Title

Distinct GAB2 signaling pathways are essential for myeloid and lymphoid transformation and leukemogenesis by BCR-ABL1

Journal

BLOOD, 127(14)

ISSN

0006-4971

Authors

Gu, Shengqing
Chan, Wayne W
Mohi, Golam
et al.

Publication Date

2016

DOI

10.1182/blood-2015-06-653006

MYELOID NEOPLASIA

Distinct GAB2 signaling pathways are essential for myeloid and lymphoid transformation and leukemogenesis by BCR-ABL1

Shengqing Gu,[1,2,*] Wayne W. Chan,[3,4,*] Golam Mohi,[5,*] Joel Rosenbaum,[3] Azin Sayad,[2] Zhibin Lu,[2] Carl Virtanen,[2] Shaoguang Li,[6] Benjamin G. Neel,[1,2] and Richard A. Van Etten[3,4]

[1]Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada; [2]Princess Margaret Cancer Center, Toronto, ON, Canada; [3]Molecular Oncology Research Institute, Tufts Medical Center, Boston, MA; [4]Program in Molecular and Cellular Physiology, Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, Boston, MA; [5]Cancer Biology Program, Division of Hematology-Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA; and [6]University of Massachusetts Medical School, Worcester, MA

Key Points

- Scaffolding adaptor protein GAB2 is required for BCR-ABL1–evoked myeloid and lymphoid leukemogenesis.
- SHP2 and p85 binding to GAB2 activate distinct signaling pathways and are required differentially for myeloid and lymphoid leukemogenesis.

Tyrosine kinase inhibitors (TKIs) directed against BCR-ABL1, the product of the Philadelphia (Ph) chromosome, have revolutionized treatment of patients with chronic myeloid leukemia (CML). However, acquired resistance to TKIs is a significant clinical problem in CML, and TKI therapy is much less effective against Ph$^+$ B-cell acute lymphoblastic leukemia (B-ALL). BCR-ABL1, via phosphorylated Tyr177, recruits the adapter GRB2-associated binding protein 2 (GAB2) as part of a GRB2/GAB2 complex. We showed previously that GAB2 is essential for BCR-ABL1–evoked myeloid transformation in vitro. Using a genetic strategy and mouse models of CML and B-ALL, we show here that GAB2 is essential for myeloid and lymphoid leukemogenesis by BCR-ABL1. In the mouse model, recipients of BCR-ABL1–transduced *Gab2*$^{-/-}$ bone marrow failed to develop CML-like myeloproliferative neoplasia. Leukemogenesis was restored by expression of GAB2 but not by GAB2 mutants lacking binding sites for its effectors phosphatidylinositol 3-kinase (PI3K) or SRC homology 2–containing phosphotyrosine phosphatase 2 (SHP2). GAB2 deficiency also attenuated BCR-ABL1–induced B-ALL, but only the SHP2 binding site was required. The SHP2 and PI3K binding sites were differentially required for signaling downstream of GAB2. Hence, GAB2 transmits critical transforming signals from Tyr177 to PI3K and SHP2 for CML pathogenesis, whereas only the GAB2–SHP2 pathway is essential for lymphoid leukemogenesis. Given that GAB2 is dispensable for normal hematopoiesis, GAB2 and its effectors PI3K and SHP2 represent promising targets for therapy in Ph$^+$ hematologic neoplasms. (*Blood*. 2016;127(14):1803-1813)

Introduction

The product of the Philadelphia (Ph) chromosome translocation, BCR-ABL1, is a constitutively active protein-tyrosine kinase that is the direct molecular cause of chronic myeloid leukemia (CML) and Ph$^+$ B-cell acute lymphoblastic leukemia (B-ALL). ABL1 tyrosine kinase inhibitors (TKIs), such as imatinib mesylate, induce hematologic and cytogenetic remissions in the majority of CML patients, but disease relapse occurs in most patients after imatinib is withdrawn,[1] and a significant proportion of patients develop resistance to TKI therapy. Furthermore, patients with advanced stage CML or B-ALL respond poorly to TKIs.[2,3] Hence, there is a need to identify and validate additional pharmacologic targets to prevent or overcome TKI resistance.

BCR-ABL1 activates multiple signaling networks in leukemic cells, including the RAS/extracellular signal-regulated kinase (ERK), signal transducer and activator of transcription (STAT), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase, and phosphatidylinositol 3-kinase (PI3K) pathways.[4] A major challenge has been determining which of these pathways are essential for leukemogenesis. Mouse models of BCR-ABL1–induced leukemia have proven useful in this effort, as both CML and Ph$^+$ B-ALL can be reproduced faithfully by expressing BCR-ABL1 in bone marrow (BM) stem/progenitor cells through retroviral transduction, followed by bone marrow transplantation (BMT). Recipients of *BCR-ABL1*–transduced BM develop a fatal CML-like myeloproliferative neoplasm (MPN) that originates from hematopoietic stem cells (HSCs),[5,6] can progress to blast crisis,[7] and is responsive to TKI therapy.[8] To model B-ALL in mice, BM is transduced in the absence of myeloid cytokines, resulting in B-lymphoblastic leukemia/lymphoma in transplant recipients.[9,10] This disease originates from early lymphoid progenitors[5] and is characterized by a block in B-cell differentiation at the pre-B stage.[9]

The contribution of the BCR region of BCR-ABL1 to leukemogenesis, specifically the autophosphorylation site at tyrosine 177 (Y177), has been the subject of intensive study. In CML cell lines, BCR-ABL1 is phosphorylated at Y177, forming a binding site for the SH2 domain of the adapter protein GRB2, which is critical for its recruitment to BCR-ABL1.[11,12] We and others have shown that the BCR-ABL1-Y177F mutant is greatly attenuated in the ability to induce

Submitted June 20, 2015; accepted January 4, 2016. Prepublished online as *Blood* First Edition paper, January 15, 2016; DOI 10.1182/blood-2015-06-653006.

*S.G., W.W.C., and G.M. contributed equally to this work.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

CML-like MPN in the murine BMT model.[13-15] These findings demonstrate that Y177 and the signaling pathway(s) downstream of this site are critical for myeloid and perhaps also contribute to lymphoid leukemogenesis by BCR-ABL1.

Earlier, we reported a critical role for a GRB2/GAB2 complex in BCR-ABL1–induced transformation in vitro that is mediated by Y177.[16] The GAB family (GAB1, GAB2, GAB3) consists of the pleckstrin homology (PH) domain containing scaffolding/adaptor proteins involved many signaling pathways.[17,18] GAB2 undergoes phosphorylation on several tyrosine residues in response to multiple cell stimuli.[19-23] Moreover, GAB2 is constitutively tyrosyl-phosphorylated in BCR-ABL1–transformed cells,[19,20] forming binding sites for other SH2-containing signal relay proteins, including the p85 regulatory subunit of type IA PI3K and the protein-tyrosine phosphatase SHP2.[20,24,25] *Gab2*$^{-/-}$ mice are viable and fertile, but have defects in their allergic response and in mast cell development.[21,23] Although GAB2-deficient mice exhibit normal baseline hematopoiesis, long-term multilineage repopulation by HSCs is impaired, concomitant with defects in the PI3K and ERK signaling cascades.[26] Roles for GAB2 in the pathogenesis of several other malignancies have also been reported.[27-31]

Here, we use a genetic strategy in mice to define the role of GAB2 in leukemogenesis by BCR-ABL1. Our results indicate an essential role for GAB2 in BCR-ABL1–induced MPN and B-ALL, but demonstrate that distinct GAB2-mediated signaling pathways are required for these 2 hematopoietic malignancies.

Methods

Mice

Gab2$^{-/-}$ mice were produced by homologous recombination in embryonic stem cells as described[21] and backcrossed for >5 generations onto the Balb/c and C57Bl/6J backgrounds, respectively.

Retroviral constructs and stocks

The retroviral vectors p210MIGR1 and MINVneo were described previously.[32,33] An HA-tagged *Gab2WT* cDNA was introduced into p210MIGR1 and p210MINV in place of the green fluorescent protein (GFP) or neomycin resistance genes, respectively, yielding p210-IRES$_{wt}$-*Gab2WT* and p210-IRES$_{mut}$-*Gab2WT* (Figure 2A). Site-specific mutagenesis was used to create the *Gab2Δp85* (Y441/465/574F), *Gab2ΔShp2* (Y604/633F), *Gab2ΔPH* (deletion of Gab2 amino acids 2-100), and *Gab2ΔGrb2* (delP348-K354; P498/503A) constructs. Ecotropic retroviral stocks were generated by transient transfection of 293 cells using the *kat* packaging system,[10] and stocks were matched for titer by transduction of NIH3T3 cells. Transduction frequency was assessed by flow cytometric detection of GFP[34] or, for bicistronic viruses coexpressing BCR-ABL1 and GAB2, by intracellular staining for HA epitope-tagged GAB2 proteins (see below), as well as by Southern blot analysis of proviral DNA content in genomic DNA.[34] Titers determined by either method were concordant.

Bone marrow transduction, transformation, and transplantation

For myeloid transformation and leukemogenesis assays, BM was collected from *Gab2*$^{+/+}$ and *Gab2*$^{-/-}$ mice and transduced twice with retrovirus, as previously described.[34] Transduced cells were plated in cytokine-free methylcellulose (M3234; Stem Cell Technologies) for myeloid colony assays or injected intravenously into lethally irradiated recipients for myeloid leukemogenesis, as previously described.[9] For B-lymphoid transformation/leukemogenesis, BM from *Gab2*$^{+/+}$ or *Gab2*$^{-/-}$ donors was transduced once with *BCR-ABL1* retrovirus in the absence of cytokines. B-lymphoid transformation was assessed by pre-B-cell colony formation in agarose and stroma-dependent growth, as previously described.[35,36] For lymphoid leukemogenesis assays, transduced cells were injected into sublethally irradiated recipients, as previously described.[10] The clinical features and histopathology of *BCR-ABL1*–induced CML-like disease, B-ALL, T-cell lymphoblastic acute leukemia/lymphoma (T-ALL), and histiocytic sarcoma were described previously.[10] All mouse experiments were approved by the Institutional Animal Use and Care Committees of Tufts Medical Center and University Health Network.

Immunoblotting

To characterize signaling downstream of GAB2 in BCR-ABL1 transformation, myeloid- or B lymphoid-enriched BM cells were transduced with viruses, as indicated. GFP$^+$ or red fluorescent protein (RFP)$^+$GFP$^+$ cells were recovered by fluorescence-activated cell sorter, starved in Iscove's Modified Dulbecco's Medium 2% fetal bovine serum for 2 hours, and lysed in radioimmunoprecipitation assay buffer. Lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted, as previously described.[10] For details on antibodies and sources, see supplemental Methods, available on the *Blood* Web site.

Southern blotting

Southern blot analysis of proviral integration and leukemia-initiating cell engraftment was performed as previously described,[10] except that genomic DNA was digested with *Bam*HI, and blots were hybridized with a radioactive probe from the 5′ end of the *BCR* gene.

Statistical analysis

Survival comparisons were performed by log-rank test. Band intensities in immunoblots from myeloid cells were compared by 2-sided Student *t* test; the null hypothesis was that the logarithm of the intensity ratio was 0. For lymphoid cell blots, band intensities were compared by 1-sided Student *t* test, owing to the results of the myeloid signaling experiments. Multiple comparison *P* value correction was performed with the Benjamini-Hochberg method.

Additional methods are found in the supplemental Methods.

Results

BCR-ABL1 cannot induce CML-like MPN in the absence of GAB2

When expressed in mouse myeloid progenitors, BCR-ABL1 can induce myeloid colony formation in the absence of exogenous cytokines.[37] We showed earlier that progenitors from *Gab2*$^{-/-}$ BM donors (mixed 129Sv;C57Bl/6J background) are resistant to transformation by BCR-ABL1 in vitro.[16] To assess the role of GAB2 in CML-like MPN, we bred the *Gab2*$^{-/-}$ mutation onto the C57Bl/6J and Balb/c backgrounds for >5 generations. For each strain, donor BM from congenic *Gab2*$^{-/-}$ mice or *Gab2*$^{+/+}$ littermates was harvested, transduced with p210MIGFP retrovirus that coexpresses p210 BCR-ABL1 and GFP,[32] and transplanted into lethally irradiated *Gab2*$^{+/+}$ recipients. Hematopoietic stem/progenitor cells from *Gab2*$^{-/-}$ mice have deficient responses to cytokines[26] and to cytotoxic drugs (G.M. and B.G.N., unpublished observations, June 2000), so donor mice were not treated with 5-fluorouracil (5-FU) prior to BM harvest.[9]

In C57Bl/6J mice, recipients of *BCR-ABL1*–transduced BM from *Gab2*$^{+/+}$ donors efficiently developed fatal CML-like leukemia within 5 weeks of transplantation (supplemental Figure 1A), characterized by leukocytosis, splenomegaly, and infiltration of the spleen, liver, and lung parenchyma with maturing GFP$^+$ myeloid cells (supplemental Figure 1B-E).[9] By contrast, recipients of BCR-ABL1–transduced BM from *Gab2*$^{-/-}$ littermate donors did not develop clinical MPN. Instead, beginning about 3 months after transplantation, they succumbed to precursor T-ALL, characterized by thymic enlargement and mesenteric lymphadenopathy with infiltration of GFP$^+$CD90$^+$ lymphoblasts (supplemental Figure 1F).

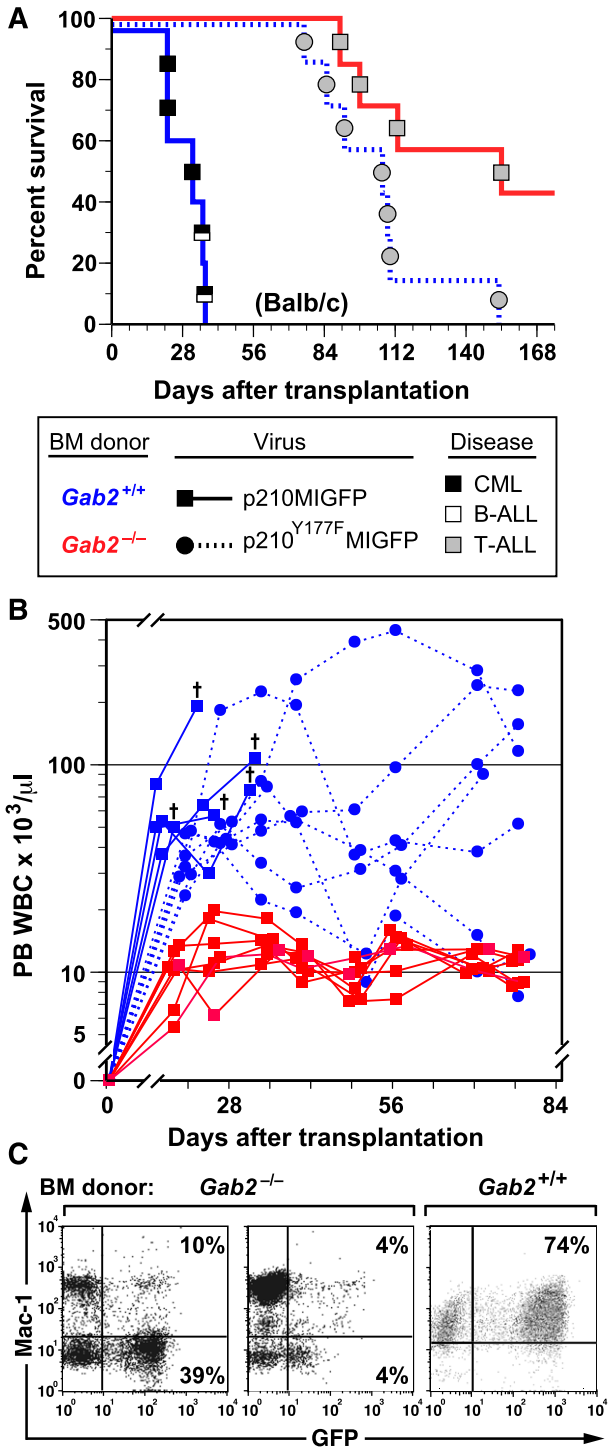

Figure 1. GAB2 is required for induction of CML-like MPN by BCR-ABL1. (A) Kaplan-Meier (K-M) survival curves for recipients of BM from *Gab2*$^{-/-}$ donors in the Balb/c background (>F9 generation backcross) transduced with p210MIGFP (red line, n = 7) or p210Y^{177F}MIGFP (blue dotted line, n = 7) retrovirus and from wild-type Balb/c donors (blue solid line, n = 5) transduced with p210MIGFP retrovirus. Symbols represent individual mice (squares for recipients of *Gab2*$^{+/+}$ BM, circles for recipients of *Gab2*$^{-/-}$ BM); disease phenotype is indicated by shading (black, CML-like MPN; white, B-ALL; gray, T-ALL). For p210MIGFP-transduced BM, the difference in survival between the *Gab2*$^{-/-}$ and *Gab2*$^{+/+}$ recipient cohorts was significant (P = .0003, Wilcoxon test). There was no significant survival difference between recipients of p210MIGFP-transduced *Gab2*$^{-/-}$ BM and p210Y^{177F}MIGFP-transduced *Gab2*$^{+/+}$ BM (P = .058). (B) Scatter plot of PBL counts in the 3 cohorts from the transplants in A at various times after transplantation. The cross indicates death of a recipient of p210MIGFP-transduced *Gab2*$^{+/+}$ BM. Note the attenuated and nonfatal leukocytosis induced in recipients of p210Y^{177F}MIGFP-transduced *Gab2*$^{+/+}$ BM. (C) Flow cytometric plot of expression of GFP (x-axis) and the myeloid marker Mac-1 (y-axis) in PBLs from 2 representative recipients of p210MIGFP-transduced BM from *Gab2*$^{-/-}$ donors (left) and from a recipient of p210MIGFP-transduced *Gab2*$^{+/+}$ BM with CML-like MPN (right), obtained 1 month after transplantation.

In parallel, we transplanted recipients with p210MIGFP-transduced BM from *Gab2*$^{-/-}$ and *Gab2*$^{+/+}$ littermate donors on the Balb/c background and also transplanted a cohort of recipients with *BCR-ABL1-Y177F*–transduced *Gab2*$^{+/+}$ BM. Balb/c recipients of *BCR-ABL1*–transduced *Gab2*$^{+/+}$ donor BM also efficiently developed CML-like MPN, either alone or concomitant with B-ALL (Figure 1A). As reported previously,[13-15] BCR-ABL1 Y177F, which lacks a GRB2 binding site, induced attenuated, nonfatal MPN in most recipients (Figure 1A-B). Similar to the results obtained using B6 mice, recipients of Balb/c *BCR-ABL1*–transduced *Gab2*$^{-/-}$ donor BM did not develop overt MPN, but tended to succumb to T-ALL after a prolonged latent period (Figure 1A). Although peripheral blood leukocyte (PBL) counts in recipients of *BCR-ABL1*–transduced *Gab2*$^{-/-}$ donor BM were predominantly within the normal range (Figure 1B), most had circulating GFP^{+} cells at 6 to 8 weeks after transplantation (Figure 1C), indicating engraftment of retrovirus-transduced donor cells. These results demonstrate that GAB2-deficient HSCs are completely resistant to induction of CML-like MPN by BCR-ABL1. The fact that donor *Gab2* deficiency phenocopies the effects of BCR-ABL1-Y177F indicates that critical leukemogenic signals from Y177 of BCR-ABL1 are probably transmitted through a GRB2/GAB2 complex.

Complementation of the *Gab2*$^{-/-}$ mutation by retroviral GAB2 expression

The failure of recipients of *BCR-ABL1*–transduced BM from *Gab2*$^{-/-}$ donors to develop MPN could indicate that signal(s) emanating from GAB2 is/are required for the massive expansion of BCR-ABL1–expressing hematopoietic stem/progenitor cells (HSPCs). Alternatively, GAB2 deficiency could decrease the number of target cells for BCR-ABL1–evoked MPN, resulting in smaller initial numbers of leukemia-initiating cells.[5,38]

To distinguish between these possibilities, we tested whether retroviral coexpression of wild-type (WT) GAB2 and BCR-ABL1 could restore leukemogenic potential to *Gab2*$^{-/-}$ cells. We designed a series of retroviral vectors wherein *BCR-ABL1* and *Gab2* are co-expressed via an internal ribosome entry site (IRES) (Figure 2A), and screened them for their ability to restore efficient transformation of *Gab2*$^{-/-}$ BM (Figure 2B). We first tested an MINV-based vector[33] with *Gab2* placed 5′ of a mutant form of the encephalomyocarditis virus IRES (IRES$_{mut}$) and BCR-ABL1 in the 3′ position, a configuration permitting 5- to 10-fold higher levels of expression of the 5′ gene. Not only did this virus fail to restore BCR-ABL1–evoked transformation of *Gab2*$^{-/-}$ BM, it significantly compromised transformation of *Gab2*$^{+/+}$ BM (Figure 2B). By contrast, when *Gab2* was positioned 3′ of IRES$_{mut}$ or IRES$_{wt}$ in the MINV- or MSCV-based vector, we observed rescue of myeloid transformation in vitro (Figure 2B), although the p210-IRES$_{wt}$-*Gab2* vector was more efficient. Finally, we compared the vectors for their ability to rescue CML-like MPN in vivo (Figure 2C). Both *Gab2*-expressing vectors restored CML-like MPN to recipients of transduced *Gab2*$^{-/-}$ donor BM, although p210-IRES$_{wt}$-*Gab2* was more efficient, inducing CML-like MPN in half of recipients. Therefore, we chose this vector for all subsequent experiments. Immunoblots of primary myeloid leukemic cell extracts demonstrated that p210-IRES$_{wt}$-*Gab2* virus led to GAB2 levels approximately

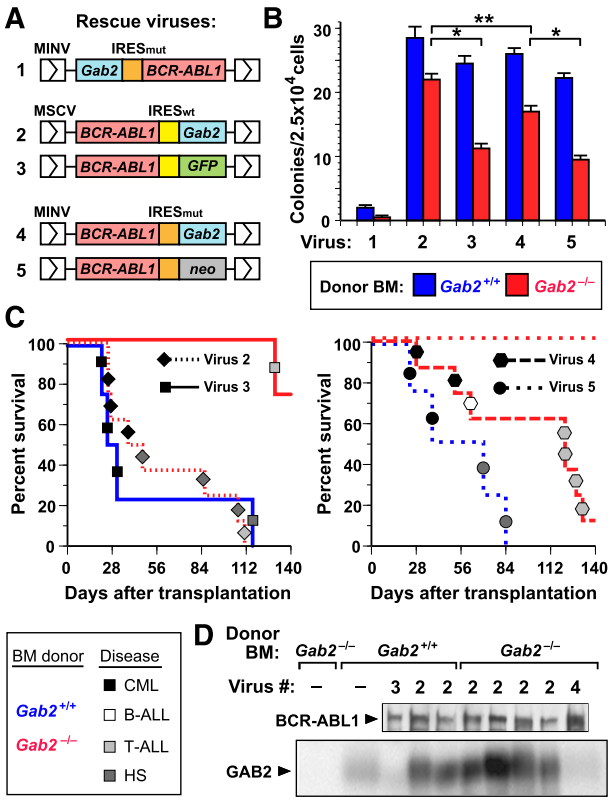

Figure 2. Complementation of *Gab2* deficiency by retroviral GAB2 expression. (A) Schematic of retroviral constructs coexpressing BCR-ABL1 and GAB2 or a reporter gene via IRES sequences. A mouse *Gab2* cDNA (blue box) was cloned either 5′ or 3′ of a mutant form of the encephalomyocarditis virus IRES ($IRES_{mut}$, orange box) in the retroviral vector Minnal virus (MINV)[33] or 3′ of the wild-type IRES ($IRES_{wt}$, yellow box) in murine stem cell virus (MSCV)-IRES-GFP (MIGR1)[32]; the *BCR-ABL1* cDNA was placed in the other position. As a control vector, either GFP (for $IRES_{wt}$) or the neomycin resistance gene (for $IRES_{mut}$) was cloned in place of *Gab2*. (B) Coexpression of GAB2 and BCR-ABL1 restores efficient myeloid cell transformation in vitro. BM from the indicated Balb/c donor mice (blue, $Gab2^{+/+}$; red, $Gab2^{-/-}$) was transduced with the indicated viral stock from A and plated in methylcellulose without cytokines. The number of cytokine-independent mixed myeloid colonies (CFU-G, -M, -GM, and -GEMM) from $Gab2^{-/-}$ BM was increased by transduction with viruses 2 or 4, relative to viruses 3 or 5 ($P < .0001$ and $P = .0005$, respectively, Student *t* tests), whereas there was no significant difference in the efficiency of transformation of $Gab2^{+/+}$ BM with viruses 2 to 5. Virus 2 (p210-$IRES_{wt}$-*Gab2*) induced significantly more colonies from $Gab2^{-/-}$ BM than virus 4 (p210-$IRES_{mut}$-*Gab2*; $P = .0082$, Student *t* test), whereas the difference in transformation of $Gab2^{+/+}$ and $Gab2^{-/-}$ BM by virus 2 was not significant ($P = .167$, Student *t* test). In these experiments, the number of cytokine-independent colonies induced in *Gab2*-deficient BM was somewhat higher than previously reported,[16] possibly due to lower retroviral titers or the different genetic background (129;B6) in our earlier study. (C) Rescue of CML-like MPN by coexpression of GAB2 and BCR-ABL1 in BM. K-M curves for recipients of BM from $Gab2^{+/+}$ (blue lines) or $Gab2^{-/-}$ (red lines) donors, transduced with p210-$IRES_{wt}$-*Gab2* or p210-$IRES_{wt}$-*GFP* retroviruses (viruses 2 and 3 from A, respectively; left) or with p210-$IRES_{mut}$-*Gab2* or p210-$IRES_{mut}$-neo retroviruses (viruses 4 and 5 from A, respectively; right). Symbol nomenclature is as in Figure 1A. Histiocytic sarcoma is a BCR-ABL1–induced disease of monocyte/macrophage proliferation that can be seen in recipients of BCR-ABL1–transduced BM from WT donors that have not been treated with 5-FU.[9] (D) Immunoblot of protein extracts from primary MPN cells from the transplants in C probed with anti-ABL1 (upper) and anti-GAB2 (lower) antibodies.

threefold higher than endogenous GAB2 levels in normal BM (Figure 2D). Southern blot analysis of leukemic cell genomic DNA revealed that the MPN in recipients of BCR-ABL1– and BCR-ABL1-Y177F–transduced $Gab2^{+/+}$ BM, as well as p210-*Gab2WT*–transduced $Gab2^{-/-}$ BM, derived from 1 to 2 distinct proviral clones (supplemental Figure 2, lanes 1-12), demonstrating repopulation of these cohorts with similar numbers of leukemia-initiating cells. These results show that comparable numbers of target cells for BCR-ABL1–induced MPN are present in $Gab2^{-/-}$ BM and WT BM and establish that GAB2 is required for BCR-ABL1 to induce MPN.

Multiple GAB2 domains are required for BCR-ABL1–evoked MPN

The ability to "rescue" the CML phenotype in *Gab2*-deficient BM provided an opportunity to identify the structural features of GAB2 required for leukemogenesis. We cloned cDNAs encoding GAB2 with deletion of its Pleckstrin homology domain (*ΔPH*), mutations in the bipartite proline-rich binding sites for the GRB2 SH3 domains (*ΔGrb2*),[39] or Tyr-to-Phe substitutions in its SH2 binding sites[40] for the p85 subunit (*Δp85*) of PI3K or for SHP2 (*ΔShp2*) into the 3′ position of p210-$IRES_{wt}$-*Gab2* (vector 2 in Figure 2A). All GAB2 constructs also encoded tandem influenza hemagglutinin (HA) epitopes at their COOH termini (Figure 3A). Transduction of $Gab2^{-/-}$ BM with the *Gab2WT* virus efficiently restored cytokine-independent myeloid colony formation; by contrast, none of the *Gab2*-mutant viruses transformed $Gab2^{-/-}$ BM in vitro (Figure 3B).

Recipients of $Gab2^{-/-}$ BM (Balb/c background) transduced with the *Gab2WT* virus also efficiently developed leukemia on transplantation, with most recipients developing mixed MPN and B-ALL. By contrast, MPN was not observed in recipients of $Gab2^{-/-}$ BM transduced with any of the *Gab2*-mutant viruses (Figure 3C; supplemental Figure 3). These recipients tended to succumb principally to T-ALL, beginning ~3 months after transplantation.

To reduce the incidence of recipients with mixed disease, we repeated these experiments but lineage depleted the donor BM prior to transplantation[41] to remove early lymphoid progenitors, which are the target cells for BCR-ABL1–induced B-ALL.[5] As expected, we observed more efficient rescue of CML-like MPN on coexpression of GAB2 and BCR-ABL1 in $Gab2^{-/-}$ BM, and about half the recipients succumbed to CML-like disease without evidence of B-ALL (Figure 3D-E). As before, coexpression of BCR-ABL1 and GAB2 lacking its p85 or SHP2 binding sites failed to cause MPN, as reflected by modestly elevated PBL counts that were consistently ~10 to $15 \times 10^3/\mu L$ (Figure 3E). Southern blot analysis of PBL DNA showed that, similar to recipients of p210-*Gab2WT*–transduced $Gab2^{-/-}$ BM, evaluable recipients of p210-*Gab2Δp85*– or p210-*Gab2ΔShp2*–transduced $Gab2^{-/-}$ BM were repopulated with provirus-containing cells (supplemental Figure 2). Also, flow cytometry and intracellular staining for the HA epitope tag revealed similar levels of expression of virus-derived GAB2 proteins in circulating myeloid cells from all cohorts (Figure 3F). These results are consistent with comparable levels of engraftment by *Gab2*-deficient BM coexpressing BCR-ABL1 and the different GAB2 mutants and demonstrate that the PH domain, the GRB2 SH3 domain-binding site, and the binding sites for PI3K and SHP2 SH2 domains are each necessary for GAB2 to mediate signals required for BCR-ABL1–evoked MPN.

To analyze signaling downstream of GAB2 in the CML-like MPN induced by BCR-ABL1, we infected lineage-depleted WT or $Gab2^{-/-}$ BM cells with p210MIGFP virus and immunoblotted extracts from transduced (GFP^+) cells. Compared with WT cells, $Gab2^{-/-}$ cells had lower levels of phospho-ERK1/ERK2, phospho–Ak strain transforming (AKT) (S473), phospho-STAT5 (Y694), phospho-SFK (Y416), and phospho-S6 (S235/236) (Figure 4A-B; supplemental Figure 4A). By contrast, phosphorylation of CRKL, a direct substrate of BCR-ABL1, was unaffected by GAB2 deficiency (Figure 4A-B). We then assessed signaling events affected by mutation of the GAB2 binding sites for SHP2 and p85. We cotransduced lineage-depleted $Gab2^{-/-}$ BM cells with pMSCV-BCR/ABL1-IRES-RFP and pMSCV-GAB2-IRES-GFP (*Gab2WT*, *Gab2ΔShp2*, or *Gab2Δp85*) viruses

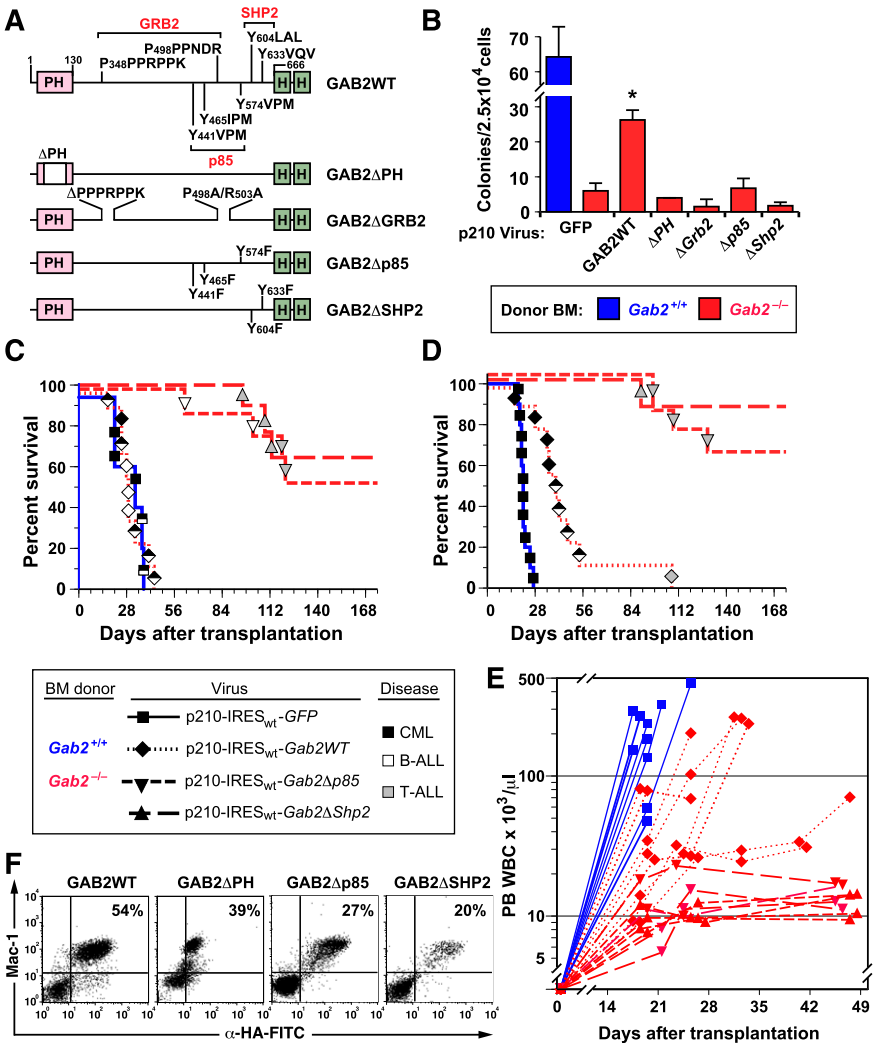

Figure 3. GAB2 structural domains required for induction of CML-like MPN by BCR-ABL1. (A) Scheme showing GAB2 mutants used in this experiment. PH, pleckstrin homology domain; HA, hemagglutinin epitope tag. Binding sites for the GRB2 SH3 domains and SH2 domains of p85 PI3K and SHP2 are indicated. (B) GAB2 mutants fail to rescue myeloid cell transformation in vitro. BM from the indicated donor mice (blue, *Gab2*$^{+/+}$; red, *Gab2*$^{-/-}$) was transduced with retrovirus coexpressing BCR-ABL1 and the different GAB2 variants depicted in A and plated in methylcellulose without exogenous cytokines. The difference in colony numbers between *Gab2*$^{-/-}$ BM transduced with p210-IRES$_{wt}$-*Gab2WT* virus, compared with all *Gab2* mutant viruses, was significant ($P \leq .004$, Student *t* tests). (C) K-M curves of recipients of BM from *Gab2*$^{+/+}$ littermates (blue solid line, n = 5) transduced with p210MIGFP retrovirus and of *Gab2*$^{-/-}$ donors (red lines), transduced with p210-IRES$_{wt}$-*Gab2WT* retrovirus (dotted line, n = 9) or the indicated *Gab2* mutant retrovirus (dashed lines, n = 8 for each cohort). There was no difference in survival between recipients of p210MIGFP-transduced *Gab2*$^{+/+}$ BM and p210-IRES$_{wt}$-*Gab2WT*–transduced *Gab2*$^{-/-}$ BM. All recipients of *Gab2* mutant-transduced BM showed prolonged survival ($P < .0001$, Mantel-Cox tests). (D) Survival curve as in C, except that the transduced BM was lineage depleted prior to transplantation, as described in Methods. (E) Scatter plot of peripheral blood (PB) leukocyte counts in the 4 cohorts from the transplant in D vs time after transplantation. (F) Flow cytometric detection of the retrovirally expressed HA-tagged GAB2 proteins in PBLs from the indicated recipients of transduced *Gab2*$^{-/-}$ BM. Note the discrete populations of Mac-1^{+}HA^{+} myeloid cells. Leukocytes from nontransduced *Gab2*$^{-/-}$ donors had no detectable fluorescein isothiocyanate fluorescence (data not shown). Detection of the HA epitope tag on the GAB2ΔPH protein by intracellular antibody staining was consistently less efficient than for the other GAB2 proteins, despite equivalent protein expression (Figure 5C).

and immunoblotted extracts from doubly infected cells. Compared with *Gab2WT*-reconstituted cells, *Gab2ΔShp2*-reconstituted cells had lower levels of phospho-ERK1/ERK2, phospho-AKT, phospho-STAT5, and phospho-S6; by contrast, only phospho-AKT levels were reduced in *Gab2Δp85*-reconstituted cells. Notably, phospho-AKT levels in the latter cells were even lower than those found in *Gab2ΔShp2*-reconstituted cells (Figure 4C-D; supplemental Figure 4B). Thus, GAB2 is a critical mediator of multiple pathways downstream of BCR-ABL1, and the SHP2 and p85 binding sites on GAB2 play distinct roles: SHP2 binding is required for full activation of multiple downstream effectors (especially ERK and STAT5), whereas p85 binding is more specifically and critically required for full activation of the AKT pathway.

Different requirements for GAB2 binding proteins in BCR-ABL1–induced B-lymphoid transformation

We then analyzed the impact of GAB2 deficiency on the ability of BCR-ABL1 to transform immature lymphoid progenitors in vitro using 2 quantitative assays: colony formation in agarose[42] and stroma-dependent growth.[43] In the colony assay, GAB2-deficient BM was profoundly refractory to BCR-ABL1 transformation, giving rise to only ~10% of the colonies as WT BM (Figure 5A). Transformation of GAB2-deficient BM was restored by transduction with p210-*Gab2WT* retrovirus, but not by *Gab2* mutants lacking either their p85 or SHP2 binding sites. In the second assay, BCR-ABL1 stimulates the outgrowth of B-lymphoid progenitors that are stroma dependent and not highly leukemogenic in mice.[36,43] As shown earlier,[16] *BCR-ABL1*–transduced *Gab2*$^{-/-}$ BM showed much lower outgrowth than transduced *Gab2*$^{+/+}$ BM (Figure 5B). Coexpression of BCR-ABL1 and *Gab2WT* restored efficient outgrowth of *Gab2*$^{-/-}$ BM, whereas coexpression of the *Gab2ΔShp2* mutant did not. Interestingly, the *Gab2Δp85* mutant also rescued B-lymphoid transformation in vitro, albeit to a lesser extent than *Gab2WT* (Figure 5B). Immunoblot analysis of extracts from the transformed cells demonstrated that the various retrovirally encoded GAB2 proteins were expressed and phosphorylated on the appropriate sites (Figure 5C).

BCR-ABL1 induces B-ALL through the GAB2-SHP2 signaling pathway

We also tested the role of GAB2 in B-lymphoid leukemogenesis by transducing BM from Balb/c *Gab2*$^{+/+}$ and *Gab2*$^{-/-}$ donors without cytokine stimulation and transplanting transduced cells into lethally irradiated recipients. In this model, recipients of BCR-ABL1–transduced *WT* BM develop B-ALL, characterized by involvement of BM, spleen, and lymph nodes, along with malignant pleural effusions containing B220^{+} lymphoblasts.[10] In contrast to the effect of GAB2 deficiency on myeloid leukemogenesis, wherein no recipient of BCR-ABL1–transduced *Gab2*$^{-/-}$ BM developed CML-like MPN

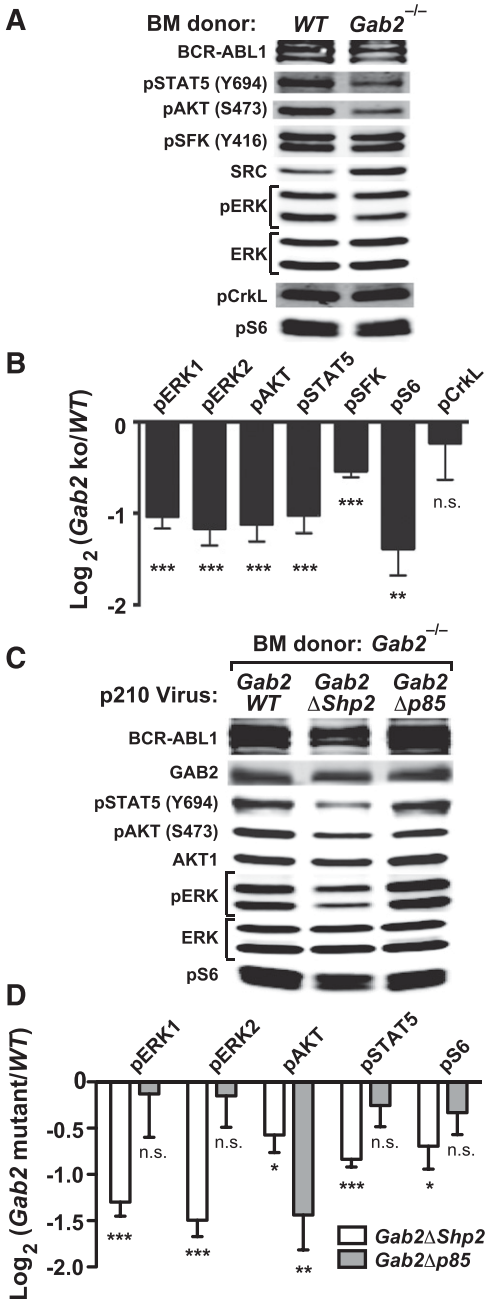

Figure 4. Analysis of signaling events in primary BCR-ABL1–expressing myeloid cells. (A) Representative immunoblot of primary Lin^-GFP^+ cell extracts from *WT* or $Gab2^{-/-}$ BM cells transduced with BCR-ABL1 p210MIGFP retrovirus; similar results were obtained in ≥6 independent biological replicates. (B) Statistical analysis of signaling events in A, pooled from ≥6 biological replicates (***$P < .001$, **$P < .01$, *$P < .05$, n.s., not significant; 2-sided Student *t* tests). (C) Representative immunoblot of primary $Lin^-GFP^+RFP^+$ cell extracts from $Gab2^{-/-}$ BM cells cotransduced with p210-IRES-RFP and *Gab2*-IRES-GFP viruses, as indicated. Similar results were obtained in ≥5 biological replicates. (D) Statistical analysis of signaling events pooled from ≥5 biological replicates of the experiment shown in C (***$P < .001$, **$P < .01$, *$P < .05$, n.s., not significant; all vs *Gab2WT* cotransduced samples; 2-sided Student *t* tests).

(Figure 1), about half of the recipients of $Gab2^{-/-}$ BM developed B-ALL, but only after a significantly prolonged latent period (Figure 5D). Coexpression of WT *Gab2WT* or the *Gab2Δp85* mutant with BCR-ABL1 restored induction of B-ALL in all recipients of $Gab2^{-/-}$ BM, although the survival of the *Gab2Δp85* cohort was significantly longer. By contrast, the *Gab2ΔShp2* mutant failed to rescue the lymphoid leukemogenesis defect. Together with the results of the in vitro growth assays (Figure 5B), these data show that GAB2 is not absolutely required for BCR-ABL1–induced B-lymphoid transformation or leukemogenesis, but GAB2-SHP2 signaling contributes significantly to the pathogenesis of BCR-ABL1–induced B-ALL.

To analyze GAB2-dependent signaling pathways in BCR-ABL1–transformed B-lymphoid cells, we performed immunoblot analysis. Similar to its effects in myeloid transformation, GAB2 was required for full activation of ERK, AKT, STAT5, SFK, and S6 (Figure 6A-B; supplemental Figure 5A). We also performed RNA-seq analysis to assess whether these changes in signaling pathways altered downstream transcriptional events. Unsupervised hierarchical clustering revealed good separation of *WT* and $Gab2^{-/-}$ samples (Figure 7A), consistent with significant effects of *Gab2* deficiency on the transcriptome. Supervised comparison revealed ~6000 differentially expressed genes in *WT* and $Gab2^{-/-}$ pre-B cells (Figure 7B). Consistent with the observed alterations in proximal signaling events, multiple downstream transcriptional targets of the ERK, AKT, and STAT5 pathways were significantly altered (Figure 7C-E), including *Etv5*, *Dusp6*, and *Cdc6* (ERK), *Foxo3*, *Bcl2l11*, and *Bcl2* (AKT), and *Gtf2h*, *Bcl6*, and *Lif* (STAT5). Gene Ontology enrichment analysis showed that genes involved in RNA processing and DNA replication also were significantly downregulated in $Gab2^{-/-}$ cells (Figure 7F-G). Finally, we used Enrichr, coupled with the Encyclopedia of DNA Elements (ENCODE) ChIP-seq database, to identify downregulation of potential targets of transcription factors (TFs) including MYC, ELK, FOS, FOXM1, and STAT5 (supplemental Tables 1 and 2), reflecting the observed changes in BCR-ABL1 signaling (Figure 6A-B).

Reconstitution studies using *Gab2* mutants indicated that SHP2 binding was required for ERK, AKT, S6, and possibly STAT5 activation, whereas p85 binding was required for activation of AKT (Figure 6C-D; supplemental Figure 5B). Hence, GAB2 activates similar downstream signaling events in BCR-ABL1–transformed myeloid and lymphoid cells, but the requirement of these pathways for transformation differs in the 2 hematopoietic neoplasms.

Discussion

Substantial effort has been made to identify key signaling pathways in Ph^+ leukemias whose blockade can prevent or overcome TKI resistance and perhaps lead to cures.[44] Mouse models provide invaluable tools for such studies, in part because they allow genetic manipulation of leukemic cells that is difficult to achieve using human cell lines or leukemia cells from patients. Here, we studied the effects of GAB2 deficiency in mouse models of CML and B-ALL and found that by acting through distinct signaling pathways, this scaffolding adaptor is required for myeloid and B-lymphoid leukemogenesis by BCR-ABL1.

We found that $Gab2^{-/-}$ BM was resistant to induction of CML-like MPN by BCR-ABL1, suggesting that signals from GAB2 are essential for the characteristic marked expansion of myeloid cells in this disease. GAB2 deficiency might have other effects that abrogate leukemogenesis in this model, including altered stem cell abundance, transduction efficiency, or engraftment ability. Several lines of evidence argue against these possibilities. We observed equivalent engraftment of provirus-positive cells (supplemental Figure 2), and circulating GFP^+ myeloid cells (Figure 1C) were present in recipients of transduced $Gab2^{-/-}$ BM, indicating that they had been repopulated with retrovirus-transduced cells. Most importantly, the mouse *Gab2*-null allele was complemented by coexpression of GAB2 and BCR-ABL1 in transduced stem cells, which resulted in efficient restoration of MPN (Figure 3C-D).

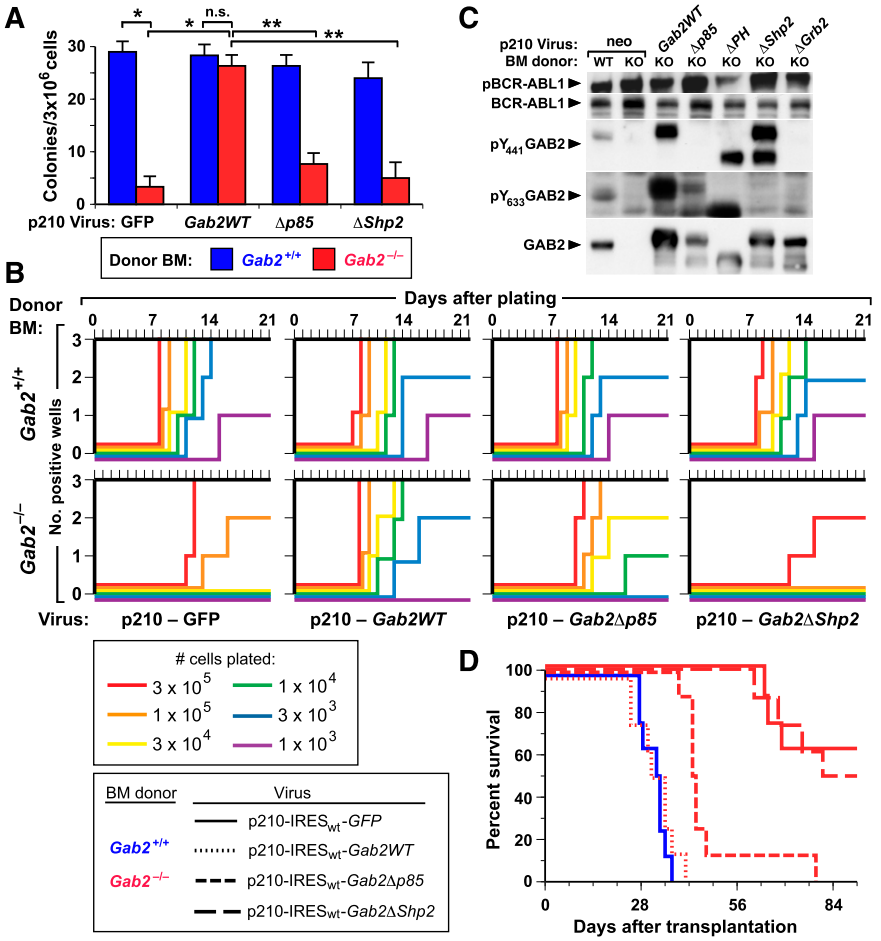

Figure 5. In vitro lymphoid transformation and in vivo leukemogenesis evoked by BCR-ABL1 are dependent on GAB2. (A) Agarose colony assays. BM from the indicated Balb/c donor (blue, *Gab2*$^{+/+}$; red, *Gab2*$^{-/-}$) mice was transduced with retrovirus coexpressing p210 BCR-ABL1 and either GFP or the indicated GAB2 variant. Transduced cells were plated directly in agarose as described in Methods, and transformed pre-B-lymphoid colonies were counted at day 10. The differences in colony numbers between p210-GFP–transduced *Gab2*$^{+/+}$ and *Gab2*$^{-/-}$ BM and between p210-GFP–transduced and p210-*Gab2WT*–transduced *Gab2*$^{-/-}$ BM, were significant ($P < .0001$ and $P = .001$, respectively, unpaired Student *t* tests), whereas there was no difference between p210-GFP–transduced *Gab2*$^{+/+}$ and p210-*Gab2WT*–transduced *Gab2*$^{-/-}$ BM ($P = .224$). The difference in colony numbers between *Gab2*$^{-/-}$ BM transduced with p210-*Gab2WT* retrovirus and either p210-*Gab2Δp85* or p210-*Gab2ΔShp2* retrovirus was significant ($P = .0004$ and $P = .0002$, respectively, unpaired Student *t* tests), whereas the difference between *Gab2*$^{-/-}$ BM transduced with p210-*Gab2Δp85* and p210-*Gab2ΔShp2* retrovirus did not reach significance ($P = .0647$, unpaired Student *t* test). (B) Whitlock-Witte assays. Freshly harvested Balb/c BM from *Gab2*$^{+/+}$ (top) and *Gab2*$^{-/-}$ (bottom) donors was transduced with retrovirus coexpressing p210 BCR-ABL1 and either GFP or the indicated GAB2 variant, and plated in triplicate at the indicated cell numbers per well. Nontransduced cells were added to provide 10^6 total cells for stromal support. Wells were scored as positive when cell number reached 10^6 viable nonadherent cells per well, as described in Methods. (C) Extracts from immortalized BCR-ABL1–transformed B-lymphoid cell lines (from Figure 5B) from *Gab2*$^{+/+}$ (WT) or *Gab2*$^{-/-}$ (knockout) donors were analyzed for GAB2 protein expression and phosphorylation, as described in Methods. (D) K-M curves of recipients of BM from *Gab2*$^{+/+}$ littermates transduced with p210MIGFP retrovirus (blue solid line, n = 8), and of *Gab2*$^{-/-}$ donors (red lines) transduced with p210-IRES$_{wt}$-*Gab2WT* retrovirus (dotted line, n = 8) or the indicated Gab2 mutant retrovirus (dashed lines, n = 8 for each cohort). All deaths were due to B-ALL. There was no significant difference in survival between recipients of p210MIGFP-transduced *Gab2*$^{+/+}$ BM and p210-IRES$_{wt}$-*Gab2WT*–transduced *Gab2*$^{-/-}$ BM ($P = .493$) or between recipients of p210MIGFP-transduced *Gab2*$^{-/-}$ BM and p210-IRES$_{wt}$-*Gab2ΔShp2*–transduced *Gab2*$^{-/-}$ BM ($P = .734$). By contrast, the survival of recipients of p210MIGFP-transduced *Gab2*$^{+/+}$ BM and p210MIGFP-transduced *Gab2*$^{-/-}$ BM, and of recipients p210-IRES$_{wt}$-*Gab2WT*–transduced and p210-IRES$_{wt}$-*Gab2Δp85*–transduced *Gab2*$^{-/-}$ BM, was different ($P < .0001$ and $P = .001$, respectively, Mantel-Cox tests).

The GRB2 binding site at Y177 of BCR-ABL1 is required for efficient induction of CML-like MPN in mice, and subsequent analyses have confirmed that pY177 has an analogous role in BCR-ABL1–expressing primary human CD34$^+$ cells.[45] Our previous studies demonstrated that GAB2 phosphorylation was diminished in leukemic cells expressing BCR-ABL1 Y177F, and in vitro transformation of GAB2-deficient myeloid and lymphoid progenitors by BCR-ABL1 also was decreased. The results here demonstrate that GAB2 is required for both myeloid and lymphoid leukemogenesis by BCR-ABL1 and argue that the critical signals flow through a pY177-GRB2-GAB2 complex. Although our data clearly show that GRB2/GAB2 complexes are essential, we do not exclude a role for SOS, another GRB2 effector, which mediates RAS activation, in CML pathogenesis. A direct role for GAB2 in human CML is also suggested by the demonstration that siRNA knockdown of *GAB2* selectively impairs colony formation by CML progenitors in vitro.[46] As hematopoiesis is essentially normal in *Gab2*$^{-/-}$ mice, these results suggest that inhibiting GAB2 function might be an effective treatment of Ph$^+$ leukemia with little adverse effects on normal blood cells. Other work suggests that GAB2 might also be a therapeutic target in AML and some solid tumors.[27-31]

In CML patients, leukemic stem cells are relatively resistant to TKI therapy.[47-49] Several observations suggest that GAB2 might be required for maintenance of BCR-ABL1$^+$ leukemic stem cells. Although we were able to detect provirus-containing, *Gab2*-deficient myeloid cells in peripheral blood at 4 to 12 weeks after transplantation, these cells generally disappeared from the circulation at longer time points, and we were unable to serially transplant BCR-ABL1–expressing *Gab2*-deficient BM (data not shown). Although further studies are needed,

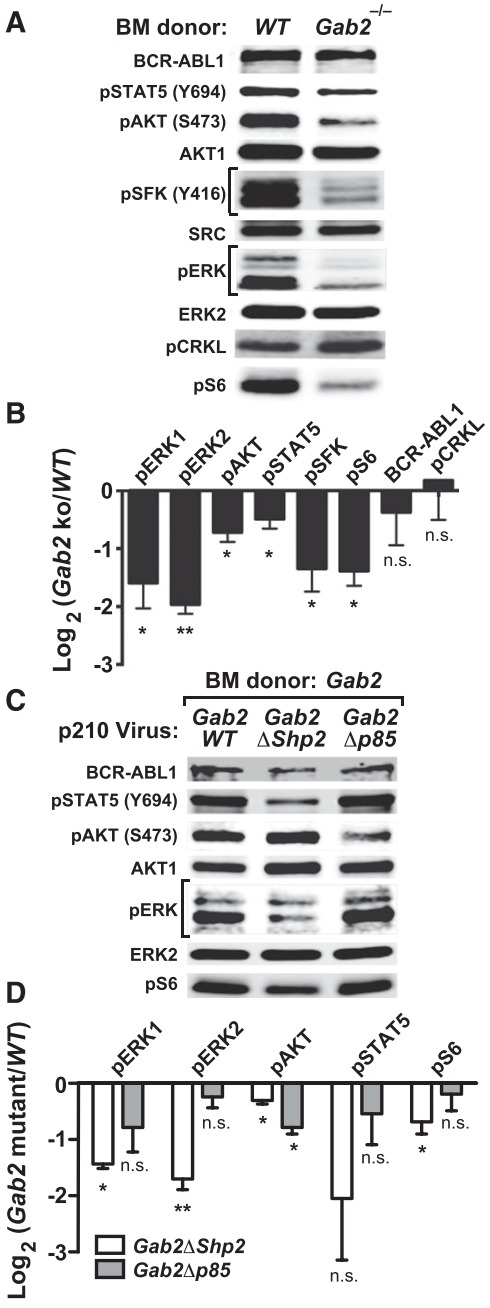

Figure 6. Analysis of signaling events in primary BCR-ABL1–expressing B-lymphoid progenitors. (A) Representative immunoblot of primary CD127$^+$GFP$^+$ cell extracts from *Gab2*$^{+/+}$ (WT) or *Gab2*$^{-/-}$ BM cells transduced with BCR-ABL1 p210MIGFP retrovirus; similar results were obtained in 3 independent biological replicates. (B) Statistical analysis of signaling events pooled from 3 biological replicates of the experiment shown in A (**$P < .01$, *$P < .05$; 1-sided Student *t* tests). (C) Representative immunoblot of primary CD127$^+$GFP$^+$RFP$^+$ cell extracts from *Gab2*$^{-/-}$ BM cells cotransduced with p210-IRES-RFP and *Gab2*-IRES-GFP viruses, as indicated. Similar results were obtained in 3 biological replicates. (D) Statistical analysis of signaling events pooled from 3 biological replicates of the experiment shown in C (**$P < .01$, *$P < .05$; $P = .1$ for p-STAT5 between *Gab2Δ Shp2* and *Gab2WT*; 1-sided Student *t* tests).

these observations suggest that targeting GAB2 or GAB2-dependent pathways might be an effective strategy for eradication of CML, in addition to reversing the myeloproliferative phenotype.

Previous studies of BCR-ABL1 leukemogenesis have suggested that different signaling pathways are involved in CML and B-ALL pathogenesis.[8,10] The ability to rescue BCR-ABL1 leukemogenesis in *Gab2*-deficient BM by coexpression of GAB2 allowed us to ask which functional domains of GAB2 are required. In the CML model, rescue of leukemogenesis by GAB2 required GRB2 binding, the PH domain, and SH2 domain binding sites for both p85 and SHP2. By contrast, B-lymphoid transformation and leukemogenesis were largely restored by the *Gab2Δp85* mutant but not by *Gab2ΔShp2*. Together, these results emphasize that distinct signaling pathways are essential for the pathogenesis of BCR-ABL1$^+$ myeloid and lymphoid leukemias. In particular, our results validate SHP2, which is required for the pathogenesis of both leukemia types, as an important new target for therapy of Ph$^+$ leukemia. In parallel, another study investigated the role of GAB2 overexpression in conferring resistance of CML cells to ABL1 TKIs and found that GAB2-mediated TKI resistance is also dependent on its interaction with SHP2 and p85.[50]

Analysis of GAB2-dependent signals in BCR-ABL1–expressing primary myeloid and B-lymphoid cells revealed distinct roles for the GAB2 SHP2 and p85 binding sites in BCR-ABL1–induced transformation. In agreement with our previous observations,[16] AKT and ERK activation were decreased in *Gab2*$^{-/-}$ myeloid cells expressing BCR-ABL1. We also found that GAB2 is required for full activation of SFKs and STAT5 by BCR-ABL1. As a direct target of SFKs, decreased STAT5 activation in *Gab2*-deficient cells could be explained by lower SFK activation. Alternatively, SHP2 might promote STAT5 activation by evoking cytokine production and consequent activation of JAK kinases; indeed, we have found that expression of a leukemogenic *PTPN11* mutant in TF-1 myeloid cells promotes STAT activation via such an autocrine pathway (S.G. and B.G.N., unpublished data, August 2014). Conversely, activation of pathways downstream of GAB2 by activated STAT5 in myeloid leukemia cells has also been reported.[51]

RNA-seq analysis of *WT* and *Gab2*$^{-/-}$ BCR-ABL1$^+$ pre-B cells revealed that these signaling changes led to corresponding alterations in downstream transcription. ENCODE TF-target enrichment analysis indicated that the activities of transcription factors such as MYC, FOS, ELK, FOXM1, and STAT5 are downregulated in *Gab2*$^{-/-}$ lymphoblasts, consistent with the lower phospho-ERK, phospho-AKT, and phospho-STAT5 in these cells. Gene Ontology term enrichment analysis indicated that biological processes such as RNA processing and DNA replication are downregulated in *Gab2*$^{-/-}$ cells. Changes in RNA processing might be attributable to lower MYC activity in *Gab2*$^{-/-}$ cells,[52] whereas DNA replication can additionally be regulated by other transcription factors, such as FOS, FOXM1, and STAT5.[53-55]

We also discriminated the roles of SHP2 and PI3K binding to GAB2 in BCR-ABL1 signaling; whereas SHP2 binding is required for activation of ERK, S6, and STAT5, PI3K binding is required only for AKT activation. The failure of *Gab2Δp85* to rescue myeloid leukemogenesis clearly implicates type IA PI3K as a potential therapeutic target in this disease, but whether AKT is also a valid therapeutic target in CML is unclear. In this regard, we have found that CML-like MPN can be induced efficiently in BM from mice with deficiency in any 1 of the 3 AKT isoforms (W.W.C. and R.A.V.E., unpublished observations, January 2013). Notably, our data indicate that p85 binding to GAB2 is less critical for BCR-ABL1–evoked lymphoid leukemogenesis, although type IA PI3Ks are required for this process.[56] Conceivably, myeloid progenitors might have a higher dependence on GAB2 for activation of PI3K than do B-lymphoid progenitors, due to parallel mechanisms for type 1A PI3K activation in lymphoid, but not myeloid, progenitors. Alternatively, myeloid cells might require higher levels of PI3K activity than lymphoid cells to be transformed by BCR-ABL1, such that residual levels of activation in GAB2-deficient lymphoid cells are sufficient.

In conclusion, we identified a critical role for GAB2 in the pathogenesis of CML and Ph$^+$ B-ALL and showed that leukemogenic

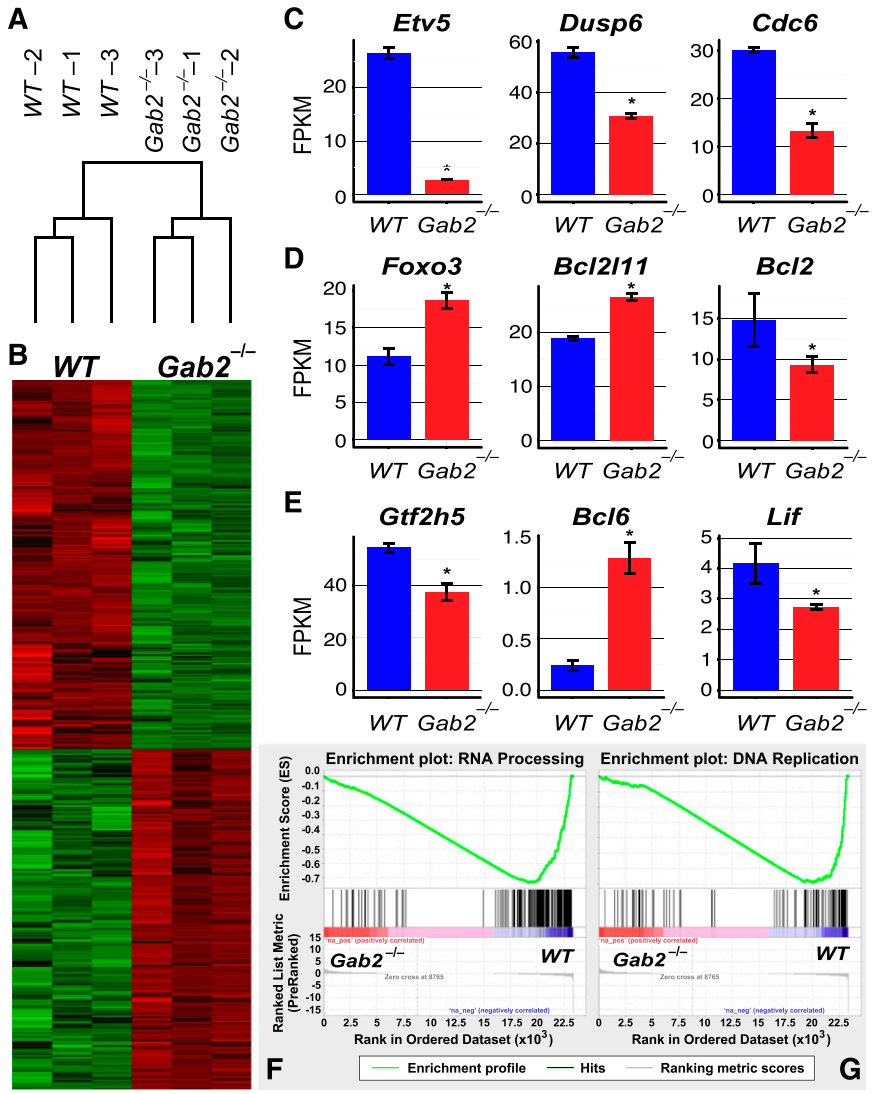

Figure 7. GAB2 deficiency affects transcription of downstream effectors. RNA-seq was performed on BCR-ABL1–transformed *WT* or *Gab2*$^{-/-}$ pre-B cells. (A) Unsupervised clustering of samples based on expression of all genes. (B) Heat map showing differential gene expression (supervised analysis) in *WT* and *Gab2*$^{-/-}$ cells. (C-E) mRNA levels of typical transcriptional targets downstream of the (C) ERK, (D) AKT, or (E) STAT5 pathway were altered (**FDR* < 0.05, CuffDiff). (F-G) gene set enrichment analysis of Gene Ontology term enrichment in *Gab2*$^{-/-}$ compared with *WT* cells. Gene sets in biological processes of (F) RNA processing and (G) DNA replication were significantly enriched in downregulated genes in *Gab2*$^{-/-}$ cells. *FDR* < 0.01 for both (F) and (G).

signals from Y177 of BCR-ABL1 flow through distinct pathways in these hematologic malignancies. Our results validate GAB2 and downstream signaling molecules, particularly SHP2, as novel targets for therapy, whose inhibition might complement TKIs in the effort to prevent relapse of leukemia and permanently cure patients.

Acknowledgments

The authors thank Katherine Lazarides and Monica Betancur for excellent technical assistance and Princess Margaret Cancer Center for RNA-seq analyses.

This work was supported by grants from the National Institutes of Health, National Cancer Institute (R01 CA090576) and the Leukemia & Lymphoma Society to R.A.V.E., from the Leukemia & Lymphoma Society and Worldwide Cancer Research to G.M., and by National Institutes of Health, National Cancer Institute grants R01 CA11494 and R37 CA049152 to B.G.N. Work in the B.G.N. laboratory was partially supported by the Ontario Ministry of Health and Long Term Care and the Princess Margaret Hospital Foundation. B.G.N. also was a Tier I Canada Research Chair.

Authorship

Contribution: S.G., W.W.C., G.M., J.R., B.G.N., and R.A.V.E. designed and performed the experiments; A.S., Z.L., and C.V. analyzed RNA-seq data; S.L. provided essential advice; and S.G., W.W.C., G.M., B.G.N., and R.A.V.E. wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

The current affiliation for G.M. is State University of New York System (SUNY) Upstate Medical University, Syracuse, NY.

The current affiliation for J.R. is University of Pittsburgh, Pittsburgh, PA.

The current affiliation for B.G.N. is Laura and Isaac Perlmutter Cancer Center, NYU Langone Medical Center, New York, NY.

The current affiliation for R.A.V.E. is Chao Family Comprehensive Cancer Center, University of California, Irvine, CA.

Correspondence: Richard A. Van Etten, Chao Family Comprehensive Cancer Center, University of California, Irvine, 839 Medical Sciences Court, Sprague Hall 124, Irvine, CA 92697; e-mail: vanetten@uci.edu; and Benjamin G. Neel, Laura & Isaac Perlmutter Cancer Center, 522 First Ave, Smilow Building 12th Floor, Suite 1201, New York, NY 10016; e-mail: benjamin.neel@nyumc.org.

References

1. Mahon F-X, Réa D, Guilhot J, et al; Intergroupe Français des Leucémies Myéloïdes Chroniques. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol.* 2010; 11(11):1029-1035.
2. Druker BJ, Sawyers CL, Kantarjian H, et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med.* 2001;344(14): 1038-1042.
3. Sawyers CL, Hochhaus A, Feldman E, et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood.* 2002;99(10): 3530-3539.
4. Quintás-Cardama A, Cortes J. Molecular biology of bcr-abl1-positive chronic myeloid leukemia. *Blood.* 2009;113(8):1619-1630.
5. Hu Y, Swerdlow S, Duffy TM, Weinmann R, Lee FY, Li S. Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for improved treatment of Ph+ leukemia in mice. *Proc Natl Acad Sci USA.* 2006;103(45): 16870-16875.
6. Huntly BJP, Shigematsu H, Deguchi K, et al. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer Cell.* 2004; 6(6):587-596.
7. Daley GQ, Van Etten RA, Baltimore D. Blast crisis in a murine model of chronic myelogenous leukemia. *Proc Natl Acad Sci USA.* 1991;88(24): 11335-11338.
8. Hu Y, Liu Y, Pelletier S, et al. Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. *Nat Genet.* 2004;36(5):453-461.
9. 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(9):1399-1412.
10. Roumiantsev S, de Aos 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(1): 4-13.
11. Pendergast AM, Quilliam LA, Cripe LD, et al. BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein. *Cell.* 1993;75(1):175-185.
12. Puil L, Liu J, Gish G, et al. Bcr-Abl oncoproteins bind directly to activators of the Ras signalling pathway. *EMBO J.* 1994;13(4):764-773.
13. He Y, Wertheim JA, Xu L, et al. The coiled-coil domain and Tyr177 of bcr are required to induce a murine chronic myelogenous leukemia-like disease by bcr/abl. *Blood.* 2002;99(8): 2957-2968.
14. Million RP, Van Etten RA. The Grb2 binding site is required for the induction of chronic myeloid leukemia-like disease in mice by the Bcr/Abl tyrosine kinase. *Blood.* 2000;96(2): 664-670.
15. Zhang X, Subrahmanyam R, Wong R, Gross AW, Ren R. The NH(2)-terminal coiled-coil domain and tyrosine 177 play important roles in induction of a myeloproliferative disease in mice by Bcr-Abl. *Mol Cell Biol.* 2001;21(3):840-853.
16. Sattler M, Mohi MG, Pride YB, et al. Critical role for Gab2 in transformation by BCR/ABL. *Cancer Cell.* 2002;1(5):479-492.
17. Gu H, Neel BG. The "Gab" in signal transduction. *Trends Cell Biol.* 2003;13(3):122-130.
18. Wöhrle FU, Daly RJ, Brummer T. Function, regulation and pathological roles of the Gab/DOS docking proteins. *Cell Commun Signal.* 2009; 7:22.
19. Gu H, Griffin JD, Neel BG. Characterization of two SHP-2-associated binding proteins and potential substrates in hematopoietic cells. *J Biol Chem.* 1997;272(26):16421-16430.
20. Gu H, Pratt JC, Burakoff SJ, Neel BG. Cloning of p97/Gab2, the major SHP2-binding protein in hematopoietic cells, reveals a novel pathway for cytokine-induced gene activation. *Mol Cell.* 1998; 2(6):729-740.
21. Gu H, Saito K, Klaman LD, et al. Essential role for Gab2 in the allergic response. *Nature.* 2001; 412(6843):186-190.
22. Lynch DK, Daly RJ. PKB-mediated negative feedback tightly regulates mitogenic signalling via Gab2. *EMBO J.* 2002;21(1-2):72-82.
23. Nishida K, Wang L, Morii E, et al. Requirement of Gab2 for mast cell development and KitL/c-Kit signaling. *Blood.* 2002;99(5):1866-1869.
24. Holgado-Madruga M, Emlet DR, Moscatello DK, Godwin AK, Wong AJA. A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature.* 1996;379(6565):560-564.
25. Wolf I, Jenkins BJ, Liu Y, et al. Gab3, a new DOS/Gab family member, facilitates macrophage differentiation. *Mol Cell Biol.* 2002; 22(1):231-244.
26. Zhang Y, Diaz-Flores E, Li G, et al. Abnormal hematopoiesis in Gab2 mutant mice. *Blood.* 2007; 110(1):116-124.
27. Zatkova A, Schoch C, Speleman F, et al. GAB2 is a novel target of 11q amplification in AML/MDS. *Genes Chromosomes Cancer.* 2006;45(9): 798-807.
28. Bentires-Alj M, Gil SG, Chan R, et al. A role for the scaffolding adapter GAB2 in breast cancer. *Nat Med.* 2006;12(1):114-121.
29. Brummer T, Schramek D, Hayes VM, et al. Increased proliferation and altered growth factor dependence of human mammary epithelial cells overexpressing the Gab2 docking protein. *J Biol Chem.* 2006;281(1):626-637.
30. Horst B, Gruvberger-Saal SK, Hopkins BD, et al. Gab2-mediated signaling promotes melanoma metastasis. *Am J Pathol.* 2009; 174(4):1524-1533.
31. Dunn GP, Cheung HW, Agarwalla PK, et al. In vivo multiplexed interrogation of amplified genes identifies GAB2 as an ovarian cancer oncogene. *Proc Natl Acad Sci USA.* 2014; 111(3):1102-1107.
32. Pear WS, Miller JP, Xu L, et al. Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood.* 1998; 92(10):3780-3792.
33. Hawley RG, Lieu FH, Fong AZ, Hawley TS. Versatile retroviral vectors for potential use in gene therapy. *Gene Ther.* 1994;1(2): 136-138.
34. Gavrilescu LC, Van Etten RA. Murine retroviral bone marrow transplantation models for the study of human myeloproliferative disorders. *Curr Protoc Pharmacol.* 2008;43: 14.10.1-14.10.28.
35. Warren D, Griffin DS, Mainville C, Rosenberg N. The extreme carboxyl terminus of v-Abl is required for lymphoid cell transformation by Abelson virus. *J Virol.* 2003;77(8): 4617-4625.
36. Smith KM, Yacobi R, Van Etten RA. Autoinhibition of Bcr-Abl through its SH3 domain. *Mol Cell.* 2003;12(1):27-37.
37. Gishizky ML, Witte ON. Initiation of deregulated growth of multipotent progenitor cells by bcr-abl in vitro. *Science.* 1992;256(5058):836-839.
38. Neering SJ, Bushnell T, Sozer S, et al. Leukemia stem cells in a genetically defined murine model of blast-crisis CML. *Blood.* 2007; 110(7):2578-2585.
39. Lock LS, Royal I, Naujokas MA, Park M. Identification of an atypical Grb2 carboxyl-terminal SH3 domain binding site in Gab docking proteins reveals Grb2-dependent and -independent recruitment of Gab1 to receptor tyrosine kinases. *J Biol Chem.* 2000;275(40): 31536-31545.
40. Pratt JC, Igras VE, Maeda H, et al. Cutting edge: gab2 mediates an inhibitory phosphatidylinositol 3′-kinase pathway in T cell antigen receptor signaling. *J Immunol.* 2000; 165(8):4158-4163.
41. Jiang X, Stuible M, Chalandon Y, et al. Evidence for a positive role of SHIP in the BCR-ABL-mediated transformation of primitive murine hematopoietic cells and in human chronic myeloid leukemia. *Blood.* 2003;102(8):2976-2984.
42. Rosenberg N, Baltimore D. A quantitative assay for transformation of bone marrow cells by Abelson murine leukemia virus. *J Exp Med.* 1976; 143(6):1453-1463.
43. McLaughlin J, Chianese E, Witte ON. Alternative forms of the BCR-ABL oncogene have quantitatively different potencies for stimulation of immature lymphoid cells. *Mol Cell Biol.* 1989;9(5): 1866-1874.
44. Van Etten RA. Mechanisms of transformation by the BCR-ABL oncogene: new perspectives in the post-imatinib era. *Leuk Res.* 2004;28(Suppl 1): S21-S28.
45. Chu S, Li L, Singh H, Bhatia R. BCR-tyrosine 177 plays an essential role in Ras and Akt activation and in human hematopoietic progenitor transformation in chronic myelogenous leukemia. *Cancer Res.* 2007;67(14):7045-7053.
46. Scherr M, Chaturvedi A, Battmer K, et al. Enhanced sensitivity to inhibition of SHP2, STAT5, and Gab2 expression in chronic myeloid leukemia (CML). *Blood.* 2006;107(8): 3279-3287.
47. Graham SM, Jørgensen HG, Allan E, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. *Blood.* 2002; 99(1):319-325.
48. Corbin AS, Agarwal A, Loriaux M, Cortes J, Deininger MW, Druker BJ. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J Clin Invest.* 2011;121(1):396-409.
49. Jiang X, Zhao Y, Smith C, et al. Chronic myeloid leukemia stem cells possess multiple unique features of resistance to BCR-ABL targeted therapies. *Leukemia.* 2007;21(5):926-935.
50. Wöhrle FU, Halbach S, Aumann K, et al. Gab2 signaling in chronic myeloid leukemia cells confers resistance to multiple Bcr-Abl inhibitors. *Leukemia.* 2013;27(1):118-129.
51. Harir N, Pecquet C, Kerenyi M, et al. Constitutive activation of Stat5 promotes its cytoplasmic localization and association with PI3-kinase in

myeloid leukemias. *Blood.* 2007;109(4): 1678-1686.

52. Kress TR, Sabò A, Amati B. MYC: connecting selective transcriptional control to global RNA production. *Nat Rev Cancer.* 2015;15(10): 593-607.
53. Murakami Y, Satake M, Yamaguchi-Iwai Y, Sakai M, Muramatsu M, Ito Y. The nuclear protooncogenes c-jun and c-fos as regulators of DNA replication. *Proc Natl Acad Sci USA.* 1991; 88(9):3947-3951.
54. Liu Y, Gong Z, Sun L, Li X. FOXM1 and androgen receptor co-regulate CDC6 gene transcription and DNA replication in prostate cancer cells. *Biochim Biophys Acta.* 2014;1839(4):297-305.
55. Watanabe S, Zeng R, Aoki Y, Itoh T, Arai K. Initiation of polyoma virus origin-dependent DNA replication through STAT5 activation by human granulocyte-macrophage colony-stimulating factor. *Blood.* 2001;97(5): 1266-1273.
56. Kharas MG, Janes MR, Scarfone VM, et al. Ablation of PI3K blocks BCR-ABL leukemogenesis in mice, and a dual PI3K/mTOR inhibitor prevents expansion of human BCR-ABL+ leukemia cells. *J Clin Invest.* 2008;118(9): 3038-3050.