Prognostic, therapeutic, and mechanistic implications of a mouse model of leukemia evoked by Shp2 (*PTPN11*) mutations

M. Golam Mohi,[1,*] Ifor R. Williams,[2] Charles R. Dearolf,[3] Gordon Chan,[1] Jeffery L. Kutok,[4] Sarah Cohen,[5] Kelly Morgan,[5] Christina Boulton,[5] Hirokazu Shigematsu,[6] Heike Keilhack,[1] Koichi Akashi,[6] D. Gary Gilliland,[5] and Benjamin G. Neel[1]

[1]Cancer Biology Program, Department of Medicine, Beth Israel Deaconess Medical Center, 77 Avenue Louis Pasteur, NRB-1030, Boston, Massachusetts 02115
[2]Department of Pathology, Emory University, Atlanta, Georgia 30322
[3]Department of Pediatrics, Massachusetts General Hospital, Boston, Massachusetts 02114
[4]Department of Pathology
[5]Division of Hematology, Brigham and Women's Hospital, Boston, Massachusetts 02115
[6]Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, Massachusetts 02115
*Correspondence: gmohi@bidmc.harvard.edu

Summary

The SH2-containing tyrosine phosphatase Shp2 (*PTPN11*) is required for growth factor and cytokine signaling. Germline Shp2 mutations cause Noonan Syndrome (NS), which is associated with increased risk of juvenile myelomonocytic leukemia (JMML). Somatic Shp2 mutations occur in sporadic JMML and other leukemias. We found that Shp2 mutants associated with sporadic leukemias transform murine bone marrow cells, whereas NS mutants are less potent in this assay. Transformation requires multiple domains within Shp2 and the Shp2 binding protein Gab2, and is associated with hyperactivation of the Erk, Akt, and Stat5 pathways. Mutant Shp2-transduced BM causes a fatal JMML-like disorder or, less commonly, lymphoproliferation. Shp2 mutants also cause myeloproliferation in *Drosophila.* Mek or Tor inhibitors potently inhibit transformation, suggesting new approaches to JMML therapy.

Introduction

Many hematopoietic signaling pathways are controlled by tyrosyl phosphorylation, which is regulated by protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). Activating mutations in PTKs are causally implicated in leukemogenesis. For example, the fusion kinase BCR-ABL, expressed as a consequence of t(9;22), causes chronic myelogenous leukemia (Sawyers, 1999). Similarly, fusions of the PDGFRα, PDGFRβ, or FGFR1 to oligomerization motifs by chromosomal translocation contributes to the pathogenesis of other chronic myeloproliferative disorders (MPD) (Cross and Reiter, 2002; Pardanani and Tefferi, 2004), whereas activating mutations in FLT3 occur in >30% of acute myelogenous leukemia (AML) (Gilliland and Griffin, 2002).

Shp2 (*PTPN11*) is the first protein-tyrosine phosphatase (PTP) implicated in leukemogenesis. Germline mutations in Shp2 cause the autosomal dominant disorder Noonan syndrome (NS), characterized by short stature, facial dysmorphia, and cardiac defects (Tartaglia et al., 2001). A possible association between NS and leukemia, particularly juvenile myelomonocytic leukemia (JMML), had been noted (Bader-Meunier et al., 1997; Choong et al., 1999; Side and Shannon, 1997), suggesting that Shp2 mutations might be leukemogenic. Subsequently, somatic Shp2 mutations were reported in ~35% of sporadic JMML, and at lower incidence in pediatric AML and myelodysplastic syndrome (MDS) (Loh et al., 2004; Tartaglia et al., 2003). Shp2 mutations also have been found in ~10% of cases of the most common form of childhood leukemia, B cell acute lymphoblastic leukemia (B-ALL) (Tartaglia et al., 2004), and in adult AML (~5%) and a small percentage of solid tumors (Bentires-Alj et al., 2004).

JMML is a rapidly progressive, clonal MPD characterized by overproduction of tissue-invading myeloid lineage cells (Arico et al., 1997; Emanuel et al., 1996). JMML bone marrow (BM)

SIGNIFICANCE

Genetic studies implicate mutations in Shp2 in NS and various forms of leukemia, but the effects of Shp2 mutants on hematopoiesis have not been evaluated. We show that leukemia-associated Shp2 mutations directly transform primary myeloid progenitors and cause leukemia in mice, thereby establishing a causal link between Shp2 mutations and oncogenesis. Shp2 mutants also cause myeloproliferation in flies, indicating that they subvert a highly conserved pathway regulating myeloid cell proliferation/survival and providing potential genetic approaches to delineating their pathogenic mechanism. Furthermore, we find that NS- and leukemia-associated mutations have intrinsically different potencies for myeloid transformation, which may have prognostic implications for NS patients. Finally, analysis of perturbed cytokine signaling mediated by mutant Shp2 suggests new therapeutic approaches to these disorders.

cells form monocytic colonies in the absence of exogenous growth factors, and exhibit selective hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF). Approximately 50% of JMML cases have activating *Ras* mutations or homozygotic inactivation of the neurofibromatosis type I gene, *NF1*, whose gene product, neurofibromin (NF-1), is a Ras-GTPase activating protein (Ras-GAP) (Cichowski et al., 2003). Hence, JMML caused by activating *Ras* or inactivating *Nf1* mutation is thought to result from increased Ras signaling (Arico et al., 1997; Emanuel et al., 1996). Indeed, BM cells from JMML patients (Bollag et al., 1996) or in murine models of *Nf1* deficiency (Bollag et al., 1996; Largaespada et al., 1996; Zhang et al., 1998; Le et al., 2004) show increased Ras activation. Furthermore, the Ras/Erk and PI3K/Akt pathways are hyperactive in mast cells from $Nf1^{+/-}$ mice (Ingram et al., 2001), and in immortalized $Nf1^{-/-}$ fetal liver (FL) cells (Donovan et al., 2002). This ability of hyperactive Ras to transform hematopoietic cells is highly conserved across evolution, as Ras mutants cause increased proliferation of *Drosophila* hemocytes (Asha et al., 2003). Nearly all JMML cases without *Ras* or *NF1* mutations have Shp2 mutations. As these mutations usually are mutually exclusive, they may perturb the same myeloid cell signaling pathway(s) (Loh et al., 2004; Tartaglia et al., 2003).

Shp2 is a nonreceptor PTP containing two SH2 domains (N-SH2, C-SH2), a PTP domain, and a C-tail with tyrosyl phosphorylation sites and a proline-rich motif. In RTK and cytokine signaling, Shp2 is required for full activation of the Ras/Erk pathway and for multiple receptor-evoked functions, including cell proliferation, differentiation, and migration (Neel et al., 2003). In RTK signaling, Shp2 controls recruitment of c-Src kinase (Csk) by dephosphorylating the Csk regulators Pag/Cbp and paxillin (Ren et al., 2004; Zhang et al., 2004). These data suggest that Shp2 is required to activate Src family kinases (SFKs), which then promote Ras activation. Others have argued that Shp2 regulates recruitment of p120Ras-GAP to RTKs (Agazie and Hayman, 2003; Klinghoffer and Kazlauskas, 1995) or the tyrosyl phosphorylation and activity of Sprouty proteins, a family of Ras inhibitors (Hanafusa et al., 2004). Shp2 also regulates other signaling pathways in a cell- and/or receptor-type-specific manner. Some studies indicate that Shp2 is required for Jak/Stat activation, whereas others suggest an inhibitory role (Van Vactor et al., 1998). Shp2 negatively regulates the PI3K/Akt pathway in EGFR and CSF-1R signaling, but is required for normal PDGF-and IGF-evoked Akt activation (Zhang et al., 2002; W. Yang and B.G.N., unpublished data).

All known actions of Shp2 require its PTP activity (Neel et al., 2003), which is tightly regulated (Barford and Neel, 1998). In the basal state, the "backside loop" of the N-SH2 domain is wedged into the PTP domain, preventing catalysis. Binding of a phosphotyrosyl (pTyr) peptide ligand alters the conformation of the N-SH2 domain, preventing its interaction with the PTP domain and activating the enzyme (Barford and Neel, 1998; Hof et al., 1998). Mutations of key contacts between the N-SH2 and PTP domains yield biochemically "activated" mutants of Shp2 that enhance growth factor action in vivo (O'Reilly et al., 1999). Phosphorylation of one or both of its C-terminal tyrosine residues also is required for Shp2 action in some, but not all, RTK signaling pathways (Araki et al., 2003).

Most human Shp2 mutations are found in the N-SH2 or PTP domains, affecting residues that participate in basal inhibition. Leukemia- and NS-associated mutations often affect the same residue, but typically, leukemia mutations are less conservative. For example, D61G and E76D are found in NS, whereas the two most common leukemia-associated mutants, D61Y and E76K, are nonconservative substitutions. Immune complex assays of transiently expressed Shp2 mutants (Tartaglia et al., 2003) or in vitro studies of recombinant proteins (H.K. and B.G.N., manuscript in preparation) show that the latter mutations are more enzymatically active than the former.

These data suggest that low levels of Shp2 activation result in NS, whereas higher levels of activity may be required for leukemogenesis (Loh et al., 2004; Tartaglia et al., 2003). Consistent with this notion, a knockin mouse model of the NS mutant D61G exhibits the major features of NS, and develops MPD (Araki et al., 2004). However, in contrast to the phenotypes of JMML and other Shp2 mutant-associated leukemias in humans (Arico et al., 1997; Tartaglia et al., 2003, 2004), or the murine MPDs caused by *K-Ras* activation (Braun et al., 2004; Chan et al., 2004) or homozygotic *Nf1* deletion (Bollag et al., 1996; Largaespada et al., 1996; Le et al., 2004), the MPD in D61G/+ mice is well tolerated, with no progression to fatal MPD or acute leukemia over ~10 months. Yet whether there are intrinsic differences in the biological properties of different disease-associated mutants of Shp2 is unclear, and the specific roles of Shp2 catalytic activity and its other domains in leukemogenesis remain unknown.

We used retroviral gene transduction to evaluate the biological effects, structure/function determinants, and signaling consequences of Shp2 mutants. We found that leukemia-associated Shp2 mutants are more potent transforming alleles than NS mutants, cause a fatal systemic MPD resembling JMML in mice, and thus are potential therapeutic targets for human malignancies associated with Shp2 mutations. Furthermore, the ability of Shp2 mutants to transform blood cells is conserved in *Drosophila*, providing potential genetic approaches to analyze Shp2-evoked transformation.

Results

Disease-associated Shp2 mutants cause myeloid transformation

Disease-associated mutations in Shp2 (Bentires-Alj et al., 2004; Loh et al., 2004; Tartaglia et al., 2001, 2002, 2003, 2004) fall into three general classes (Figure 1A). Some occur only in NS patients ("NS" mutants), others are seen only in patients with JMML or other neoplasms ("leukemia" mutants), and a third class is found either in those rare NS patients who develop leukemia or independently as germline mutations in NS patients and somatic mutations in sporadic neoplasms in unrelated individuals ("NS/leukemia" mutants). NS- and Shp2-associated neoplasms are uncommon, so it is not clear whether segregation of specific mutations into apparent pathogenic classes is coincidental or reflects intrinsic differences in their biological properties.

To address this issue, we first assessed the effects of the mutations D61Y and E76K, found only in JMML and other neoplasms, on hematopoietic cells. We introduced these mutants or wild-type (WT) Shp2 into the retroviral expression vector MSCV-IRES-EGFP (Hawley et al., 1994), and transduced factor-dependent Ba/F3 cells, but observed no consistent biological effects of WT Shp2 or either mutant (data not shown). In contrast, retroviral-mediated expression of E76K or D61Y, but not WT Shp2 or the parental retroviral vector in primary murine BM

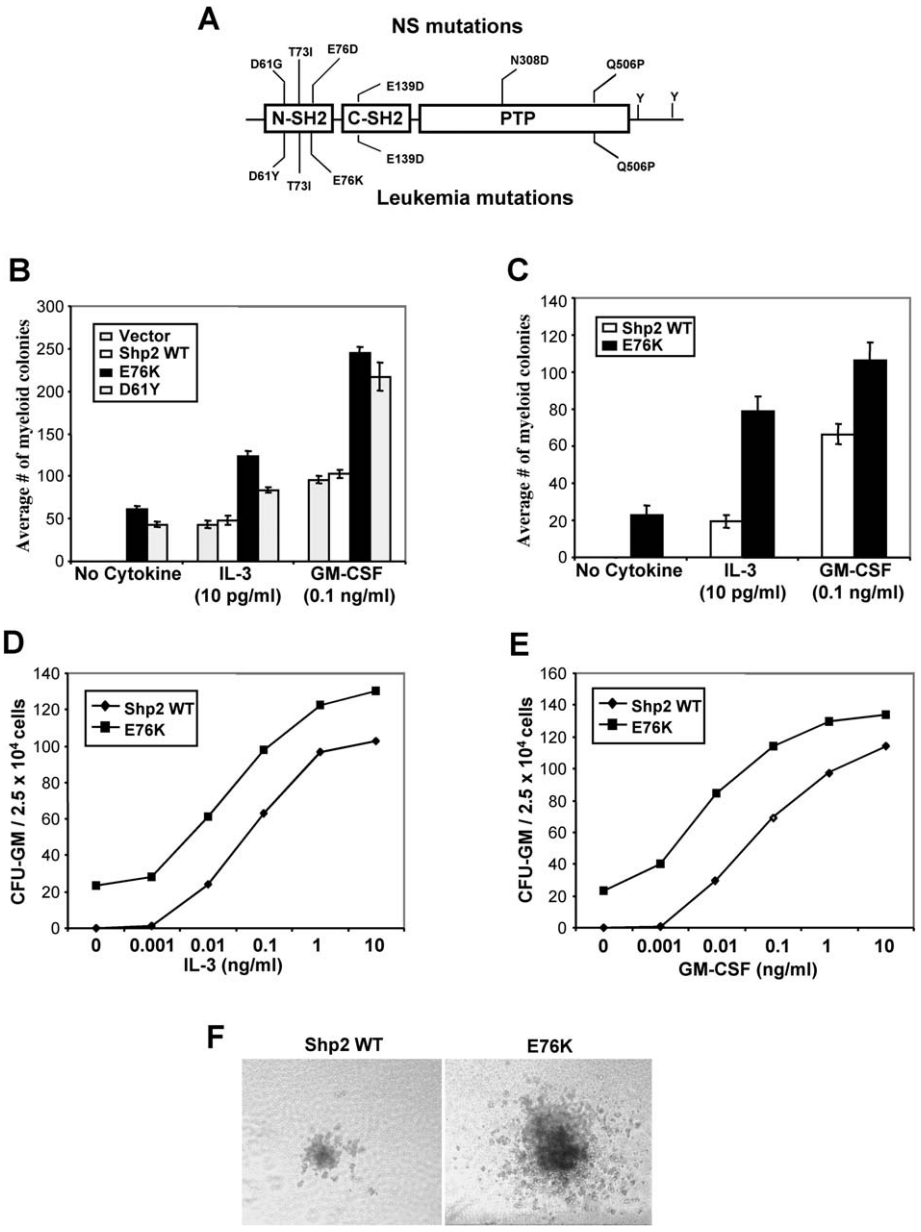


Figure 1. Leukemia-associated Shp2 mutants transform murine BM cells

A: Schematic of disease-associated Shp2 mutants. NS mutants are above the diagram; those in leukemia and other neoplasms are below. Some mutations are found in both types of disorder.

B: Normal murine BM infected with MSCV-IRES-GFP-based retroviruses expressing WT Shp2, E76K, or D61Y or parental control virus (vector) were plated in duplicate in methylcellulose media. The number of colonies was counted in the absence or presence of limiting murine IL-3 (10 pg/ml) or GM-CSF (0.1 ng/ml). Values are the average of three independent experiments; bars ± SD.

C: FL cells (E13.5) were infected with retroviruses expressing Shp2 WT or E76K, and myeloid colonies were quantified as in **B**. Values represent the average of two independent experiments; bars ± SD.

D and E: Myeloid colonies from Shp2 WT-or E76K-transduced BM cells at various concentrations of IL-3 (**D**) or GM-CSF (**E**).

F: Colony morphology produced by Shp2 WT- or E76K-transduced BM cells in the presence of GM-CSF (0.1 ng/ml).

(Figure 1B) or FL (Figure 1C), cells consistently evoked cytokine-independent myeloid colonies. Cells transduced with either mutant also formed more colonies with limiting doses of either interleukin-3 (IL-3) or GM-CSF (Figures 1B and 1C). E76K-transduced BM cells showed a leftward shift in dose-response to either cytokine, although GM-CSF sensitivity was reproducibly more enhanced (Figures 1D and 1E). Shp2 mutant-expressing myeloid colonies grown in the presence of cytokines were also larger than normal CFU-GM (Figure 1F). Normal CFU-GM, as well as the CFU-GM generated by E76K-transformed BM cells in the presence of cytokines, contained mature granulocytes and monocyte/macrophage cells. In contrast, factor-independent E76K-evoked colonies were primarily comprised of monocytic cells (data not shown).

Differential transforming potency of disease-associated Shp2 mutants

We next compared the ability of different classes of Shp2 mutants to transform murine BM cells. The leukemia-associated mutants E76K and D61Y gave rise to larger numbers of cytokine-independent myeloid colonies than the "pure" NS mutants D61G, N308D, and E76D, whereas NS/leukemia mutants (T73I, Q506P, E139D) evoked intermediate numbers of colonies (Figure 2A). Analysis of variance (ANOVA) showed highly significant differences between all groups (i.e., leukemia versus leukemia/NS, leukemia versus NS, NS/leukemia versus NS), but no significant difference among alleles in the same group (Figure 2A, legend). Flow cytometric analysis for EGFP expression confirmed comparable levels of retroviral transduction (Figure 2B).

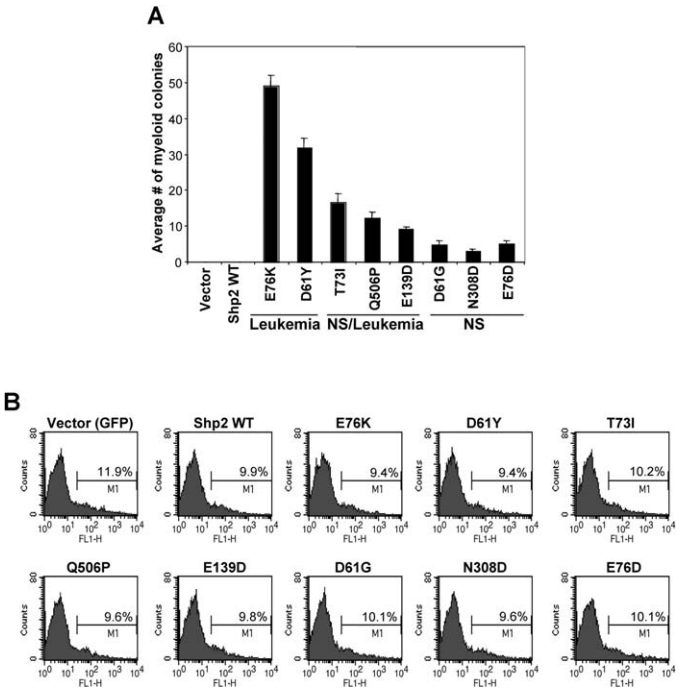


Figure 2. Differential transforming potency of disease-associated Shp2 mutants

A: Shp2 mutants found exclusively in leukemia or other neoplasms (E76K and D61Y), in NS and leukemia (T73I, Q506P, and E139D), and in NS alone (D61G, E76D, and N308D) were transduced into murine BM cells, and factor-independent myeloid colonies were quantified at day 12. Data represent mean colony number ± SD from three independent experiments. Differences in transforming potency within and between classes of Shp2 mutants were analyzed by two-way ANOVA. Leukemia-associated mutants are more transforming than NS ($p < 0.0001$) or NS/leukemia ($p < 0.005$). NS/leukemia mutants are more transforming than NS mutants ($p < 0.01$). Individual mutants within a given group showed no difference in transforming potency.

B: GFP expression in BM cells infected with the mutants in **A.** Note comparable level of infection with each retrovirus.

These data suggest that different classes of Shp2 mutants have distinct intrinsic myeloid-transforming ability.

Structural requirements for myeloid transformation by leukemogenic Shp2 mutants

To identify subdomains within leukemogenic Shp2 mutants required for transformation, we generated second site mutations in E76K (Figure 3A) and expressed these mutants in murine BM. Mutating the canonical arginine residue required for pTyr peptide binding (Pawson and Scott, 1997) in either the N- (E76K/R32K) or C- (E76K/R138K) SH2 domain of E76K ablated myeloid transforming ability (Figure 3B). Mutation of the conserved arginine residue within the E76K PTP domain "signature motif" (Denu et al., 1996) to methionine (E76K/R465M), which substantially reduces catalysis by Shp2 (data not shown) and other PTPs (Flint et al., 1997), also eliminated myeloid transformation (Figure 3B). The R465M mutation was chosen to avoid the potentially confounding effects (Tonks and Neel, 2001) of "substrate-trapping" mutants (e.g., C459S). Tyrosines 542 and 580 undergo phosphorylation in response to many, but not all, growth factors, and Shp2 is also tyrosyl phosphorylated in response to cytokines such as IL3 and GM-CSF in factor-dependent cell lines (Bennett et al., 1994; Li et al., 1994; Welham et al., 1994). Both sites can bind Grb2, and although Y542 is the major Grb2 binding site in RTK signaling (Araki et al., 2003; Bennett et al., 1994), both sites are required for optimal Shp2 function in some RTK pathways (Araki et al., 2003). Both Y542 and Y580 were phosphorylated in IL-3 stimulated BM cells (Figure 3C), but whereas Y542 mutation (E76K/Y542F) markedly impaired transformation, Y580 mutation (E76K/Y580F) had little effect. However, mutation of both tyrosines (E76K/Y542F/Y580F) eliminated E76K-evoked transformation, suggesting that Y580 phosphorylation accounts for residual transformation by E76K/Y542F (Figure 3D). Shp2 also has a proline-rich domain juxtaposed between the two C-terminal tyrosyl residues, and the recent identification of a NS mutation in this region (Sarkozy et al., 2003) suggests that it might have an important, if as yet undefined, role. Nevertheless, a proline-rich domain deletion in E76K (E76K/Δ 559–568) retained full transforming ability (Figure 3D). The efficiency of transduction with each mutant-expressing retrovirus, as assessed by flow cytometry for EGFP, was comparable to WT Shp2 virus (Figure 3E), and each mutant protein was expressed at comparable levels in hematopoietic cells (Figure 3F). Thus, the observed differences in colony formation reflect intrinsic alterations in transforming properties of the second site mutants.

Gab2 is required for transformation of primary murine myeloid cells by Shp2 E76K

E76K is fully activated catalytically, even in the absence of pTyr peptide binding (Tartaglia et al., 2003; H.K. and B.G.N., manuscript in preparation). The loss of transforming potential following mutation of the pTyr binding pocket of either the N- or C-SH2 domain in E76K implies that proper targeting of leukemogenic Shp2 mutants to one or more pTyr proteins is required for transformation. Previous studies identified the scaffolding adaptor Gab2 (Gu and Neel, 2003; Liu and Rohrschneider, 2002) as a major Shp2 binding protein in response to cytokine stimulation, and Gab2 binding is mediated by the Shp2 SH2 domains (Gu et al., 1997). Furthermore, Gab2 is essential for myeloid transformation (Sattler et al., 2002) and leukemogenesis (M.G.M., S. Li, R.A. Van Etten, and B.G.N., unpublished data) by BCR/ABL.

We compared the ability of E76K to transform primary myeloid cells from BM of WT (*Gab2*$^{+/+}$) and *Gab2*$^{-/-}$ mice. *Gab2*$^{-/-}$ mice have normal steady state hematopoiesis (Gu et al., 2001; Nishida et al., 2002), and their BM cells have normal susceptibility to retroviral infection (Sattler et al., 2002). As expected, E76K transformed *Gab2*$^{+/+}$ BM, resulting in cytokine-independent myeloid colonies. However, E76K was unable to transform BM cells from *Gab2*$^{-/-}$ mice (Figure 3E). Notably, WT and *Gab2*$^{-/-}$ BM cells were comparably susceptible to transformation by the Tel-Jak2 fusion protein (J. Frantsve, M.G.M., B.G.N., and D.G.G., unpublished data). Together with the effects of the SH2 domain mutants of E76K, these data suggest that E76K signals essential for myeloid transformation are transmitted by a Gab2/mutant Shp2 complex.

Leukemogenic Shp2 mutants cause fatal leukemias

To assess the leukemogenic potential of Shp2 mutants, we transduced WT Shp2, the leukemia-associated Shp2 mutants

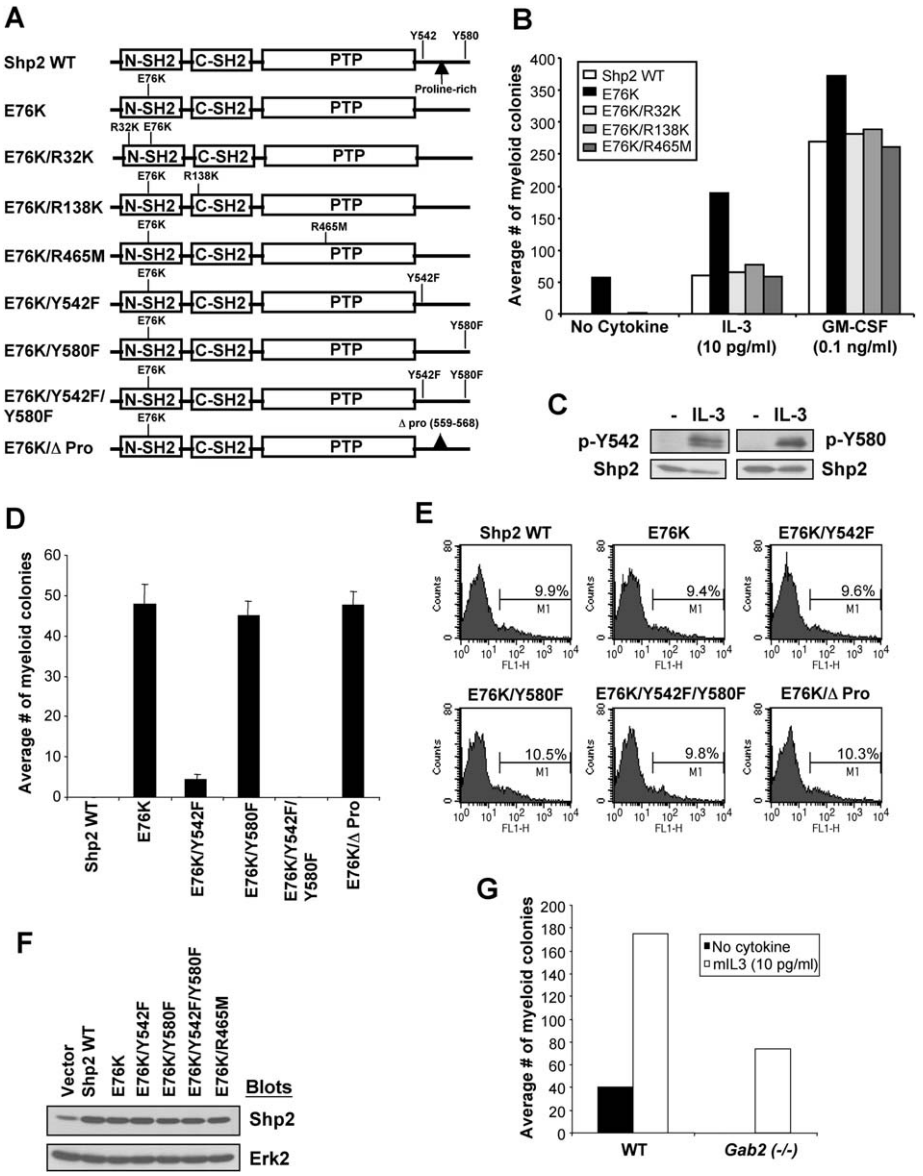


Figure 3. Structural determinants for myeloid transformation

A: Schematic of E76K and location of second site mutants.

B: SH2 and PTP domains are required for myeloid transformation. The indicated mutants in **A** were introduced into MSCV-IRES-GFP, and myeloid colony formation in the presence or absence of cytokines was analyzed. A representative experiment from three independent assays yielding comparable results is shown.

C: Shp2 C-terminal tyrosines are phosphorylated in cytokine-stimulated BM cells. Short-term BM cultures were factor-depleted for 5 hr, restimulated for 10 min with IL-3 (5 ng/ml), and analyzed by immunoblotting with phosphospecific antibodies to Shp2-Y542 or -Y580. Blots were reprobed with Shp2 antibodies to control for loading.

D: Role of C-terminal tyrosines and proline-rich domain in E76K-evoked transformation. Data represent mean colony number ± SD from two independent experiments.

E: Comparable GFP expression levels in BM cells infected with the mutants in **B** and **D**.

F: Shp2 mutants are expressed at comparable levels in hematopoietic cells. Ba/F3 cells were infected with viral supernatants used for experiments in **B** and **D**, sorted for GFP+, and analyzed by immunoblotting for Shp2.

G: Gab2 is required for myeloid transformation by E76K. BM cells from *Gab2*$^{-/-}$ and littermate WT mice were infected with E76K-expressing retroviruses, and myeloid colony formation was assessed. A representative experiment from two independent assays that yielded comparable results is shown.

(E76K or D61Y), or parental retrovirus into BM from Balb/c mice primed with 5-fluorouracil (5-FU), and performed bone marrow transplants (BMT) into lethally irradiated syngeneic recipients. Neither parental retrovirus- (data not shown) nor WT Shp2-infected BM (Figure 4) caused any abnormalities. In contrast, mice transplanted with E76K- or D61Y-transduced BM developed leukocytosis within 3 months (Figure 4A), due both to neutrophilia (Figure 4B) and usually mild, but occasionally marked, monocytosis (Figure 4C). The leukocytosis was progressive, with some mice exhibiting white blood cell counts (WBC) of 40-70,000 per μl within six months, although there was considerable variation in disease progression among individual BMT recipients (Figures 4A–4C). Some mice also exhibited lymphocytosis at 3 and/or 6 months. RBC and platelet counts typically remained within the normal range, but mice with higher WBC eventually developed anemia and/or thrombocytopenia (data not shown). BM recovered from E76K- or D61Y-transplanted mice exhibited increased ability to generate cytokine-independent colonies, and, like directly transduced BM cells, IL-3 or GM-CSF hypersensitivity (Figure 4D). Beginning at 6–7 months, a significant percentage of D61Y- or E76K-transplanted mice (~50%) appeared moribund (requiring sacrifice) or were found dead. Transplanted mice continued to die over the succeeding 12–18 months, with >90% eventually succumbing. There were no deaths among recipients of WT Shp2- or parental retrovirus-transduced BM during this time (Figure 4E).

Histology revealed two types of neoplastic pathology caused by D61Y and E76K. Most mice (~60%) had severe MPD (Figure 5A). Blood smears showed substantially increased neutrophils, a smaller proportionate increase in monocytes, and in some cases, occasional (<10%) blasts (Figure 5A, top left panel and data not shown). The BM showed a marked myeloid hyperplasia, predominantly consisting of granulocytic forms at various stages of differentiation (Figure 5A, upper middle panel).

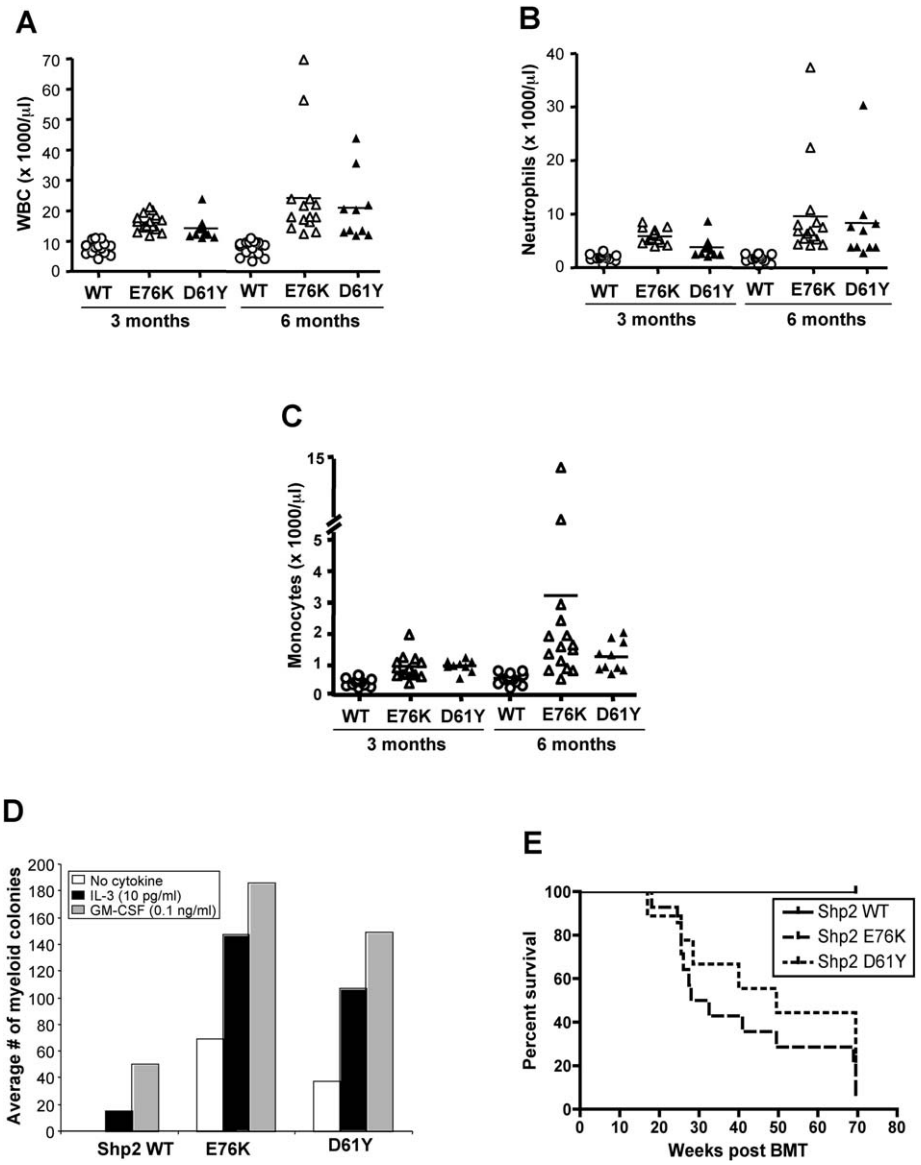


Figure 4. Shp2-transduced BM causes fatal hematopoietic disease upon BMT

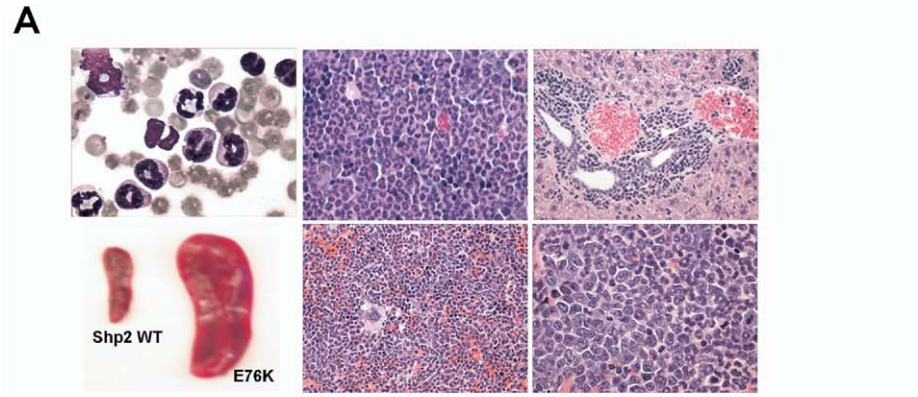
A–C: WBC (**A**), neutrophil (**B**), and monocyte (**C**) counts from mice transplanted with BM transduced with the indicated retroviruses and analyzed at 3 or 6 months.

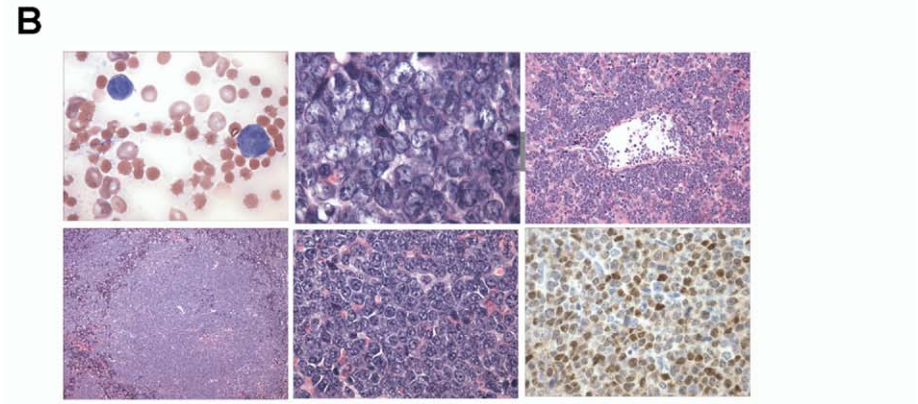
D: Increased factor-independent colony formation in BM from transplanted mice. BM was recovered from the indicated transplanted mice at 4–5 months, and myeloid colony assays performed. Shown is a representative experiment from four independent assays with similar results.

E: Kaplan-Meier curves of mice transplanted with D61Y- (n = 10) or E76K- (n = 15) transduced BM. Survival of Shp2 mutant-transduced recipients was substantially diminished compared to Shp2 WT (n = 15) ($p < 0.0001$). Although there was a trend toward decreased survival of E76K compared with D61Y, this did not reach statistical significance ($p < 0.255$).

There was marked splenomegaly with extensive infiltration of maturing myeloid cells similar to that seen in the BM (Figure 5A, lower panels), as well as perivascular infiltration of neutrophils in the liver (Figure 5A, right upper panel). This fatal, tissue-infiltrating MPD contrasted with the chronic MPD found in Shp2 D61G/+ mice (Araki et al., 2004). Most remaining mice developed lymphoblastic leukemia/lymphoma (~20%) or a mixture of lymphoblastic leukemia/lymphoma and MPD (~20%). Mice with lymphoproliferative disease had enlarged lymph nodes and thymuses (data not shown), with lymphoblasts in their peripheral blood (Figure 5B, top left panel) that also infiltrated the BM (Figure 5B, upper middle panel), splenic white pulp (Figure 5B, lower panels), and liver (Figure 5B, upper right panel).

Flow cytometry of BM and spleen cells showed marked expansion of donor-derived cells, as indicated by EGFP expression, in E76K- and D61Y-transplanted mice (Figures 6A and 6C). Thus, E76K or D61Y-transduced BM cells had a survival and/or proliferation advantage in vivo, as in ex vivo colony assays (Figures 1 and 2). Mice with histological MPD (as defined in Figure 5A) had increased numbers of immature myelomonocytic cells (Mac-1^{hi}/Gr-1^{lo}) in BM and spleen (Figures 6B and 6C). BM progenitor analysis showed expansion of the IL-7Rα-Lin-Sca-1-c-Kit+CD34+FcγR+ population that corresponds to the CMP/GMP (common myeloid progenitor/granulocyte macrophage progenitor) populations (Akashi et al., 2000). In contrast, mice with pure lymphoproliferative disease had no increase in myeloid cells (Figure 6D). Instead, CD4–/CD8+ cells (and in one case, CD4+/CD8+ cells; data not shown) were increased markedly in BM, spleen, and lymph nodes of these mice (Figure 6D). The CD4–/CD8+ cells were immature T lymphoid precursors with downregulated CD4 expression, as they expressed terminal deoxyribonucleotidyl transferase (TdT) uniformly (Figure 5B; lower right panel). Therefore, the Shp2-evoked lymphoproliferative disease is best characterized as T acute lymphoblastic leukemia (T-ALL)/lymphoma.





C

Larval Genotype	# Larvae examined	Plasmatocyte Concentration (x 10^6 cells/ml)
CgGAL4/+	8	4.1 ± 1.2
UAS-E76K/+	7	1.9 ± 0.8
CgGAL4/UAS-E76K	8	26.3 ± 8.3

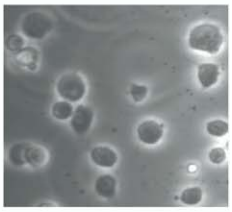


Figure 5. Shp2 mutants cause leukemia in mice and flies

A: Shp2-evoked MPD. Top row, left panel: Blood smear (1,000×) from mouse with Shp2-evoked MPD showing marked neutrophilia. Top row, middle panel: BM section (400×) showing prominent hypercellularity with mature myeloid (granulocytic) predominance. Top row, right panel: Liver section (200×) showing periportal vascular invasion of myeloid cells. Bottom row, left panel: massive splenomegaly in E76K-transplanted mice. Bottom row, middle and right panels: Spleen section at 200× (middle) and 400× (right) showing infiltration of spleen with myeloid elements.

B: Shp2 mutants also cause lymphoproliferation. Top row, left panel: Blood smear (1,000×) showing increased lymphocytes and occasional lymphoblasts. Top row, middle panel: BM section (1,000×) showing hypercellularity with large number of lymphoblasts. Top row, right panel: Liver section (100×) showing periportal invasion with immature lymphoid cells. Bottom row: Spleen section at 40× (left) and 400× (middle) showing massive expansion of white pulp with large numbers of blasts. Right panel shows spleen section (400×) demonstrating effacement of architecture with abnormal lymphoid elements and TdT immunoreactivity.

C: E76K causes MPD in *D. melanogaster*. A strain expressing Shp2 E76K under the control of the GAL4 UAS (UAS-E76K/+) was crossed to a driver strain (GcGAL4) that expresses GAL4 in larval, and plasmatocyte concentrations (mean ± SEM) were determined. Right panel: E76K-evoked plasmatocytes have normal morphology but vary in size. CgGAL4/ UAS-E76K larvae were bled into a drop of halocarbon oil, and examined under a phase contrast microscope. The size variation suggests expansion of a more immature population.

Following adoptive transfer to sublethally irradiated recipients, spleen cells from mice with E76K- or D61Y-evoked MPD (n = 4 each) did not transfer disease over a nine-month period. In contrast, T-ALL/lymphoma was readily transplantable, with all recipients (n = 8) dying of a similar disease within one month. Southern blot analysis of proviral integration sites from mice with E76K- or D61Y-evoked MPD or T lymphoid leukemia indicated that both disorders were oligoclonal, with 3–7 integration sites per sample (data not shown). Oligoclonality, and the significant lag before the appearance of frank disease, suggests that additional events may collaborate with Shp2 mutations to cause MPD/leukemia.

Leukemogenic Shp2 mutations cause MPD in *Drosophila*

Activating Ras mutations cause a model MPD in *D. melanogaster* (Asha et al., 2003), and flies express a highly conserved Shp2 ortholog (Freeman et al., 1992; Perkins et al., 1992). Therefore, we assessed the effects of E76K expression in hemocytes using the GAL4 system (Brand and Perrimon, 1993). UAS-E76K stable transgenic strains, in which E76K expression is controlled by yeast GAL4 activation sequences, were mated to a CgGAL4 transgenic strain (Asha et al., 2003), which strongly expresses the GAL4 transcription factor in larval plasmatocytes and fat body tissues. The UAS-E76K strain or the CgGAL4 strain alone, crossed to WT Oregon R flies, served as

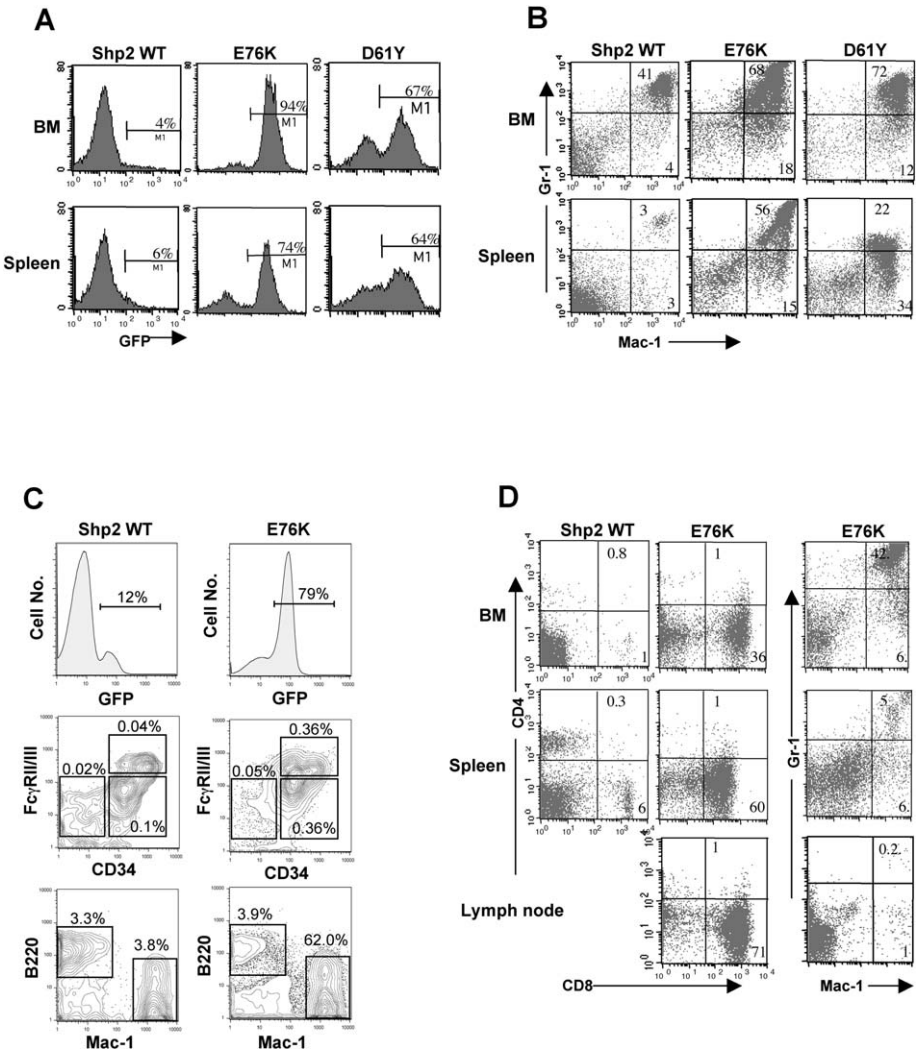


Figure 6. Flow cytometric analysis of Shp2-evoked leukemias

A–C: Shp2-evoked MPD. **A:** Marked expansion of GFP+ (virally infected donor cell) population in E76K- and D61Y-transplanted recipients. **B:** Expanded population of immature myeloid cells in recipients of E76K- and D61Y-transduced BM. **C:** Myeloid progenitor analysis of BM of transplanted mice. Note expansion of IL-7Rα-Lin-Sca-1-c-Kit+CD34+FcγR+ population corresponding to CMP/GMP in E76K-evoked MPD (middle panel). Expanded GFP+ and Mac1+ population also is evident, whereas B220+ cells are unaffected.

D: Subset of recipients develop T-ALL/lymphoma. Cells from the indicated tissues were analyzed for T cell (CD4, CD8) and myeloid (Mac-1, Gr-1) markers. Note expansion of a CD4−/CD8+ population in all tissues.

controls. E76K expression caused an ~6-fold increase in the number of plasmatocytes, which are myeloid-like cells that comprise ~95% of circulating hemocytes in normal larvae (Dearolf, 1998; Meister, 2004). Mutant plasmatocytes had normal morphology but varied in size, suggesting that many of the cells are immature (Figure 5C). Overall, the effects of E76K overexpression were qualitatively similar to the effects of activated Ras or Raf mutants. However, in previous experiments using the same driver strain, Ras or Raf gain-of-function mutants caused accumulation of circulating larval blood cells to ~200 × 10^6 cells/ml (Asha et al., 2003). Neither E76K nor activated Ras increased the numbers of differentiated lamellocytes, another form of larval blood cell, in contrast to the effects of dominant forms of the Janus kinase (Jak) ortholog Hopscotch (Harrison et al., 1995; Luo et al., 1995).

Leukemia-associated mutants of Shp2 activate multiple signaling pathways

The Ras/Erk and PI3K/Akt pathways are hyperactive in immortalized *Nf1*$^{-/-}$ FL cells (Donovan et al., 2002) and *Nf1*$^{+/-}$ mast cells (Ingram et al., 2001). To assess the effects of leukemogenic Shp2 on cytokine signaling, BM from mice transduced with WT Shp2, D61Y, or E76K was cultured in IL-3. Under these conditions, BM normally gives rise to mast cells (BMMC), whose proliferation slows by 5–6 weeks and eventually ceases. In contrast, D61Y- or E76K-BMMC remained highly proliferative at 6 weeks (Figure 7A), although still dependent on IL-3 for survival and proliferation. Such cultures also immortalized spontaneously (without crisis) to generate IL-3 dependent lines with mast cell characteristics, as demonstrated by uniform staining with toluidine blue and strong degranulation responses to IgE receptor stimulation (data not shown).

We compared the response to IL-3 stimulation of BMMC (at 6 weeks, before immortalization) expressing WT Shp2 and leukemogenic mutants. Marked differences were observed in tyrosyl phosphoproteins from basal (factor-starved) and IL-3-stimulated WT and mutant-expressing BMMC. Several proteins exhibited reproducibly decreased tyrosyl phosphorylation, suggesting that they might be substrates of the activated mutants (Figure 7B, open arrowheads). Others showed increased tyrosyl phosphorylation (Figure 7B, closed arrowheads), consistent with activation of one or more PTKs by leu-

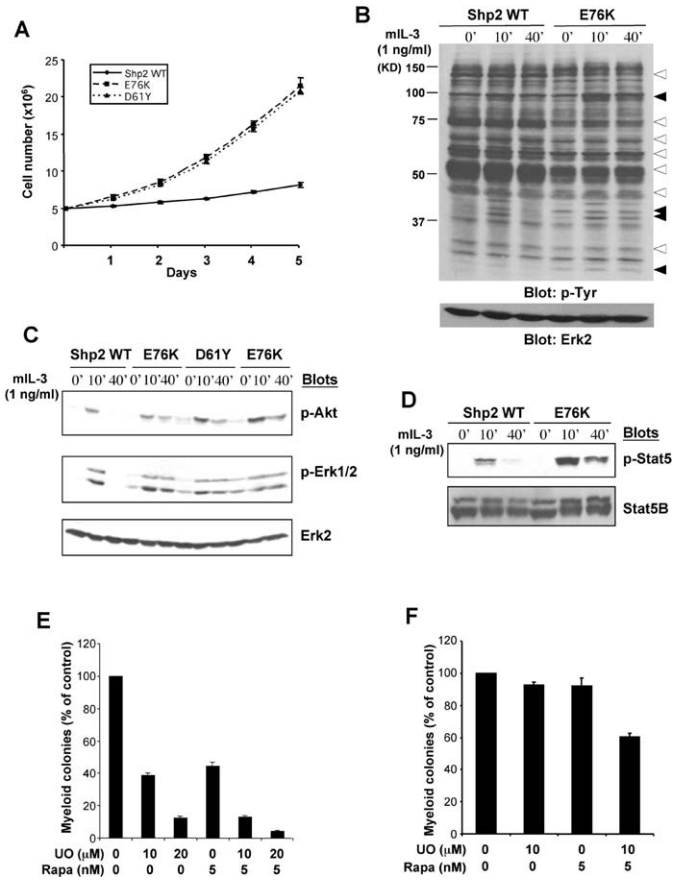


Figure 7. Effects of leukemogenic Shp2 mutations on BMMC

A: Proliferation of BMMC (six weeks) derived from recipients of BM transduced with WT Shp2 or the indicated mutants in response to IL-3 (5 ng/ml).

B–D: BMMC were starved, stimulated with IL-3 (1 ng/ml) for the indicated times, and total cellular phosphotyrosine, and Erk, Akt, and Stat-5 activity was assessed by immunoblotting. Specific decreases (open arrowheads) in several phosphotyrosyl proteins, some of which may be direct substrates, and increased tyrosyl phosphorylation of others (closed arrowheads) are seen in cells expressing E76K (**B**). Note increased basal and stimulated activity of Erk and Akt **(C),** and increased Stat5 activation **(D)** caused by leukemogenic Shp2 mutants.

E: Effects of the Mek inhibitor U0126 (U0) and/or the Tor inhibitor rapamycin (Rapa) on E76K-evoked myeloid colony formation.

F: Effects of U0126 and/or rapamycin on colony formation by WT BM cells in the presence of IL-3 (5 ng/ml).

kemogenic mutants. Compared to WT BMMC, basal Erk and Akt activity, as monitored by phosphospecific antibody immunoblotting, was increased in BMMC-expressing leukemogenic Shp2 mutants, and their activation in response to IL-3 stimulation was sustained (Figure 7C). Stat5 activation also was increased and sustained in leukemogenic mutant-transformed BMMC, consistent with increased activity of at least one PTK in these cells (Figure 7D). Thus, leukemia-associated Shp2 mutants activate multiple downstream signaling pathways known to be important for transformation.

Current treatments for hematopoietic diseases caused by Shp2 mutations are often ineffective. Given the effects of leukemogenic mutants on cytokine signaling (Figures 7B–7D), we tested the effects of inhibiting Erk activation by using the Mek inhibitor UO126, the mTOR arm of the PI3K/Akt pathway, using the mTOR inhibitor rapamycin, or both pathways on E76K-evoked myeloid transformation (Figure 7E). Mek inhibition alone (at 20 μM) caused ~85% inhibition of colony formation. Rapamycin at the lower end of the therapeutic range for immunosuppression (5 nM) also inhibited transformation. Coadministration of these two drugs resulted in superadditive inhibition. The doses used resulted in inhibition of their target signaling pathways (p-Erk, p-S6) in E76K-expressing cells (data not shown). Furthermore, consistent with previous studies (Mohi et al., 2004), these drugs alone or in combination have little effect on normal BM cells (Figure 7F). Because our structure-function analyses showed that Shp2 catalytic activity was required for transformation by leukemogenic Shp2 mutants, we also tested the nonspecific PTP inhibitor sodium orthovanadate. At concentrations corresponding to ~one-tenth the serum vanadium levels achieved in a clinical trial of sodium metavanadate as an anti-diabetogenic agent (Goldfine et al., 1995), sodium orthovanadate completely prevented myeloid colony formation by E76K. However, this effect was not specific for Shp2 mutant-transformed cells, as normal myeloid colony formation was also inhibited at these doses (data not shown).

Discussion

We addressed several questions about the Shp2 mutations found in NS and in some types of myeloid and lymphoid leukemias: (1) what are the effects of leukemia-associated Shp2 mutations on primary hematopoietic cells ex vivo and in vivo; (2) do NS- and leukemia-associated mutants have intrinsic differences in bioactivity; (3) what domains within the Shp2 molecule contribute to leukemogenesis by Shp2 mutants; and (4) how do leukemogenic mutants perturb hematopoietic cell signaling? We find that leukemia-associated mutant(s) transform myeloid progenitors to factor independence and increase their cytokine sensitivity. Transformation requires Shp2 catalytic activity, intact C-terminal tyrosines, and the ability of both SH2 domains to bind pTyr peptides. The latter probably reflects a need for mutant Shp2 proteins to bind Gab2, which is itself required for myeloid transformation by some PTK-fusion oncogenes (Sattler et al., 2002). Leukemia-associated Shp2 mutant-transformed BM causes a fatal, tissue-infiltrating MPD that resembles human JMML and the MPD evoked by activated *Ras* or *Nf1* deficiency, and/or T-ALL/lymphoma. Leukemia-associated mutants aberrantly activate multiple hematopoietic signaling cascades, including the Ras/Erk, PI3K/Akt, and Stat5 pathways, and blocking two of these pathways abrogates myeloid transformation ex vivo. Our results thereby establish a causal role for Shp2 mutations in leukemogenesis and suggest new therapeutic approaches to these disorders.

Shp2 mutants evoke cytokine independence and increase cytokine-evoked colony size (Figure 1). Moreover, like the factor-independent colonies from human JMML BM (Emanuel et al., 1996), Shp2 mutant colonies (in the absence of cytokines) are predominantly monocytic (data not shown). Thus, leukemogenic Shp2 mutants promote myeloid cell survival (factor-independence) and proliferation (colony size), and preferentially enhance monocytoid differentiation. In contrast to a previous study (Loh et al., 2004), we failed to observe effects of D61Y or E76K on factor-dependent cell lines (e.g., Ba/F3). Instead, our data suggest that Shp2 mutants exhibit cell type-

selective transforming ability, reminiscent of the tissue-specific effects of the NS mutation D61G, in knockin mice. Although the molecular basis for cell selectivity remains unknown, biological efficacy of the D61G allele in mice correlates with its ability to promote Erk hyperactivation in specific tissues (Araki et al., 2004). Notably, Erk activity is unaffected in Ba/F3 cells expressing leukemogenic Shp2 mutants (Loh et al., 2004; M.G.M. and B.G.N., unpublished data), but increased in mutant Shp2-expressing BMMC (Figure 7C).

Ras, *NF1*, and Shp2 mutations are almost always mutually exclusive in leukemia (Loh et al., 2004; Tartaglia et al., 2003), suggesting that they affect a common pathway. Yet although the effects of leukemogenic Shp2 mutants are similar to those of *Ras* and *Nf1* mutations, there are potentially important differences in murine models. *NF1* deficiency reportedly results in selective GM-CSF hypersensitivity (Bollag et al., 1996; Largaespada et al., 1996; Le et al., 2004), although others observed more pleiotropic effects on cytokine/growth factor responsiveness (Zhang et al., 1998). In contrast, leukemogenic Shp2 mutants (Figure 1) and activated K-Ras (Braun et al., 2004) cause increased sensitivity to both IL-3 and GM-CSF. Human and murine myeloid progenitors may have some fundamental differences in their response to Shp2, *Ras*, and *NF1* mutations, as studies of large numbers of JMML cases (which likely included examples of each type of mutation) showed selective GM-CSF hypersensitivity in all (Arico et al., 1997; Emanuel et al., 1996). However, the murine MPD caused by *Nf1* deficiency (Le et al., 2004) appears less severe than that caused by activated K-Ras (Braun et al., 2004), raising the possibility that JMML caused by activating *Ras* (and possibly, Shp2) mutations may be more aggressive than MPD associated with *NF1* deficiency.

Approximately 25% of NS patients develop evidence of mild MPD (Bader-Meunier et al., 1997; Choong et al., 1999; Side and Shannon, 1997), the overwhelming majority of which resolve. Nevertheless, for unclear reasons, a very small number of such patients develop JMML and/or other Shp2 mutant-associated leukemias. Our data show that Shp2 mutations have intrinsic differences in leukmogenicity (Figure 2). Using transformation to factor independence as an assay, it may be possible to prospectively classify the relative leukemogenic risk of a given Shp2 mutation in NS patients and potentially intervene before overt leukemia develops.

Transplantation of E76K- or D61Y-transduced BM results in an oligoclonal MPD (Figure 4E). Oligoclonality and the fairly long latency of this disorder suggest that additional events besides Shp2 mutation may be required to cause tissue-invasive MPD. There also is a lag before MPD develops in mice with induced expression of mutant *K-Ras* (Braun et al., 2004; Chan et al., 2004) or *Nf1* deletion (Le et al., 2004). One attractive possibility is that a second event results in autocrine production of a cytokine that signals through the IL-3 receptor; indeed, transformed $Nf1^{-/-}$ FL cells produce GM-CSF (Donovan et al., 2002). Alternatively, latency and oligoclonality could also be explained if the target cell for Shp2-evoked MPD is rare. Although a differentiated progenitor population (CMP/GMP) is expanded in Shp2 mutant-evoked MPD (Figure 6C), identification of the cell type transformed by Shp2 will require prospective purification and infection of HSC and/or progenitors with leukemogenic Shp2 mutants.

The leukemia-associated mutant E76K (and D61Y; data not shown), like activated Ras (Asha et al., 2003), evokes MPD in *Drosophila* (Figure 5C). These data indicate that *Ras* and Shp2 mutants engage a highly conserved signaling mechanism(s) to transform hematopoietic cells. But whereas the effects of Shp2 and *Ras* mutants on fly hemocytes are qualitatively similar, Ras evoke more (20- to 50-fold) hemocyte proliferation. Although we cannot exclude the possibility that E76K functions less effectively in fly hemocytes than in mammalian myeloid progenitors, these findings may provide further evidence of important differences in leukemogenesis by Ras and Shp2 mutations. Hyperactivation of the Jak-Stat pathway also leads to hematopoietic abnormalities in flies (Harrison et al., 1995; Luo et al., 1995). However, hyperactive Hop Jak kinase activity enhances the encapsulation response of blood cells, leading to a large increase in lamellocytes. The lack of a lamellocyte response in E76K-expressing flies suggests that E76K does not act by globally increasing Jak signaling.

Some mice transduced with Shp2 mutants develop T-ALL/lymphoma. A recent study did not identify Shp2 mutations in small numbers of human T-ALL patients (Tartaglia et al., 2004), but a larger survey may be warranted. We also failed to observe the other hematopoietic disorders (AML, B-ALL) associated with human Shp2 mutations. Most likely, as suggested previously (Gilliland and Tallman, 2002), Shp2 mutants must cooperate with other mutations to cause B-ALL or AML. Translocations involving the homeobox gene *MLL* are common in AML associated with Shp2 mutations (Tartaglia et al., 2004), so it will be interesting to see the effects of leukemia-associated Shp2 mutants in murine models of abnormal MLL function (Armstrong et al., 2003).

Our results provide insight into how leukemogenic Shp2 mutants transform myeloid cells. SH2 domain function is required (Figure 3B), most likely to direct binding to Gab2 (Figure 3G). Gab2 also is required for myeloid transformation by BCR/ABL (Sattler et al., 2002), and resides within a fairly common amplicon in AML (Zatkova et al., 2004), suggesting a potential role for Gab/Shp2 signaling in AML pathogenesesis. Because PTP activity also is essential (Figure 3B), leukemogenic Shp2 presumably must dephosphorylate one or more key targets to effect transformation. Candidates include proposed substrates of normal Shp2 (see Introduction) and/or the multiple hypophosphorylated pTyr-proteins in BMMC expressing leukemia-associated mutants (Figure 7B). Y542 also is required for optimal transformation by leukemogenic Shp2, whereas Y580 has a minor role (Figure 3D). Although both Y542 and Y580 bind Grb2, and have been suggested to promote Ras activation via recruitment of the guanine nucleotide exchange protein Sos (Bennett et al., 1994; Li et al., 1994), Y580 can enhance Shp2 action without any effect on Grb2 binding (Araki et al., 2003). Another suggested function for Shp2 tyrosine phosphorylation is stimulation of Shp2 catalytic activity (Lu et al., 2001). However, E76K is fully activated in the absence of any pTyr peptide, so this model cannot explain the requirement for the C-terminal tyrosines in Shp2-evoked transformation. If the proline-rich domain has a function in transformation, it most likely is inhibitory, as E76KΔPro had unperturbed transforming ability (Figure 3D).

Similar to the effects of *Nf1* deficiency (Donovan et al., 2002; Ingram et al., 2001; Le et al., 2004), leukemogenic Shp2 increases BMMC proliferation (Figure 7A) and enhances Erk and Akt activation (Figure 7C). The latter probably reflects increased Ras activation, as Ras activates Erk via the Ras/Raf/

Mek pathway (Howe et al., 1992; Leevers and Marshall, 1992), and binds and stimulates the PI3K p110 catalytic subunit (Khwaja et al., 1997). Shp2 mutant-expressing BMMC also have increased Stat5 phosphorylation (Figure 7D), most likely due to increased Jak2 or SFK activity. We favor the latter, given our finding that Shp2 promotes SFK activation in RTK signaling (Zhang et al., 2004) and the effects of Shp2 mutants in *Drosophila* (see above). Indeed, preliminary data show an increased phosphorylation of Src Tyr416 in E76K-expressing BMMC (M.G.M. and B.G.N., unpublished data).

These signaling abnormalities suggest new therapeutic approaches. Mek inhibitors, as well as inhibitors of the upstream kinase Raf, are in clinical trials (Sebolt-Leopold, 2000; Wilhelm et al., 2004); our data (Figure 7E) suggest that they may be efficacious against mutant Shp2-transformed cells. Rapamycin inhibits one arm of the Akt pathway, and is FDA-approved for immunosuppresion (Bjornsti and Houghton, 2004). Rapamycin doses well within the typical therapeutic serum levels achieved in patients (MacDonald et al., 2000) also inhibited Shp2-evoked myeloid transformation, and combining Mek and Tor inhibition was even more potent (Figure 7E). Given the often fatal outcome of JMML and other Shp2-associated neoplasms, clinical trials of Mek and/or Tor inhibitors should be considered, at least until selective Shp2 inhibitors are developed. Our murine model of Shp2-evoked MPD may provide a useful system to test these and other potential therapeutic approaches.

Experimental procedures

Retroviral transduction and BM colony-forming assays

High-titer retroviral stocks of WT or mutant Shp2 were prepared by transient transfection of 293T cells (Million and Van Etten, 2000). BM transduction and colony assays were performed using MethoCult M3234 medium (Stem Cell Technologies, Vancouver) in the presence or absence of cytokines, as described (Sattler et al., 2002). In some experiments, E13.5 FL cells were transduced with Shp2-expressing retroviruses and assayed for colony formation.

BMT, histopathology and immunocytochemistry

BM cells (10^6) from 5-fluorouracil (5FU)-primed Balb/c mice transduced with Shp2 WT- or E76K- or D61Y-expressing retroviruses were injected into tail veins of lethally irradiated (2 × 450 cGy) female recipients, as described (Schwaller et al., 1998). The method of Kaplan and Meier was used to generate survival curves, and significance was evaluated by log rank test.

Murine organs were collected and fixed in 10% neutral buffered formalin for at least 72 hr and embedded in paraffin. Tissue sections (4 μm) were deparaffinized in xylene, rehydrated, and stained with hematoxylin and eosin (H&E). For TdT immunostaining, slides were pretreated with Peroxidase Block (DAKO, Carpinteria, CA) for 5 min, followed by blocking of nonspecific binding sites with goat serum, incubation with anti-TdT antibodies (DAKO, Carpinteria, CA), and detection with anti-rabbit horseradish peroxidase-conjugated antibodies (Envision plus, DAKO). Immunoperoxidase stains were developed using a DAB chromogen (DAKO), and counterstained with Harris hematoxylin.

Flow cytometry

Single cell suspensions from BM, spleen, and LN were prepared as described previously (Schwaller et al., 1998), and washed in PBS containing 0.1% NaN_3 and 0.1% BSA. Fc receptor-mediated binding was blocked by preincubation with 2.4G2 hybridoma supernatants (anti-CD16/CD32; ATCC, Rockville, MD). Aliquots (0.5–1.0 × 10^6 cells) were stained with monoclonal antibodies specific for Mac-1 (CD11b), Gr-1, CD4, CD8α, Thy1.2, or B220 (CD45R), and conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or biotin. All antibodies were from Pharmingen (San Diego, CA). Flow cytometry was performed with a FACSCalibur (Becton-Dickinson, Mountain View, CA) and analyzed with CELLQUEST software. HSC/progenitor analysis was performed as described (Akashi et al., 2000) to quantify IL-7Rα-Lin-Sca-1-c-Kit+CD34+FcγRII/III^{lo} common myeloid progenitors (CMP), IL-7Rα-Lin-Sca-1-c-Kit+CD34+FcγRII/III^{hi} granulocyte-monocyte progenitors (GMP), and IL-7Rα-Lin-Sca-1-c-Kit+CD34-FcγRII/III^{lo} megakaryocyte-erythrocyte progenitors (MEP).

Generation of transgenic flies

E76K cDNA, cloned into the *Drosophila* P element transformation vector pUAST, was injected (Duke University Model System Genomics Facility), and two independent transgenic strains (identified by eye color) were recovered. Both were homozygous viable and fertile, and behaved comparably. Flies were maintained at 18°C on standard cornmeal/agar/molasses/yeast/Tegosept medium. Crosses were carried out at 25°C. Circulating plasmatocyte concentrations were measured as described (Zinyk et al., 1993).

BMMC and short-term BM assays

BMMC were generated as described previously (Gu et al., 2001). After ~5 weeks, a homogeneous population of nonadherent cells was apparent. Cell aliquots were stained with Wright-Giemsa and toluidene blue to confirm their mast cell phenotype, and used for assays. For proliferation assays, 5 × 10^6 cells were seeded into 10 cm dishes and viable cells were quantified over 5 days. For biochemical analyses, BMMC were deprived of serum and growth factors for 5 hr, stimulated with mIL-3 (1 ng/ml) for the indicated times, washed 2× with PBS, and lysed in boiling SDS-PAGE sample buffer. Immunoblotting was performed using phosphospecific antibodies against p44/42 Erk, Akt Ser 473, or Stat5-Tyr 694 (Cell Signaling Technology, Beverly, MA). Detection was by enhanced chemiluminescence (ECL). To control for equal loading, blots were reprobed with antibodies against total Erk2 (Santa Cruz Biotechnology).

WT BM cells were cultured for 10 days in IL-3-containing IMDM medium, factor-depleted for 5 hr, and restimulated for 10 min with IL-3 (5 ng/ml). Cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific antibodies to Shp2-Tyr542 or -Tyr580 (Cell Signaling Technology, Beverly, MA).

Statistical analysis

Paired data were evaluated by Student's t test. Simultaneous comparisons between multiple groups were performed using two-way ANOVA, using commercially available software (GraphPad Software Inc., San Diego, CA).

Acknowledgments

This work was supported by NIH grants R01 DK50693 (B.G.N.), P01 CA66996 (D.G.G.), and DK50654 (D.G.G.), and grants 6207-04 (B.G.N.) and 7059 (D.G.G.) from the Leukemia and Lymphoma Society. Histopathology services were supported by NIH grant P01 DK50654 to J.L.K. D.G.G. is an investigator of the HHMI. M.G.M was supported by a Hood Postdoctoral Fellowship from the Medical Foundation. C.R.D. was supported in part by NIH R24 RR15061.

Received: November 18, 2004
Revised: January 16, 2005
Accepted: January 19, 2005
Published: February 14, 2005

References

Agazie, Y.M., and Hayman, M.J. (2003). Molecular mechanism for a role of SHP2 in epidermal growth factor receptor signaling. Mol. Cell. Biol. *23*, 7875–7886.

Akashi, K., Traver, D., Miyamoto, T., and Weissmann, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *404*, 193–197.

Araki, T., Nawa, H., and Neel, B.G. (2003). Tyrosyl phosphorylation of Shp2 is required for normal ERK activation in response to some, but not all, growth factors. J. Biol. Chem. *278*, 41677–41684.

Araki, T., Mohi, M.G., Ismat, F.A., Bronson, R.T., Williams, I.R., Kutok, J.L., Yang, W., Pao, L.I., Gilliland, D.G., Epstein, J.A., and Neel, B.G. (2004). Mouse model of Noonan syndrome reveals cell type- and gene dosage-dependent effects of Ptpn11 mutation. Nat. Med. *10*, 849–857.

Arico, M., Biondi, A., and Pui, C.-H. (1997). Juvenile myelomonocytic leukemia. Blood *90*, 479–488.

Armstrong, S.A., Golub, T.R., and Korsmeyer, S.J. (2003). MLL-rearranged leukemias: Insights from gene expression profiling. Semin. Hematol. *40*, 268–273.

Asha, H., Nagy, I., Kovacs, G., Stetson, D., Ando, I., and Dearolf, C.R. (2003). Analysis of Ras-induced overproliferation in Drosophila hemocytes. Genetics *163*, 203–215.

Bader-Meunier, B., Tchernia, G., Mielot, F., Fontaine, J.L., Thomas, C., Lyonnet, S., Lavergne, J.M., and Dommergues, J.P. (1997). Occurrence of myeloproliferative disorder in patients with Noonan syndrome. J. Pediatr. *130*, 885–889.

Barford, D., and Neel, B.G. (1998). Revealing mechanisms for SH2 domain-mediated regulation of the protein tyrosine phosphatase SHP-2. Structure *6*, 249–254.

Bennett, A., Tang, T., Sugimoto, S., Walsh, C., and Neel, B. (1994). Protein-tyrosine-phosphatase SHPTP2 couples platelet-derived growth factor receptor beta to Ras. Proc. Natl. Acad. Sci. USA *91*, 7335–7339.

Bentires-Alj, M., Paez, J.G., David, F.S., Keilhack, H., Halmos, B., Naoki, K., Maris, J.M., Richardson, A., Bardelli, A., Sugarbaker, D.J., et al. (2004). Activating mutations of the Noonan Syndrome-associated SHP2/PTPN11 gene in human solid tumors and adult AML. Cancer Res. *64*, 8816–8820.

Bjornsti, M.A., and Houghton, P.J. (2004). The TOR pathway: A target for cancer therapy. Nat. Rev. Cancer *4*, 335–348.

Bollag, G., Clapp, D.W., Shih, S., Adler, F., Zhang, Y., Thompson, P., Lange, B.J., Freedman, M.H., McCormic, F., Jacks, T., and Shannon, K.M. (1996). Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in hematopoietic cells. Nat. Genet. *12*, 144–148.

Brand, A.H., and Perrimon, P. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Braun, B.S., Tuveson, D.A., Kong, N., Le, D.T., Kogan, S.C., Rozmus, J., Le Beau, M.M., Jacks, T.E., and Shannon, K.M. (2004). Somatic activation of oncogenic Kras in hematopoietic cells initiates a rapidly fatal myeloproliferative disorder. Proc. Natl. Acad. Sci. USA *101*, 597–602.

Chan, I.T., Kutok, J.L., Williams, I.R., Cohen, S., Kelly, L., Shigematsu, H., Johnson, L., Akashi, K., Tuveson, D.A., Jacks, T., and Gilliland, D.G. (2004). Conditional expression of oncogenic K-ras from its endogenous promoter induces a myeloproliferative disease. J. Clin. Invest. *113*, 528–538.

Choong, K., Freedman, M.H., Chitayat, D., Kelly, E.N., Taylor, G., and Zipursky, A. (1999). Juvenile myelomonocytic leukemia and Noonan Syndrome. J. Pediatr. Hematol. Oncol. *21*, 523–527.

Cichowski, K., Santiago, S., Jardim, M., Johnson, B.W., and Jacks, T. (2003). Dynamic regulation of the ras pathway via proteolysis of the NF1 tumor suppressor. Genes Dev. *17*, 449–454.

Cross, N.C., and Reiter, A. (2002). Tyrosine kinase fusion genes in chronic myeloproliferative diseases. Leukemia *16*, 1207–1212.

Dearolf, C.R. (1998). Fruit fly "leukemia.". Biochim. Biophys. Acta *1377*, M13–M23.

Denu, J.E., Stuckey, J.A., Saper, M.A., and Dixon, J.E. (1996). Form and function in protein dephosphorylation. Cell *87*, 361–364.

Donovan, S., See, W., Bonifas, J., Stokoe, D., and Shannon, K.M. (2002). Hyperactivation of protein kinase B and ERK have discrete effects on survival, proliferation and cytokine expression in Nf1-deficient myeloid cells. Cancer Cell *2*, 507–514.

Emanuel, P.D., Shannon, K.M., and Castleberry, R.P. (1996). Juvenile myelomonocytic leukemia: Molecular undrestanding and prospects for therapy. Mol. Med. Today *2*, 468–475.

Flint, A.J., Tiganis, T., Barford, D., and Tonks, N.K. (1997). Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. Proc. Natl. Acad. Sci. USA *94*, 1680–1685.

Freeman, R.M., Jr., Plutzky, J., and Neel, B.G. (1992). Identification of a human *src*-homology 2 (SH2) containing tyrosine phosphatase: A putative homolog of *Drosophila corkscrew*. Proc. Natl. Acad. Sci. USA *89*, 11239–11243.

Gilliland, D.G., and Griffin, J.D. (2002). Role of FLT3 in leukemia. Curr. Opin. Hematol. *9*, 274–281.

Gilliland, D.G., and Tallman, M.S. (2002). Focus on acute leukemias. Cancer Cell *1*, 417–420.

Goldfine, A.B., Simonson, D.C., Folli, F., Patti, M.-E., and Kahn, C.R. (1995). Metabolic effects of sodium metavanadate in humans with insulin-dependent and noninsulin-dependent diabetes mellitus in vivo and in vitro studies. J. Clin. Endocrinol. Metab. *80*, 3311–3319.

Gu, H., and Neel, B.G. (2003). The 'Gab' in signal transduction. Trends Cell Biol. *13*, 122–130.

Gu, H., Griffin, J.D., and Neel, B.G. (1997). Characterization of two SHP-2-associated binding proteins and potential substrates in hematopoietic cells. J. Biol. Chem. *272*, 16421–16430.

Gu, H., Saito, K., Klaman, L.D., Shen, J., Fleming, T., Wang, Y., Pratt, J.C., Lin, G., Lim, B., Kinet, J.-P., and Neel, B.G. (2001). Essential role for Gab2 in the allergic response. Nature *412*, 186–190.

Hanafusa, H., Torii, S., Yasunaga, T., Matsumoto, K., and Nishida, E. (2004). Shp2, an SH2-containing protein-tyrosine phosphatase, positively regulates receptor tyrosine kinase signaling by dephosphorylating and inactivating the inhibitor Sprouty. J. Biol. Chem. *279*, 22992–22995.

Harrison, D.A., Binari, R., Nahreini, S., Gilman, M., and Perrimon, N. (1995). Activation of a Drosophila Janus Kinase (JAK) causes hematopoietic neoplasia and developmental defects. EMBO J. *14*, 2857–2865.

Hawley, R.G., Lieu, F.H.L., Fong, A.Z.C., and Hawley, T.S. (1994). Versatile retroviral vectors for potential use in gene therapy. Gene Ther. *1*, 136–138.

Hof, P., Pluskey, S., Dhe-Paganon, S., Eck, M.J., and Shoelson, S.E. (1998). Crystal structure of the SH2 domain phosphatase SHP-2. Cell *98*, 441–450.

Howe, L.R., Leevers, S.J., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C.J. (1992). Activation of the MAP kinase pathway by the protein kinase raf. Cell *71*, 335–342.

Ingram, D.A., Hiatt, K., King, A.J., Fisher, L., Shivakumar, R., Derstine, C., Wenning, M.J., Diaz, B., Travers, J.B., Hood, A., et al. (2001). Hyperactivation of $p21^{ras}$ and the hematopoietic-specific Rho GTPase, Rac2, cooperate to alter the proliferation of neurofibromin-deficient mast cells in vivo and in vitro. J. Exp. Med. *194*, 57–69.

Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P.H., and Downward, J. (1997). Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J. *16*, 2783–2793.

Klinghoffer, R.A., and Kazlauskas, A. (1995). Identification of a putative Syp substrate, the PDGFB receptor. J. Biol. Chem. *270*, 22208–22217.

Largaespada, D.A., Brannan, C.I., Jenkins, N.A., and Copeland, N.G. (1996). Nf1 deficiency causes Ras-mediated granulocyte/macrophage colony stimulating factor hypersensitivity and chronic myeloid leukemia. Nat. Genet. *12*, 137–143.

Le, D.T., Kong, N., Zhu, Y., Lauchle, J.O., Aiyigari, A., Braun, B.S., Wang, E., Kogan, S.C., Le Beau, M.M., Parada, L., and Shannon, K.M. (2004). Somatic inactivation of Nf1 in hematopoietic cells results in a progressive myeloproliferative disorder. Blood *103*, 4243–4250.

Leevers, S.J., and Marshall, C.J. (1992). Activation of extracellular signal-regulated kinase, ERK2, by p21ras oncoprotein. EMBO J. *11*, 569–574.

Li, W., Nishimura, R., Kashishian, A., Batzer, A.G., Kim, W.J.H., Cooper, J.A., and Schlessinger, J. (1994). A new function for a phosphotyrosine phosphatase: Linking GRB2-Sos to a receptor tyrosine kinase. Mol. Cell. Biol. *14*, 509–517.

Liu, Y., and Rohrschneider, L.R. (2002). The Gift of Gab. FEBS Lett. *515*, 1–7.

Loh, M.L., Vattikuti, S., Schubbert, S., Reynolds, M.G., Carlson, E., Lieuw, K.H., Cheng, J.W., Lee, C.M., Stokoe, D., Bonifas, J.M., et al. (2004). Mutations in PTPN11 implicate the SHP-2 phosphatase in leukemogenesis. Blood *103*, 2325–2331.

Lu, W., Gong, D., Bar-Sagi, D., and Cole, P.A. (2001). Site-specific incorporation of a phosphotyrosine mimetic reveals a role for tyrosine phosphorylation of SHP-2 in cell signaling. Mol. Cell *8*, 759–769.

Luo, H., Hanratty, W.P., and Dearolf, C.R. (1995). An amino acid substitution in the Drosophila hop^{tum-l} Jak kinase causes leukemia-like hematopoietic defects. EMBO J. *14*, 1412–1420.

MacDonald, A., Scarola, J., Burke, J.T., and Zimmerman, J.J. (2000). Clinical pharmacokinetics and therapeutic drug monitoring of sirolimus. Clin. Ther. *22*, B101–B121.

Meister, M. (2004). Blood cells of Drosophilla: Cell lineages and role in host defence. Curr. Opin. Immunol. *16*, 10–15.

Million, R.P., and Van Etten, R.A. (2000). The Grb2 binding site is required for the induction of chronic myeloid leukemia-like disease in mice by the Bcr/Abl tyrosine kinase. Blood *96*, 664–670.

Mohi, M.G., Boulton, C., Gu, T.-L., Sternberg, D.W., Neuberg, D., Griffin, J.D., Gilliland, D.G., and Neel, B.G. (2004). Combination of rapamycin and protein tyrosine kinase (PTK) inhibitors for the treatment of leukemias caused by oncogenic PTKs. Proc. Natl. Acad. Sci. USA *101*, 3130–3135.

Neel, B.G., Gu, H., and Pao, L. (2003). The "Shp"ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. Trends Biochem. Sci. *28*, 284–293.

Nishida, K., Wang, L., Morii, E., Park, S.J., Narimatsu, M., Itoh, S., Yamasaki, S., Fujishima, M., Ishihara, K., Hibi, M., et al. (2002). Requirement of Gab2 for mast cell development and KitL/c-Kit signaling. Blood *99*, 1866–1869.

O'Reilly, A.M., Pluskey, S., Shoelson, S.E., and Neel, B.G. (1999). Activated mutants of SHP-2 preferentially induce elongation of *Xenopus* animal caps. Mol. Cell. Biol. *20*, 299–311.

Pardanani, A., and Tefferi, A. (2004). Imatinib targets other than bcr/abl and their clinical relevance in myeloid disorders. Blood *104*, 1931–1939.

Pawson, T., and Scott, J.D. (1997). Signaling through scaffold, anchoring and adapter proteins. Science *278*, 2075–2080.

Perkins, L.A., Larsen, I., and Perrimon, N. (1992). corkscrew encodes a putative tyrosine phosphatase that functions to transduce the terminal signal from the receptor tyrosine kinase torso. Cell *70*, 225–236.

Ren, Y., Meng, S., Mei, L., Zhao, Z.J., Jove, R., and Wu, J. (2004). Roles of Gab1 and SHP2 in paxillin tyrosine dephosphorylation and Src activation in response to epidermal growth factor. J. Biol. Chem. *279*, 8497–8505.

Sarkozy, A., Conti, E., Seripa, D., Digilio, M.C., Grifone, N., Tandoi, C., Fazio, V.M., Ciommo, V.D., Marino, B., Pizzuti, A., and Dallapiccola, B. (2003). Correlation between PTPN11 gene mutations and congenital heart defects in Noonan and LEOPARD syndromes. J. Med. Genet. *40*, 704–708.

Sattler, M., Mohi, M.G., Pride, Y.B., Quinnan, L.R., Malouf, N.A., Podar, K., Gesbert, F., Iwaski, H., Li, S., Van Etten, R.A., et al. (2002). Critical role for Gab2 in transformation by BCR/ABL. Cancer Cell *1*, 479–492.

Sawyers, C.L. (1999). Chronic myeloid leukemia. N. Engl. J. Med. *340*, 1330–1340.

Schwaller, J., Frantsve, J., Aster, J., Williams, I.R., Tomasson, M.H., Ross, T.S., Peeters, P., Van Rompaey, L., Van Etten, R.A., Ilaria, R., Jr., et al. (1998). Transformation of hematopoietic cell lines to growth-factor independence and induction of a fatal myelo- and lymphoproliferative disease in mice by retrovirally transduced TEL/JAK2 fusion genes. EMBO J. *17*, 5321–p5333.

Sebolt-Leopold, J. (2000). Development of anticancer drugs targeting the MAP kinase pathway. Oncogene *19*, 6594–6599.

Side, L.E., and Shannon, K.M. (1997). Myeloid disorders in infants with Noonan syndrome and a resident's "rule" recalled. J. Pediatr. *130*, 857–859.

Tartaglia, M., Mehler, E.L., Goldberg, R., Zampino, G., Brunner, H.G., Kremer, H., van der Burgt, I., Crosby, A.H., Ion, A., Jeffery, S., et al. (2001). Mutations in PTPN11, encoding protein tyrosine phosphatase SHP-2, cause Noonan syndrome. Nat. Genet. *29*, 465–468.

Tartaglia, M., Kalidas, K., Shaw, A., Song, X., Musat, D.L., van der Burgt, I., Brunner, H.G., Bertola, D.R., Crosby, A., Ion, A., et al. (2002). PTPN11 mutations in Noonan syndrome: Molecular spectrum, genotype-phenotype correlation, and phenotypic heterogeneity. Am. J. Hum. Genet. *70*, 1555–1563.

Tartaglia, M., Niemeyer, C.M., Fragale, A., Song, X., Buechner, J., Jung, A., Hahlen, K., Hasle, H., Licht, J.D., and Gelb, B.D. (2003). Somatic mutations in *PTPN11* in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nat. Genet. *34*, 148–150.

Tartaglia, M., Martinelli, S., Cazzaniga, G., Cordeddu, V., Iavarone, I., Spinelli, M., Palmi, C., Carta, C., Pession, A., Arico, M., et al. (2004). Genetic evidence for lineage-related and differentiation stage-related contribution of somatic PTPN11 mutations to leukemogenesis in childhood acute leukemia. Blood *104*, 307–313.

Tonks, N.K., and Neel, B.G. (2001). Combinatorial control of specificity by protein-tyrosine phosphatases. Curr. Opin. Cell Biol. *13*, 182–195.

Van Vactor, D., O'Reilly, A.O., and Neel, B.G. (1998). Genetic analysis of protein tyrosine phosphatases. Curr. Opin. Genet. Dev. *8*, 112–126.

Welham, M.J., Dechert, U., Leslie, K.B., Jirik, F., and Schrader, J.W. (1994). Interleukin (IL)-3 and granulocyte/macrophage colony-stimulating factor, but not IL-4, induce tyrosine phosphorylation, activation, and association of SHPTP2 with Grb2 and phosphatidylinositol 3′-kinase. J. Biol. Chem. *269*, 23764–23768.

Wilhelm, S.M., Carter, C., Tang, L., Wilkie, D., McNabola, A., Rong, H., Chen, C., Zhang, X., Vincent, P., McHugh, M., et al. (2004). BAY 43–9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res. *64*, 7099–7109.

Zatkova, A., Ullmann, R., Rouillard, J.M., Lamb, B.J., Kuick, R., Hanash, S.M., Schnittger, S., Schoch, C., Fonatsch, C., and Wimmer, K. (2004). Distinct sequences on 11q13.5 and 11q23–24 are frequently coamplified with MLL in complexly organized 11q amplicons in AML/MDS patients. Genes Chromosomes Cancer *39*, 263–276.

Zhang, Y., Vik, T.A., Ryder, J.W., Srour, E.F., Jacks, T., Shannon, K., and Clapp, D.W. (1998). Nf1 regulates hematopoietic progenitor cell growth and Ras signaling in response to multiple cytokines. J. Exp. Med. *187*, 1893–1902.

Zhang, S.Q., Tsiaris, W.G., Araki, T., Wen, G., Minichiello, L., Klein, R., and Neel, B.G. (2002). Receptor-specific regulation of phosphatidyl 3′-kinase activation by the protein tyrosine phosphatase Shp2. Mol. Cell. Biol. *22*, 4062–4072.

Zhang, S.Q., Yang, W., Kontaridis, M.I., Bivona, T.G., Wen, G., Araki, T., Luo, J., Thompson, J.A., Schraven, B.L., Philips, M.R., and Neel, B.G. (2004). Shp2 regulates SRC family kinase activity and Ras/Erk activation by controlling Csk recruitment. Mol. Cell *13*, 341–355.

Zinyk, D.L., McGonnigal, B.G., and Dearolf, C.R. (1993). *Drosophila awd^{K-pn}*, a homologue of the metastasis suppressor gene *nm23*, suppresses the *Tum-l* hematopoietic oncogene. Nat. Genet. *4*, 195–201.