a subset of these genes (including *Ighm*, *Slp65*, and *Ikaros*) was substantially down-regulated upon full leukemic transformation, as determined by GeneChip analysis (Fig. 2 D) and quantitative RT-PCR validation (Fig. 2 E). Together, these results suggest that active signaling from a functional pre–B cell receptor and the BCR-ABL1 kinase are mutually exclusive.

Reconstitution of pre–B cell receptor signaling in *Slp65*$^{-/-}$ and *Ighm*$^{-/-}$ *BCR-ABL1*–transformed pre–B ALL cells suppresses leukemic growth

To test this hypothesis in a formal experiment, we reconstituted pre–B cell receptor signaling in *BCR-ABL1*–transformed bone marrow B cell precursors from mice carrying pre–B cell receptor defects. To this end, we transformed bone marrow B cell precursors from *Slp65*$^{-/-}$ and *Ighm*$^{-/-}$ mice with retroviral BCR-ABL1 (Fig. 3). These mice lack expression of the pre–B cell receptor linker molecule Slp65 (Flemming et al., 2003) or fail to express a μ chain because of deletion of the Cμ transmembrane domain. In one set of experiments, we reconstituted *Slp65* expression in *BCR-ABL1*–transformed *Slp65*$^{-/-}$ pre–B ALL cells either constitutively (Fig. 3 A) or inducibly (Fig. 3 B; Meixlsperger et al., 2007). In both systems, we observed selective depletion of *Slp65*-reconstituted *BCR-ABL1* ALL cells within 9 d after Slp65 reconstitution. We also studied one case of primary human Ph^+ ALL with *SLP65* deficiency (Jumaa et al., 2003), reconstitution of which had a similar effect (Fig. 3 C).

To investigate the consequences of pre–B cell receptor reconstitution in vivo, *Slp65*$^{-/-}$ *BCR-ABL1*–transformed pre–B cells were labeled with lentiviral firefly luciferase and transduced with Slp65-GFP or GFP alone. NOD/SCID mice were sublethally irradiated and injected with Slp65-GFP^+ or GFP^+ ALL cells. Starting 1 wk after tail vein injection, engraftment was monitored by bioluminescence imaging (Fig. 3 D). As opposed to mice injected with Slp65-GFP–transduced leukemia cells, the mice that were inoculated with GFP-transduced leukemia cells showed signs of terminal disease and weight loss 36 d after injection. At this time, all mice were sacrificed; mice injected with GFP-transduced, but not Slp65-GFP–transduced, *BCR-ABL1* ALL cells showed increased white blood cell counts and expansion of peripheral blood B cell precursors (Table S3). Compared with GFP-transduced leukemia cells, reconstitution of *Slp65* expression in the *Slp65*$^{-/-}$ *BCR-ABL1*–transformed pre–B ALL cells reduced the leukemia cell burden by ∼15-fold in the bone marrow, 5-fold in the spleen, and >100-fold in the peripheral blood (Fig. 3 E and Table S3). We conclude that inactivation of the pre–B cell receptor–related signaling molecule Slp65 not only represents a frequent feature in human ALL cells (Jumaa et al., 2003; Fig. 1 C), but also represents a requirement for leukemic growth of *BCR-ABL1*–transformed mouse pre–B cells in vivo.

In an alternative approach, we transduced *BCR-ABL1*–transformed *Ighm*$^{-/-}$ ALL cells with a retroviral vector encoding a functional μ chain (*Ighm*) together with CD8 or CD8 alone (control). Enrichment or depletion of $\mu/CD8^+$ cells versus $CD8^+$ cells was monitored for 10 d by flow cytometry (Fig. 4 A). We observed progressive loss of $\mu/CD8^+$ cells, whereas the population of $CD8^+$ cells remained stable. We next reconstituted μ chain expression in a primary case of human Ph^+ ALL, in which both *IGHM* alleles were nonproductively rearranged. Also in this case, reconstitution of μ chain expression rapidly suppressed leukemic growth in primary human Ph^+ ALL cells (Fig. 4 B). We conclude that reconstitution of pre–B cell receptor signaling effectively prevents leukemic growth of human Ph^+ ALL.

Mechanism of pre–B cell receptor–induced suppression of leukemic growth

To clarify how reconstitution of pre–B cell receptor signaling in Ph^+ ALL cells suppresses leukemic growth, we tested the effect of pre–B cell receptor signaling on the survival and proliferation of leukemia cells (Fig. 4, C and D, and Fig. 5).

To this end, we reconstituted μ chain–negative *BCR-ABL1* leukemia cells either with a μ chain/CD8 expression vector or a CD8 empty vector control and measured cell death in μ chain/CD8$^+$ and CD8$^+$ cells (Fig. 4, C and D). μ Chain reconstitution resulted in a modest induction of cell death, and most of the leukemia cells remained viable 5 d after reconstitution (Fig. 4, C and D). It should be noted that this analysis was gated on μ chain/CD8$^+$ and CD8$^+$ cells, which underestimates the degree of cell death induction because dying cells lose μ chain/CD8 or CD8 surface expression. In a complementary system that allows for inducible activation of pre–B cell receptor signaling in all B cell precursors (Hess et al., 2001), we further studied the role of pre–B cell receptor signaling in *BCR-ABL1* leukemia cells. Such B cell

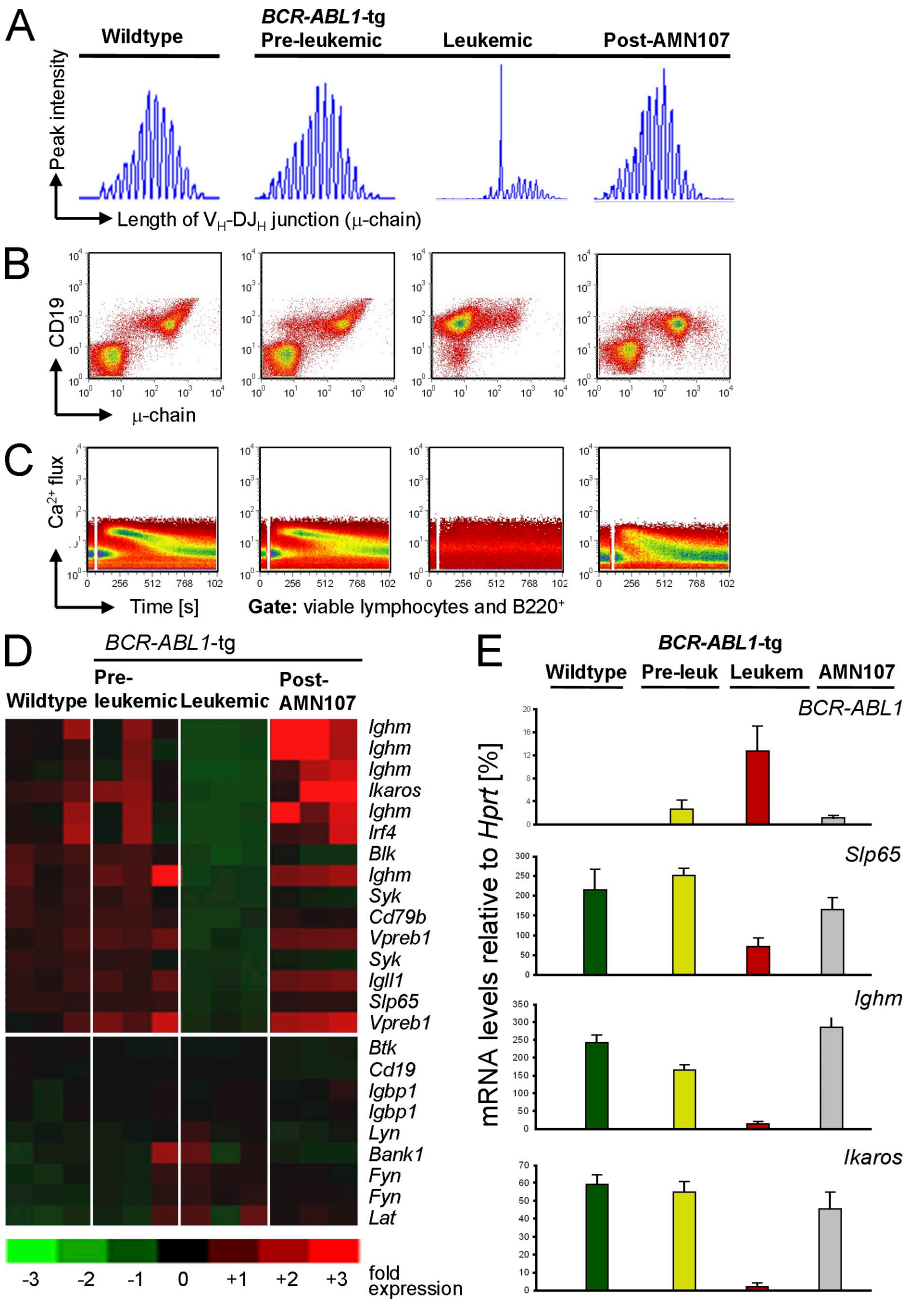

Figure 2. Progressive leukemic transformation of B cell precursors in *BCR-ABL1* transgenic mice. B cell precursors from wild-type and *BCR-ABL1* transgenic mice were analyzed for their clonality using spectratyping of Ig gene rearrangements (A), μ chain expression (B), pre–B cell receptor responsiveness (C), and gene expression pattern (D), and RT-PCR validation (E; three experiments). Data in A–C are representative of results from six independent experiments with at least two mice per group. B cell precursor populations were compared from wild-type animals, preleukemic *BCR-ABL1* transgenic mice (age <60 d), *BCR-ABL1* transgenic mice with full-blown leukemia (age >90 d), and after treatment of mice with AMN107 (75 mg/kg/d) for 7 d.

precursors from *Rag2*$^{-/-}$ tetracycline (Tet)-controlled transactivator (tTA)/μ chain transgenic mice (Hess et al., 2001) were transformed with *BCR-ABL1*. *Rag2*$^{-/-}$ tTA/μ chain transgenic B cell precursors are unable to endogenously express a pre–B cell receptor, but carry a Tet-inducible μ chain gene (Fig. 5 A). We verified induction of μ chain expression in *Rag2*$^{-/-}$ tTA/μ chain transgenic *BCR-ABL1* ALL cells by flow cytometry (Fig. 5 A). Because μ chain expression in this system is regulated by endogenous transcriptional control elements, levels of μ chain expression are low, as typically observed in normal pre–B cells (Hess et al., 2001; Fig. 5 A). Withdrawal of Tet in *BCR-ABL1*–transformed Rag2$^{-/-}$ tTA/μ chain transgenic B cell precursors resulted in μ chain expression and subsequent suppression of leukemic growth in cell culture (Fig. 5, B and C), indicating that the tumor suppressor function of the pre–B cell receptor involves induction of cell cycle exit.

IKAROS function is required for the ability of the pre–B cell receptor to induce cell cycle arrest in Ph$^+$ ALL cells

We then focused our investigation of the mechanism of pre–B cell receptor–induced leukemia-suppression on the *IKAROS* transcription factor based on the following rationale. Two recent studies showed that pre–B cell receptor signaling in normal B cell precursors results in up-regulation of *IKAROS*, which subsequently induces cell cycle arrest (Thompson et al., 2007; Ma et al., 2008). In addition, a recent landmark study demonstrated that deletions of the *IKZF1* (*IKAROS*) gene represents a defining feature of Ph$^+$ ALL (deletions in >80% of the cases; Mullighan et al., 2008). Validation by quantitative RT-PCR confirmed previous findings (Thompson et al., 2007; Ma et al., 2008) that inducible pre–B cell receptor activation resulted in a 6–8-fold up-regulation of full-length *IKAROS* mRNA levels within 24 h (Fig. 6 A). Within 42 h after induction of pre–B cell receptor expression,

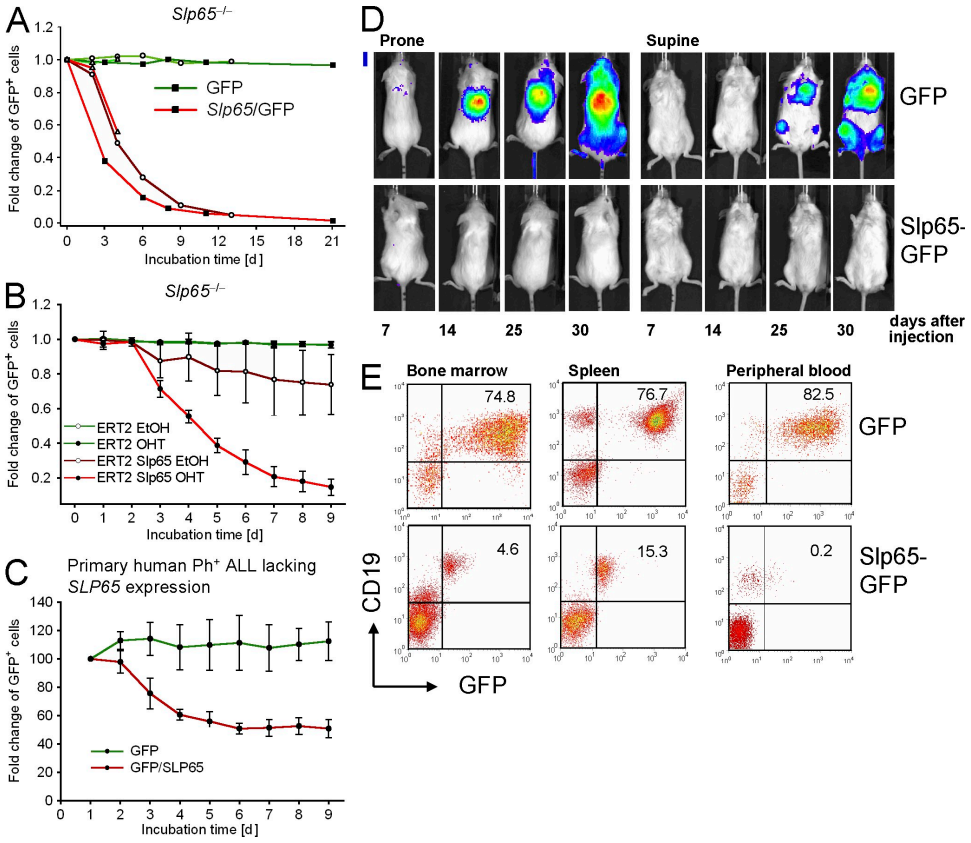

Figure 3. Reconstitution of Slp65 deficiency in *BCR-ABL1* ALL cells suppresses leukemic growth *BCR-ABL1*–transformed *Slp65*$^{-/-}$. ALL cells were reconstituted with *Slp65*/GFP or a GFP empty vector control (A; single measurements in three independent experiments). For inducible *Slp65* reconstitution, we fused *Slp65* to the estrogen receptor ligand-binding domain (ERT2). Either ERT2 fused to the N terminus of Slp65 (ERT2-Slp65/GFP) or ERT2 alone (ERT2/GFP) were expressed in the *Slp65*$^{-/-}$ *BCR-ABL1*–transformed ALL cells and activated by addition of 1 μmol/l 4-hydroxy-tamoxifen (OHT) using ethanol (EtOH) as vehicle control (B; triplicate measurements, the experiment was repeated once). Primary human leukemia cells from one case of Ph$^+$ ALL cells lacking expression of SLP65 were cultured on OP9 stroma in the presence of IL-7 and transduced with SLP65/GFP or a GFP empty vector control (three independent transductions) and monitored by flow cytometry (C). *BCR-ABL1*–transformed *Slp65*$^{-/-}$ ALL cells were labeled by lentiviral firefly luciferase, transduced with retroviral vectors encoding either Slp65/GFP or GFP alone and injected into five sublethally irradiated NOD/SCID mice per group. Engraftment and leukemic growth was monitored by luciferase-bioimaging (D; experiment repeated once). The blue scale bar in (D) corresponds to 1 cm in length. Leukemic infiltration (CD19$^+$ GFP$^+$) of bone marrow, spleen, and peripheral blood (three independent experiments) was documented by flow cytometry (E; representative data from two experiments).

close to 70% of viable *BCR-ABL1* ALL cells were arrested in G0/G1 (Fig. 5, B and C, and Fig. 6, B and C). To investigate whether *IKAROS* function is required for pre–B cell receptor–induced cell cycle arrest, we transduced *BCR-ABL1* ALL cells with IK6, a dominant-negative splice variant of *IKAROS* (Iacobucci et al., 2008; Klein et al., 2006; Reynaud et al., 2008). The IK6 splice variant neutralizes *IKAROS* function by forming nonfunctional heterodimers with full-length *IKAROS* (Reynaud et al., 2008) and is abundantly expressed in most if not all cases of Ph$^+$ ALL (Klein et al., 2006; Iacobucci et al., 2008; Mullighan et al., 2008). To test this hypothesis, pre–B cell receptor signaling was induced in *BCR-ABL1*–transformed *Rag2*$^{-/-}$ tTA/μ chain transgenic ALL cells that were transduced with the dominant-negative IK6 splice variant (IK6/GFP) or an empty vector control (GFP; Fig. 6, B–D). Whereas IK6/GFP-overexpression did not confer a significant

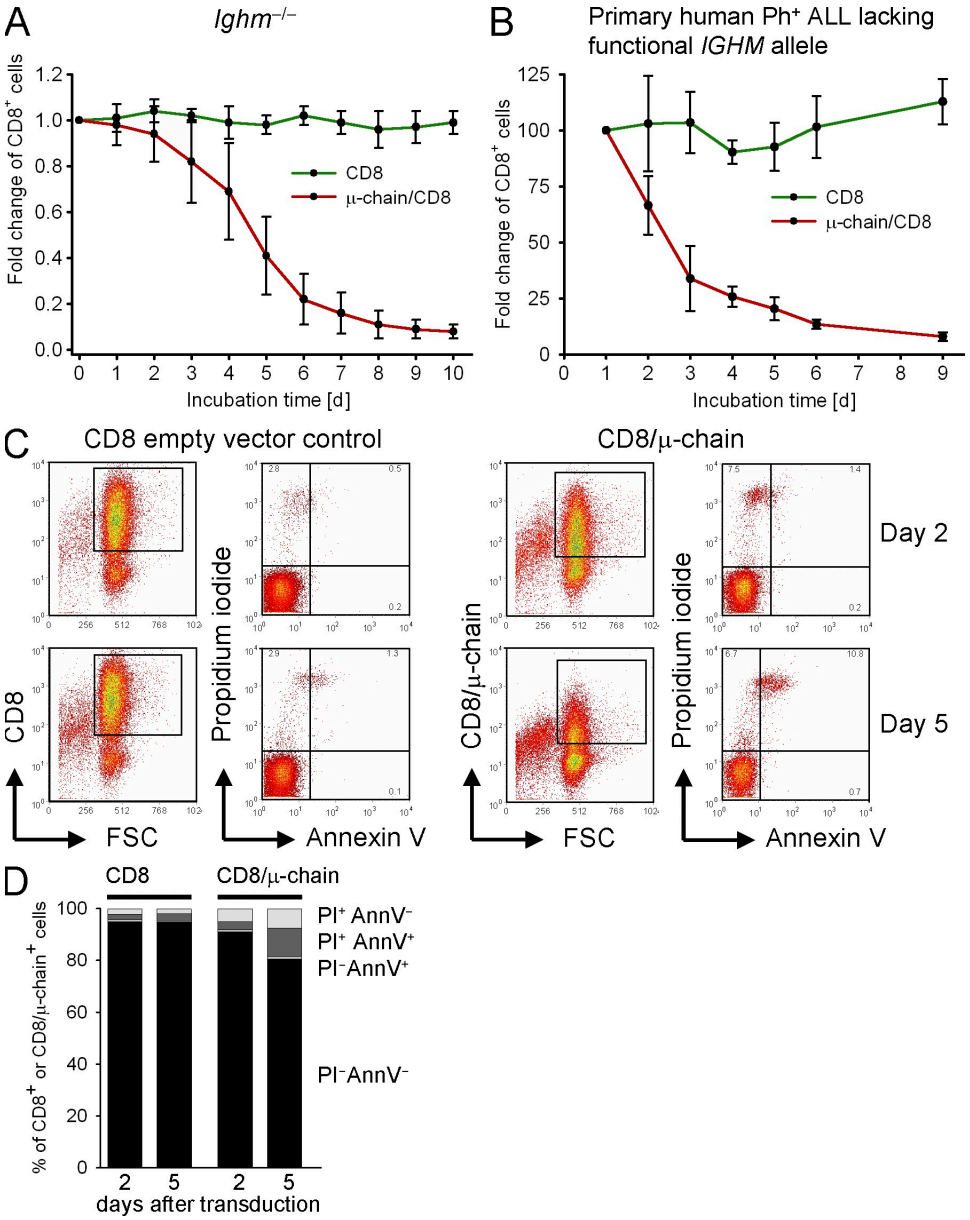

Figure 4. Reconstitution of μ chain expression results in leukemia suppression *BCR-ABL1*–transformed *Ighm*$^{-/-}$. ALL cells were transduced with retroviral expression vectors encoding either CD8 alone or CD8 with a functional μ heavy chain. Enrichment or depletion of CD8$^+$ and μ/CD8$^+$ cells was monitored over 10 d (A; three independent transductions). Primary human Ph$^+$ ALL cells lacking coding capacity for expression of a μ chain were cultured on OP9 stroma in the presence of IL-7 and transduced with μ chain/CD8 or CD8 alone and monitored by flow cytometry (B; three independent transductions). *BCR-ABL1* transgenic mouse B cell lineage leukemia cells lacking μ chain expression were transduced with retroviral expression vectors encoding either CD8 alone or CD8/μ chain (C and D). Percentages of Annexin V$^+$ and propidium iodide$^+$ cells were determined after 2 and 5 d of transduction and means of three experiments are indicated in (D).

growth advantage over GFP background levels in pre–B cell receptor–negative *BCR-ABL1*–transformed ALL cells, pre–B cell receptor induction resulted in an up to 25-fold increase of IK6/GFP-transduced leukemia cells (Fig. 6 D; a 5-fold increase was measured in one repeat experiment). Previous studies demonstrated that *IKAROS* negatively regulates the G1–S phase transition of the cell cycle in pre–B cells (Gómez-del Arco et al., 2004; Kathrein et al., 2005), mainly through histone H3 acetylation and up-regulation of negative cell cycle regulators, including *CDKN1B* (Kathrein et al., 2005; Fig. S1). Consistent with these findings, we observed that pre–B cell receptor/*IKAROS*-induced cell cycle arrest can be reversed by dominant-negative IK6 (Fig. 6, B and C).

IKAROS cooperates with SLP65 as tumor suppressor even in the absence of a pre–B cell receptor

If *IKAROS* function was indeed required for the ability of the pre–B cell receptor to induce cell cycle arrest in Ph$^+$ ALL, one would expect that presence or absence of *IKAROS* (*IKZF1*) deletions in Ph$^+$ ALL predicts sensitivity of these leukemia clones to reconstitution of pre–B cell receptor signaling. We reconstituted pre–B cell receptor signaling (SLP65 and μ chain) in three established human Ph$^+$ ALL clones carrying either wild-type *IKAROS* (*IKAROS*$^{+/+}$), a heterozygous deletion leading to the expression of dominant-negative *IKAROS* (*IKAROS*$^{+/DN}$), or biallelic deletions of *IKAROS* (large genomic deletion at 7p12 on one allele, and a small deletion leading to expression of dominant-negative *IKAROS* on the other (*IKAROS*$^{-/DN}$). Whereas *IKAROS*$^{+/+}$ Ph$^+$ ALL cells are sensitive to pre–B cell receptor reconstitution (SLP65 and μ chain), *IKAROS*$^{+/DN}$ and *IKAROS*$^{-/DN}$ Ph$^+$ ALL cells are largely resistant to pre–B cell receptor–induced leukemia suppression (Fig. 7 A). Retroviral expression of the functional *IKAROS* form IK1, however, induced cell cycle arrest in all three Ph$^+$ ALL cases (Fig. 7 A), even though leukemia cells in none of these cases expressed a functional pre–B cell receptor. These findings demonstrate that *IKAROS* function is required for pre–B cell receptor–mediated cell cycle arrest in pre–B cell–derived Ph$^+$ ALL cells. However, these observations also raise the question of how *IKAROS* can function as a tumor suppressor downstream of the pre–B cell receptor, if pre–B cell receptor signaling is inactivated in most, if not all, cases of Ph$^+$ ALL. Although none of the three Ph$^+$ ALL cases studied in Fig. 7 A express a functional pre–B cell receptor (Fig. 1 B), overexpression of the pre–B cell receptor downstream molecule SLP65 was sufficient to induce leukemia suppression, unless IKAROS function was compromised by genomic deletions (Fig. 7 A).

IKAROS redirects BCR-ABL1–mediated SRC-kinase activation to SLP65, a tumor suppressor downstream of the pre–B cell receptor

To elucidate how IKAROS intersects signaling from the pre–B cell receptor downstream molecules (e.g., SLP65), we studied how IKAROS reconstitution in IKAROS/pre–B

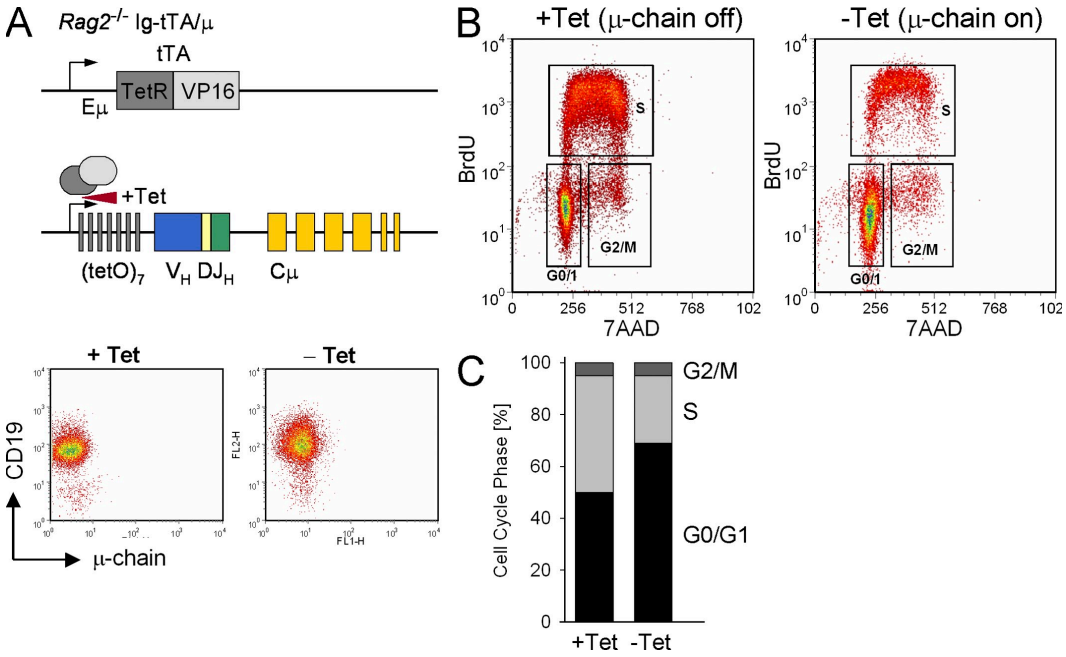

Figure 5. Reconstitution of pre–B cell receptor signaling in *BCR-ABL1* ALL cells induces cell cycle arrest. *Rag2*$^{-/-}$ tTA/μ chain transgenic mice are unable to express an endogenous μ chain but carry a functionally prerearranged μ chain under control of Tet operator (tetO) sequences. These mice express a tTA under control of endogenous μ chain regulatory elements and withdrawal of Tet results in activation of μ chain expression (A; routinely performed quality control). The effect of Tet-inducible activation of μ chain expression in *BCR-ABL1*–transformed ALL cells on leukemic growth was measured in a detailed cell cycle analysis (B-C). Percentages of cells in G0/G1, S and G2/M phases of the cell cycle were calculated and means of three experiments are indicated in C.

cell receptor double-deficient leukemia cells affects the activity of pre–B cell receptor downstream molecules (Fig. 7 B). Unlike other subtypes of ALL, Ph$^+$ ALL is driven by a constitutively active tyrosine kinase that is encoded by the *BCR-ABL1* oncogene. Therefore, tyrosine phosphorylation of pre–B cell receptor downstream molecules in Ph$^+$ ALL is not necessarily initiated from the pre–B cell receptor and may also originate from the oncogenic BCR-ABL1 kinase. Indeed, the oncogenic BCR-ABL1 tyrosine kinase in Ph$^+$ ALL phosphorylates downstream signaling components of the pre–B cell receptor, thereby mimicking survival and proliferation signals from the pre–B cell receptor (Feldhahn et al., 2005).

As shown by us and others, the pre–B cell receptor–related molecules LYN (Hu et al., 2004; Ptasznik et al., 2004), HCK, FGR (Hu et al., 2004), BTK (Bäckesjö et al., 2002; Feldhahn et al., 2005), and CBL (Sattler et al., 2002) are all tyrosine phosphorylated by BCR-ABL1. These molecules belong to the SRC kinase pathway downstream of the pre–B cell receptor, which mainly promotes survival and proliferation through activation of NF-κB (Saijo et al., 2003; Hendriks and Kersseboom, 2006). SRC kinase signaling is distinct from proximal pre–B cell receptor signaling components (e.g., SYK and SLP65; Hendriks and Kersseboom, 2006; Sprangers et al., 2006). For instance, SRC kinases, but not SYK and SLP65, are required for pre–B cell proliferation and NF-κB activation in pre–B cells (Saijo et al., 2003). Likewise, SRC kinase-driven pre–B cell proliferation is even increased in the absence of SLP65 (Flemming et al., 2003). Conversely, pre–B cell differentiation depends on SYK/SLP65, but not on SRC kinase signaling (Cheng et al., 1995; Jumaa et al., 1999; Flemming et al., 2003). SRC kinase and SYK/SLP65-dependent pathways intersect at the level of the ubiquitin ligase CBL. SRC kinases, including LYN, activate CBL, which then ubiquitinates SYK for proteasomal degradation (Ota and Samelson, 1997). Because activation of SLP65 through phosphorylation at SLP65-Y96 requires SYK (Fu et al., 1998), SRC kinase/CBL–induced degradation of SYK compromises SLP65-mediated pre–B cell differentiation (Song et al., 2007) and tumor suppression (Flemming et al., 2003; Hayashi et al., 2003; Fig. S1). In Ph$^+$ ALL, LYN, HCK, FGR, BTK, and CBL are all tyrosine-phosphorylated by BCR-ABL1, but not SYK and SLP65 (Hu et al., 2004; Ptasznik et al., 2004; Feldhahn et al., 2005; Fig. 7 B). Whereas the SRC kinase pathway is critical for the survival of Ph$^+$ ALL cells (Hu et al., 2004; Ptasznik et al.,

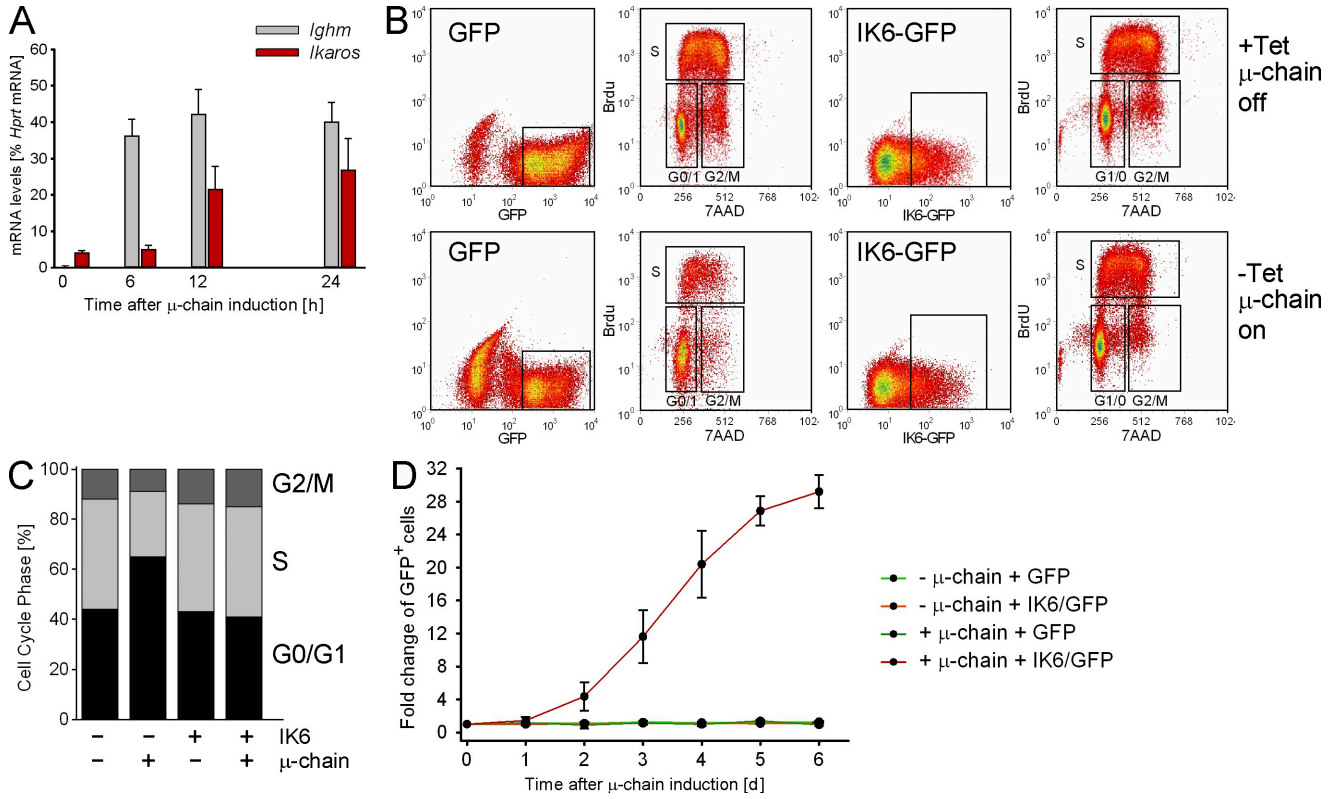

Figure 6. Pre–B cell receptor–mediated suppression of leukemic growth requires IKAROS function. mRNA levels of *Ikaros* after μ chain induction were measured by quantitative RT-PCR (A; three experiments). *BCR-ABL1*–transformed ALL cells were retrovirally transduced with the dominant-negative IKAROS splice variant IK6/GFP or a GFP empty vector control (B–D). Leukemia cells transduced with IK6/GFP (+IK6) or an empty vector control (-IK6) were studied in the presence or absence of pre–B cell receptor activation (+/− μ chain). IK6/GFP- and GFP-transduced leukemia cells were subjected to cell cycle analysis in the presence or absence of inducible pre–B cell receptor signaling (B–D). The percentages of cells in G0/G1, S and G2/M phase are indicated and means of three experiments are indicated in C. In D, the relative increase or decrease of IK6/GFP$^+$ versus GFP$^+$ leukemia cells in the presence or absence of pre–B cell receptor activation (± μ chain) was measured. The experiment was repeated once.

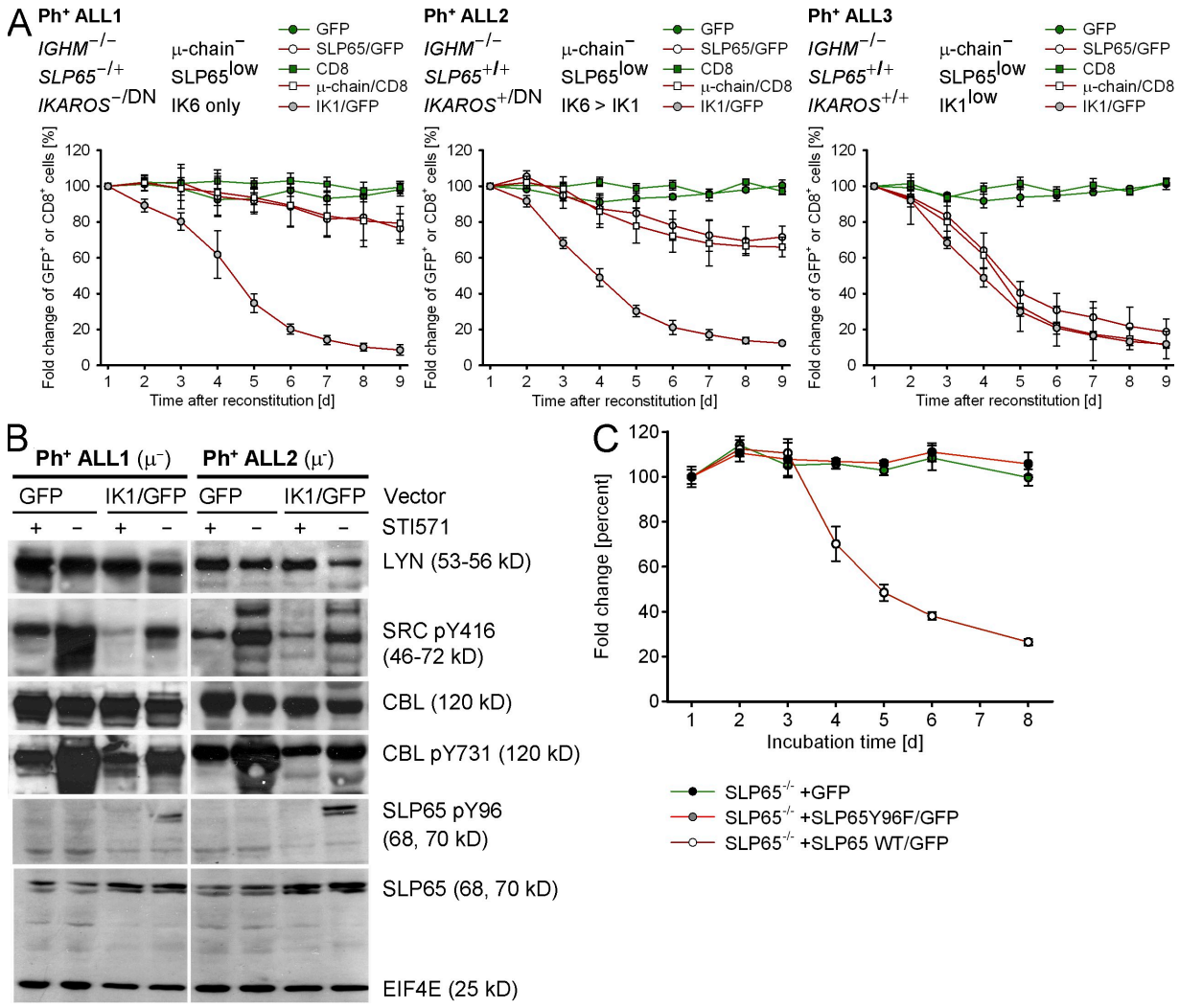

Figure 7. Reconstitution of IKAROS expression in Ph+ ALL cells results in BCR-ABL1-mediated activation of SLP65 even in the absence of a pre–B cell receptor. Genotype and expression of *IGHM* (μ chain), *SLP65*, and *IKAROS* (both functional IK1 and dominant-negative IK6 forms of *IKAROS*) were studied in three cases of Ph+ ALL. Ph+ ALL cells were transduced once in each case with retroviral vectors encoding SLP65/GFP, IKAROS (IK1)/GFP and μ chain/CD8 or GFP- and CD8-empty vector controls. Enrichment or depletion of GFP+ and CD8+ cells was monitored by flow cytometry (A; three cases were studied). IKAROS-deficient leukemia cells from Ph+ ALL cases 1 and 2 were transduced with a retroviral expression vector for IKAROS (IK1)/GFP and a GFP empty vector control. 2 d after transduction, GFP+ cells were sorted and cultured in the presence or absence of 10 μmol/liter Imatinib for 12 h (B). Protein lysates from these cells were then subjected to Western blot analysis using antibodies against the SRC family kinase LYN, activated SRC kinases (including LYN) that are phosphorylated at Y416, the ubiquitin-ligase CBL, activated CBL phosphorylated at Y731, SLP65, and activated SLP65 phosphorylated at Y96. Antibodies against EIF4E were used as a loading control. Experiments on two cases were performed. In (C), pre–B cells isolated from bone marrow of $Slp65^{-/-}$ mice were transformed by a retrovirus encoding BCR-ABL1/Neo. In three experiments, BCR-ABL1–transformed $Slp65^{-/-}$ ALL cells were reconstituted with retroviral expression vectors encoding wild-type SLP65/GFP or the mutant SLP65Y96F/GFP or transduced with a GFP empty vector control (C).

2004), SYK and SLP65 are frequently down-regulated and occasionally deleted in Ph+ ALL (Fig. 1 C), and SLP65 functions as a potent tumor suppressor downstream of the pre–B cell receptor (Fig. 3, 7A).

Notably, a recent study demonstrated the ability of *IKAROS* to redirect B cell receptor signaling in DT40 B cell lymphoma from preferential tyrosine phosphorylation of LYN/CBL to SYK/SLP65 (Nera et al., 2006). Whereas LYN/CBL (at the expense of SYK/SLP65) are the main substrates of B cell receptor–induced phosphorylation in IKAROS-deficient DT40 B cell lymphoma, reconstitution of IKAROS reinstates SYK/SLP65 signaling and dephosphorylates LYN/CBL (Nera et al., 2006). For this reason, we examined whether IKAROS can play a similar role in Ph+ ALL, where BCR-ABL1 phosphorylates LYN, CBL and other molecules of the SRC kinase pathway (Hu et al., 2004; Ptasznik et al., 2004; Feldhahn et al., 2005), but not SYK and the tumor suppressor SLP65 (Feldhahn et al., 2005 and Fig. 7 B). To this end, we studied the effect of IKAROS-reconstitution on protein expression and tyrosine phosphorylation of LYN/CBL and

SLP65 in two IKAROS-/μ chain double-deficient Ph+ ALL cases (Fig. 7 B). IKAROS reconstitution (IK1) had no significant effect on global protein levels of LYN, CBL, and SLP65. However, tyrosine phosphorylation of SRC family kinases (including LYN) at Y416 and CBL at Y731 was substantially reduced upon IKAROS reconstitution, as previously observed in DT40 chicken B cells. In contrast, IKAROS reconstitution resulted in de novo tyrosine phosphorylation of the tumor suppressor SLP65 at Y96. This phosphorylation event was sensitive to BCR-ABL1 kinase inhibition by Imatinib (Fig. 7 B), suggesting that oncogenic BCR-ABL1 kinase activity is indeed redirected to SLP65-Y96. Importantly, a recent study demonstrated that Y96 of SLP65 is crucial for the ability of SLP65 to bind and, hence, inhibit JAK3–STAT5 (Nakayama et al., 2009), a pathway that is also activated in oncogenic BCR-ABL1 signaling (Danial et al., 1995). Inhibition of JAK3–STAT5 signaling through interaction with SLP65 Y96 leads to activation of p27 (CDKN1B) and rapid cell cycle arrest (Nakayama et al., 2009). Consistent with a tumor suppressor pathway that requires interaction of SLP65-Y96 with JAK3–STAT5 proteins, we observed that SLP65-Y96F, in contrast to wild-type SLP65, failed to suppress BCR-ABL1–driven leukemic growth (Fig. 7 C).

DISCUSSION

Previous work by our group showed that leukemia cells in Ph+ ALL often lack coding capacity for a pre–B cell receptor (Klein et al., 2004) and down-regulate pre–B cell receptor–related signaling molecules (Klein et al., 2004; Klein et al., 2006). These findings suggested that leukemia clones in Ph+ ALL either bypass pre–B cell receptor selection or are even selected against expression of a pre–B cell receptor.

Here, we demonstrate that pre–B cell receptor signaling suppresses proliferation of Ph+ ALL cells via up-regulation of *IKAROS* and *IKAROS*-mediated cell cycle arrest (Fig. 5). Specifically, upstream components of the pre–B cell receptor signaling complex, including the μ chain (Fig. 4) and SLP65 (Fig. 3), function as potent tumor suppressors and induce cell cycle exit through an *IKAROS*-dependent mechanism (Fig. 6 and Fig. 7). Consistent with a tumor suppressor role of the pre–B cell receptor, its function is compromised in most if not all cases of Ph+ ALL (Fig. 1). This raises the question how *IKAROS* can function as a tumor suppressor downstream of the pre–B cell receptor, if μ chain expression is compromised in most, if not all, cases of Ph+ ALL. In this respect, Ph+ ALL differs from other subtypes of ALL by its particularly high frequency of IKAROS deletions (>80%, Mullighan et al., 2008) and by the fact that Ph+ ALL is driven by a constitutively active tyrosine kinase (BCR-ABL1) that activates critical signaling molecules (SRC kinases, BTK, and CBL) downstream of the pre–B cell receptor (Hu et al., 2004; Ptasznik et al., 2004; Feldhahn et al., 2005). Here, we show that these two characteristics of Ph+ ALL are linked to each other because pre–B cell receptor signaling induced up-regulation of IKAROS (Fig. 6) and reconstitution of IKAROS redirects oncogenic BCR-ABL1 tyrosine kinase signaling from SRC kinase activation to SLP65 (Fig. 7 B), a critical tumor suppressor downstream of the pre–B cell receptor. We propose that even in the absence of a functional μ chain, *IKAROS* functions as a tumor suppressor in Ph+ ALL through its ability to divert oncogenic BCR-ABL1 kinase signaling to the pre–B cell receptor downstream linker molecule SLP65. According to this scenario, SLP65 is at the center of an IKAROS-dependent pathway of tumor suppression in Ph+ ALL (Fig. S1). Activation of SLP65 (phosphorylation at Y96) is either initiated from the pre–B cell receptor (μ chain, SYK; Fig. S1 A) or through IKAROS-mediated diversion of BCR-ABL1, when pre–B cell receptor signaling is compromised (Fig. S1 B). In both cases, SLP65, when phosphorylated at Y96 would inhibit STAT5–JAK3 signaling (Nakayama et al., 2009) and induce cell cycle arrest through up-regulation of IKAROS (Fig. 6) and p27 (CDKN1B; Gómez-del Arco et al., 2004; Kathrein et al., 2005). If both pre–B cell receptor and IKAROS signaling are compromised, however, this pathway of tumor suppression is inactivated (Fig. S1 C). In this case, SLP65 is no longer activated downstream of the pre–B cell receptor or through IKAROS-mediated rerouting of BCR-ABL1 kinase activity. Instead, BCR-ABL1 activates SRC kinases (LYN, HCK, and FGR; Hu et al., 2004; Ptasznik et al., 2004), BTK (Feldhahn et al., 2005) and STAT5–JAK3 (Ilaria and Van Etten., 1996), which promote oncogenic survival signaling (Ilaria and Van Etten, 1996; Hu et al., 2004; Ptasznik et al., 2004; Feldhahn et al., 2005). In addition, BCR-ABL1–mediated phosphorylation of LYN leads to activation of CBL, which ubiquitinates SYK for proteasomal degradation (Ota and Samelson, 1997; Fig. S1C). Because SYK kinase activity is required for activation of SLP65 (phosphorylation of SLP65-Y96; Fu et al., 1998), BCR-ABL1/LYN/CBL-induced proteasomal degradation of SYK also prevents further activation of the SLP65 tumor suppressor (Fig. S1 C). As a consequence, SRC kinase signaling downstream of BCR-ABL1 induces survival signaling through activation of NF-κB (Saijo et al., 2003; Fig. S1 C), whereas BCR-ABL1–mediated activation of STAT5–JAK3 induces proliferation via up-regulation of MYC (Tsuruyama et al., 2002; Fig. S1 C). Although our findings do not exclude other important functions of the IKAROS tumor suppressor in Ph+ ALL, we propose that IKAROS induces cell cycle arrest downstream of the pre–B cell receptor and also reroutes oncogenic BCR-ABL1 kinase activity into the pre–B cell receptor signaling pathway, even if signaling from the μ chain is compromised. Future experiments will focus on the mechanism through which IKAROS diverts BCR-ABL1 kinase activity from the SRC kinase pathway (survival, proliferation) to SYK/SLP65 (differentiation, cell cycle control).

MATERIALS AND METHODS

Patient samples, human cells, and cell lines. Normal human pro–B cells ($CD19^+$ $CD34^+$ $VpreB^-$), pre–B cells ($CD19^+$ $CD34^-$ $VpreB^+$) and immature B cells ($CD19^+$ $CD10^+$ $CD20^+$) were sorted from human bone marrow (from four healthy donors; Cambrex, Verviers, Belgium) by flow cytometry using antibodies from BD and a FACSVantage SE cell sorter (BD). Single cells from each population were sorted into PCR reaction tubes for single-cell PCR analysis of Ig V_H-DJ_H gene rearrangements.

V_H-DJ_H gene rearrangements were analyzed from 111 ALL cases, including 57 cases of Ph^+ ALL and 54 cases of Ph-negative ALL. For 82 ALL cases, we amplified and sequenced clonal V_H-DJ_H gene rearrangements from leukemia-derived cDNA, and for 9 cases, we reanalyzed primary data provided by M.J.S. Dyer (Institute of Cancer Research, Sutton, England, UK). For 10 additional cases of Ph^+ ALL, sequence data from EMBL/GenBank/DDBJ (M.J. Brisco, Flinders University, Adelaide, Australia; accession nos. L77971.1, L77974.1, L77980.1, L77982.1–L77987.1, L77989.1) were reanalyzed. Fresh leukemic blasts were isolated from patient-derived bone marrow samples with Ph^+ ALL. Primary leukemia cells were cultured on OP9 stroma cells in the presence of 10 ng/ml human IL-7. Patient samples were provided from the Departments of Hematology and Oncology, University Hospital Benjamin Franklin, Berlin, Germany (W.K. Hoffman), the University of Bologna, Bologna, Italy (G. Martinelli), and the Department of Medical Biosciences, Pathology, Umea University, Umea, Sweden (A. Li) 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 Internal Review Board of the University of Southern California Health Sciences Campus). The human ALL cell lines 380, 697, BV173, Kasumi-2, MHH-Call3, Nalm1, Nalm6, RCH-ACV, SD1, SEM, SUP-B15, and TOM1 were purchased from DSMZ.

Single-cell PCR and sequence analysis of V_H-DJ_H gene rearrangements. Single pro–, pre–, and immature B cells were FACS sorted into individual PCR reaction tubes containing 20 µl 1X PCR reaction buffer. Single cells were then digested using proteinase K and subjected to a whole-genome preamplification step, as previously described (Müschen et al., 2000). Aliquots from these reactions were used for two rounds of PCR amplification using the primer sets detailed in Table S4 in 35 and 45 cycles, respectively.

SNP mapping assay for genomic deletions. Genomic DNA was extracted from 22 bone marrow Ph^+ ALL samples, in which blast counts were >80%, using the DNA Blood Mini kit (QIAGEN) and quantified using a NanoDrop Spectrophotometer. The SNP mapping assay (Affymetrix) started with the digestion of 250 ng of DNA sample (*Nsp*I), followed by ligation of a common primer and amplification by PCR. The PCR conditions were optimized for the selective amplification of fragments that are 250–2,000 nt in length. The amplicons were fragmented by DNaseI digestion and labeled using TdT and a biotinylated nucleotide analogue, and then hybridized to 250K *Nsp*I arrays (Affymetrix). After washing and staining, the arrays were scanned for data analysis using the GeneChip Scanner 3000 (Affymetrix). CEL files were generated using GeneChip Genotyping Analysis Software (GTYPE) version 4.0. SNP calls were generated using GTYPE. Affymetrix CEL files were analyzed for genomic copy number variations using Partek Genomic Suite V and are available from GEO under accession no. GSE13612. The underlying algorithm of CNAG strongly improves the signal-to-noise ratios of the final copy number output by correcting for length and GC content of the individual PCR products using quadratic regressions and by providing fully automated optimal sample selection. Copy number aberrations were scored using the Hidden Markow Model and by visual inspection. All aberrations were calculated with respect to a set of 48 HapMap normal individuals.

Affymetrix GeneChip analysis. $CD19^+$ $AA4.1^+$ bone marrow B cell precursors were FACS sorted from each three wild-type, *BCR-ABL1* transgenic, preleukemic mice with full-blown ALL and from leukemic mice after 7 d of treatment with the BCR-ABL1 kinase inhibitor AMN107. Total RNA from cells used for microarray or RT-PCR analysis was isolated by RNeasy (QIAGEN) purification. Double-strand complementary DNA was generated from 5 µg of total RNA using a poly(dT) oligonucleotide that contains a T7 RNA polymerase initiation site and the SuperScript III reverse transcription (Invitrogen). Biotinylated cRNA was generated and fragmented according to the Affymetrix protocol and hybridized to 430 mouse microarrays (Affymetrix). To determine relative signal intensities, the ratio of intensity for each sample in a probeset was calculated by normalizing to the mean value. Ratios were exported in Gene Cluster and visualized as a heat map with Java TreeView. Cel files from GeneChip arrays are available from GEO under accession no. GSE7182 and were imported to the 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. Affymetrix U133A2.0 GeneChip data from four human Ph^+ ALL cell lines (BV173, Nalm1, SUP-B15, and TOM1) generated by us are accessible through GEO accession GSE7182.

Quantitative RT-PCR analysis. For quantitative RT-PCR analysis of human *BCR-ABL1*, mouse *Hprt*, *Slp65*, *Ighm*, and *Ikaros*, PCR primers are listed in Table S4. Quantitative real-time PCR performed with the SYBR GreenER mix (Invitrogen) was performed according to standard PCR conditions and an ABI7900HT (Applied Biosystems) real-time PCR system.

BCR-ABL1 kinase inhibitors STI571 and AMN107. STI571 and AMN107 were obtained from Novartis Pharmaceuticals. Leukemic *BCR-ABL1* transgenic mice were fed a mixture of 8 parts peanut butter and 2 parts vegetable oil, with 75 mg of AMN107/kg body weight added to the same peanut/oil mixture daily. After 7 d of treatment, mice were sacrificed and leukemia cells were analyzed in the peripheral blood, bone marrow, and spleen.

BCR-ABL1 transgenic mice. The *BCR-ABL1* transgenic mouse model was described previously (Heisterkamp et al., 1990). In the C57BL/6J background, mean age at death for the f10-f15 generation ($n = 127$) was 100 d (range, 38–265 d). In the experiments described here, healthy and phenotypically normal BCR-ABL1 transgenic mice at an age <60 d were considered preleukemic.

***Slp65*$^{-/-}$ mice.** Targeting of the Slp65 locus in BALB/c mice resulted in replacement of the *Slp65* exon 4 by a Neomycin selection cassette in opposite transcriptional orientation (Jumaa et al., 1999). These mice lack expression of functional *Slp65*, but exhibit Neo-*Slp65* fusion transcripts. Bone marrow–derived B cells from these mice are arrested at the pre–B cell stage of development and proliferated vigorously under cell culture conditions in the presence of 10 ng/ml mouse IL-7.

***Ighm*$^{-/-}$ mice.** The transmembrane domain of the constant µ region was targeted in these mice (The Jackson Laboratory). B cell precursors in these mice can undergo V(D)J recombination of Ig variable genes, but they fail to express a µ chain on the cell surface and therefore lack pre–B cell receptor function. B cell precursors in *Ighm*$^{-/-}$ mice are arrested at the pro–B cell stage of development. Bone marrow from *Ighm*$^{-/-}$ mice was isolated and B cell precursors were propagated in the presence of 10 ng/ml mouse IL-7.

***Rag2*$^{-/-}$ tTA/µ chain transgenic mice.** *Rag2*$^{-/-}$ tTA/µ chain transgenic mice (Hess et al., 2001) are unable to express an endogenous µ chain because they lack Rag2-dependent V(D)J recombination, but carry a functionally prerearranged µ chain under control of Tet operator sequences in the germline. In addition, *Rag2*$^{-/-}$ tTA/µ chain transgenic mice express a tTA under control of endogenous µ chain regulatory elements.

Reconstitution of pre–B cell receptor defects in Ph^+ ALL and BCR-ABL1–transformed mouse ALL cells. Human Ph^+ ALL cells lacking µ chain, SLP65 or IKAROS expression were transduced with retroviral vectors (MSCV backbone described in Pear et al., 1998) encoding a functional µ chain/CD8, the active IKAROS form IK1/GFP or SLP65/GFP. CD8 and GFP empty vector controls were used. Bone marrow cells from *Rag2*$^{-/-}$ tTA/µ chain transgenic, *Ighm*$^{-/-}$, and *Slp65*$^{-/-}$ mice were transduced with retroviral vectors encoding p190 and p210 BCR-ABL1 (Pear et al., 1998) in the presence of 10 ng/ml IL-7, which results in the outgrowth of pre–B ALL (Li et al., 1999). After transformation, IL-7 was no longer supplemented. *Ighm*$^{-/-}$ ALL cells were transduced with retroviral constructs encoding mouse CD8 alone or CD8 and a functional mouse µ chain. In a different set of experiments, *Slp65*$^{-/-}$ ALL cells were

transduced with a retroviral vector encoding GFP alone (GFP), wild-type murine Slp65 (Slp65/GFP), and mutant Slp65Y96F (Slp65Y96F/GFP) and GFP. In a loss-of-function analysis for IKAROS, IK6 a dominant-negative form of IKAROS was overexpressed (IK6/GFP) using a GFP empty vector control (GFP).

Inducible reconstitution of Slp65 in *Slp65*$^{-/-}$ BCR-ABL1–transformed pre–B ALL cells. To test the effect of *Slp65*-reconstitution on *Slp65*$^{-/-}$ BCR-ABL1–transformed pre–B ALL cells using an inducible model, we fused the *Slp65* to the mutated estrogen receptor ligand-binding domain (Meixlsperger et al., 2007). Either ERT2 fused to the N terminus of *Slp65* (ERT2-Slp65/GFP) or ERT2 alone (ERT2/GFP) were expressed in the *Slp65*$^{-/-}$ BCR-ABL1–transformed pre–B ALL cells. Addition of 1 μmol/l 4-hydroxy-tamoxifen dissolved in ethanol resulted in full activation of ERT2-Slp65 fusion molecules. Ethanol was used as a vehicle control.

In vivo model for BCR-ABL1–transformed ALL and bioluminescence imaging. Pre–B cells from *Slp65*$^{-/-}$ mice were transformed with a retroviral vector encoding *BCR-ABL1* and labeled with lentiviral firefly luciferase. After luciferase-labeling, *BCR-ABL1*–transformed pre–B cells were transduced with retroviral vectors encoding either GFP or *Slp65*/GFP, and 3×10^6 GFP$^+$ cells were injected into sublethally irradiated (250 cGy) NOD/SCID. After the injection of cells, the mice were imaged at different time points using an in vivo IVIS 100 bioluminescence/optical imaging system (Xenogen). D-Luciferin (Xenogen) dissolved in PBS was injected i.p. at a dose of 2.5 mg per mouse 15 min before measuring the light emission. General anesthesia was induced with 5% isoflurane and continued during the procedure with 2% isoflurane introduced via a nose cone. Mice were monitored by bioimaging on a weekly basis and also monitored for weight loss and other signs of disease. When the first mice became terminally ill because of leukemia, all mice in the ongoing experiment were sacrificed. Mice were studied for potential enlargement of the spleen and bone marrow, spleen, and peripheral blood were analyzed by flow cytometry to quantitate leukemic infiltration. All mouse experiments were subject to institutional approval by Childrens Hospital Los Angeles Institutional Animal Care and Use Committee.

Clonality analysis and spectratyping of B cell populations. V_H-DJ_H gene rearrangements from B cell populations were amplified using PCR primers specific for the J558 V_H region gene together with a primer specific for the Cμ constant region gene. Using a FAM-labeled Cμ constant region gene-specific primer in a run-off reaction, PCR products were labeled and subsequently analyzed on an ABI3100 capillary sequencer by fragment length analysis. Sequences of primers used are given in Table S4.

Flow cytometry and cell cycle analysis. Peripheral blood, spleen, and bone marrow samples from C57BL/6 mice and *BCR-ABL1* transgenic mice were stained using FACS antibodies against CD19, CD23, B220, κ and λ light chains, IL-7rα, μ chain and AA4.1 (antibodies from BD). For cell cycle analysis in *BCR-ABL1* ALL cells, the BrdU flow cytometry kit for cell cycle analysis (BD) was used according to manufacturer's instructions. BrDU incorporation (APC-labeled anti-BrdU antibodies) was measured together with DNA content (7-amino-actinomycin-D) in fixed and permeabilized cells. The analysis was either gated on viable cells that were identified either based on scatter morphology or GFP or IK6/GFP expression. Because individual experiments showed variations, means of percentages of cells in G0/G1, S and G2/M phase were calculated and depicted as box plots. The leukemia cells studied here have a cell cycle duration of ~48 h (estimate based on cell culture growth kinetics). Long incubation of the cells in BrdU may lead to an overestimate of the fraction of cells in cycle, because cells that incorporated BrdU during the incubation with the nucleotide will remain BrdU$^+$ even after the cells returned into the G0/G1 phase of the cell cycle. For this reason, BrdU-incubation was limited to 30 min as recommended by the manufacturer.

Western blot. Polyclonal antibodies against LYN, SRC phospho-Y416, CBL, CBL phospho-Y731, SLP65, SLP65 phospho-Y96, and a monoclonal antibody against EIF4E (C46H6) were purchased from Cell Signaling Technologies. Western blot analysis was performed using the Western breeze kit (Invitrogen). Imatinib (Gleevec, STI571) was a gift from Novartis.

Measurement of Ca^{2+} release in response to pre–B cell receptor engagement. CD19$^+$ cells from peripheral blood and bone marrow of healthy donors were purified using immunomagnetic beads (Miltenyi Biotech). Human and mouse B cell and ALL populations were treated with antibodies specific for the μ chain of human (Jackson ImmunoResearch Laboratories) and mouse (SouthernBiotech) origin. Ca^{2+} release from cytoplasmic stores in response to (pre–) B cell receptor engagement was measured by loading of the cells with the lipophilic Fluo-4 dye (Invitrogen).

Online supplemental material. Table S1 presents the amino acid translation of the *IGHM* VDJ junction sequence of single sorted pro– and pre–B cells and immature B cells from healthy donors (A) and 57 cases of Ph$^+$ ALL (B). Table S1 also includes a description of our statistical model for the comparison of functional versus nonfunctional *IGHM* V-DJ gene rearrangements. Table S2 presents data on white blood counts, counts of peripheral blood B cell precursors, and splenic weights in BCR-ABL1 transgenic mice during progressive transformation. Table S3 presents data on white blood counts, counts of peripheral blood B cell precursors, and splenic weights in a different mouse model, in which NOD/SCID mice were injected with SLP65$^{-/-}$ pre–B cells that were transformed by retroviral *BCR-ABL1*. Table S4 presents a summary of oligonucleotides used for single-cell PCR, quantitative RT-PCR and Ig spectratyping. Fig. S1 presents a schematic rendition of our scenario for the cooperation between pre–B cell receptor signaling and IKAROS as tumor suppressors in Ph$^+$ ALL. Fig. S2 presents details of our normalization algorithm and comparison of two previously published microarray analyses (Ross et al., 2003; van Zelm et al., 2005) in one meta-analysis (Fig. 1 C). Fig. S3 shows experimental data on verification of *VPREB1* gene deletions in Ph$^+$ ALL by FISH and genomic quantitative PCR. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090004/DC1.

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