The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein

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Summary

The oncogenic BCR/ABL kinase activity induces and maintains chronic myelogenous leukemia (CML). We show here that, in BCR/ABL-transformed cells and CML blast crisis (CML-BC) progenitors, the phosphatase activity of the tumor suppressor PP2A is inhibited by the BCR/ABL-induced expression of the PP2A inhibitor SET. In imatinib-sensitive and -resistant (T315I included) BCR/ABL$^+$ cell lines and CML-BC progenitors, molecular and/or pharmacological activation of PP2A promotes dephosphorylation of key regulators of cell proliferation and survival, suppresses BCR/ABL activity, and induces BCR/ABL degradation. Furthermore, PP2A activation results in growth suppression, enhanced apoptosis, restored differentiation, impaired clonogenic potential, and decreased in vivo leukemogenesis of imatinib-sensitive and -resistant BCR/ABL$^+$ cells. Thus, functional inactivation of PP2A is essential for BCR/ABL leukemogenesis and, perhaps, required for blastic transformation.

Introduction

A tight control of kinase and phosphatase activity is fundamental for normal cell growth, survival, and differentiation (Hunter, 1995). Chronic myelogenous leukemia (CML) is the best example for understanding how central the "reversible phosphorylation" process is in the regulation of normal hematopoiesis. In fact, the deregulated tyrosine kinase activity of the BCR/ABL oncoprotein induces and maintains the leukemic phenotype and contributes to transition from an indolent "chronic phase" (CML-CP) to the aggressive "blast crisis" (CML-BC) (Calabretta and Perrotti, 2004) by recruiting and activating pathways that transduce oncogenic signals leading to growth factor-independent proliferation, increased survival, and altered differentiation of hematopoietic progenitors (Calabretta and Perrotti, 2004; Wong and Witte, 2004). Dependence on BCR/ABL expression is not only a feature of CML-CP; in fact, BCR/ABL levels increase during disease progression (Barnes et al., 2005; Elmaagacli et al., 2000; Gaiger et al., 1995; Jamieson et al., 2004), and sustained BCR/ABL expression in myeloid progenitors induces phenotypic changes characteristic of CML-BC (Calabretta and Perrotti, 2004). Accordingly, inhibition of BCR/ABL kinase activity with imatinib mesylate is therapeutically effective not only in CML-CP (O'Brien et al., 2003) but also, albeit temporarily, in CML-BC (Sawyers et al., 2002), in which resistance and relapses are contingent upon BCR/ABL reactivation (Shah and Sawyers, 2003).

In BCR/ABL-expressing myeloid progenitor cells, high levels

SIGNIFICANCE

A tight control of kinase and phosphatase activity is fundamental for normal cell growth, survival, and differentiation. Leukemia is often associated with expression of oncoproteins with aberrant kinase activity. By contrast, PP2A, a phosphatase regulating many cellular functions, is genetically inactivated in many types of cancer. Here, we show that PP2A activity is suppressed in blast crisis but not chronic phase CML cells through the BCR/ABL-induced expression of its inhibitor, SET. Restoration of PP2A activity inhibits BCR/ABL expression/activity, hence impairing wild-type and T315I BCR/ABL leukemogenesis. These findings establish an unexpected link between an oncogenic kinase and a phosphatase with tumor suppressor activity and indicate that pharmacologic enhancement of PP2A represents a possible therapeutic strategy for blast crisis and imatinib-resistant CML.

of BCR/ABL, like those observed in CML-BC, arrest differentiation and enhance survival in part by enhancing the expression and activity of specific RNA binding proteins (Iervolino et al., 2002; Perrotti et al., 1998, 2002; Trotta et al., 2003). In particular, expression of hnRNP A1 is induced by BCR/ABL in a kinase-dependent manner and increases in CML-BC (Iervolino et al., 2002). In addition, hnRNP A1 mRNA export activity is required for proliferation, survival, and tumorigenesis of acute phase CML blasts and BCR/ABL$^+$ myeloid precursor cell lines (Iervolino et al., 2002).

In this study, we identified SET, a nucleus/cytoplasm-localized phosphoprotein overexpressed in solid tumors and leukemias (Carlson et al., 1998; Fornerod et al., 1995), as a novel BCR/ABL target whose mRNA is associated with hnRNP A1 in Ph^1 cells. Notably, SET is a potent inhibitor of the tumor suppressors NM23-H1 (Fan et al., 2003) and protein phosphatase 2A (PP2A) (Li et al., 1996). The latter is a phosphatase that regulates cell proliferation, survival, and differentiation (Janssens and Goris, 2001; Wang et al., 2004) and whose loss of function has been associated with cell transformation (Janssens et al., 2005; Schonthal, 2001). We herein show that in BCR/ABL$^+$ cell lines and in patient-derived CML (CP and BC) CD34$^+$ cells, SET expression is enhanced by BCR/ABL and increases during CML disease progression. This, in turn, results in progressive loss of PP2A tumor-suppressive activity. Restoring PP2A activity in myeloid CML-BC^{CD34+} patient cells and BCR/ABL$^+$ cell lines impairs in vitro and in vivo BCR/ABL leukemogenic potential by inducing inactivation and consequent downregulation of the BCR/ABL oncoprotein itself. Thus, the BCR/ABL-dependent SET-mediated inhibition of PP2A tumor-suppressive activity may, therefore, favor CML disease progression.

Results

SET association with hnRNP A1 and effect of BCR/ABL on SET expression

Using Ribonomics (Tenenbaum et al., 2002), SET mRNA was found specifically associated with cytoplasmic hnRNP A1 in Ph^1 K562 cells (data not shown). To validate the SET mRNA-hnRNP A1 association, RT-PCR was carried out on anti-hnRNP A1- and anti-FLAG-immunoprecipitated cytoplasmic mRNAs using a pair of primers spanning a 224 bp segment of the human *SET* cDNA. As shown, high levels of SET mRNA transcripts, similar to those present in the mRNP-enriched mRNA fraction, were clearly detectable in association with hnRNP A1 (Figure 1A).

In growth factor-independent BCR/ABL-expressing 32Dcl3 myeloid precursors, SET mRNA and protein levels correlated with those of hnRNP A1 and BCR/ABL (Figure 1B). Similarly, SET was upregulated in the doxycycline-treated (2 μg/ml; 3 days) TonB210.1 lymphoid precursors in which BCR/ABL expression is tetracycline inducible (Figure 1B, lanes 6 and 7). In addition, treatment (24 hr, 1 μM) with the ABL tyrosine kinase inhibitor imatinib markedly impaired SET mRNA and protein expression in both 32D-BCR/ABL and K562 cells (Figure 1B, lanes 2 and 5). Consistent with these data, SET and hnRNP A1 levels were increased upon BCR/ABL expression in CD34$^+$ mouse bone marrow cells (BMC) (Figure 1B, lanes 8 and 9).

SET protein levels correlated with BCR/ABL activity and were higher in myeloid CML-BC^{CD34+} (n = 3) than CML-CP^{CD34+} (n = 3) patient-derived BMC, and in CML-CP^{CD34+} patient cells than in CD34$^+$ cells from normal donors (NBM) (n = 3) (Figure 1C, right panel). Similarly, SET expression was higher in myeloid CML-BC (n = 9) than in CML-CP (n = 9) patient-derived mononuclear marrow cells (Figure 1C, left and middle panels) and correlated with that of BCR/ABL and hnRNP A1 in paired CML-CP and CML-BC samples (Figure 1C, left panel). Moreover, treatment of CML-BC^{CD34+} cells with imatinib decreased SET levels (Figure 1C, lanes 5 and 6), further indicating that BCR/ABL activity is responsible for SET upregulation in CML-BC. As expected, SET expression was reduced in cytokine-deprived (12 hr) 32Dcl3 and NBM^{CD34+} cells (see Figure S1A in the Supplemental Data available with this article online) but not in cytokine-deprived BCR/ABL$^+$ cells.

Relevance of increased SET expression in BCR/ABL-transformed cells: Role of PP2A

Reportedly, SET is a potent inhibitor of the multimeric serine-threonine phosphatase PP2A (Li et al., 1996). In 32D-BCR/ABL and in CML-BC^{CD34+} patient cells (Figure 2A, left panel) and K562 cells (data not shown), levels of the PP2A catalytic subunit (PP2Ac) were not affected by BCR/ABL expression and/or kinase activity. By contrast, levels of the PP2A structural subunit PR65/A were enhanced in 32D-BCR/ABL cells and correlated with increased levels of PP2Ac phosphorylation on tyrosine 307 (Figure 2A, right panel) which, as reported, inhibits PP2A activity (Chen et al., 1992). Accordingly, PP2A activity was reduced by 83% in 32D-BCR/ABL compared to parental 32Dcl3 cells, and restored to normal levels in imatinib-treated (1 μM, 24 hr) 32D-BCR/ABL cells (Figure 2B). As expected, imatinib did not rescue PP2A activity in imatinib-resistant BaF3p210^{T315I} cells (Figure 2B). Furthermore, PP2A activity was also reduced by 94% and 52% in the CD34$^+$ fraction of myeloid CML-BC and CML-CP cells, respectively, compared to the CD34$^+$ fraction of NBM cells (Figure 2B).

To determine whether SET upregulation is responsible for suppression of PP2A activity, we interfered with SET expression by infecting parental 32Dcl3, 32D-BCR/ABL, and K562 cells with a pSuper.retro-shSET construct carrying a short hairpin RNA (shRNA) targeting nucleotides 120–138 of the *SET* cDNA. Expression of SET shRNA downregulated levels of SET, but not those of the other PP2A regulator pp32/I1PP2A (Figure 2B, inset) and restored PP2A activity to levels similar to those of 32Dcl3 or imatinib-treated 32D-BCR/ABL cells (Figure 2B) without affecting PP2Ac expression (Figure 2A). Interestingly, levels of PP2Ac^{TYR307} were markedly reduced upon imatinib treatment and SET downregulation (Figure 2C). Moreover, in BCR/ABL-expressing cells, expression of ectopic HA-PP2Ac strongly increased PP2A activity (Figure 2B) and diminished PP2Ac^{TYR307} levels (Figure 2C), while no effects were observed in vector-transduced cells (data not shown). PP2A activity also was not significantly affected in parental 32Dcl3 cells by SET shRNA expression (Figure S1B). By contrast, ectopic FLAG-SET expression augmented PP2A^{TYR307} levels in HA-PP2Ac-expressing BCR/ABL cells (Figure 2C) and lowered PP2A activity in 32Dcl3 cells to levels similar to those of 32D-BCR/ABL cells (Figure S1B). Accordingly, SET was associated with PP2Ac in 32D-BCR/ABL and CML-BC^{CD34+} cells but not in 32Dcl3 cells (Figure S2C).

Because several PP2A targets are essential for BCR/ABL leukemogenic potential, we assessed the importance of the SET-PP2A interplay in the regulation of the BCR/ABL signaling

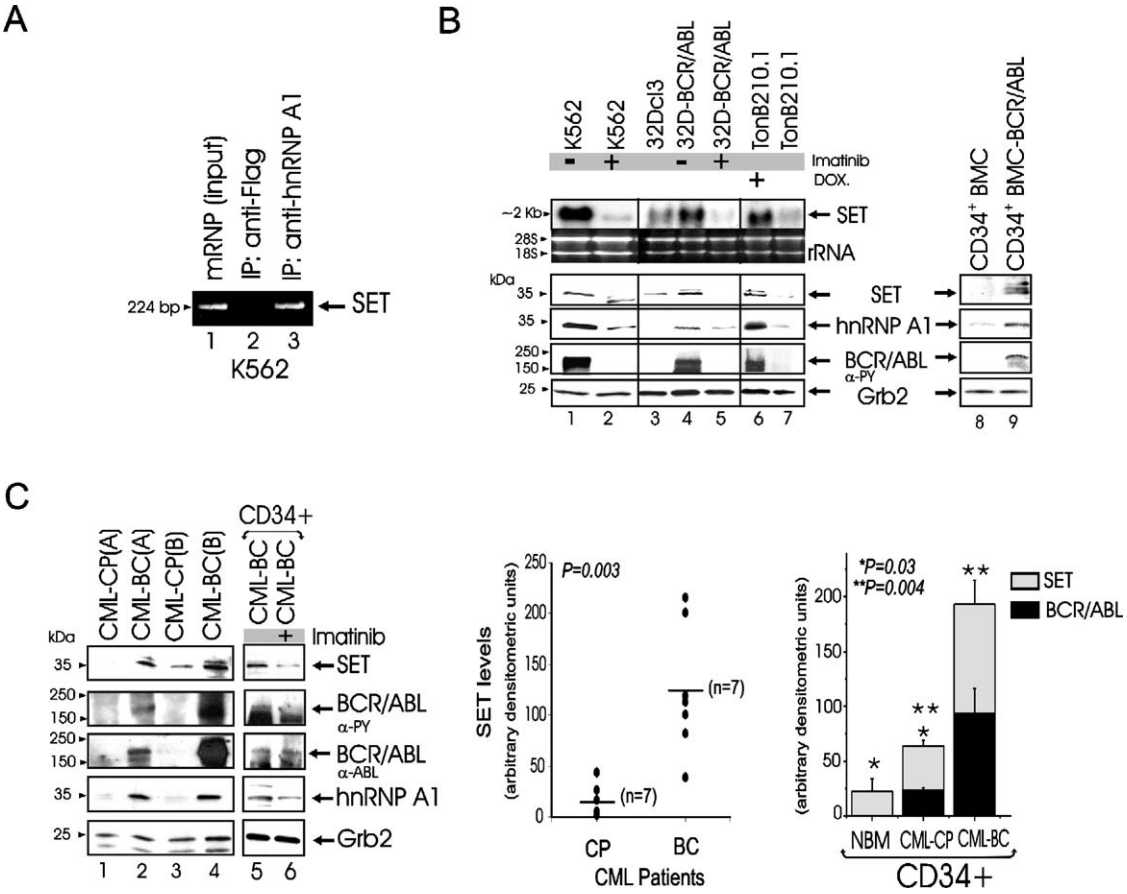


Figure 1. Effect of BCR/ABL on SET expression

A: RT-PCR shows association of SET mRNA with hnRNP A1 in mRNP-enriched lysates, anti-FLAG-IP (negative control), and anti-hnRNP A1-IP from K562 cells.

B: SET RNA (first row) and protein (third row) levels in 32Dcl3, untreated and imatinib-treated 32D-BCR/ABL and K562 cells, doxycycline (DOX)-treated or untreated BCR/ABL-inducible TonB210.1 cells, and vector- and BCR/ABL-infected mouse CD34$^+$/lin$^-$ bone marrow cells (BMC). hnRNP A1, BCR/ABL, and Grb2 protein levels were detected as control.

C: Left panel: SET, hnRNP A1, and BCR/ABL protein levels in mononuclear marrow (BM) cells from two paired CML-CP and -BC patient samples, and in untreated and imatinib-treated CML-BC^{CD34+} cells; middle panel: scatter plots show SET protein levels in BM cells from seven CML-CP and -BC patient specimens ($p = 0.003$; Wilcoxon rank sum test); right panel: graph shows SET (light bars) and BCR/ABL (dark bars) protein expression (expressed as mean ± SE of densitometric units after normalization with Grb2 levels) in the CD34$^+$ fraction from bone marrow of healthy donors (NBM) (n = 3), CML-CP (n = 3), and CML-BC (n = 3) patients (NBM versus CML-CP, $p = 0.03$; CML-CP versus CML-BC, $p = 0.004$; paired samples t test).

network by determining the levels and/or activity of distinct BCR/ABL and PP2A targets in SET shRNA- and HA-PP2Ac-expressing 32D-BCR/ABL cells. In these cells, enhanced PP2A activity induced by imatinib treatment, by inhibition of SET, and to a greater extent, by PP2Ac overexpression decreased levels of hyperphosphorylated Rb, suppressed Myc expression, and STAT5, ERK1/2, Akt, BAD, and Jak2 phosphorylation (Figure 2C, lanes 2–4) without affecting their expression (Figure S2D). Consistent with the role of PP2A as a potent BCR/ABL antagonist, transduction of FLAG-SET in HA-PP2A-expressing 32D-BCR/ABL cells restored the expression/activity of these BCR/ABL targets (Figure 2C, lane 5).

BCR/ABL is a target of PP2A activity

Expression of SET shRNA or HA-PP2A in 32D-BCR/ABL cells suppressed p210$^{BCR/ABL}$ tyrosine phosphorylation and expression (Figure 3A, lanes 2 and 3). This effect, also observed in K562 cells (data not shown), was dependent on enhanced PP2A phosphatase activity because both SET overexpression and treatment with the phosphatase inhibitor okadaic acid (0.25 nM; 1 hr) and calyculin A (data not shown), used at concentrations that inhibit PP2A but not other phosphatases (Saydam et al., 2003), restored BCR/ABL activity and expression in HA-PP2Ac-transduced (Figure 3A, lanes 4 and 7) and in SET shRNA-transduced (data not shown) cells. Conversely, exposure (18–24 hr) of BCR/ABL$^+$ cell lines and CML-BC^{CD34+} marrow blasts to the PP2A activators (Feschenko et al., 2002) forskolin (40 μM), butyryl-forskolin (water soluble), or 1,9-dideoxy-forskolin (lacks adenylate cyclase-activating function) enhanced PP2A activity (Figure S3A), abolished BCR/ABL phosphorylation, and to a different extent, induced downregulation of BCR/ABL (Figure 3B) with EC_{50} of 18 μM and 25 μM in imatinib-sensitive and -resistant cells, respectively. Interestingly, the cAMP inducer theophylline (100 μM; 24 hr) did not affect BCR/ABL expres-

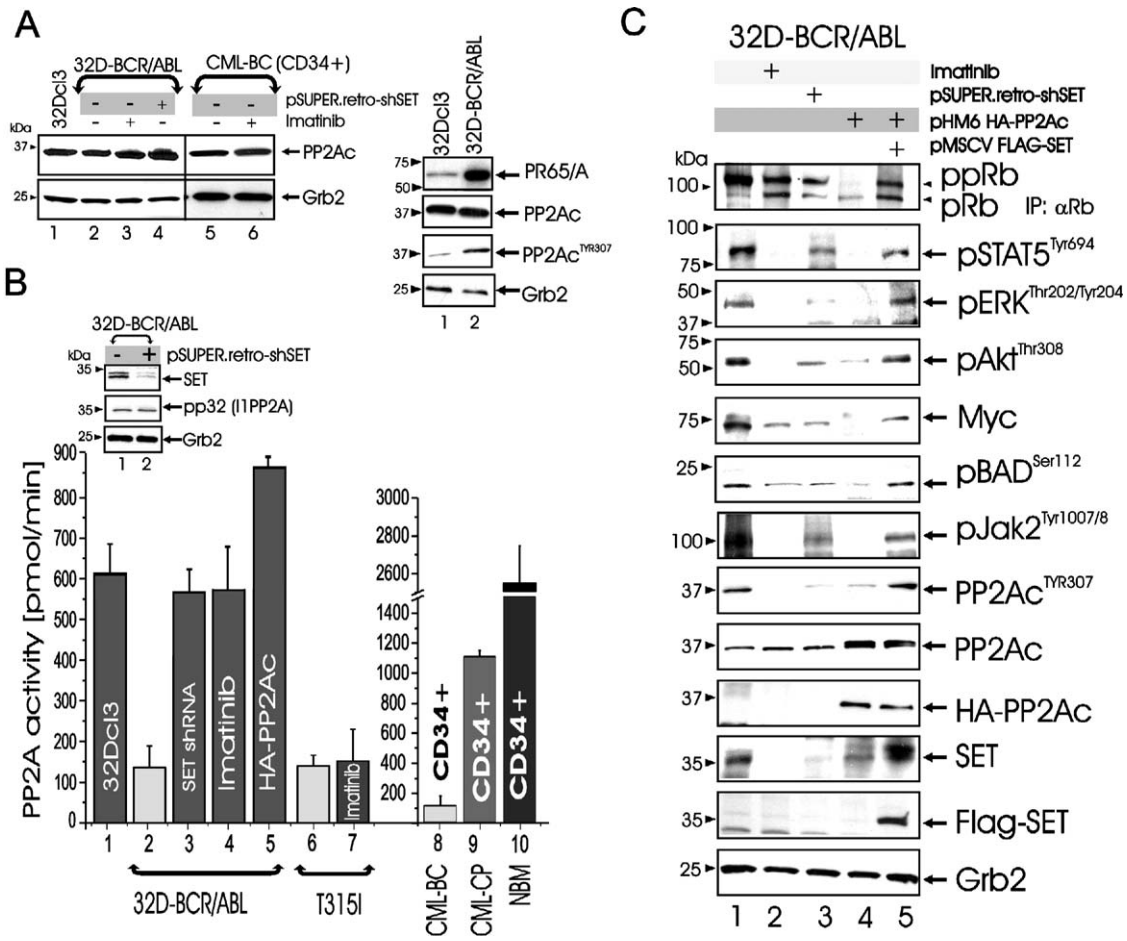


Figure 2. Effect of BCR/ABL on PP2A activity

A: Left panel: protein levels of PP2Ac and Grb2 in the following: 32Dcl3, untreated and imatinib-treated 32D-BCR/ABL and primary CML-BC^{CD34+} marrow cells, and SET shRNA-expressing 32D-BCR/ABL; right panel: levels of PR65/A, PP2Ac, PP2Ac^{TYR307}, and Grb2 in 32Dcl3 and 32D-BCR/ABL cells.

B: PP2A phosphatase assay in 32Dcl3, 32D-BCR/ABL, BaF3-p210(T315I), CML-BC^{CD34+}, CML-CP^{CD34+}, and NBM^{CD34+}cells, and in BCR/ABL cells expressing the SET shRNA or HA-PP2Ac or treated with imatinib. Bars represent the PP2A activity (expressed as mean ± SE of three independent experiments performed with the indicated cell lines, and with NBM^{CD34+} (n = 3), CML-CP^{CD34+} (n = 3), and CML-BC^{CD34+} (n = 3) cells. Inset: effect of the SET shRNA on SET and pp32 protein levels; representative of two experiments performed in triplicate.

C: Effect of imatinib and of ectopic SET shRNA, HA-PP2Ac, or HA-PP2A/FLAG-SET expression on the levels of the indicated BCR/ABL and PP2A targets in 32D-BCR/ABL cells. Endogenous and ectopic SET and PP2Ac and PP2Ac^{TYR307} levels are shown (rows 8–12).

sion/activity, and the PKA inhibitor myristoylated-PKI(14-22)-amide (4 μM; 24 hr) did not inhibit the forskolin-induced BCR/ABL downregulation (Figure 3C).

PP2A-dependent mechanisms regulating BCR/ABL expression/activity

To determine whether PP2A-induced BCR/ABL dephosphorylation renders BCR/ABL more susceptible to proteolysis, we treated PP2A-expressing 32D-BCR/ABL cells with the proteasome inhibitor ALLN (25 μM; 8 hr). As shown, ALLN restored BCR/ABL expression and, as expected, did not prevent PP2A-induced BCR/ABL tyrosine dephosphorylation (Figure 4A), which precedes and, likely, is required for its degradation.

In anti-Abl Western blot on anti-PP2Ac immunoprecipitates, we found p210$^{BCR/ABL}$ associated with PP2Ac in SET shRNA-expressing but not in parental 32D-BCR/ABL cells (Figure 4B, lanes 3 and 4). Interestingly, the BCR/ABL-associated SHP-1 tyrosine phosphatase (Liedtke et al., 1998) was also present in the same PP2A-containing complex (Figure 4B), and restored BCR/ABL phosphorylation was observed after treatment of SET shRNA- and HA-PP2A-expressing 32D-BCR/ABL cells with sodium stibogluconate (SS) (Figure 4C), a specific SHP-1 inhibitor (Pathak and Yi, 2001). Furthermore, the PP2A activator forskolin (40 μM; 18 hr) inhibited BCR/ABL expression and tyrosine phosphorylation and decreased by ~70% the clonogenic potential of BCR/ABL-transduced wild-type (+/+) but not *SHP-1*-deficient (Kozlowski et al., 1993) (–/–) *moth eaten viable* CD34$^+$ mouse marrow cells (Figures 4D and 4E). Note that the clonogenicity of *SHP-1*$^{-/-}$ cells was higher than that of wild-type cells (Figure 4E).

Increased PP2A activity suppresses BCR/ABL oncogenic potential in vitro

To assess whether increased PP2A activity affects proliferation and survival of BCR/ABL-transformed cells, parental, SET shRNA-expressing, and HA-PP2A-expressing 32D-BCR/ABL

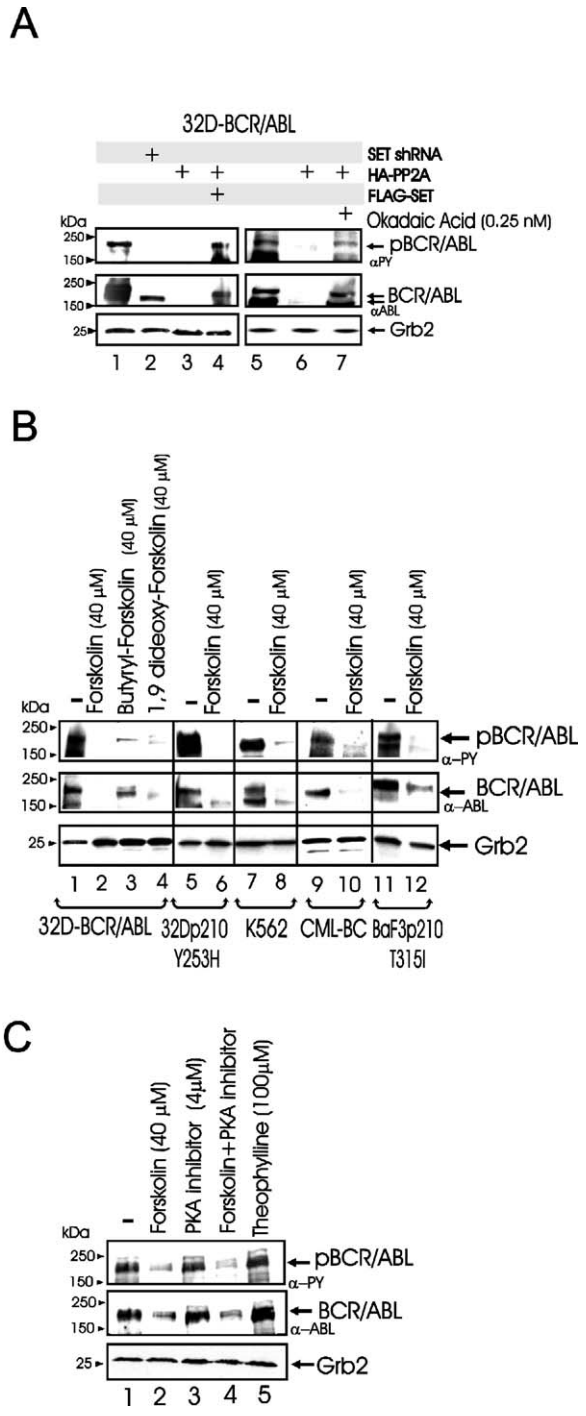


Figure 3. Effect of PP2A on BCR/ABL kinase activity and expression

A: $p210^{BCR/ABL}$ activity (αPY) and expression (α-Abl) in 32D-BCR/ABL and in its derivative SET shRNA-, HA-PP2A-, and HA-PP2A/FLAG-SET-expressing cell lines, and in HA-PP2A 32D-BCR/ABL cells treated with okadaic acid.

B: Effect of the PP2A activators forskolin, butyryl-forskolin, and 1,9-dideoxy-forskolin on BCR/ABL activity and expression in primary CML-BC and imatinib-sensitive and -resistant BCR/ABL cell lines.

C: Effects of forskolin, theophylline, and the PKA inhibitor myristoylated PKI (14-22) amide on BCR/ABL expression/activity. Grb2 levels were detected as controls.

cells were grown in the presence or absence of IL-3. In IL-3-containing medium, there were no differences in the growth of 32D-BCR/ABL and its derivative cell lines (data not shown). Between 36 and 48 hr after IL-3 withdrawal, an ~60% decrease in the number of viable cells was visible in SET shRNA- and HA-PP2A-expressing 32D-BCR/ABL when compared to parental cultures (Figure 5A). Moreover, SET shRNA- and, to a greater extent, HA-PP2A-expressing 32D-BCR/ABL cells underwent granulocytic differentiation upon exposure (9–12 days) to G-CSF (Figure 5B). As expected, forced FLAG-SET expression conferred a proliferation advantage and inhibited differentiation of HA-PP2A-expressing cells (Figures 5A and 5B). Similarly, reduced viability and spontaneous erythroid differentiation, observed in K562 cells ectopically expressing SET shRNA or HA-PP2A, was counteracted by SET overexpression (data not shown).

Note that SET overexpression did not confer cytokine-independent growth and slightly enhanced proliferation of 32Dcl3 cells (Figure S1C). Similarly, SET downregulation in 32Dcl3 cells did not alter IL-3-dependent proliferation, consistent with the observation that SET shRNA expression did not affect PP2A activity in 32Dcl3 cells (Figures S1B and S1C).

The importance of suppression of PP2A activity for BCR/ABL leukemogenesis was further investigated by assessing the colony-forming ability of SET shRNA-, HA-PP2A-, and HA-PP2A/FLAG-SET-expressing BCR/ABL^{+} cell lines. Similarly, $CML\text{-}BC^{CD34+}$ patient cells transduced with a MigRI-HA-PP2Ac or insert-less retrovirus were GFP sorted and plated in methylcellulose in the presence of cytokines. 32D-BCR/ABL (Figure 5C) and K562 (data not shown) cells formed high numbers of colonies in the absence of growth factors (Figure 5C). By contrast, the colony-forming ability of SET shRNA- and HA-PP2A-expressing cells was markedly suppressed (60%–65% inhibition) (Figure 5C), and the colony size was much smaller than that of parental cells (data not shown). Consistent with its role as a PP2A inhibitor, SET overexpression completely rescued the cytokine-independent colony-forming ability of HA-PP2A-expressing BCR/ABL cell lines (Figure 5C). Accordingly, HA-PP2Ac expression restored PP2A activity back to normal levels (Figure 5C, inset in right panel) and reduced by ~90% the clonogenic potential of $CML\text{-}BC^{CD34+}$ cells (Figure 5C, right panel).

Antileukemic effects of forskolin in primary $CML\text{-}BC^{CD34+}$ cells and in imatinib-sensitive and -resistant BCR/ABL cell lines

To investigate whether inhibition of BCR/ABL activity/expression by pharmacological activation of PP2A (Figure 3B) correlates in vitro with impaired BCR/ABL leukemogenic potential, 32Dcl3, NBM^{CD34+}, 32D-BCR/ABL, K562, the imatinib-resistant 32D-$p210^{Y253H}$ and BaF3-$p210^{T315I}$, and $CML\text{-}BC^{CD34+}$ patient cells were treated with the PP2A activator forskolin (40 μM; 96 hr). Forskolin strongly augmented PP2A phosphatase activity in all BCR/ABL cell lines and primary $CML\text{-}BC^{CD34+}$ blasts (Figure 6A) but not in 32Dcl3 (Figure S1B) or in NBM^{CD34+} cells (Figure 6A). While untreated cells grew with normal kinetics (Figure 6A, light bars), forskolin treatment resulted in marked suppression of cell growth and enhanced apoptosis (Figure 6A, dark bars) of cytokine-deprived imatinib-sensitive and -resistant BCR/ABL^{+} cell lines and of IL-3/IL-6/Flt-3 ligand/c-Kit ligand-cultured $CML\text{-}BC^{CD34+}$ patient cells.

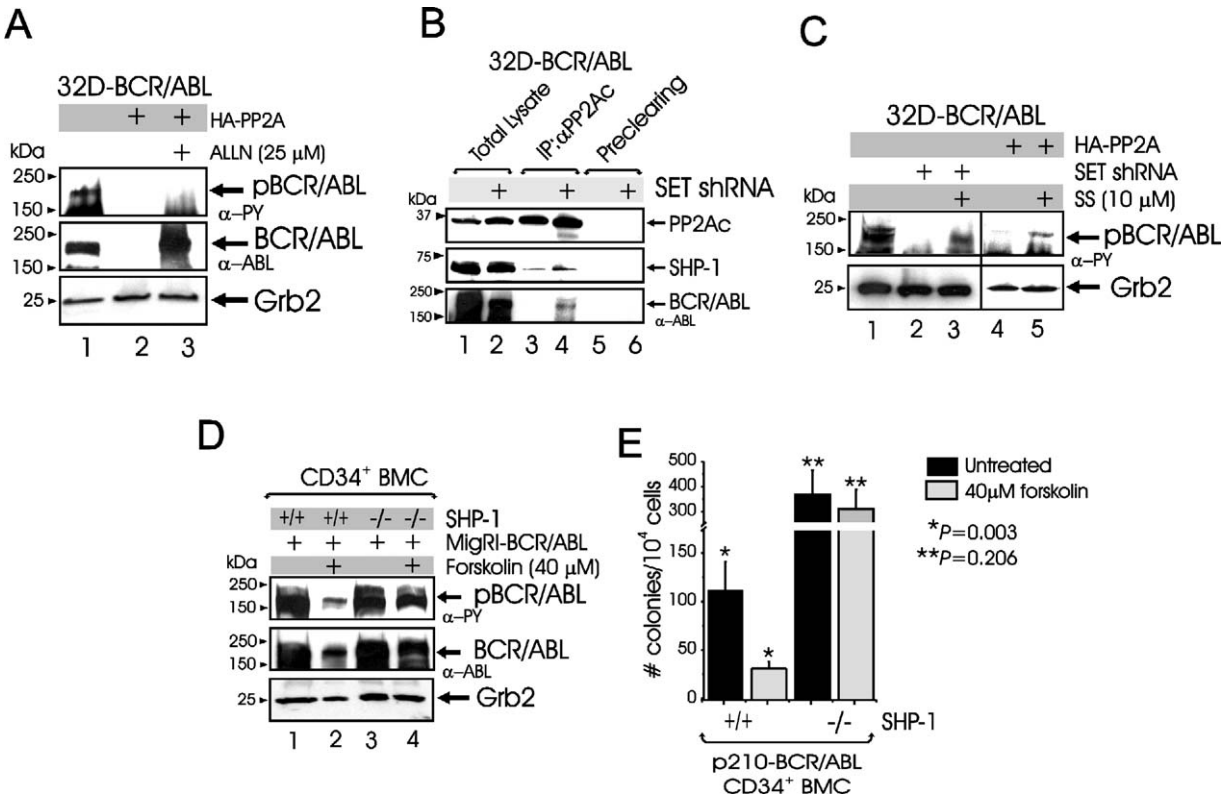


Figure 4. PP2A-dependent regulation of BCR/ABL activity/expression

A: $p210^{BCR/ABL}$ activity and expression in parental and untreated or ALLN-treated HA-PP2A-expressing 32D-BCR/ABL cells. **B:** Association of PP2A with BCR/ABL and SHP-1 in parental and SET shRNA-expressing 32D-BCR/ABL cells. **C:** Effect of the SHP-1 inhibitor sodium stibogluconate (SS) on BCR/ABL activity in parental and HA-PP2A- and SET shRNA-expressing 32D-BCR/ABL cells. Effect of forskolin on BCR/ABL expression/phosphorylation (**D**) and clonogenicity (**E**) of BCR/ABL-expressing wild-type (+/+) and *SHP-1*-deficient "viable moth eaten" (−/−) $CD34^+$ mouse bone marrow cells (BMC). Bars represent the mean ± SE of the colony number from four clonogenic assays with untreated and forskolin-treated BCR/ABL-expressing wild-type and viable moth eaten $CD34^+$ marrow cells (untreated$^{+/+}$ versus drug-treated$^{+/+}$, p = 0.003; untreated$^{-/-}$ versus drug-treated$^{-/-}$ p = 0.206; paired samples t test).

As expected, the effect of imatinib (1 μM) paralleled that of forskolin in imatinib-sensitive, but not resistant, BCR/ABL^+ cells (Figure 6A, red lines). Note that, after 48 hr of forskolin treatment, the percentage of apoptosis (annexin V^+ cells) in BCR/ABL^+ cell lines and primary CML-BC cells was ≥80% and ≥95%, respectively (Figure S3B). By contrast, forskolin treatment neither inhibited IL-3-dependent growth nor induced apoptosis of 32Dcl3 cells (Figures S1C and S3B). Notably, in primary NBM^{CD34+} cells, forskolin partially inhibited cytokine-dependent proliferation to the same extent as imatinib (Figure 6A) without inducing apoptosis (Figure S3B). Indeed, cell cycle analysis of forskolin-treated (24 hr) NBM^{CD34+} cells revealed an increased number of cells in G1 and a reduced number in S phase (data not shown).

Restored differentiation was another feature exhibited by forskolin-treated cells; after 9–12 days of exposure to G-CSF and forskolin, cell death was evident in 30%–40% of both imatinib-sensitive 32D-BCR/ABL and -resistant 32D-$p210^{Y253H}$ cell cultures, whereas the remaining 60%–70% showed signs (e.g., segmented nuclei) of terminal neutrophilic differentiation (Figure 6B, right panels). By contrast, cells exposed only to G-CSF remained blasts and proliferated (Figure 6B, left panels). Seemingly, forskolin-treated K562 cells underwent erythroid differentiation (Figure 6B).

Regardless of the degree of responsiveness to imatinib, both sensitive and resistant BCR/ABL^+ cell lines showed inhibition (average: 75%) of clonogenic potential when exposed to forskolin (Figure 6C). Accordingly, forskolin strongly abolished (80%–90% inhibition) the ability of primary $CML\text{-}BC^{CD34+}$ and $CML\text{-}CP^{CD34+}$ marrow cells to form IL-3-derived colonies in semisolid medium (Figure 6C). In addition, the colonies derived from forskolin-treated cells were also reduced in size (data not shown), thus resembling the effects of PP2A overexpression on the colony-forming ability of BCR/ABL^+ cells. Moreover, levels of BCR/ABL phosphorylation in residual colonies derived from forskolin-containing cultures were comparable to those from colonies of untreated BCR/ABL cells (data not shown), suggesting that a decrease in forskolin activity occurring in 15 days of semisolid culture accounts for the presence of residual colonies.

Although forskolin did not induce apoptosis of NBM^{CD34+} cells, a 40%–50% decrease in their IL-3-dependent clonogenicity was observed upon exposure to forskolin (Figure 6C). Interestingly, the PP2A activator 1,9-dideoxy forskolin, which lacks adenylate cyclase-activating function, had only a modest (≤15%) effect on cytokine-driven colony formation of $CD34^+$ myeloid progenitors, whereas it induced a 85%–90% decrease in the clonogenic potential of $CML\text{-}BC^{CD34+}$cells (Figure 6C).

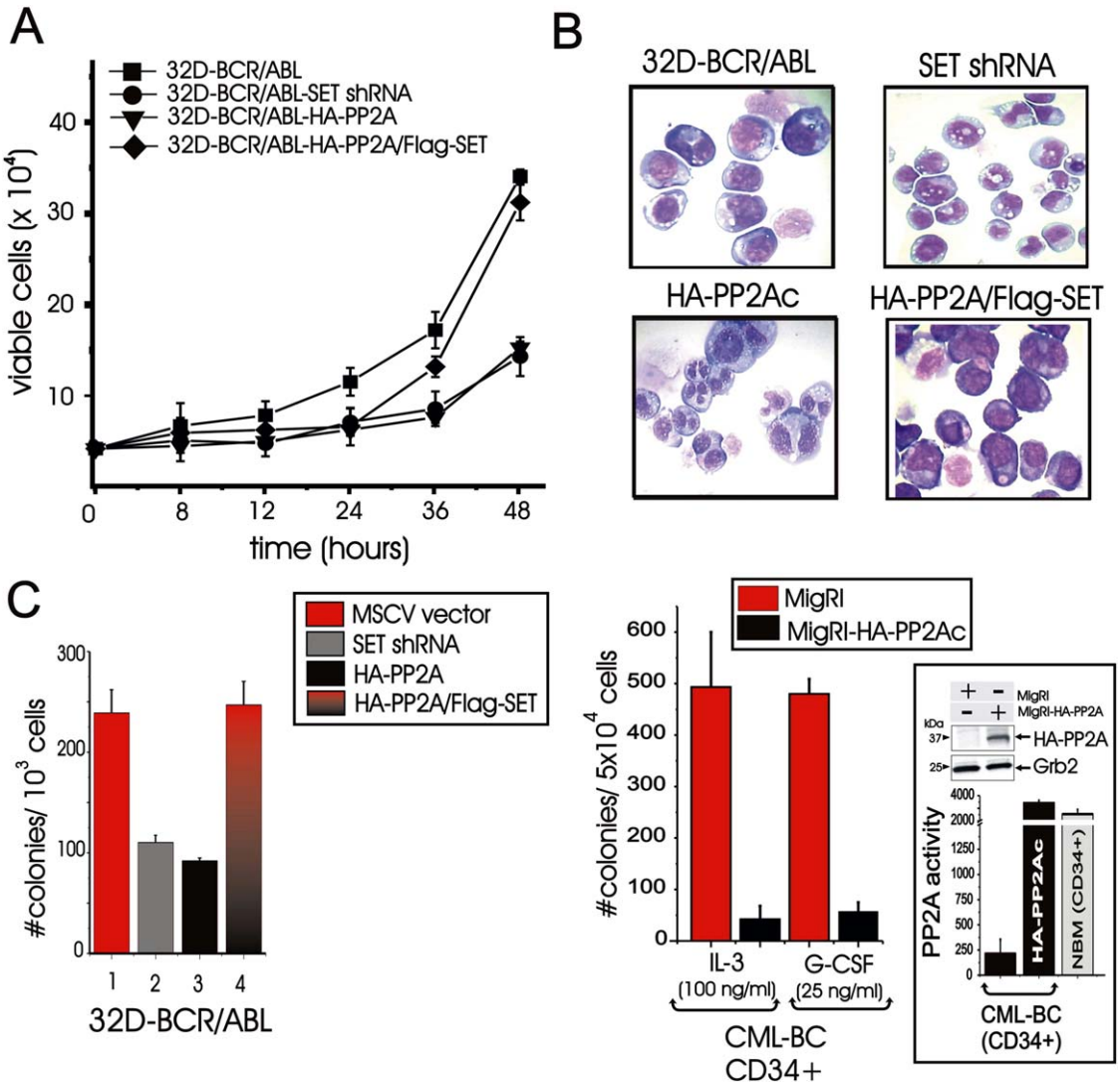


Figure 5. In vitro effects of PP2Ac overexpression

A: Effect of ectopic SET shRNA, HA-PP2A, and HA-PP2A/FLAG-SET expression on the cytokine-independent growth of 32D-BCR/ABL. Each point of the graph represents the mean ± SE of the number of viable cells from three independent experiments.

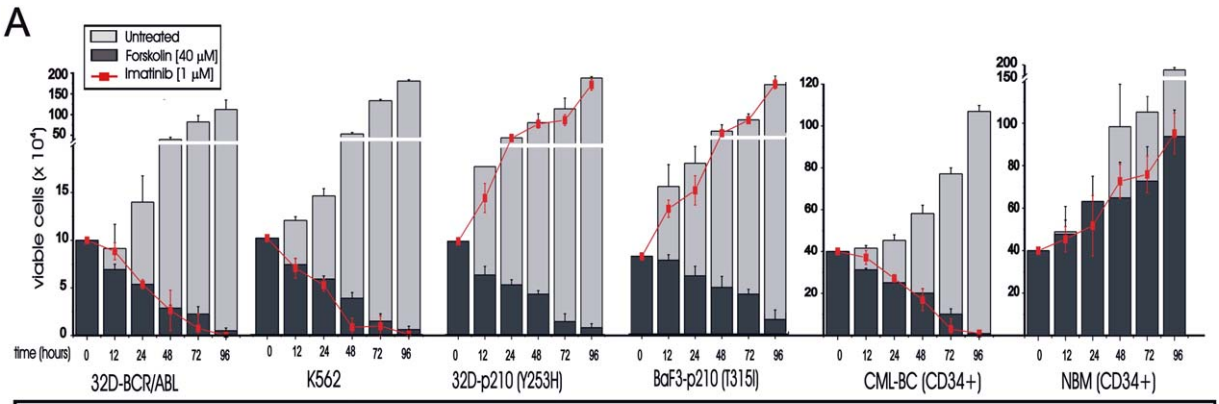
B: May-Grunwald/Giemsa staining of cytospins of G-CSF-treated (9–12 days) 32D-BCR/ABL and derivative cell lines.

C: Growth factor-independent colony-forming ability of parental cells and SET shRNA-, HA-PP2A-, and HA-PP2A/FLAG-SET-expressing 32D-BCR/ABL, and effect of ectopic PP2Ac expression on the IL-3- and G-CSF-driven colony formation of CML-BC^{CD34+} (dark bars). Red bars indicate colonies from vector-transduced cells. Bars represent the mean ± SD of three independent experiments (primary cells: n = 2). Inset: expression of ectopic PP2Ac in GFP^{+} CML-BC^{CD34+} and PP2A activity in MigRI- and MigRI-HA-PP2Ac-transduced CML-BC^{CD34+}, and in CD34^{+} normal marrow cells (NBM).

Increased PP2A activity impairs in vivo BCR/ABL leukemogenesis

To investigate the effect of PP2A on BCR/ABL tumorigenesis, SCID mice (n = 5 per group) were injected subcutaneously with parental cells or SET shRNA-, HA-PP2Ac-, or HA-PP2A/FLAG-SET-expressing 32D-BCR/ABL cells. 32D-BCR/ABL and 32D-BCR/ABL-HA-PP2A/FLAG-SET cells formed tumors (incidence: 5/5) in 6–7 days, whereas tumors from SET shRNA-expressing 32D-BCR/ABL cells were palpable (incidence: 5/5) only after 10–12 days postinjection (Figure 7A). By contrast, tumors from HA-PP2A-expressing 32D-BCR/ABL cells were at most barely palpable (incidence: 3/5) and, at 15 days postinjection, weighed 86%–90% less than the tumors derived from parental or HA-PP2A/FLAG-SET-expressing 32D-BCR/ABL cells (Figure 7A). Likewise, a 75% decrease in tumor mass was observed in mice injected with SET shRNA-expressing 32D-BCR/ABL cells.

To determine if forskolin ($LD50_{i.p.}$ = 67 mg/kg) can attenuate BCR/ABL leukemogenesis, SCID mice were intraperitoneally (i.p.) treated with forskolin (4 mg/kg) 1 hr prior to and 7 days after the intravenous (i.v.) injection of 32D-BCR/ABL cells (5 × 10^5 cells/mouse) and compared with mice injected either with cells or with forskolin only. Four weeks later, various organs obtained from untreated, treated, and control groups were evaluated by visual inspection and light microscopy. Mice in-



PP2A activity [pmol/min]	32D-BCR/ABL	K562	32D-p210 (Y253H)	BaF3-p210 (T315I)	CML-BC (CD34+)	NBM (CD34+)
untreated:	150.9± 12.9	124.9± 16.6	438.3±19.5	112.3±67.6	152.3±30.8	2029.3±61.3
+Forskolin:	1776.1± 36.1	1456.3± 61.5	1832.0±66.5	1096.9±94.8	1487.6± 23.2	2403.6±37.3

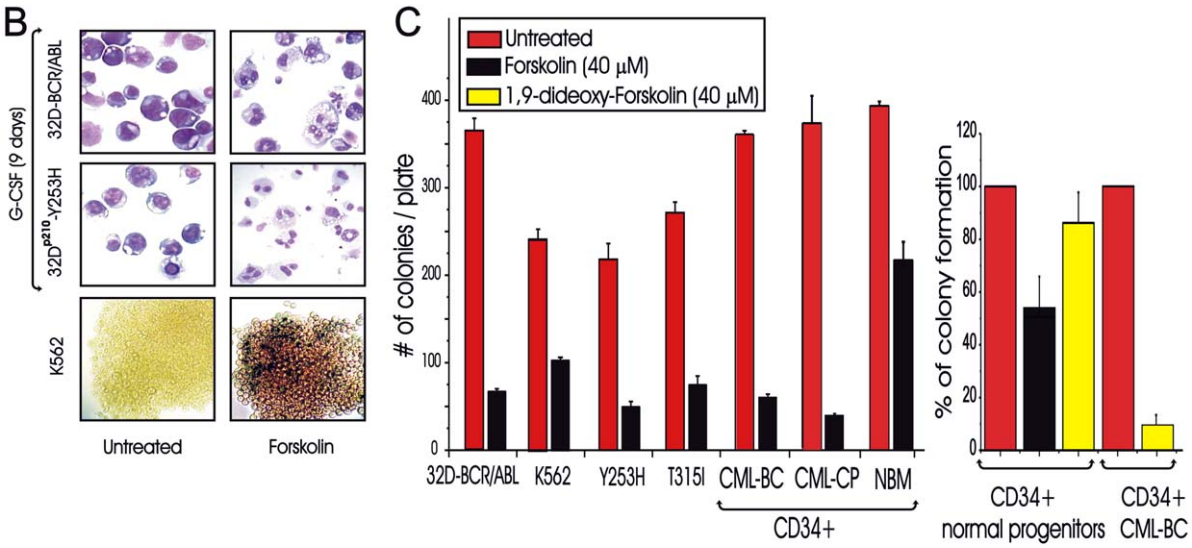


Figure 6. In vitro effects of forskolin on BCR/ABL oncogenic potential

A: Effect of the PP2A activator forskolin (dark bars) and of imatinib (red lines) on the growth of imatinib-sensitive and -resistant BCR/ABL^{+} cell lines and of primary CML-BC^{CD34+} (n = 2) and NBM^{CD34+} (n = 2) cells; untreated cells (light bars). Effect of forskolin on PP2A activity is shown below each graph. Viable cells and phosphatase activity in untreated and drug-treated cell lines and primary cells (n = 2) are expressed as mean ± SE of three independent experiments.

B: May-Grunwald/Giemsa staining of G-CSF-treated 32D-BCR/ABL and imatinib-resistant 32D-p210^{Y253H} cells; o-Dianisidine staining of untreated and forskolin-treated (48 hr) K562 cells.

C: Methylcellulose colony formation (expressed as mean ± SE of the colony number from three clonogenic assays) of untreated (red bars), forskolin-treated (black bars), and 1,9-dideoxy-forskolin-treated (yellow bars) imatinib-sensitive and -resistant BCR/ABL cell lines, and cytokine-driven colony formation of primary CML-BC^{CD34+} (n = 2), CML-CP^{CD34+} (n = 2), and normal (NBM) (n = 2) CD34^{+} cells.

jected with parental 32D-BCR/ABL cells (n = 3) showed massive splenomegaly, whereas morphology of spleens from forskolin-treated 32D-BCR/ABL-injected mice (n = 3) resembled that of control age-matched (n = 2) or forskolin-only (n = 2) treated mice (Figure 7B). Hematoxylin/eosin-stained sections of spleen, marrow, and liver of vehicle-treated 32D-BCR/ABL-injected mice showed extensive infiltration of blasts with a low degree of myeloid maturation typical of an overt acute myeloid leukemia-like process (Figure 7B). In contrast, histopathology of organs from the forskolin-treated 32D-BCR/ABL-injected group (Figure 7B) was similar to that of the age-matched and forskolin-injected control groups. Consistent with these findings, the survival of forskolin-treated mice injected with 32D-BCR/ABL cells was significantly longer (median: 8 weeks) than that of mice injected with cells only (median: 4 weeks) (Figure 7C). At 15 weeks postinjection, 40% of forskolin-treated cell-injected mice and 100% of control mice that received only forskolin were still alive with no sign of toxic side effects (data

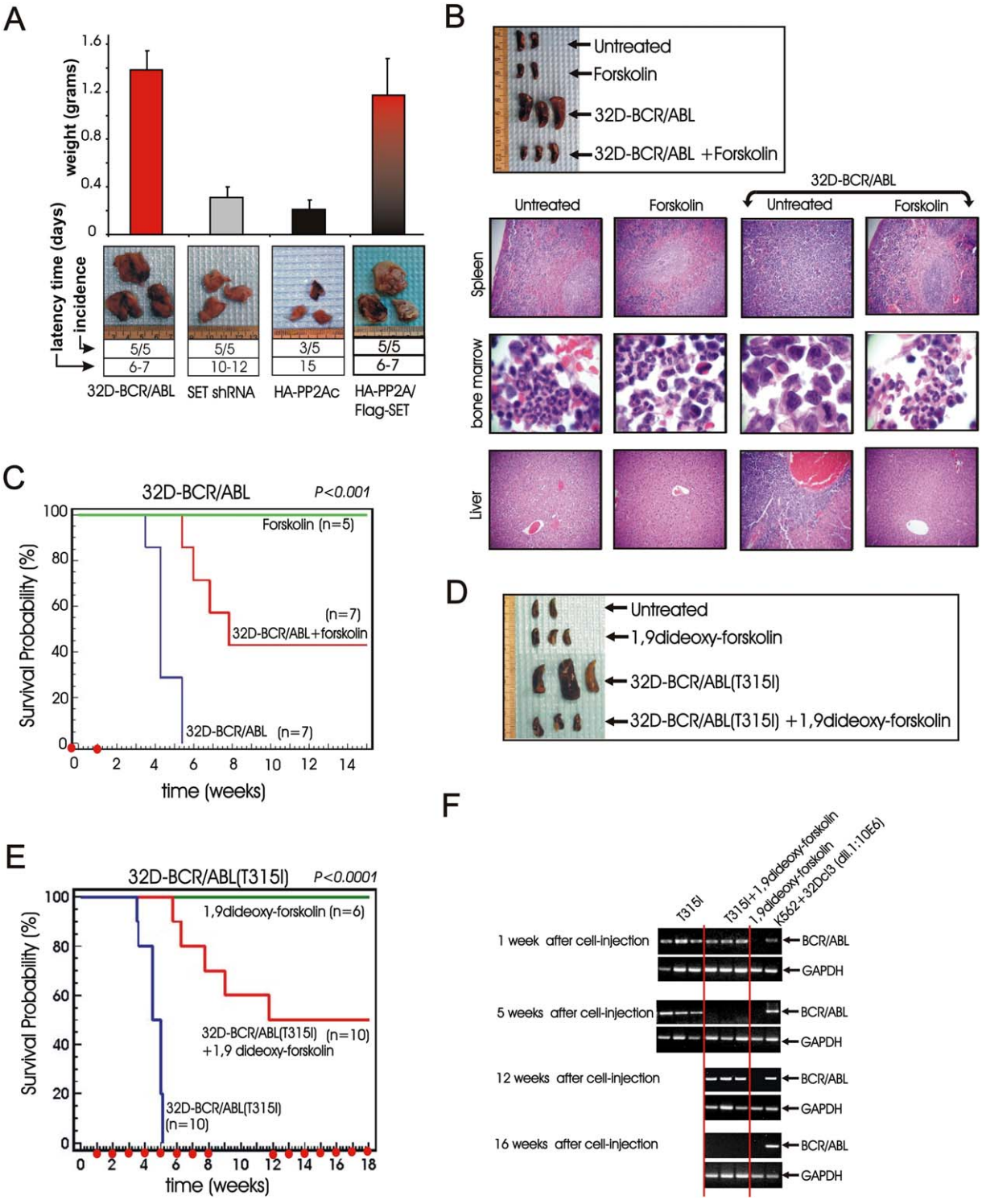


Figure 7. In vivo effects of PP2A activation on wild-type and T315I BCR/ABL leukemogenesis

A: Tumors in SCID mice arising from subcutaneous injection of parental and derivative 32D-BCR/ABL cell lines. Latency time, incidence, and tumor weight (mean ± SD) were calculated. ($p < 0.01$; Kruskal-Wallis test). **B** (top) and **D:** Representative visual analysis of spleens of untreated and drug-treated mice injected or not injected with BCR/ABL$^+$ cells. **B** (bottom): Hematoxylin/eosin staining of tissue sections from spleen, marrow, and liver. **C:** Effect of forskolin on survival of SCID mice i.v. injected with 32D-BCR/ABL cells. Estimate survival probabilities were calculated by the Kaplan-Meier method (overall: $p < 0.001$; 32D-BCR/ABL versus 32D-BCR/ABL + forskolin: $p < 0.001$; log-rank test). **E:** Effect of 1,9-dideoxy forskolin on BCR/ABL(T315I)-mediated leukemogenesis. SCID mice were treated 1–8 and 12–18 weeks after i.v. injection with 5×10^5 cells. Mice injected with cells or drug only served as controls. Survival probability was calculated by the Kaplan-Meier method ($p < 0.0001$; log-rank test). **F:** Nested RT-PCR for BCR/ABL indicates the presence of 32D-BCR/ABL(T315I) cells in peripheral blood of untreated and drug-treated mice. The last two lanes (negative and positive control, respectively) show nested RT-PCR performed with RNA from blood of mice treated with drug only and from K562:32Dcl3 cells. Detection of *GAPDH* mRNA was used as control. Red dots indicate the time of drug injection.

not shown). By contrast, modest to severe splenomegaly and leukemic infiltration of hematopoietic organs were evident in drug-treated BCR/ABL mice that died 5 or more weeks after cell injection (data not shown).

In similar experiments, 26 SCID mice were i.v. injected (5×10^5 cells/mouse) with 32D-BCR/ABL cells expressing the imatinib-, AMN107-, and BMS354825-resistant T315I BCR/ABL mutant. After 8 days, treatment with 1,9-dideoxy forskolin (8 mg/kg/week; i.p.; LD_{50} = 68 mg/kg) was initiated on 13 mice upon determining the presence of circulating BCR/ABL$^+$ cells by nested RT-PCR-mediated detection of *BCR/ABL* transcripts in the peripheral blood (Figure 7F, first panel). After 4 weeks of treatment, drug-treated leukemic mice were all alive and BCR/ABL negative (Figures 7E and 7F), whereas only 3 untreated leukemic mice were alive but appeared lethargic and were BCR/ABL$^+$ (Figure 7F, second panel). Thus, a representative sample of each group of mice was sacrificed, and various organs were evaluated for signs of leukemia. Splenomegaly (Figure 7D) and infiltration of hematopoietic organs (data not shown) was observed in mice injected with T315I cells. By contrast, organ infiltration (data not shown) and splenomegaly were not present in drug-treated cell-injected mice (Figure 7D). Because 70% of these mice were alive after 7 weeks of treatment, administration of the drug was suspended (Figure 7E). Unexpectedly, 20% of mice relapsed and died of an acute leukemia-like disease process within 4 weeks from the end of treatment (Figure 7E), and the remaining 50% tested BCR/ABL$^+$ (Figure 7F, third panel). After 4 weeks from restarting therapy, 100% of treated mice (50% of cell-injected mice) became BCR/ABL negative and were alive 18 weeks after cell injection with no signs of toxic side effects (Figure 7E) like the control age-matched or drug only-treated mice.

Discussion

Although the mechanisms responsible for CML disease progression are still largely unclear, growing evidence suggests that the phenotype of CML-BC cells depends on the unrestrained activity of BCR/ABL and on the genetic or functional inactivation of genes with tumor suppressor activity (Calabretta and Perrotti, 2004). Here, we report that the phosphatase activity of the tumor suppressor PP2A (Janssens et al., 2005) is modestly affected in CML-CP^{CD34+} progenitors but markedly impaired in myeloid CML-BC^{CD34+} cells. We demonstrated that PP2A inactivation results from increased expression of SET, a physiological inhibitor of PP2A (Li et al., 1996), which is induced by BCR/ABL in a dose- and kinase-dependent manner and, like BCR/ABL (Barnes et al., 2005; Elmaagacli et al., 2000; Gaiger et al., 1995; Jamieson et al., 2004), progressively increases during transition to blast crisis. In fact, imatinib treatment and SET downregulation restored PP2A activity back to normal levels. Interestingly, BCR/ABL might also inactivate PP2A by inducing Jak2-dependent (Yokoyama et al., 2001) and/or src-dependent (Chen et al., 1992) PP2Ac phosphorylation on tyrosine 307. Indeed, a portion of PP2Ac is phosphorylated on tyrosine 307 in BCR/ABL-expressing cells, and PP2A^{TYR307} levels decrease upon SET downregulation or inhibition of BCR/ABL activity. Thus, it is possible that BCR/ABL also affects the ability of PP2A to reactivate itself by autodephosphorylation (Chen et al., 1992) and/or that the SET/PP2A association favors the recruitment of a BCR/ABL-activated tyrosine kinase that inhibits PP2A through phosphorylation of the PP2Ac tyrosine 307. Alternatively, SET overexpression and src and/or Jak2 activity might independently contribute to PP2A inactivation in BCR/ABL cells. Interestingly, the increased expression of the PP2A subunit PR65/A observed in BCR/ABL$^+$ cells may also contribute to PP2A inactivation, as PR65/A overexpression could sequester the catalytic or variable subunits or act as a PP2Ac inhibitor (Kamibayashi et al., 1992; Wera et al., 1995). Suppression of PP2A activity appears to be a common event in malignant transformation; in fact, mutations in the genes encoding the β-isoform of the structural $PP2A_A$ and some regulatory $PP2A_B$ subunits have been found in several human cancers (Janssens et al., 2005). Moreover, cellular transformation by the SV40 viral oncoprotein small t Ag requires inactivation of PP2A (Yang et al., 1991), and PP2Ac overexpression reduces Ha-RAS-induced cell transformation (Baharians and Schonthal, 1999).

The tumor-suppressing activity of PP2A depends on its ability to interact with and dephosphorylate several factors implicated in the regulation of cell cycle progression, proliferation, survival, and differentiation (Sontag, 2001). Remarkably, several targets are shared by BCR/ABL kinase and PP2A phosphatase. Among these, expression and/or activity of the PP2A substrates Myc, STAT5, MAPK, Akt, BAD, and Rb (Avni et al., 2003; Chiang et al., 2003; Gomez and Cohen, 1991; Sato et al., 2000; Yeh et al., 2004; Yokoyama et al., 2001) are either essential for BCR/ABL leukemogenesis or have been found altered in CML-BC (Calabretta and Perrotti, 2004; Cortez et al., 1997). In 32D-BCR/ABL cells, inhibition of SET expression and, to a greater extent, forced expression of PP2Ac led to inhibition of MAPK, STAT5 and Akt phosphorylation, decreased Myc expression, and increased levels of proapoptotic BAD. Since ectopic SET expression antagonized the effects of exogenous PP2A, it is possible that, in CML-BC progenitors, SET-dependent suppression of PP2A activity represents one of the main mechanisms used by BCR/ABL to prevent inactivation of mitogenic and survival signals. The importance of PP2A inhibition for CML disease progression is also supported by the ability of PP2A to induce Rb dephosphorylation (Avni et al., 2003) and to suppress Jak2 kinase, which was found activated in CML-BC (Xie et al., 2002).

By using chemical inhibitors and activators of PP2A and by interfering with the SET/PP2A interplay (i.e., PP2Ac overexpression, SET knockdown), we provide evidence that BCR/ABL is a bona fide target of PP2A in imatinib-sensitive and -resistant BCR/ABL cell lines and in CML-BC^{CD34+} patient cells. In fact, the ability of PP2A to markedly reduce BCR/ABL tyrosine phosphorylation and expression is consistent with its capacity to trigger inactivation and induce proteolysis of other oncogenic tyrosine kinases (i.e., v-src) (Yokoyama and Miller, 2001). Mechanistically, downregulation of BCR/ABL levels induced by ectopic PP2Ac expression depends on increased proteasome degradation of nonphosphorylated BCR/ABL protein that was found physically associated to active PP2A and SHP-1 tyrosine phosphatase. By using a specific SHP-1 inhibitor and BCR/ABL-transduced SHP-1-decifient CD34$^+$ marrow cells, we demonstrated that the PP2A-induced BCR/ABL dephosphorylation and degradation are mediated by SHP-1. Because SHP-1 associates with BCR/ABL and its tyrosine phosphatase activity counteracts BCR/ABL leukemogenic potential (Liedtke et al., 1998; Lim et al., 2000), it is likely that PP2A

associates with and activates SHP-1 which, in turn, dephosphorylates BCR/ABL and makes it prone to proteasome-dependent proteolysis. Accordingly, we showed that loss of SHP-1 abolished the inhibitory effects of PP2A activation on BCR/ABL-dependent clonogenic potential. Restoration of PP2A activity back to normal levels via SET downregulation, PP2Ac overexpression, or treatment with the potent PP2A activator forskolin (Feschenko et al., 2002) induced marked apoptosis, reduced proliferation, impaired colony formation, inhibited tumorigenesis, and restored differentiation of BCR/ABL-transformed cells regardless of their degree of sensitivity to imatinib (note that also T315I BCR/ABL$^+$ cells were inhibited by PP2A activation). Because ectopic SET expression neutralized the tumor-suppressive activity of PP2A both in vitro and in vivo, our data indicate that the antileukemic effects of SET downregulation are highly dependent on increased PP2A activity. However, we cannot exclude that interference with the ability of SET to regulate nucleosome assembly (Fan et al., 2003) and to function as an acetyltransferase inhibitor (Seo et al., 2001) might, in part, account for the phenotype of shRNA-SET-expressing cells.

Consistent with the effects of PP2A in BCR/ABL cell lines, reestablishment of PP2A activity in myeloid CML-BC^{CD34+} cells resulted in marked inhibition of proliferation and induction of apoptosis (>95% annexin V$^+$). Similarly, the clonogenic potential of CML-BC^{CD34+} cells was also dramatically reduced by ectopic PP2A expression (90% inhibition) and by forskolin or 1,9-dideoxy-forskolin (lacks adenylate cyclase inducing activity) treatment (80%–95% inhibition). Interestingly, the clonogenic potential of CML-CP^{CD34+} patient cells was also strongly inhibited by activation of PP2A, consistent with the effect of PP2A on signal transducers (e.g., Akt) essential for survival of CML-CP progenitors. Importantly, in vivo administration of the PP2A activators forskolin and/or 1,9-dideoxy forskolin severely impacted and efficiently modulated the development of wild-type and T315I BCR/ABL-induced acute leukemia-like disease process, consistent with the reported ability of forskolin to prevent tumor colonization and metastasis (Agarwal and Parks, 1983) and inhibit growth and induce apoptosis of myeloid and lymphoid leukemic cells (Gutzkow et al., 2002; Moon and Lerner, 2003; Taetle and Li-en, 1984). Remarkably, forskolin but not its derivative, 1,9-dideoxy-forskolin, partially inhibited cytokine-driven colony formation and proliferation of NBM^{CD34+} cells without inducing either apoptosis or PP2A activity. Thus, the effects of forskolin on NBM cells most likely depend on its ability to activate adenylate cyclase and induce cAMP (Seamon and Daly, 1981), which inhibits cytokine-dependent proliferation of normal myeloid progenitors (Derigs et al., 1989; Lu et al., 1986). By contrast, the antileukemic effects of forskolin appear to depend on induction of PP2A activity rather than increased intracellular cAMP and/or PKA activation (Ding and Staudinger, 2005), as exposure of BCR/ABL-transformed cells to the cAMP inducer theophylline (Yasui et al., 1997) or to a PKA inhibitor (Rimon and Rubin, 1998) did not alter BCR/ABL expression/activity.

In summary, we showed that functional loss of PP2A tumor suppressor activity occurs in myeloid CML-BC through the effect of BCR/ABL on SET expression and that reestablishment of normal PP2A activity antagonizes both in vitro and in vivo BCR/ABL leukemogenesis. Thus, enhancement of PP2A tumor suppressor activity may represent a novel therapeutic strategy for blast crisis and imatinib-resistant CML patients.

Experimental procedures

Cell cultures and primary cells

The Ph^1 K562, the BCR/ABL-expressing 32Dcl3 and BaF3 cells, and their derivative lines were maintained in culture in IMDM/10% FBS/2 mM L-glutamine. The SET shRNA-, HA-PP2Ac-, and HA-PP2Ac/FLAG-SET-expressing cells were generated by retroviral infection followed by either antibiotic-mediated selection or FACS-mediated sorting of the GFP$^+$ (green fluorescent protein) cells as described (Perrotti et al., 2002). The following cells were kind gifts: 32D-p210^{Y253H} (B. Calabretta; TJU, Philadelphia, PA); BaF3-p210^{T315I} (B. Druker; OHSU, Portland, OR); TonB210.1 (G. Daley; Harvard University, Cambridge, MA). Frozen samples of CD34$^+$ bone marrow cells (NBM) from different healthy donors were from AllCells LLC (Berkeley, CA). Frozen samples of mononuclear hematopoietic cells from bone marrow or peripheral blood of unidentifiable CML patients were Ficoll separated and used for Western blot analysis or to isolate the CD34$^+$ fraction by using the CD34 MultiSort kit (Miltenyi Biotec). Before being used in the different assays, CD34$^+$ NBM and CML cells were kept overnight in IMDM supplemented with 50% FBS, 2 mM glutamine, and rhIL-3 (20 ng/ml), rhIL-6 (20 ng/ml), rhFlt-3 ligand (100 ng/ml), and rhKL (100 ng/ml) (Stem Cell Technologies Inc.). Retroviral infection with MSCV-based GFP-containing bicistronic constructs were carried out as described (Iervolino et al., 2002), and infected cells were isolated by FACS-mediated sorting of GFP$^+$ cells. All studies performed with human specimens obtained from The Ohio State University Leukemia Tissue Bank (Columbus, OH); from the Division of Hematology, Maisonneuve-Rosemont Hospital (Montreal, Canada); and from Dr. Gambacorti-Passerini (Division of Experimental Oncology, NCI Milan, Italy) were done with approval from The Ohio State University Institutional Review Board. The percentage of CML-CP Ph$^+$ cells by FISH ranged from 91% to 100%. The CML-BC samples were all myeloid blast crisis and, mostly, with complex karyotype; however, no one CML-BC presented deletions of the der9q. Granulocytic differentiation of 32Dcl3-derivative cell lines was induced as described (Perrotti et al., 2002). Erythroid differentiation of K562 and derivative cell lines was assessed by o-Dianisidine (Fast Blue B; Sigma) staining of methylcellulose colonies. Green/brown-stained cells indicate presence of hemoglobin.

Normal murine hematopoietic marrow cells were obtained from the femurs of wild-type and SHP-1-deficient "viable moth eaten" mice (The Jackson Laboratory). After hypotonic lysis and Ficoll separation, cells were used for the isolation of the CD34$^+$/lin$^-$ fraction (Miltenyi Biotech). CD34$^+$ cells were kept for 2 days in complete IMDM supplemented with murine IL-3 (2 ng/ml), IL-6 (1.2 ng/ml), and KL (10 ng/ml) prior to infection with MigRI or MigRI-BCR/ABL (W. Pear, University of Pennsylvania, Philadelphia, PA). GFP$^+$ (Iervolino et al., 2002) wild-type and *SHP-1*$^{-/-}$ CD34$^+$ cells (10^6) were treated or not with forskolin (40 μM; 48 hr) and used in clonogenic assay or processed for Western blot. Where indicated, cells were treated with the following: ALLN, okadaic acid, calyculin A, SS, and butyryl-forkolin (EMD Bioscience Inc.); forskolin, 1,9-dideoxy-forskolin, and myristoylated PKI-(14-22) amide (BioMol); imatinib (Novartis); and theophylline (Sigma). Cells in liquid culture were treated with forskolin or imatinib at 40 μM and 1 μM, respectively, and a half dose of forskolin or imatinib was added every 18 hr.

Plasmids

pHM6-HA-PP2Ac

pHM6-HA-PP2Ac contains the HA-tagged PP2A catalytic subunit under the control of the CMV promoter (P.B. Rothman, Columbia University, New York, NY).

MigRI-HA-PP2Ac

The HA-tagged PP2Ac cDNA was PCR amplified from pHM6-HA-PP2Ac and subcloned into the HpaI/EcoRI sites of the bicistronic GFP-containing MigRI vector.

pSuper.retro-shSET

The shRNA SET construct was obtained by subcloning the double-stranded 60 mer oligonucleotide containing the SET target sequence (5′-TGAAATA GACAGACTTAAT-3′) into the pSuper.retro.neo+GFP vector (OligoEngine Inc.).

MSCV-FLAG-SET

The human *SET* cDNA was obtained from K562 mRNA by RT-PCR using an upstream primer containing a HpaI site, the FLAG epitope, and the first 16 nucleotides of *SET* cDNA, and a downstream primer containing the last 21 nucleotides of SET linked to an EcoRI restriction site. The HpaI/EcoRI-digested PCR product was subcloned into the MSCV-puro vector.

RT-PCR of immunoprecipitated RNA

K562 cells (10^8) were lysed in 2 pellet/vol of 10 mM HEPES (pH 7.0), 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.5% Nonidet P-40, 100 U/ml RNase OUT (Invitrogen), 0.2 mM PMSF, 1 mg/ml pepstatin A, 5 mg/ml bestatin, and 20 mg/ml leupeptin and clarified (100,000 × g, 2.5 hr). RNP-containing particles were purified by centrifugation (300,000 × g; 3 hr) and resuspended in 1 ml of 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 0.05% NP-40, 100 U/ml RNase OUT, 0.2 mM PMSF, and 20 mM EDTA. Immunoprecipitation (IP) and extraction of the hnRNP A1 bound mRNA was performed as described (Tenenbaum et al., 2002). The presence of *SET* transcripts was assessed by RT-PCR performed on an equal amount of RNA, extracted from RNP-enriched lysates (input) as well as from the hnRNP-A1 or FLAG immunoprecipitates. To amplify SET mRNA, the following primers were used: 5′-GAGGTCAGAATTGATCGCCAAAATC-3′ and 5′-TCAGATG AAATTCTTTGGAGAGAAC-3′.

Northern blot analysis and RT-PCR

Total RNA was isolated using the acid phenol-guanidinium-mediated extraction (Tri-Reagent; Invitrogen). For Northern blot, total RNA (5 μg) was hybridized to ^{32}P-labeled *hSET* cDNA. To detect *BCR/ABL* transcripts, RNA was extracted (QIAamp RNA blood kit; Qiagen) from mouse peripheral blood (100 μl). RNA from K562:32Dcl3 cells (ratio 1:10^6) served as positive control, whereas RNA from blood of mice that were not injected with BCR/ABL$^+$ cells was used as negative control. Total RNA (1 μg) was reverse transcribed in 20 μl, and cDNA (5 μl) was used to detect BCR/ABL transcripts by nested PCR using a first set of primers spanning the *bcr* exon 1/*abl* exon 3 and a second set amplifying a *bcr* exon 2/*abl* exon 3 fragment. The nested RT-PCR conditions were previously described (Cross et al., 1993). GAPDH levels were monitored as control for equal amplification.

Western blot analysis and IP

Cells (10^7) were lysed in 100 μl of RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 50 mM Tris [pH 8.0]) supplemented with 1 mM PMSF, 25 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml pepstatin A, 5 mM benzamidine, 1 mM Na_3VO_4, 50 mM NaF, 10 mM β-glycerol-phosphate. After incubation on ice (30 min), lysates were clarified (12,000 × g, 15 min, 4°C), denatured, and subjected to SDS-PAGE and Western blot (Iervolino et al., 2002). The antibodies used were goat polyclonal anti-SET(I2PP2A), -pp32(I1PP2A), and -hnRNP A1 and rabbit polyclonal anti-SHP-1 (Santa Cruz Biotechnology); monoclonal anti-Abl (Ab-3), -c-*myc* (Ab-2), -phosphotyrosine PY20 (Ab-4), and -pBAD^{S112} (Ab-1) (EMD); monoclonal anti-hnRNP A1 (9H10) (G. Dreyfuss, University of Pennsylvania, Philadelphia, PA); monoclonal anti-GRB2 and -Rb (BD TransLab Inc.); monoclonal anti-HA (Covance); monoclonal anti-PP2Ac, clone 1D6 (Upstate Inc.); monoclonal anti-FLAG, clone M2 (Sigma); monoclonal anti-pERK$^{T202/Y204}$ and rabbit polyclonal anti-pAKT^{T308}, -pSTAT5^{Y694}, -pJak2$^{Y1007/1008}$, -Jak2, -BAD, -Akt, and -ERK1/2 (Cell Signaling Technology Inc.); monoclonal anti-STAT5 (Invitrogen); rabbit monoclonal anti-pPP2A^{Y307} (Epitomics); rabbit polyclonal anti-PR65/A (Calbiochem). IPs were carried out in 20 mM HEPES (pH 7.0), 150 mM NaCl, and 0.2% NP-40 supplemented with protease and phosphatase inhibitors. Lysates were precleared and immunoprecipitated with Ab-coated beads for 3 hr at 4°C. After washings, IPs were subjected to SDS-PAGE and Western blot.

Phosphatase assays

PP2A phosphatase assays were carried out using the PP2A IP phosphatase assay kit (Upstate). Briefly, protein lysates (50 μg) in 100 μl of 20 mM HEPES (pH 7.0)/100 mM NaCl, 5 μg of PP2Ac antibody (Upstate), and 25 μl of Protein A-agarose were added to 400 μl of 50 mM Tris (pH 7.0)/100 mM $CaCl_2$, and IPs were carried out at 4°C for 2 hr. IPs were washed and used in the phosphatase reaction according to the manufacturer's protocol. Specificity of the PP2A assay was assessed by titration with okadaic acid and by ascertaining the absence of PP1α in the PP2A immunoprecipitates (Figure S2A). As internal control, the amount of immunoprecipitated PP2A was also monitored by anti-PP2Ac Western blots (Figure S2B).

Clonogenic assays

Methylcellulose colony formation assays were carried out by plating 10^3 or 5×10^4 cells from BCR/ABL cell lines or primary human CML and mouse BMC, respectively, in 0.9% MethoCult H4230 (Stem Cell Technologies Inc.). Where indicated, cells were plated in the presence of rhIL-3 (100 ng/ml) or rhG-CSF (25 ng/ml). Colonies (> 125 μm) from cell lines and primary cells were scored 7 and 15 days later, respectively.

Leukemogenesis in SCID mice

Parental cells and shRNA SET-, HA-PP2A-, and HA-PP2A/FLAG-SET-expressing 32D-BCR/ABL cells were injected (5×10^6 cells/mouse) subcutaneously into 5-week-old SCID mice (Fox Chase SCID ICR; Taconic). Tumor growth was monitored every other day. Mice were sacrificed 15 days postinjection, and excised tumors were fixed in phosphate-buffered formalin. To determine the effect of forskolin and 1,9-dideoxy forskolin on in vivo BCR/ABL leukemogenesis, 6-week-old SCID mice were i.v. injected with 5×10^5 32D-BCR/ABL or 32D-BCR/ABL (T315I) cells (10 to 13 mice/group) and treated with forskolin (100 μg/ml of 2% DMSO/PBS) or 1,9-dideoxy forskolin (200 μg/ml of 2% DMSO/PBS) using the indicated schedule. Age-matched mice or mice injected with cells or drugs only (6 to 7 mice) served as controls. Four weeks postinjection, 2 to 3 mice from each group were sacrificed, and organs were analyzed for the presence of leukemia. At the indicated times, the disease process was monitored by nested RT-PCR-mediated *BCR/ABL* detection using peripheral blood collected by lateral tail-vein incision. For pathological examination, tissue sections from bone marrow, spleen, and liver were fixed in formalin, embedded in paraffin blocks, and hematoxylin/eosin stained. The remaining mice were used for survival studies, which were terminated 15–18 weeks postinjection. The in vitro and in vivo animal studies were conducted with the approval of the Ohio State University Institutional Lab Animal Care and Use Committee and in accordance with the National Institute of Health guidelines for animal care.

Supplemental data

The Supplemental Data include four supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/8/5/355/DC1/.

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