

Phosphoinositide 3-kinase signaling is essential for *ABL* oncogene-mediated transformation of B-lineage cells

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***BCR-ABL* and *v-ABL* are oncogenic forms of the Abl tyrosine kinase that can cause leukemias in mice and humans. *ABL* oncogenes trigger multiple signaling pathways whose contribution to transformation varies among cell types. Activation of phosphoinositide 3-kinase (PI3K) is essential for *ABL*-dependent proliferation and survival in some cell types, and global PI3K inhibitors can enhance the antileuke-**

mia effects of the Abl kinase inhibitor imatinib. Although a significant fraction of *BCR-ABL*-induced human leukemias are of B-cell origin, little is known about PI3K signaling mechanisms in B-lineage cells transformed by *ABL* oncogenes. Here we show that activation of class I_A PI3K and downstream inactivation of FOXO transcription factors are essential for survival of murine pro/pre-B cells

transformed by *v-ABL* or *BCR-ABL*. In addition, analysis of mice lacking individual PI3K genes indicates that products of the *Pik3r1* gene contribute to transformation efficiency by *BCR-ABL*. These findings establish a role for PI3K signaling in B-lineage transformation by *ABL* oncogenes. (Blood. 2004;103:4268-4275)

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Introduction

Altered forms of the tyrosine kinase c-Abl are potent oncogenes. *v-ABL* is the transforming gene of Abelson murine leukemia virus (Ab-MuLV).¹ Neonatal mice infected with Ab-MuLV predominantly develop B-cell leukemias corresponding to the pro-B or pre-B-cell stages. Infection of murine bone marrow or fetal liver also results in preferential transformation of pro/pre-B cells. Expression of the *v-ABL* oncogene can transform certain other cell types in vitro, including myeloid cells and certain fibroblast lines. *BCR-ABL* is a fusion oncogene expressed in human leukemia cells with a (9;22) translocation known as the Philadelphia (Ph) chromosome.^{2,3} Depending on the specific breakpoint, Bcr-Abl proteins exist in p190, p210, or p230 forms that are associated with different clinical syndromes. p210-Bcr-Abl is found in nearly all cases of chronic myelogenous leukemia (CML) and some patients with Ph⁺ acute lymphoid leukemia (ALL). p190-Bcr-Abl is almost exclusively found in ALL. Retroviral transduction of murine bone marrow cells with p210 or p190 forms can yield either myeloid or B-lymphoid tumors in recipient mice, depending on the experimental model used.⁴

The Abl tyrosine kinase inhibitor imatinib (Gleevec, STI-571) has been shown to elicit remarkable clinical responses with minimal toxicity in CML and Ph⁺ ALL patients. Unfortunately, resistance eventually develops and is usually associated with mutations or amplifications of *BCR-ABL*.^{5,6} Targeting multiple signaling steps therefore may generate more lasting remissions in patients with *ABL*-dependent leukemias. Expression of *v-Abl* or Bcr-Abl activates many of the same signaling intermediates including the small G proteins Ras and Rac, tyrosine kinases of the Janus kinase family, protein kinase C, and phosphoinositide

3-kinase (PI3K).^{7,8} It is important to note that signaling components downstream of *ABL* oncogenes have been defined in diverse cell systems. However, it is becoming evident that many of the effects of *v-ABL* and *BCR-ABL* are cell-context specific and may not have functional significance in the transformation of primary cells. For example, signal transducer and activator of transcription 5 was shown to be dispensable for *v-ABL*- or *BCR-ABL*-mediated transformation and leukemogenesis even though it is phosphorylated in human CML cells and is required for conversion of hematopoietic cell lines to growth factor-independence.^{9,10} Thus, it is critical that the contribution of specific signaling components to *ABL*-mediated disease be confirmed in direct transformation assays done in hematopoietic cell types representative of their natural target cells.

The PI3K family of enzymes phosphorylates inositol phospholipids, thereby promoting membrane association of certain cytoplasmic proteins that specifically bind to PI3K lipid products.^{11,12} There are many subtypes of PI3K with distinct functions in cells. Class I_A PI3Ks, the subgroup that functions downstream of activated tyrosine kinases, are heterodimers composed of a catalytic subunit and a regulatory subunit. Signaling through class I_A PI3K promotes cell proliferation and survival in many cell types and is strongly associated with transformation and metastasis.¹³ There are 3 catalytic subunit isoforms (p110 α , p110 β , and p110 δ) and 5 regulatory subunit isoforms (p85 α , p55 α , p50 α , p85 β , and p55 γ ; the first 3 are transcriptional variants of a single gene, *Pik3r1*).¹¹ We and others have shown that the predominant class I_A regulatory isoform expressed in murine B cells, p85 α , is required for B-cell proliferation triggered by some but not all mitogens.^{14,15} In

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addition, p85 α -deficient mice show impaired B-cell development as judged by reduced percentages of pre-B and mature B cells.^{14,15}

Previous work on *v-Abl* activation of PI3K has been carried out mostly in model cell lines. In fibroblasts, it was shown that the transforming ability of *v-Abl* variants correlated with the ability to activate PI3K.¹⁶ In a mast cell line, a temperature-sensitive allele of *v-ABL* was used to demonstrate that activation of PI3K, and its downstream effector Akt, contributes to the suppression of apoptosis following growth factor withdrawal.¹⁷ PI3K has been implicated downstream of Bcr-Abl mainly from studies of the p210 isoform in mouse models of CML and in myeloid cell lines converted to factor independence.¹⁸⁻²² Most recently, it was shown that PI3K inhibitors synergize with imatinib to inhibit growth of human CML cells.²³ It has also been reported that PI3K and Akt are activated in murine B-lineage cells transformed by *BCR-ABL*.^{20,22} However, the importance of PI3K signaling, the specific isoforms involved, and the critical downstream targets have not been studied directly in these cells. In this work we investigate whether PI3K activation contributes to *v-ABL* and p190-*BCR-ABL* transformation of immature B-lineage cells. In addition, we test the hypothesis that *Pik3r1* gene products, previously shown to be essential for B-cell development, are required for transformation in this system. Finally, we address the roles of downstream effectors of PI3K in proliferation and survival of B cells transformed by *ABL* oncogenes. We show that PI3K signaling and FOXO inactivation are essential in this system, and that *Pik3r1* contributes quantitatively to transformation by *BCR-ABL* but not *v-ABL*.

Materials and methods

Antibodies and inhibitors

Primary antibodies used for immunoblot were rabbit antisera specific for p110 β (H-198; Santa Cruz Biotechnology, Santa Cruz, CA), p110 α and p110 δ (gift from Bart Vanhaesebroeck, Ludwig Cancer Research Institute, London, United Kingdom), phosphotyrosine (PY20; Santa Cruz Biotechnology), a monoclonal antibody (mAb) specific for p110 α (Transduction Laboratories, Lexington, KY), a rabbit antiserum that recognizes all class I_A PI3K regulatory isoforms (anti-pan-p85, 06-195; Upstate Biotechnology, Lake Placid, NY), and a mAb specific for p55 γ (V2; Abcam, Cambridge, MA). Rabbit antibodies specific for total and phosphorylated forms of glycogen synthase kinase 3 β (GSK3 β) (Ser9), Akt (Thr308), and FOXO (Ser256) were from Cell Signaling Technologies (Beverly, MA). To detect p85 β , lysates were subjected to partial immunodepletion with a mAb specific for an N-terminal epitope in p85 α (clone AB6; Upstate Biotechnology) (3 successive immunoprecipitations) before blotting with anti-pan-p85. Reagents used for flow cytometry (fluorescence activated cell sorting [FACS]) were as follows: Annexin V-phycoerythrin (PE; Caltag, Burlingame, CA), anti-B220-fluorescein isothiocyanate, and Streptavidin-Cyochrome (Becton Dickinson, San Jose, CA), anti-Thy1.1-Biotin, and anti-BP-1-PE (eBiosciences, San Diego, CA). Pharmacologic inhibitors (Calbiochem, San Diego, CA) were used at the following final concentration: LY294002, 10 μ M; rapamycin, 10 ng/mL; and imatinib mesylate, 10 μ M.

Retroviral vectors and generation of virus stocks

Bicistronic retroviral vectors were used to express exogenous genes and a marker gene from the murine stem cell virus (MSCV) long terminal repeat (LTR). pMIG (MSCV-IRES-GFP) was provided by R. Hawley (American Red Cross, Rockville, MD) and pMIT (MSCV-IRES-Thy1.1), by P. Marrack (National Jewish Hospital, Denver, CO). A cDNA encoding p190-Bcr-Abl was subcloned into pMIG, and epitope-tagged cDNAs for FOXO3a and FOXO3a(A3) (gift from B. Burgering, University Medical Center, Utrecht, The Netherlands) and Δ p85 (gift from T. Shioi, Beth Israel Deaconess Medical Center, Boston, MA) were subcloned into pMIT. High-titer helper-free retrovirus stocks were prepared by transient cotrans-

fection of 293T cells with pMIT vectors and ψ^- ecotropic packaging vector.²⁴ Viral supernatants were collected 24 to 48 hours after transfection, filter-sterilized, and stored at -80°C . Virus titers were determined by transducing murine 3T3 fibroblasts and checking marker gene expression by FACS. In each individual experiment, the same virus stock was used to infect cells of different genotypes.

Colony transformations and generation of transformed cell lines

Animal procedures were approved by the institutional animal care and use committees of University of California (UC) Irvine and UC Los Angeles (UCLA). *Pik3r1* heterozygous mice were in a mixed genetic background (129SvEv \times C57Bl/6) or were backcrossed with Balb/c (Jackson Laboratories, Bar Harbor, ME) for 8 generations. From timed matings of heterozygous animals, embryonic day-16.5 (E16.5) or E18.5 embryos were obtained and fetal livers isolated. Following preparation of single-cell suspensions in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS), cells were placed on ice during the polymerase chain reaction genotyping procedure (approximately 6 hours). *Pik3r1* null and wild-type samples were then used for *v-ABL* and *BCR-ABL* infections. Fetal liver cells were infected with AbMuLV by mixing 2×10^6 cells in 1 mL with 1 mL of P160 AbMuLV stock and polybrene (4 $\mu\text{g}/\text{mL}$). After incubation at 37°C , 5% CO₂ for 2 to 4 hours, cells (1×10^5 cells/mL in 24-well plates) were placed in a 1.2% agar medium supplemented with $1 \times$ RPMI 1640, 50 μM β -mercaptoethanol, and 20% FCS. For *BCR-ABL*, 1×10^6 cells were incubated with retroviral stocks for pMIG or pMIG-p190-*BCR-ABL* and polybrene (4 $\mu\text{g}/\text{mL}$) for 3 hours at 37°C . Cells were centrifuged at 550g (2000 rpm) at 30°C for 30 minutes. Cells (5×10^4) were then added to methylcellulose METHOCULT M3630 with recombinant human interleukin-7 (rhIL-7; StemCell Technologies, Vancouver, BC, Canada) in duplicate dishes. The remaining fractions of infected cells were used for suspension cultures that were grown initially in pre-B-cell medium (RPMI 1640, 20% heat-inactivated FCS, penicillin [100 units/mL], streptomycin [100 $\mu\text{g}/\text{mL}$], 2 mM L-glutamine, 50 $\mu\text{g}/\text{mL}$ gentamicin, 50 μM β -mercaptoethanol). Established lines were later switched to 10% FCS. *v-ABL* colony assays were counted 9 days after infection by adding the counts from all 24 wells. *BCR-ABL* colony assays were scored 7 days after transduction by adding the total counts from both dishes.

Retroviral transductions of transformed cell lines

Although the *v-ABL*-transformed cell lines were established by infection with a replication competent retrovirus (AbMuLV), we found that MSCV-based retroviral vectors could superinfect some *v-ABL* cells, as well as cell lines established with pMIG-p190-*BCR-ABL*, and provided the most reliable method of gene delivery. Cells (2×10^6) in 1 mL pre-B-cell medium were mixed with 1 mL virus and 8 $\mu\text{g}/\text{mL}$ polybrene and centrifuged in 24-well plates at 33°C for 45 minutes at 450g. After incubation for 20 to 24 hours at 37°C , cells were either analyzed by FACS or expanded into 3 mL fresh medium.

FACS analysis

Cells were analyzed for green fluorescent protein (GFP) expression and stained with various antibodies and/or Annexin V. For cell cycle analysis, cells were fixed in ethanol and stained with propidium iodide as described.^{14,25} The percentages of cells in different phases of the cell cycle were calculated with ModFit LT software (Verity Software House, Topsham, ME).

In vivo leukemogenesis assay

BCR-ABL-transformed pro/pre-B cells were harvested after 7 days in culture (at which point the live cells were $> 90\%$ GFP⁺) and intravenously introduced into syngeneic (Balb/c.scid) mice, sublethally irradiated at 6 Gy (600 rad). Animals were monitored daily for signs of illness as previously described.²⁶

Cell morphologic analyses and surface marker staining

Peripheral blood, spleen, and bone marrow cells were harvested from overtly ill animals and single-cell suspensions from these tissues were

depleted of red blood cells by hypotonic lysis and acquired on FACScan (Becton Dickinson) or cytospun onto microscope slides (Fisher, Hampton, NH). FACS data were analyzed using WinMDI version 2.8 software (Scripps Research Institute, La Jolla, CA). Dead cells were excluded from analysis based on forward- and side-scatter properties. Peripheral blood smears (nonlysed), as well as spleen and bone marrow cytopsins, were analyzed by Giemsa/Wright staining using the 2-hydroxyethyl methacrylate (HEMA) solution per the manufacturer's protocol (Biochemical Sciences, Swedesboro, NJ).

Immunoprecipitation/immunoblotting

The indicated numbers of cells were washed in $1 \times$ phosphate-buffered saline (PBS), then lysed in 40 μ L for direct immunoblot analysis, or 500 μ L for immunoprecipitation, of lysis buffer (50 mM Tris[tris(hydroxymethyl)aminomethane, pH 7.6], 150 mM NaCl, 1% Triton X-100, 10% glycerol). Immunoprecipitates were prepared by sequential one-hour incubations with antibody and protein A–Sepharose. Beads were washed 3 times with lysis buffer before boiling in $1 \times$ sample buffer. Immunoprecipitates or 40 μ g lysate was electrophoresed (7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS–PAGE]) and transferred to nitrocellulose. Immunoblotting was done as described.²⁵

PI3K activity

Lysates from 1×10^6 cells were immunoprecipitated with the pan85 antibody or lysates from 5×10^6 cells with the pTyr antibody. Immune complex PI3K assays were performed as previously described.²⁷ A phosphoimager (Molecular Dynamics, Sunnyvale, CA) was used to quantify activity.

Results

Contribution of *Pik3r1* gene products to PI3K-dependent transformation by p190–*BCR-ABL*

Addition of the global PI3K inhibitor LY294002 blocked pre-B colony formation in transformation assays with *v-ABL* or p190–*BCR-ABL* (data not shown). The compound was added at a concentration (10 μ M) that abrogates specific mitogenic signals in B cells without general toxicity.¹⁴ These results suggested a role for PI3K in transformation in this system but did not distinguish which isoforms are important. To determine if the predominant class I_A regulatory isoform was required for transformation, we studied mice with a disrupted *Pik3r1* gene, encoding p85 α and the smaller splice variants p55 α and p50 α . This mutation causes perinatal lethality,²⁸ so bone marrow could not be obtained. Instead, we used fetal liver as a source of hematopoietic progenitors for in vitro transformation assays. For *v-ABL* transformation, cells were infected with AbMuLV and plated in soft agar. For *BCR-ABL*, cells were plated in methylcellulose following infection with a replication-defective retrovirus in which the murine stem cell virus long terminal repeat (MSCV LTR) drives expression of p190–*Bcr-Abl* in a bicistronic mRNA with GFP (pMIG–p190–*BCR-ABL*).

In 4 independent experiments with AbMuLV, we observed no significant reduction in the number of colonies derived from fetal liver of *Pik3r1* null compared with wild type (Figure 1A). Clonal cell lines could be established from both genotypes following transfer of colonies into liquid culture, and bulk cell lines could be derived by plating fetal liver in liquid culture immediately after infection. We did observe a significant reduction (approximately 2-fold) in the number of pre-B-cell colonies in methylcellulose seeded with *BCR-ABL*–transduced fetal liver from *Pik3r1* null compared with wild type (Figure 1B). However, cell lines could also be derived from *BCR-ABL* transformants of both genotypes. The expression of *v-Abl* and *Bcr-Abl* in the respective cell lines

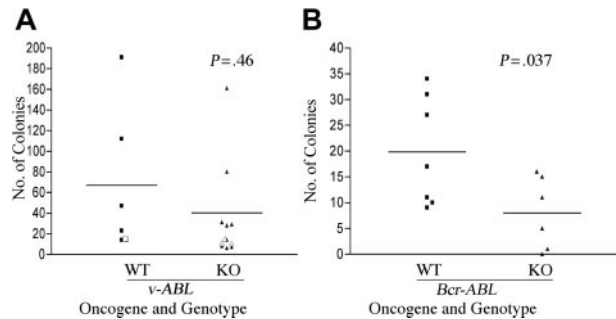


Figure 1. Partially impaired transformation of B-cell progenitors from *Pik3r1* null fetal liver by *BCR-ABL* but not *v-ABL*. Wild-type (WT) and *Pik3r1* null (KO) fetal liver cells were infected with Ab-MuLV or pMIG–p190–*BCR-ABL*. Transformation by *v-ABL* (A) and *BCR-ABL* (B) was measured by counting colonies formed in soft agar or methylcellulose. Open symbols are from a single transformation assay done in cells from a mixed genetic background (129SvEv \times C57BL/6) at embryonic day 16.5, whereas the closed symbols were from 3 independent assays done in cells from a Balb/c background at E18.5. Horizontal bars depict averages: *v-ABL* WT, 67; *v-ABL* KO, 40; *BCR-ABL* WT, 20; and *BCR-ABL* KO, 8. Data analyzed by paired *t* test revealed no difference between *v-ABL* WT and KO ($P = .46$) but showed a significant decrease in transformation of KO by p190–*BCR-ABL* ($P = .037$).

was confirmed by immunoblotting with a monoclonal antibody to *c-Abl* (data not shown). Together these results indicated that *Pik3r1* gene products are not essential for establishment or maintenance of the transformed phenotype but contribute to maximal efficiency of transformation by *BCR-ABL*.

Given the impairment of B-cell development in *Pik3r1* null mice, it was possible that the target cell for transformation differed depending on the genotype of the fetal liver. However, most of the cell lines of both genotypes were positive for the B-lineage marker B220 (Table S1; see the Supplemental Tables link at the top of the online article on the *Blood* website). Some cell lines contained mixed populations of pro/pre-B cells as determined by staining for BP-1, a marker that distinguishes early pro-B cells (BP-1[−]) from late pro-B and pre-B cells (BP-1⁺).²⁹ However, WT and *Pik3r1* null lines did not show reproducible differences in the percentages of BP-1⁺ versus BP-1[−] cells (Table S1). All cell lines were surface immunoglobulin M–negative (IgM[−]).

Delayed leukemia development in absence of *Pik3r1* gene products

Perinatal lethality in the *Pik3r1* null mice hindered our ability to use standard mouse models for leukemia caused by *v-ABL* or *BCR-ABL*. Instead, we tested the leukemogenic potential of day-18.5 fetal liver cells following initial transformation in vitro by p190–*BCR-ABL*. Cells were allowed to expand for 1 week in vitro to ensure efficient disease induction, as fetal liver transformation is less efficient than for bone marrow (Figure 1; Table S2). Equal numbers of transformed wild-type and *Pik3r1* null cells were then injected into severe combined immunodeficiency (SCID) mice and monitored for disease progression. Mice receiving wild-type cells died from pro/pre-B-cell leukemia after an average of 17 days, whereas mice receiving *Pik3r1* null cells succumbed after 23 days (Figure 2A). Although this delay was significant ($P = .018$), the phenotype of the leukemia was comparable (Figure 2B and data not shown). Cells from the spleen and bone marrow were removed and stained for B220 and BP-1, and analyzed for expression of GFP. A majority of pro/pre-B-cells from mice injected with wild-type and *Pik3r1* null cells were GFP positive (Figure 2B). One difference between the wild-type and *Pik3r1* null cells was a lower percentage of BP-1⁺ cells in the bone marrow and spleen of mice receiving *Pik3r1* null cells. This was not due to altered B-cell precursor distribution in the

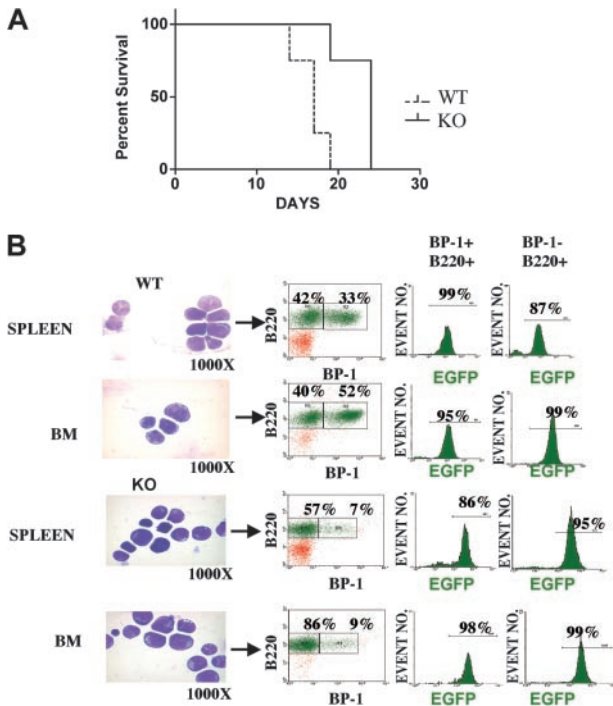


Figure 2. Delayed onset but similar phenotype of leukemic disease in mice that received transplants of p190-*BCR-ABL*-transduced fetal liver from *Pik3r1* null embryos. Wild-type and *Pik3r1* null fetal liver transduced with p190-*BCR-ABL* were allowed to expand one week in vitro prior to transplantation of 1×10^6 cells into SCID mice. (A) Kaplan-Meier plot showing number of days until leukemia development. Of the mice, 4 received wild-type cells (from 2 pooled embryos) and 4 received *Pik3r1* null cells (from one embryo). (B) Bone marrow and spleen cells were cytopspun and stained with Giemsa/Wright to show the lymphoblastic phenotype. Cells were also analyzed by FACS for expression of GFP, B220, and BP-1. Original magnifications of the panels are shown below the images.

Pik3r1 null fetal liver (Table S2), perhaps indicating a decrease in leukemogenic potential of BP-1⁺ transformants.

Proliferation requires Abl, class I_A PI3K, and mTOR but not p85 α

The results from colony assays indicated that *Pik3r1* gene products are not essential for the transformation of B-cell progenitors by *v-ABL* but contribute quantitatively to *BCR-ABL* transformation. To study growth parameters of established cell lines in more detail, we compared cell size, cell cycle profiles, and cell death by flow cytometry. The results showed no consistent differences among genotypes in the percentages of dead cells, or of live cells in G₀/G₁, S, or G₂/M phases (Figure 3A). In contrast, treatment of either wild-type or *Pik3r1* null cell lines with LY294002 for 24 hours resulted in dramatic cell cycle arrest, as judged by decreased S and increased G₀/G₁ fractions (Figure 3B). LY294002 treatment also increased apoptosis, although this effect varied among cell lines (Figure 3D). The sensitivity of wild-type and *Pik3r1* null cells to a range of LY294002 concentrations was comparable (data not shown). These results confirm that PI3K enzyme activity is required for *ABL* oncogenes to promote cell cycle progression and survival in B-lineage cells. However, *Pik3r1* gene products are not solely required for this function.

We also examined the effects of rapamycin, an immunosuppressant that targets the mammalian target of rapamycin (mTOR)/S6kinase pathway downstream of PI3K. Rapamycin also promoted cell cycle arrest, but was less effective than LY294002 in promoting apoptosis (Figure 3C). Addition of imatinib had a greater apoptotic effect than LY294002, causing more than 80% of the cells to die (Figure 3D). These results suggest that oncogenic Abl kinases promote survival via both PI3K-dependent and PI3K-independent pathways. Cell survival was also dependent on the presence of serum (Figure 3D).

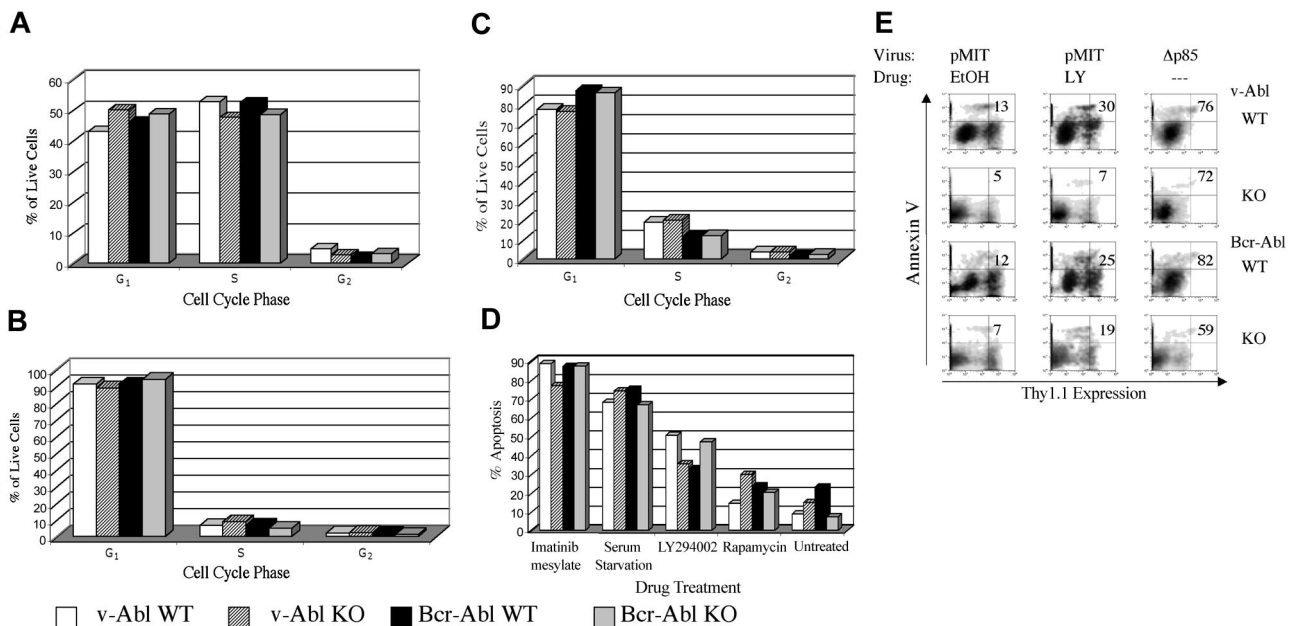


Figure 3. Comparable cell cycle, survival, and drug sensitivity in wild-type and *Pik3r1* null cell lines. *BCR-ABL*- or *v-ABL*-transformed cells of the indicated genotypes were cultured for 24 hours in either diluent alone (0.1% EtOH) (A), or treated with LY294002 (B) or rapamycin (C), then fixed and stained with propidium iodide to determine DNA content. The percentages of live cells in G₀/G₁, S, and G₂/M stages of cell cycle were calculated using ModFit LT software. (D) The percentages of apoptotic cells under the indicated conditions were assessed by subdiploid DNA content using Cell Quest plots (BD Biosciences, San Diego, CA) of propidium iodide fluorescence. Graphs are representative of at least 3 experiments per condition and at least 3 different cell lines per genotype. (E) Cell lines were retrovirally infected with pMIT or pMIT- Δ p85, then cultured in the presence of LY294002 (LY) or EtOH before analysis by FACS 20 hours after infection. The extent of apoptosis in cells with different levels of Thy1.1 expression was determined by Annexin V staining. The number in the upper-right quadrant represents the percentage of dying cells among the population expressing high levels of the Thy1.1 marker.

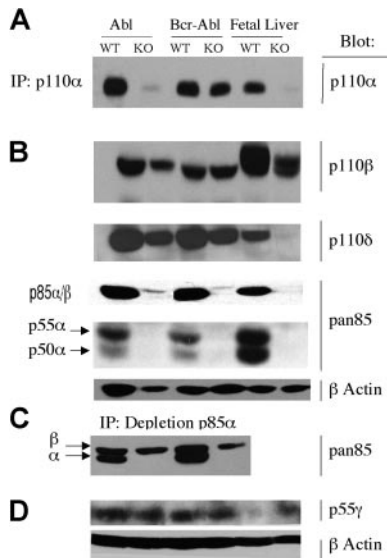


Figure 4. Expression of PI3K isoforms. Lysates or immunoprecipitates (IP) from wild-type and *Pik3r1* null (WT and KO) cells transformed by *v-ABL* or *BCR-ABL* were immunoblotted with the indicated antibodies. (A) p110 α was detected by IP and immunoblotting. (B) p110 β , p110 δ , p85 α , p85 β , p55 α , and p50 α were detected by direct immunoblotting; β -actin was used as a loading control. (C) To optimize detection of p85 β with anti-pan-p85, lysates were first subjected to partial immunodepletion of p85 α using a specific mAb. p55 γ was detected by direct immunoblotting; β -actin was used as a loading control. Isoform blots are representative of 2 to 6 cell lines per genotype.

LY294002 inhibits all classes of PI3K catalytic subunit at the concentrations used. To ascertain if class I_A PI3K function was specifically required in these cells, we used a dominant-negative construct (Δ p85) that selectively inhibits class I_A signaling. Δ p85 is a variant of p85 α lacking a portion of the p110-binding domain, and has been shown to act as a dominant-negative in other cell systems by competing with endogenous class I_A heterodimers (including those containing p85 β or p55 γ) for binding to signaling complexes.³⁰ Δ p85 was inserted into a retroviral vector (pMIT) that drives expression of the test gene from the MSCV LTR along with the surface marker Thy1.1 on a bicistronic mRNA.³¹ At 20 hours after infection, cells transduced with Δ p85 showed markedly lower levels of Thy1.1 marker gene expression compared with cells transduced with empty vector pMIT (Figure 3E, compare right panels with left panels). A selective increase in cell death was observed in the high-expressing population of Δ p85-transduced cells, as measured by staining with Annexin V (Figure 3E; note percentages in upper right quadrant). Similar patterns were observed regardless of *Pik3r1* genotype (Figure 3E). The finding that cells with high expression of Δ p85 undergo cell death suggests that some class I_A PI3K function is required for maintenance of the transformed phenotype. The reduced Thy1.1 expression was not a general effect on protein expression caused by PI3K inhibition, as LY294002 did not reduce expression of Thy1.1 in cells transduced with empty vector (Figure 3E, middle panels).

Class I_A PI3K signaling output is maintained in transformed cell lines lacking p85 α

In many tissues in *Pik3r1* null mice, loss of p85 α , p55 α , and p50 α is associated with marked reductions in the expression of the class I_A catalytic subunit isoforms (p110 α , p110 β , and p110 δ).^{28,32} This is presumed to be the result of decreased stability of the monomeric catalytic subunits.³³ Indeed, protein expression of all 3 catalytic isoforms was markedly reduced in total fetal liver populations from *Pik3r1* null

embryos (Figure 4, top 2 rows). In most transformed *Pik3r1* null lines, however, expression of p110 α , p110 β , and p110 δ was reduced to a lesser degree than in the original fetal liver populations (Figure 4A-B).

Catalytic subunit expression could be preserved by the expression of other class I_A regulatory isoforms (p85 β or p55 γ). We have found that the available antibodies are inadequate for specific detection of murine p85 β in these cells. However, p85 β can be detected weakly with an antiserum raised to the N-terminal Src homology 2 domain of p85 α , termed "anti-pan-p85." Blotting with this antiserum revealed a strong band at 85 kDa in wild-type cells that was greatly reduced in *Pik3r1* null cells, as expected (Figure 4C). Similarly, bands corresponding to p55 α and p50 α were detected in transformed wild-type cell lines but not *Pik3r1* null cells (Figure 4B). The residual signal at 85 kDa in cells lacking p85 α represents p85 β . Although the putative p85 β band migrates slightly more slowly than p85 α , in wild-type cells it is mostly obscured by the stronger p85 α signal. To better distinguish and quantitate the putative p85 β band, we analyzed lysates following partial immunodepletion of p85 α with a monoclonal antibody (Figure 4C). This strategy revealed comparable p85 β expression in *Pik3r1* null cells relative to wild type. Similarly, transformed cells of both genotypes expressed comparable amounts of the p55 γ isoform. Interestingly, this isoform was only weakly detected in total fetal liver from wild-type embryos and was up-regulated in *Pik3r1* fetal liver (Figure 4D). Together these results suggest that transformation of *Pik3r1* null cells is possible because other regulatory isoforms are expressed in *ABL*-transformed pro/pre-B cells. These alternative isoforms appear to be sufficient to stabilize p110 catalytic isoforms and maintain PI3K signaling capacity.

To test this possibility further, the amount of total class I_A PI3K activity was measured in anti-pan-p85 immunoprecipitates and compared with the activity associated with signaling complexes, as measured in antiphosphotyrosine immunoprecipitates (Figure 5). As seen previously in other tissues,²⁸ anti-pan-p85 precipitated

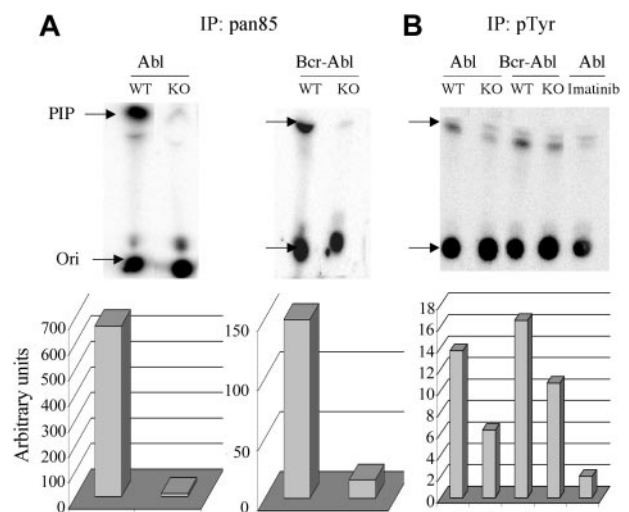


Figure 5. Measurement of total and phosphotyrosine-associated PI3K activity. Lysates from 1×10^6 cells from WT and KO cells were immunoprecipitated with pan85 antibody (A) or lysates from 5×10^6 cells with pTyr antibody (B). Imatinib was included for 15 minutes prior to cell lysis in the indicated sample (*v-ABL* WT). Immune complexes were subjected to an in vitro PI3K assay using phosphatidylinositol as substrate and the products resolved by thin-layer chromatography (Ori indicates origin). Radioactivity in the phosphatidylinositol-3-phosphate product (PIP, indicated by arrow) was quantitated by phosphoimager and graphed below. Similar results were obtained in 3 independent experiments of 2 different cell lines per genotype. The specificity of the immune complex kinase assays was verified in control experiments showing that kinase activity was completely blocked by in vitro treatment with wortmannin (50 nM), a selective PI3K inhibitor (not shown).

v-ABL cell line shown was somewhat less sensitive to the effects of FOXO3a and FOXO3a(A3). The increased death with FOXO3a(A3) relative to wild-type FOXO3a supports the model that PI3K/Akt signaling is sufficiently active in these cells to partially inactivate the overexpressed wild-type FOXO3a protein.

Discussion

Several reports have indicated that PI3K is required for *BCR-ABL*-mediated myeloid transformation, or conversion of model cell lines to growth factor-independence.^{18,19} It has been reported that PI3K and Akt are active in primary murine B lymphoblasts transformed with *BCR-ABL*^{20,22}; however, the role of PI3K signaling in the transformed phenotype was not investigated directly. We have shown that a PI3K inhibitor blocks initial transformation of pro/pre-B cells by *v-ABL* or p190-*BCR-ABL*, and inhibits proliferation and survival of established cell lines. In addition, we have used a dominant-negative p85 construct to establish a specific role for class I_A PI3K function in survival of these cells. We have shown that FOXO proteins are phosphorylated in *ABL*-transformed pro/pre-B cells and that expression of the PI3K-independent FOXO3a(A3) causes a marked increase in apoptosis. As the wild-type FOXO3a had only a partial effect, these findings support a role for continued PI3K signaling to FOXO inactivation as a mechanism for survival in B-lineage cells transformed by *ABL* oncogenes.

The ubiquitous expression and function of PI3K will likely prevent the use of global inhibitors for chronic treatment. Determining if specific PI3K isoforms are required in leukemic cells may make it possible to develop more selective inhibitors for treatment of ABL-dependent disease. A previous study demonstrated that antisense inhibition of expression of p85 α decreased proliferation and colony formation of primary human CML cells and several model cell lines expressing p210-*BCR-ABL*.¹⁸ The data presented here demonstrate that p85 α (and/or p55 α , p50 α) also contributes to optimal transformation of primary murine B-lineage cells by p190-*BCR-ABL*. However, *Pik3r1* gene products are dispensable for *v-ABL* transformation. The apparently lesser role of p85 α and its variants in murine B-lineage transformation relative to human CML cells might be the result of several factors. One variable is that v-Abl and the p190 isoform of Bcr-Abl are more active kinases with greater oncogenic potential relative to p210,⁴ so compensatory signaling pathways may be more strongly triggered. There may also be differences in expression of alternative regulatory isoforms in the different cell lineages or species studied. We do not believe that the p85 β isoform is more critical for *ABL* transformation in this system, as no defects were observed in transformation of bone marrow cells from *Pik3r2* (p85 β)-deficient mice (data not shown). Our future studies will investigate whether combined deletion of *Pik3r1* and *Pik3r2* impairs transformation. However, this might not fully impair class I_A PI3K signaling as the p55 γ isoform (*Pik3r3*) is expressed in *ABL*-transformed pro/pre-B cells regardless of *Pik3r1* genotype (Figure 4).

Class I_A regulatory subunits are required for stabilization of p110 catalytic subunits, association of the enzyme with phosphoty-

rosine-containing signaling complexes, and allosteric activation.¹¹ Consistent with the likely redundant functions of p85 β and/or p55 γ in cells lacking p85 α /p55 α /p50 α , class I_A PI3K catalytic (p110) subunit expression was relatively unaltered in the transformed cell lines when compared with the fetal liver cells taken prior to transduction. Furthermore, PI3K activity associated with phosphotyrosine-containing signaling complexes was only modestly diminished in the *Pik3r1* null cells. In addition, we found no consistent differences between wild-type and *Pik3r1* null cells in the phosphorylation of Akt, S6K (data not shown), or the Akt substrates GSK3 β and FOXO1. Thus, PI3K signaling appeared to be intact in the absence of p85 α . It should be noted that class I_B PI3K (the p110 γ isoform) might contribute to PI3K signaling output in this system, as it can be activated by ligands present in serum, whose presence was found to be required for survival of these cells (Figure 3D).

Stimulation of Akt phosphorylation is considered a hallmark of PI3K activation and has been demonstrated in a great number of cellular contexts.^{12,13,34} However, despite the requirement for continued PI3K signaling in our system, we detected very low levels of phosphorylated Akt in transformed cell lines. Of note, Akt phosphorylation was somewhat more prominent in bone marrow cells isolated from mice with leukemic disease (data not shown). In studies of *ABL* oncogenes, Akt activation has been reported in a mast cell line bearing a temperature-sensitive *v-ABL* variant, and in BaF3 cells and primary myeloid or B-lymphoblastoid cells transduced with p210-*BCR-ABL*.^{17,22} In contrast, we recently showed that in hematopoietic precursors differentiated from embryonic stem cells, inducible expression of p210-*BCR-ABL* did not lead to appreciable increases in phosphorylated Akt.²⁴ Thus, the maintenance of high-level Akt activation appears dependent on cell context. Regardless, the wild-type or *Pik3r1* null cells showed abundant LY294002-sensitive phosphorylation of 2 known Akt substrates, GSK3 β and FOXO1. It is possible that these targets may be phosphorylated by kinases of the Sgk family, which are also PI3K regulated and whose substrate selectivity overlaps with Akt.³⁹

Although we have shown that p85 α , p55 α , and p50 α regulatory isoforms are dispensable for transformation by *v-ABL*, *Pik3r1* gene products do appear to contribute quantitatively to colony formation and leukemogenesis by p190-*BCR-ABL*. It is worth testing whether other PI3K components have nonredundant functions. In particular, the catalytic isoforms p110 γ and p110 δ are specifically expressed in leukocytes and may be attractive drug targets.⁴⁰

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References

- Shore SK, Tantravahi RV, Reddy EP. Transforming pathways activated by the v-Abl tyrosine kinase. *Oncogene*. 2002;21:8568-8576.
- Deininger MW, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. *Blood*. 2000;96:3343-3356.
- Sawyers CL. Chronic myeloid leukemia. *N Engl J Med*. 1999;340:1330-1340.
- Li S, Ilaria RL Jr, Million RP, Daley GQ, Van Etten RA. The P190, P210, and P230 forms of the *BCR/ABL* oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. *J Exp Med*. 1999;189:1399-1412.
- Gambacorti-Passerini CB, Gunby RH, Piazza R, Galletta A, Rostagno R, Scapozza L. Molecular mechanisms of resistance to imatinib in Philadelphia-chromosome-positive leukaemias. *Lancet Oncol*. 2003;4:75-85.
- Hochhaus A, Kreil S, Corbin AS, et al. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. *Leukemia*. 2002;16:2190-2196.
- Calabretta B, Skorski T. *BCR/ABL* regulation of PI-3 kinase activity. *Leuk Lymphoma*. 1996;23:473-476.
- Zou X, Calame K. Signaling pathways activated by oncogenic forms of Abl tyrosine kinase. *J Biol Chem*. 1999;274:18141-18144.
- Sexl V, Piekorz R, Moriggi R, et al. Stat5a/b contribute to interleukin 7-induced B-cell precursor

- expansion, but *abl*- and *bcr/abl*-induced transformation are independent of *stat5*. *Blood*. 2000;96:2277-2283.
10. Kindler T, Breitenbuecher F, Kasper S, et al. In BCR-ABL-positive cells, STAT-5 tyrosine-phosphorylation integrates signals induced by imatinib mesylate and Ara-C. *Leukemia*. 2003;17:999-1009.
 11. Fruman DA, Meyers RE, Cantley LC. Phosphoinositide kinases. *Annu Rev Biochem*. 1998;67:481-507.
 12. Vanhaesebroeck B, Leevers SJ, Ahmadi K, et al. Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem*. 2001;70:535-602.
 13. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*. 2002;2:489-501.
 14. Fruman DA, Snapper SB, Yballe CM, et al. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. *Science*. 1999;283:393-397.
 15. Suzuki H, Terauchi Y, Fujiwara M, et al. Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. *Science*. 1999;283:390-392.
 16. Varticovski L, Daley GQ, Jackson P, Baltimore D, Cantley LC. Activation of phosphatidylinositol 3-kinase in cells expressing *abl* oncogene variants. *Mol Cell Biol*. 1991;11:1107-1113.
 17. Tang X, Downes CP, Whetton AD, Owen-Lynch PJ. Role of phosphatidylinositol 3-kinase and specific protein kinase B isoforms in the suppression of apoptosis mediated by the Abelson protein-tyrosine kinase. *J Biol Chem*. 2000;275:13142-13148.
 18. Skorski T, Kanakaraj P, Nieborowska-Skorska M, et al. Phosphatidylinositol-3 kinase activity is regulated by BCR/ABL and is required for the growth of Philadelphia chromosome-positive cells. *Blood*. 1995;86:726-736.
 19. Skorski T, Bellacosa A, Nieborowska-Skorska M, et al. Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway. *Embo J*. 1997;16:6151-6161.
 20. Roumiantsev S, de Aoz IE, Varticovski L, Ilaria RL, Van Etten RA. The src homology 2 domain of Bcr/Abl is required for efficient induction of chronic myeloid leukemia-like disease in mice but not for lymphoid leukemogenesis or activation of phosphatidylinositol 3-kinase. *Blood*. 2001;97:4-13.
 21. Sonoyama J, Matsumura I, Ezoe S, et al. Functional cooperation among Ras, STAT5, and phosphatidylinositol 3-kinase is required for full oncogenic activities of BCR/ABL in K562 cells. *J Biol Chem*. 2002;277:8076-8082.
 22. Sattler M, Mohi MG, Pride YB, et al. Critical role for Gab2 in transformation by BCR/ABL. *Cancer Cell*. 2002;1:479-492.
 23. Klejman A, Rushen L, Morrione A, Slupianek A, Skorski T. Phosphatidylinositol-3 kinase inhibitors enhance the anti-leukemia effect of ST1571. *Oncogene*. 2002;21:5868-5876.
 24. Wong S, McLaughlin J, Cheng D, Witte ON. Cell context-specific effects of the BCR-ABL oncogene monitored in hematopoietic progenitors. *Blood*. 2003;101:4088-4097.
 25. Donahue AC, Fruman DA. Proliferation and survival of activated B cells requires sustained antigen receptor engagement and phosphoinositide 3-kinase activation. *J Immunol*. 2003;170:5851-5860.
 26. Afar DE, Han L, McLaughlin J, et al. Regulation of the oncogenic activity of BCR-ABL by a tightly bound substrate protein RIN1. *Immunity*. 1997;6:773-782.
 27. Kaluz S, Kaluzova M, Chrastina A, et al. Lowered oxygen tension induces expression of the hypoxia marker MN/carbonyl anhydrase IX in the absence of hypoxia-inducible factor 1 alpha stabilization: a role for phosphatidylinositol 3'-kinase. *Cancer Res*. 2002;62:4469-4477.
 28. Fruman DA, Mauvais-Jarvis F, Pollard DA, et al. Hypoglycaemia, liver necrosis and perinatal death in mice lacking all isoforms of phosphoinositide 3-kinase p85alpha. *Nat Genet*. 2000;26:379-382.
 29. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J Exp Med*. 1991;173:1213-1225.
 30. Wennstrom S, Hawkins P, Cooke F, et al. Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. *Curr Biol*. 1994;4:385-393.
 31. Mitchell TC, Hildeman D, Kedl RM, et al. Immunological adjuvants promote activated T cell survival via induction of Bcl-3. *Nat Immunol*. 2001;2:397-402.
 32. Lu-Kuo JM, Fruman DA, Joyal DM, Cantley LC, Katz HR. Impaired kit- but not FcepsilonRI-initiated mast cell activation in the absence of phosphoinositide 3-kinase p85alpha gene products. *J Biol Chem*. 2000;275:6022-6029.
 33. Yu J, Zhang Y, McIlroy J, Rordorf-Nikolic T, Orr GA, Backer JM. Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. *Mol Cell Biol*. 1998;18:1379-1387.
 34. Franke TF, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis. *Cell*. 1997;88:435-437.
 35. Burgering BM, Kops GJ. Cell cycle and death control: long live Forkheads. *Trends Biochem Sci*. 2002;27:352-360.
 36. Tran H, Brunet A, Griffith EC, Greenberg ME. The many forks in FOXO's road. *Sci STKE*. 2003;172:RE5.
 37. Komatsu N, Watanabe T, Uchida M, et al. A member of Forkhead transcription factor FKHL1 is a downstream effector of ST1571-induced cell cycle arrest in BCR-ABL-expressing cells. *J Biol Chem*. 2003;278:6411-6419.
 38. Dijkers PF, Medema RH, Pals C, et al. Forkhead transcription factor FKHL-1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). *Mol Cell Biol*. 2000;20:9138-9148.
 39. Lang F, Cohen P. Regulation and physiological roles of serum- and glucocorticoid-induced protein kinase isoforms. *Sci STKE*. 2001;108:RE17.
 40. Wymann MP, Zvelebil M, Laffargue M. Phosphoinositide 3-kinase signalling— which way to target? *Trends Pharmacol Sci*. 2003;24:366-376.