Hyperactivation of protein kinase B and ERK have discrete effects on survival, proliferation, and cytokine expression in *Nf1*-deficient myeloid cells

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Summary

The *Nf1* tumor suppressor encodes a GTPase-activating protein for Ras. Previous work has implicated hyperactive Ras in the aberrant growth of *Nf1*-deficient cells; however, there are limited data on which effectors modulate specific phenotypes. To address this, we generated myeloid cell lines by infecting fetal liver cells with a retrovirus encoding a truncated allele of *c-Myb.* Granulocyte-macrophage colony stimulating factor (GM-CSF) promoted the survival of wild-type *Myb* cells in a dose-dependent manner. By contrast, *Nf1*-deficient myeloid cells deprived of growth factors, were resistant to apoptosis due to hyperactivation of the phosphoinositide-3-OH kinase/protein kinase B cascade. *Nf1*$^{-/-}$ cells also demonstrated growth factor-independent proliferation and upregulation of GM-CSF mRNA production that were dependent upon Raf/MEK/ERK signaling. These data link specific Ras effectors with discrete cellular phenotypes in *Nf1*-deficient cells.

Introduction

Neurofibromatosis type 1 (NF1) is the most common inherited cancer predisposition syndrome with an incidence of 1 in 3500 (Riccardi and Eichner, 1986). Affected persons are at increased risk of developing specific benign and malignant neoplasms that primarily arise in cells derived from the embryonic neural crest (Side and Shannon, 1998). In addition, children (but not adults) with NF1 are predisposed to myeloid malignancies, particularly juvenile myelomonocytic leukemia (JMML) (Bader and Miller, 1978; Shannon et al., 1992; Stiller et al., 1994). The *NF1* gene encodes neurofibromin, a GTPase-activating protein (GAP) that negatively regulates p21^{ras} (Ras) output by accelerating GTP hydrolysis on Ras (reviewed in Bernards, 1995; Boguski and McCormick, 1993; Donovan et al., 2002). Genetic and biochemical analysis of primary tumors and of tumor-derived cell lines demonstrated that *NF1* and its murine homolog *(Nf1)* function as tumor suppressor genes, and revealed aberrant activation of Ras effector pathways in *Nf1*-deficient cells (reviewed in Cichowski and Jacks, 2001; Side and Shannon, 1998).

JMML is a relentless myeloproliferative disorder (MPD) characterized by overproduction of myeloid cells that infiltrate multiple tissues (reviewed in Arico et al., 1997; Emanuel et al., 1996). JMML cells show hypersensitive growth of large colony forming unit granulocyte-macrophage (CFU-GM) progenitor colonies in methylcellulose cultures stimulated with granulocyte-macrophage colony stimulating factor (GM-CSF) (Emanuel et al., 1991b; Schiro et al., 1994). Homozygous *Nf1* mutant embryos fail around E13 with cardiovascular defects (Brannan et al., 1994; Jacks et al., 1994); however, approximately 10% of heterozygous *(Nf1*$^{+/-}$*)* mutant mice spontaneously develop an MPD that models JMML (Jacks et al., 1994). *Nf1*$^{-/-}$ fetal liver cells show hypersensitive CFU-GM progenitor growth in response to GM-CSF that is similar to human JMML cells (Bollag et al., 1996; Largaespada et al., 1996), and adoptive transfer of these mutant cells consistently induces a JMML-like MPD in irradiated recipient mice (Largaespada et al., 1996; Zhang et al., 1998). Further studies in which fetal liver cells doubly mutant at the *Gmcsf* and *Nf1* loci were generated and transferred into *Gmcsf*$^{-/-}$ or wild-type recipients have underscored the central

SIGNIFICANCE

Hyperactive Ras is among the most common biochemical abnormalities found in cancer cells and results from oncogenic *RAS* point mutations and other genetic lesions. Based on this, the Ras-activated signaling pathways are being pursued intensively as potential targets for drug discovery. However, because Ras interacts with multiple downstream effectors, the contribution of individual Ras-dependent signaling pathways to tumorigenesis is largely unknown. We show that hyperactivation of the PI3 kinase/PKB and Raf/MEK/ERK cascades coordinately deregulates the growth of myeloid cells through discrete effects on survival, proliferation, and autocrine expression of growth factors. These findings, which underscore the pleiotropic effects of hyperactive Ras on growth control, have implications for the development of therapeutics that may target specific effectors.

role of aberrant GM-CSF signaling in initiating and maintaining this MPD in vivo (Birnbaum et al., 2000).

In 1989, Gonda and coworkers (Gonda et al., 1989a) developed cell lines that resemble early granulocyte-macrophage progenitors by infecting murine fetal liver cells with a retrovirus encoding a truncated allele of *c-Myb*. Cells transformed with this REDMyb(CT3) virus are dependent on serum and hematopoietic growth factors and are especially responsive to GM-CSF (Gonda et al., 1993). Here we show that *Nf1*$^{-/-}$ *Myb* cells are hypersensitive to GM-CSF in tissue culture, resist apoptosis upon growth factor withdrawal, and proliferate in the absence of exogenous cytokines. These cells also demonstrate constitutive activation of Ras effector pathways, and we find that the phosphoinositide-3-OH kinase/protein kinase B (PI3K/PKB) is essential for survival in the absence of cytokines. *Nf1*$^{-/-}$ *Myb* cells also proliferate without exogenous growth factors and demonstrate elevated levels of GM-CSF mRNA that are dependent upon activation of the Raf/MEK/ERK cascade. Thus, the pleiotropic effects of hyperactive Ras in *Nf1*-deficient cells are mediated through discrete downstream signaling cascades, which cooperate to undermine growth control. Aberrant GM-CSF expression is likely to reinforce aberrant growth through an autocrine loop that further deregulates Ras signaling.

Results

Characterization of *Myb*-transformed cells

A retrovirus was constructed in the murine stem cell virus (MSCV) backbone to coexpress the truncated Myb-CT peptide with a green fluorescent protein (GFP) marker (see Experimental Procedures). Infections were performed by transiently transfecting Phoenix packaging cells with this *MSCV-Myb-GFP* virus, followed by co-culture with E12.5–E13.5 fetal liver cells to generate stable *Myb* lines as described previously (Gonda et al., 1989b). After 1–2 weeks in culture, *Nf1*$^{-/-}$ and wild-type *Myb* cells uniformly expressed the myelomonocytic marker Mac1 and the neutrophilic antigen Gr1 and showed variable expression of Sca1, which marks hematopoietic cells with high repopulating potential (data not shown). Stable *Myb* lines that emerged after 2–3 months were Mac1^{+}, Gr1^{-}, and Sca1^{-} (Figure 1A), and 5%–10% of these cells formed CFU-GM colonies in the presence of saturating concentrations of GM-CSF. There were marked quantitative and qualitative differences between CFU-GM colonies grown from wild-type and *Nf1*$^{-/-}$ *Myb* cells. Whereas wild-type *Myb* cells did not form CFU-GM in the absence of exogenous GM-CSF, *Nf1*$^{-/-}$ cells consistently showed cytokine-independent colony growth (Figure 1B). Wild-type cells cultured with 0.1 ng/ml of GM-CSF generated CFU-GM that were compact and contained relatively few cells, while 1 ng/ml induced the formation of larger colonies with a spreading morphology. By contrast, CFU-GM grown from *Nf1*$^{-/-}$ *Myb* cells were more diffuse, and enormous CFU-GM containing thousands of cells were observed at saturating concentrations of GM-CSF (Figure 1C). Thus, *Nf1*$^{-/-}$ *Myb* cells mimic the GM-CSF hypersensitivity and morphologic abnormalities that are in vitro hallmarks of CFU-GM colonies grown from JMML patients.

Apoptosis and proliferation of *Myb* cells

Wild-type and *Nf1*$^{-/-}$ *Myb* lines were established and maintained in culture medium supplemented with 20% fetal calf serum and 1 ng/ml of recombinant murine GM-CSF. Under these conditions, *Nf1*$^{-/-}$ *Myb* lines proliferated robustly while wild-type cells demonstrated variable rates of growth. We first compared apoptosis in wild-type and *Nf1*$^{-/-}$ *Myb* lines over a range of GM-CSF concentrations. Growing wild-type and *Nf1*$^{-/-}$ *Myb* cells were washed, incubated overnight with 0–10 ng/ml of GM-CSF, and stained with Annexin V to detect apoptosis. There was a strong dose-dependent effect of GM-CSF on the survival of wild-type *Myb* cells with >80% of the cells undergoing apoptosis in the absence of GM-CSF and ~95% surviving at 10 ng/ml (Figure 2A). By contrast, *Nf1*$^{-/-}$ *Myb* cells were resistant to apoptosis induced by GM-CSF withdrawal (Figure 2B). Using DNA laddering as an alternative method for measuring apoptosis gave similar results (data not shown). *Nf1*$^{-/-}$ *Myb* cells were cultured with inhibitors of MEK (PD184352) or PI3K (LY294002) to address if signaling though the Raf/MEK/ERK and PI3K/PKB cascades is required for the survival in the absence of exogenous GM-CSF. To reduce the potential for nonspecific interactions with other signaling molecules, we sought to expose the cells to the lowest effective doses of PD184352 and LY294002. Limited data have been published on the minimal concentration of PD184352 that is required to block activation of ERK kinase. We therefore performed a titration and showed that 10 μM restored elevated levels of phosphorylated ERK detected in *Nf1*$^{-/-}$ *Myb* cells (see following section and Figure 4B) to normal, while 1 μM did not consistently have this effect. In later experiments, we found that 2 μM of PD184352 was the minimal effective dose of PD184352. By contrast, LY294002 has been used extensively and we selected a dose of 20 μM. LY294002 efficiently induced apoptosis of *Nf1*$^{-/-}$ *Myb* cells (Figure 2C) while PD184352 was much less effective (Figure 2D). Together, these results suggest that loss of *Nf1* deregulates hematopoietic growth, at least in part, by rendering myeloid precursors less dependent upon cytokines for survival, and they show that that the PI3K/PKB pathway is integral to this phenotype. Because *Nf1*$^{-/-}$ *Myb* cells maintained in 20% fetal calf serum continued to grow (albeit at a reduced rate) in the absence of GM-CSF, bromo-dioxyuridine (Brd-U) incorporation was measured to assess progression through the cell division cycle. Remarkably, 15%–20% of *Nf1*$^{-/-}$ *Myb* cells incorporated Brd-U; this was reduced dramatically by exposure to PD184352 (Figure 3).

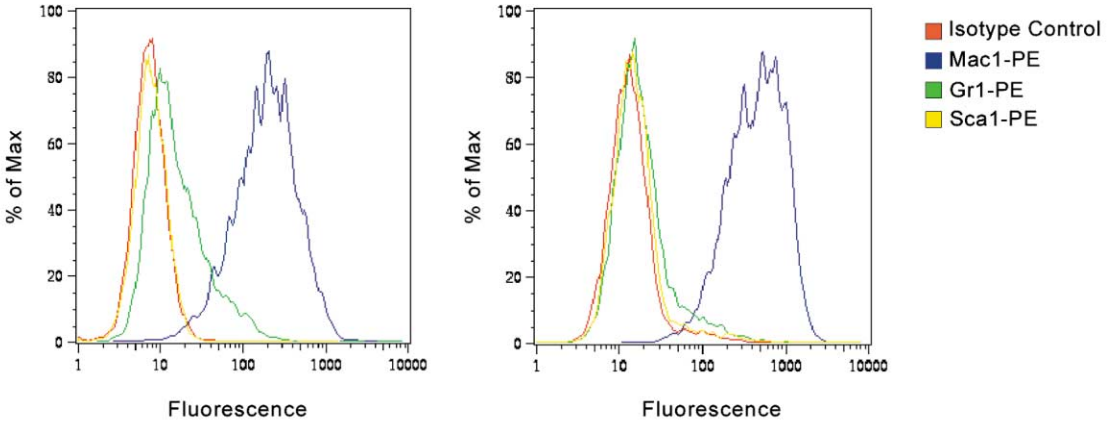
Ras activation and signaling in *Myb* cells

In pilot experiments, >80% of wild-type *Myb* cells deprived of GM-CSF for 4 hr were viable as assessed by absence of Annexin V labeling (data not shown). Signaling experiments were therefore performed 4 hr after GM-CSF removal on cells that were maintained in 20% fetal calf serum to avoid the confounding effects of cell death on biochemical endpoints. Under these conditions, *Nf1*$^{-/-}$ *Myb* cells demonstrated a marked increase in the percentage of Ras in the active, GTP bound conformation (Figure 4A). These elevated levels of Ras-GTP were associated with basal activation of ERK and PKB, and with hyperactivation following exposure to GM-CSF (Figures 4B and 4C). ERK kinase activity was also induced to a lesser degree by interleukin 3 (IL-3) (Figure 4B). *Nf1*$^{-/-}$ and wild-type *Myb* cells contained similar levels of ERK (Figure 4B); however, the total amount of PKB was higher in the *Nf1*$^{-/-}$ cells (data not shown).

Production of GM-CSF by *Nf1*$^{-/-}$ *Myb* cells

The data presented above raised the possibility that an external stimulus such as autocrine production of GM-CSF might promote

A



B

Colonies per 500 Cells

GM-CSF	Wild Type	*Nf1-/-*
0	0 ± 0	27 ± 23
1 ng/mL	68 ± 40	62 ± 33

C

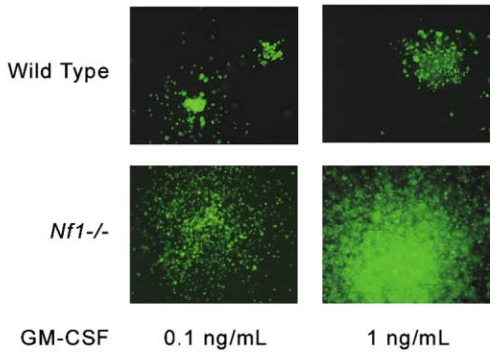


Figure 1. Characteristics of *Myb*-infected cell lines

A: Immunophenotypes of *Myb* cells. Wild-type cells are shown on the left and *Nf1*$^{-/-}$ cells are on the right.
B: CFU-GM colony growth from *Myb* cells (±SEM). Data were pooled from three experiments.
C: Morphology of CFU-GM colonies grown from *Myb* cells.

the survival and proliferation of *Nf1*$^{-/-}$ *Myb* cells. To address this question, we performed quantitative real time polymerase chain reaction (Taqman) analysis and found that GM-CSF mRNA levels were 17–113 times higher in *Nf1*$^{-/-}$ *Myb* cells versus wild-type cells (Figure 5A). To ascertain the role of signaling through the Raf/MEK/ERK and PI3K/PKB pathways on GM-CSF expression, mRNA levels were measured in *Nf1*$^{-/-}$ *Myb* cells that were treated with PD184352 (2 μM) or LY294002 (20 μM). PD184352 induced a dramatic reduction in the amount of GM-CSF mRNA, while LY294002 had inconsistent effects (Figure 5B). We also used a neutralizing rat antibody to murine GM-CSF to ascertain the functional significance of elevated levels of GM-CSF mRNA in *Nf1*$^{-/-}$ *Myb* cells. In these experiments, the cells were cultured in the absence of exogenous GM-CSF and antibody was added at the beginning of the experiment only (one dose) or at the start and 12 hr later (two doses). A single dose of the antibody had no effect; however, significant apoptosis was detected after 48 hr in cells that received two doses of the antibody (Figure 5C).

Discussion

Myb-transformed fetal liver cells provide a relevant experimental system for interrogating the cellular and biochemical consequences of *Nf1* inactivation because they manifest growth and differentiation characteristics of primary myeloid progenitors and are particularly dependent upon GM-CSF for proliferation and survival (Gonda et al., 1993, 1989b). Furthermore, leukemias that develop in heterozygous *Nf1* mutant mice infected with the BXH-2 retrovirus frequently demonstrate both inactivation of the wild-type *Nf1* allele and integrations at the *Epi1* locus, which is 30–40 kb downstream of *Myb* (Blaydes et al., 2001). These data suggest that loss of *Nf1* and deregulated expression of *Myb* cooperate in myeloid leukemogenesis in vivo. We previously detected elevated levels of Ras-GTP in JMML cells from children with NF1 (Bollag et al., 1996), and Largaespada et al. (1996) reported sustained hyperactivation of Ras-GTP in *Nf1*$^{-/-}$ *Myb* cells that were stimulated with GM-CSF. Our studies showing constitutive activation of the Raf/MEK/ERK effector pathway with hyperactivation in response to GM-CSF extend this work and are consistent with data from primary *Nf1*$^{-/-}$ c-kit-positive bone marrow cells (Zhang et al., 1998) and heterozygous *Nf1*-deficient bone marrow-derived mast cells (Ingram et al., 2000).

We extensively characterized the cellular consequences of deregulated Ras signaling in *Nf1*$^{-/-}$ *Myb* cells. GM-CSF prevented apoptosis in wild-type *Myb* cells in a dose-dependent manner, and loss of *Nf1* allowed *Myb*-transformed cells to sur-

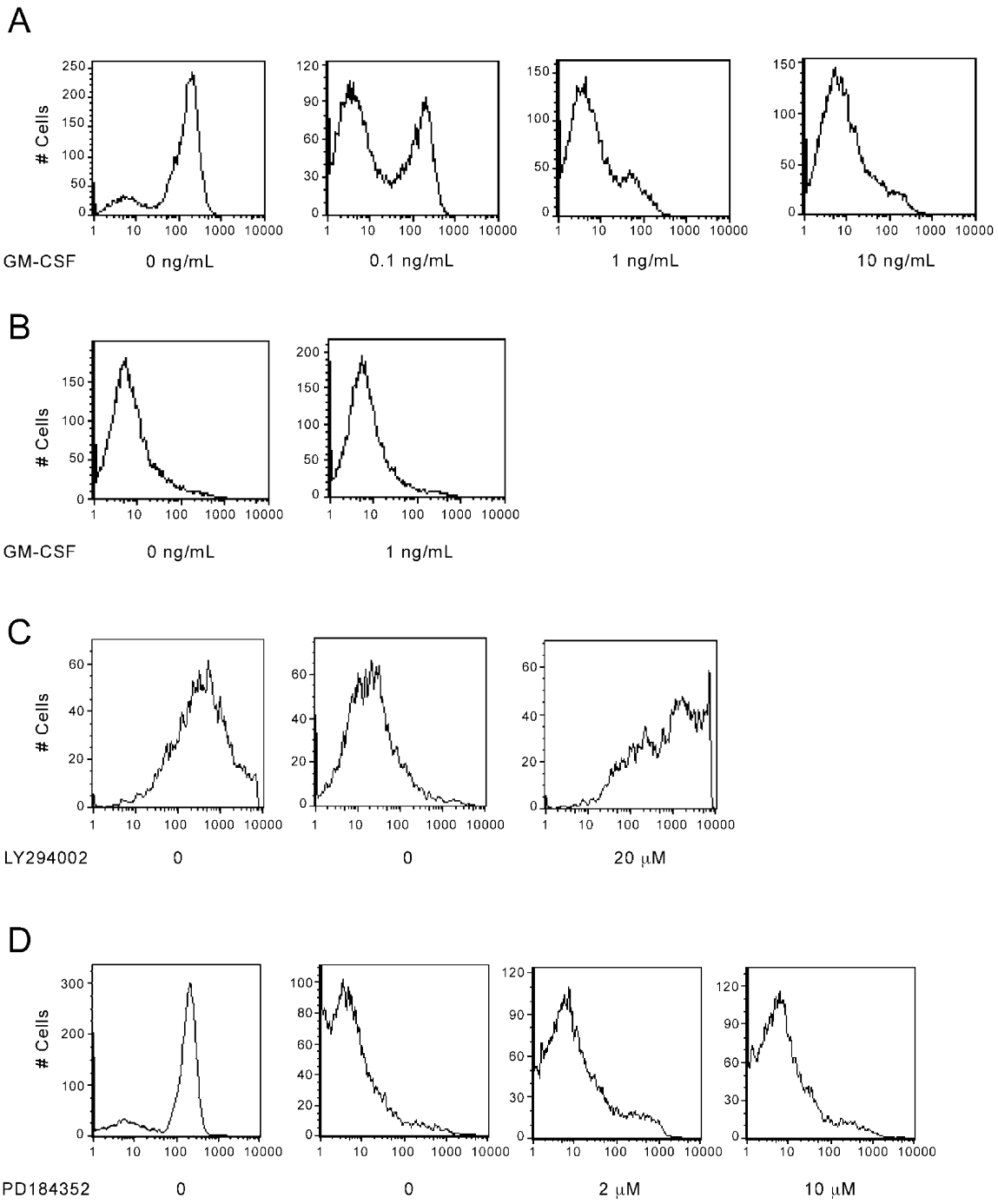


Figure 2. Apoptosis of *Myb* cells measured by annexin V staining

Increased fluorescence intensity on the horizontal axis corresponds to annexin V labeling.
A: Wild-type *Myb* cells. Note that the proportion of Annexin V-positive cells decreases with increasing GM-CSF concentrations.
B: *Nf1*$^{-/-}$ cells survive without GM-CSF.
C: LY294002 induces apoptosis of *Nf1*$^{-/-}$ *Myb* cells cultured in the absence of GM-CSF. The left plot shows wild-type cells cultured without GM-CSF (positive control), the middle plot is *Nf1*$^{-/-}$ cells, and the right plot is *Nf1*$^{-/-}$ cells cultured with the inhibitor.
D: PD184352 has limited effects on the survival of *Nf1*$^{-/-}$ *Myb* cells. As in **C**, the cells were cultured in the absence of GM-CSF. The left plot is wild-type cells (positive control), and the three right plots are *Nf1*$^{-/-}$ cells cultured in the absence or presence of PD184352.

vive without added cytokines. Experiments using small molecule inhibitors demonstrated a primary role of the PI3K/PKB pathway in conferring resistance to apoptosis in *Nf1*$^{-/-}$ *Myb* cells. Interestingly, mice deficient in the inositol 5′ phosphatase SHIP, a negative regulator of PI3K signaling, develop a MPD that resembles the disorder seen in recipients of *Nf1*$^{-/-}$ fetal liver cells (Huber et al., 1998; Liu et al., 1999). Like *Nf1*$^{-/-}$ *Myb* cells, neutrophils and mast cells from *SHIP* mutant mice show defec-

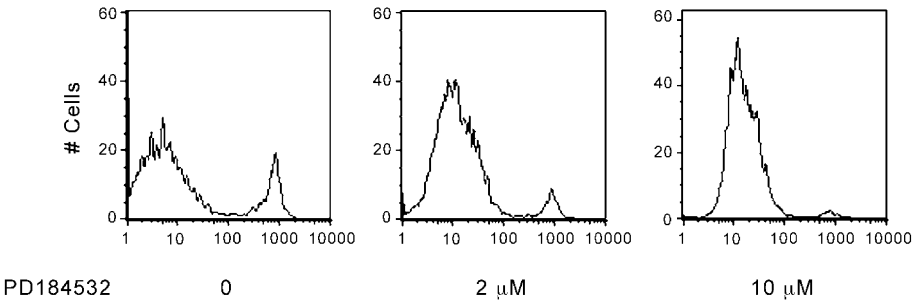


Figure 3. Proliferation of *Nf1*$^{-/-}$ *Myb* cells in the absence of GM-CSF

Peaks corresponding to Brd-U-positive cells are shifted to the right on the horizontal axis. In this experiment, 18.1% of untreated cells incorporated Brd-U versus 6.8% of cells exposed to 2 μM PD184352, and 2.3% in the presence of 10 μM of inhibitor.

tive apoptotic responses to growth factor withdrawal (Liu et al., 1999). We also found that *Nf1*$^{-/-}$ *Myb* cells proliferated in the absence of GM-CSF; this phenotype was susceptible to inhibition of the Raf/MEK/ERK cascade. These biochemical data implicate deregulation of PI3K/PKB and Raf/MEK/ERK signaling as mediating distinct and cooperative effects in *Nf1*-deficient cells.

Cultured *Nf1*-deficient embryonic neurons display neurotrophin-independent survival and growth factor hypersensitivity (Vogel et al., 1995). Other studies using dominant-negative adenoviruses implicated the PI3K/PKB pathway, but not Raf/MEK/ERK signaling, as essential for the survival of wild-type and *Nf1*$^{-/-}$ neurons (Klesse and Parada, 1998). Most recently, Li et al. (2002) detected amplification of the epidermal growth factor receptor and activation of the Raf/MEK/ERK and PI3K/PKB cascades in cell lines derived from soft tissue tumors that developed in mice heterozygous for mutations in *Nf1* and *p53*. Growth of these lines was potently inhibited by LY294002. These studies in other lineages that are affected in persons with NF1 disease suggest that growth factor hypersensitivity and defective apoptosis mediated through deregulated PI3K/PKB are common molecular defects that contribute to aberrant growth and tumorigenesis in distinct *Nf1*-deficient cell types.

Autocrine production of GM-CSF by *Nf1*$^{-/-}$ *Myb* cells is intriguing in light of the central and specific role of GM-CSF in the pathogenesis of JMML and in the corresponding murine MPD. The GM-CSF promoter contains adjacent Ets and AP-1 elements that are activated by signaling through the Raf/MEK/ERK cascade (Wang et al., 1994); this is consistent with our data showing that PD184352 treatment markedly reduced GM-CSF mRNA levels in *Nf1*$^{-/-}$ *Myb* cells. Studies in *JunB* mutant cells have provided additional insights implicating deregulated

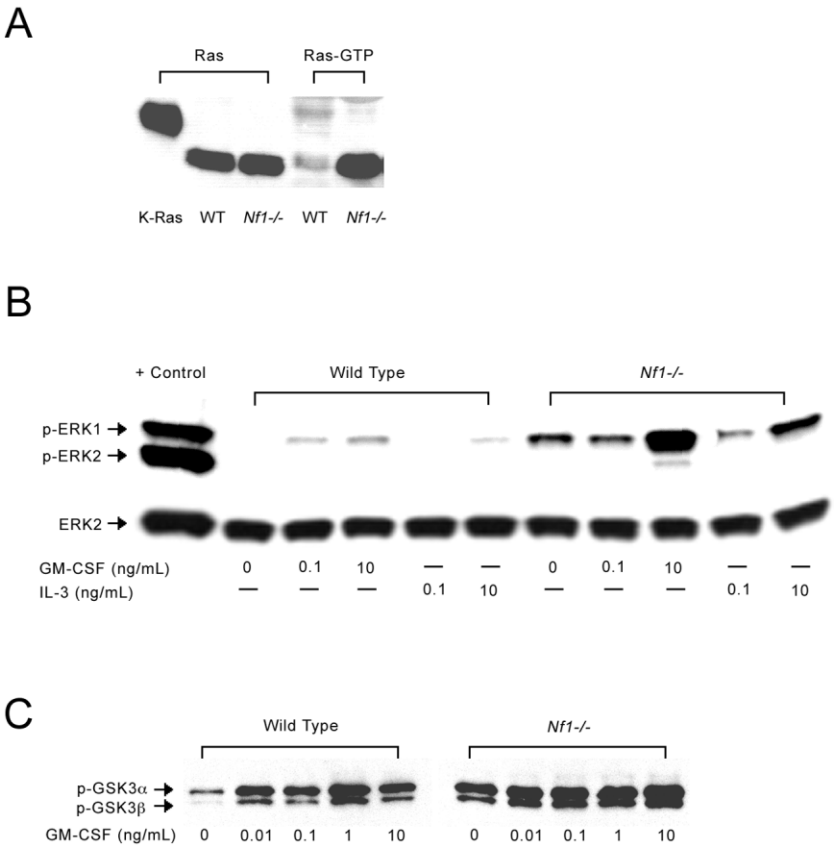


Figure 4. Activation of Ras, ERK, and PKB in wild-type and *Nf1*$^{-/-}$ *Myb* cells

The cells were assayed 4 hr after GM-CSF was removed.

A: Total Ras and Ras GTP levels. An aliquot corresponding to 1/25th of the total lysate was used to compare total Ras levels in wild-type and mutant cells. The control K-Ras peptide migrates more slowly because it contains an epitope tag.

B: Phosphorylated ERK and total ERK levels in *Myb* cells stimulated with GM-CSF or interleukin-3 (IL-3) for 10 min. The control lane is from NIH 3T3 cells stimulated with platelet-derived growth factor.

C: Basal and GM-CSF-stimulated PKB kinase activities were determined by measuring phosphorylation of a GSK substrate.

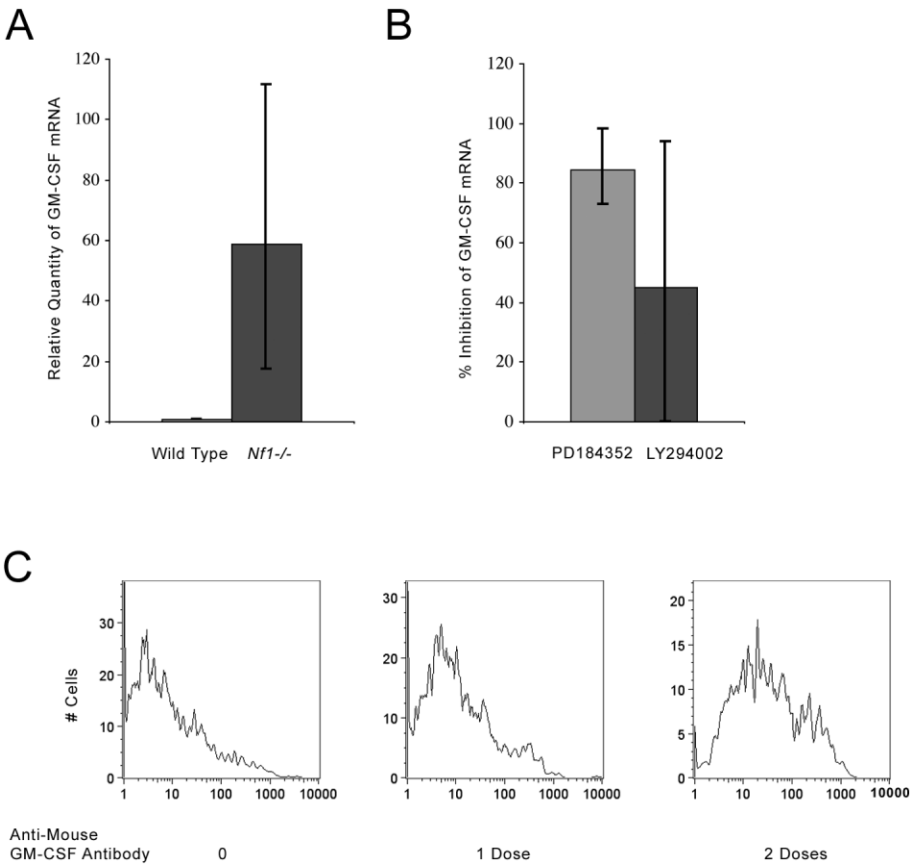


Figure 5. Expression of GM-CSF mRNA in *Myb* cells

A: GM-CSF mRNA levels in wild-type and $Nf1^{-/-}$ *Myb* cells.

B: Effects of PD184352 (2 μM) and LY29004 (20 μM) on GM-CSF mRNA levels in *Myb* cells. RNA was extracted at different times from six individual subcultures of a stable $Nf1^{-/-}$ *Myb* line to obtain the data shown in **A** and from four subcultures for **B**.

C: Apoptosis of $Nf1^{-/-}$ *Myb* cells exposed to a neutralizing rat-anti-mouse GM-CSF antibody. $Nf1^{-/-}$ Myb cells cultured in the absence of GM-CSF for 48 hr received either no antibody, a single dose of the antibody at the start of the experiment (final concentration 4 μg/ml), or this dose at the start of the experiment and a second dose 12 hr later. Annexin V staining was performed after 48 hr. The percentage of Annexin V-positive cells was low in cells that were not treated with antibody or received a single dose (14.5% and 17.5% in this experiment). By contrast, 38% of the $Nf1^{-/-}$ *Myb* cells that received both doses of antibody were stained by Annexin V.

GM-CSF signaling in the development of MPD. JunB is a component of the AP-1 complex that functionally antagonizes the effects of c-Jun. JunB has prominent repressor activity, but may also activate transcription in certain contexts (Chiu et al., 1989; Hsu et al., 1993). Homozygous *JunB* mutant embryos fail between E8.5 and E10 with vascular defects (Schorpp-Kistner et al., 1999); however, high levels of GM-CSF production by immortalized $JunB^{-/-}$ fibroblasts induce human keratinocytes to form aberrant epithelial layers in orthotopic cultures (Szabowski et al., 2000). Expressing a *JunB* transgene from the ubiquitin C promoter rescued $JunB^{-/-}$ embryos and resulted in normal development (Passegue et al., 2001). Surprisingly, the ubiqutin C transgene was silenced in myeloid cells by an epigenetic mechanism, and these $JunB^{-/-}$ *Ubi-junB* mice consistently developed MPD, which was associated with selective hypersensitivity to GM-CSF and with overexpression of the α chain of the GM-CSF receptor (Passegue et al., 2001). Similarities between the MPDs associated with inactivation of *Nf1* and *JunB* implicate misregulation of AP-1 target genes downstream of Ras-GTP as a common pathogenic mechanism.

Chronic myeloid leukemia (CML), chronic myelomonocytic leukemia (CMML), and JMML are clonal disorders in which the malignant stem cells retain the capacity to differentiate. In JMML, there is over-production of monocytes that infiltrate into the liver, spleen, and other tissues. Removing these monocytes by adherence depletion abrogates the hypersensitive pattern of CFU-GM colony growth that is a cellular hallmark of JMML (Bagby et al., 1988; Emanuel et al., 1991a; Estrov et al., 1986); this suggested a paracrine mechanism of action. Importantly, although JMML bone marrow cells show intrinsic GM-CSF hypersensitivity in colony forming assays (Emanuel et al., 1991b; Zhang et al., 1998), GM-CSF production is also increased in ~50% of JMML samples (Emanuel et al., 1991a; Freedman et al., 1992; Gualtieri et al., 1989). These observations suggest that growth factor concentrations are limiting in the bone marrow stroma and that the leukemic clone gains an additional proliferative advantage through autocrine production of GM-CSF. $Nf1^{-/-}$ *Myb* cell lines appear to model this aspect of the human disease. It is likely that loss of *Nf1* has effects on apoptosis that are independent of GM-CSF (the data in Figure 1 argue for this), and that overproduction of GM-CSF is an acquired event that cooperates in leukemogenesis. This idea is consistent with the fact that human JMML cells are universally hypersensitive to GM-CSF in culture, yet only about half of these leukemias produce excessive levels of GM-CSF.

It will be of interest to explore the contribution of autocrine mechanisms to aberrant growth in disorders such as CML, CMML, multiple myeloma, and polycythemia vera in which malignant hematopoietic cells undergo some differentiation. Finally, small molecules that either inhibit hyperactive Ras or interfere with paracrine/autocrine loops represent rational therapeutic strategies for improving the dismal prognosis in JMML, and may prove efficacious in other NF1-associated cancers. However, our data implicating coordinate hyperactivation of the PI3K/PKB and Raf/MEK/ERK cascades in the aberrant survival and proliferation of $Nf1^{-/-}$ myeloid cells also suggest that targeting a single effector cascade may not fully suppress the growth advantage found in cells with hyperactive Ras.

Experimental procedures

MSCV-Myb construction and fetal liver cell transduction

The mice used in these studies were housed in the UCSF Animal Care Facility, and the study procedures were approved by the Committee on Animal Research. *Nf1* mutant mice were provided by Dr. Tyler Jacks (Jacks et al., 1994). *Nf1*$^{+/-}$ mice on a mixed C57Bl/6 × 129/Sv background were mated to generate fetal liver cells. Single cell suspensions of E13.5 fetal liver cells were prepared and embryo genotypes were determined as described (Birnbaum et al., 2000; Zhang et al., 1998). Fetal liver cells were cultured in 50 ng/ml stem cell factor (SCF) and 50 ng/ml of IL-6 (Peprotech, Rocky Hill, New Jersey) for 2 days in 20% FBS DMEM-H21 containing 20 mM L-glutamine, 10 μg/ml streptomycin, and 10 units/ml penicillin G (UCSF Cell Culture Facility). Phoenix packaging cells were cultured until they were 60%–80% confluent, then transfected with the *MSCV-MybCT* plasmid using either calcium phosphate or Lipofectamine PLUS reagents (Invitrogen, Carlsbad, California). In our initial experiments, 1 × 10^6 fetal liver cells were infected with 1.5 ml of viral supernatant in the presence of 5 μg/ml polybrene and centrifuged for 45 min at 500 × g. Later infections were performed by co-culturing fetal liver cells with Phoenix cells for 2 days in the presence of 100 ng/ml of stem cell factor, 20 ng/ml of IL-6, and 5 μg/ml polybrene. The cells were removed and placed in fresh media containing 1–5 ng/ml GM-CSF (Peprotech) for 2 days, then analyzed by flow cytometry for EGFP expression to determine the percent of cells infected. Infected pools were examined daily for the first 2–4 weeks in culture and the medium was changed every 1–3 days depending on the rate of growth. Typically, cultures expanded for the first 2 weeks. This was either followed by a decline in cell numbers with death of the culture or by a plateau phase followed by resumption in growth by 2 months. At this time, the cells were used for experiments and/or aliquots were frozen. Most of the data presented in the figures is from an extensive analysis of a pair of *Myb* lines (one wild-type and one *Nf1*$^{-/-}$) performed over the past year. *Nf1*$^{-/-}$ fetal liver cells infected on multiple occasions also demonstrated enhanced proliferation and survival with increased levels of phosphorylated ERK.

CFU-GM assay

Wild-type and *Nf1*$^{-/-}$ *Myb* cells were plated in duplicate at a density of 500 cells/ml in methylcellulose culture medium (MethoCult M3231, Stem Cell Technologies, Vancouver, British Columbia), over a range of GM-CSF concentrations. CFU-GM colony growth was scored after 8 days by light microscopy.

Apoptosis and proliferation of *Myb* cells

Myb cells (1 × 10^6 per data point) were cultured in 20% FBS DMEM-H21 at 37°C. Apoptosis analysis was performed using the TACs Annexin-V Apoptosis Detection Kit protocol (R&D Systems, Minneapolis, Minnesota) and analyzed by flow cytometry. Proliferation assays were performed by labeling cells with 10 μM Brd-U (Pharmingen, San Diego, California) for 30 min according to the protocol available at http://scooter.cyto.purdue.edu/pucl_cd/flow/vol14/15_apop/data/capri1.htm. Flow cytometry was performed to detect the percent of viable cells that incorporated Brd-U.

Ras-GTP assay

Cells (1 × 10^7 of each genotype) were washed three times with PBS and suspended in 20% FBS DMEM-H21 without GM-CSF for 4 hr at 37°C. The cells were lysed in 25 mM Hepes (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate buffer supplemented with 1 μg/ml of Complete, a protease inhibitor cocktail (Roche, Indianapolis, Indiana). Ras-GTP was precipitated with a fusion protein consisting of glutathione-S-transferase and the Ras binding domain (RBD) of Raf (Taylor and Shalloway, 1996). The lysates were incubated overnight with RBD beads at 4°C. The beads were washed three times and boiled in 2× Laemmli buffer for 5 min to release bound Ras protein. Ras was detected by Western blotting using a pan-Ras antibody (Catalog #OP40-100UG, Oncogene Research Products, Boston, Massachusetts). Total Ras levels were determined by Western blot analysis of an aliquot of the lysates removed before immunoprecipitation.

ERK and PKB activation

Myb cells suspended at 1 × 10^6 per ml were washed as described above for the Ras-GTP assay and cultured without GM-CSF in 20% FBS DMEM-H21 at 37°C in 5% CO_2 for 4 hr. Aliquots of cells (1 × 10^6 per condition) were stimulated with growth factors for 10 min before lysis in Holstrom buffer (Holmstrom et al., 1998). Phospho-ERK levels were measured by Western blotting using a phospho-p44/42 MAPK antibody (Catalog #9101, Cell Signaling). The blots were stripped and probed with anti-ERK antibody (Catalog #sc-154-G, Santa Cruz Biotechnologies, Santa Cruz, California) to control for protein loading. PKB/Akt kinase activity was detected using the Akt Kinase Assay Kit (Cell Signaling), which utilizes GSK fusion protein as a PKB substrate. Phospho-GSK levels were detected by Western blotting according to the manufacturer's instructions.

GM-CSF production by cultured Myb cells and antibody treatment

Cells suspended at 1 × 10^6 per ml in 2 ml wells were washed to remove GM-CSF and cultured as described above. After 4 hr, the cells were centrifuged and lysed in 1 ml of Trizol (Invitrogen). RNA prepared according to the manufacturer's instructions was reverse transcribed into cDNA using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, California). Real time polymerase chain reaction analysis was performed on an ABI PRISM 7700 Sequence Detection System using murine GM-CSF Taqman Assay Reagents (PE Biosystems, Foster City, California) according to the manufacturer's instructions. GM-CSF mRNA expression was normalized to GAPDH. A cell line that expresses constitutively high levels of murine GM-CSF was used as a positive control. We used a rat anti-mouse GM-CSF antibody from abcam (Catalog #ab7377-50; Cambridge, United Kingdom), which was added at a final concentration of 4 μg/ml to *Myb* cultures as described in the text. We based the dose of antibody on published data (O'Garra et al., 1989).

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References

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

Bader, J.L., and Miller, R.W. (1978). Neurofibromatosis and childhood leukemia. J. Pediatr. *92*, 925–929.

Bagby, G.C.J., Dinarello, C.A., Neerhout, R.C., Ridgway, D., and McCall, E. (1988). Interleukin 1-dependent paracrine granulopoiesis in chronic granulocytic leukemia of the juvenile type. J. Clin. Invest. *82*, 1430–1436.

Bernards, A. (1995). Neurofibromatosis type 1 and Ras-mediated signaling: filling in the GAPs. Biochim. Biophys. Acta *1242*, 43–59.

Birnbaum, R.A., O'Marcaigh, A., Wardak, Z., Zhang, Y.Y., Dranoff, G., Jacks, T., Clapp, D.W., and Shannon, K.M. (2000). Nf1 and Gmcsf interact in myeloid leukemogenesis. Mol. Cell *5*, 189–195.

Blaydes, S.M., Kogan, S.C., Truong, B.T., Gilbert, D.J., Jenkins, N.A., Copeland, N.G., Largaespada, D.A., and Brannan, C.I. (2001). Retroviral integration at the Epi1 locus cooperates with Nf1 gene loss in the progression to acute myeloid leukemia. J. Virol. *75*, 9427–9434.

Boguski, M., and McCormick, F. (1993). Proteins regulating Ras and its relatives. Nature *366*, 643–653.

Bollag, G., Clapp, D.W., Shih, S., Adler, F., Zhang, Y., Thompson, P., Lange, B.J., Freedman, M.H., McCormick, F., Jacks, T., and Shannon, K. (1996).

Loss of *NF1* results in activation of the Ras signaling pathway and leads to aberrant growth in murine and human hematopoietic cells. Nat. Genet. *12*, 144–148.

Brannan, C.I., Perkins, A.S., Vogel, K.S., Ratner, N., Nordlund, M.L., Reid, S.W., Buchberg, A.M., Jenkins, N., Parada, L., and Copeland, N. (1994). Targeted disruption of the neurofibromatosis type 1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. Genes Dev. *8*, 1019–1029.

Chiu, R., Angel, P., and Karin, M. (1989). Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun. Cell *59*, 979–986.

Cichowski, K., and Jacks, T. (2001). NF1 tumor suppressor gene function: narrowing the GAP. Cell *104*, 593–604.

Donovan, S., Shannon, K.M., and Bollag, G. (2002). GTPase activating poteins: critical regulators of intracellular signaling. Biochim. Biophys. Acta *1602*, 23–45.

Emanuel, P.D., Bates, L.J., Ahu, S.W., Castleberry, R.P., Gualtieie, R.J., and Zuckerman, K.S. (1991a). The role of monocyte-derived hemopoietic growth factors in the regulation of myeloproliferation in juvenile chronic myelogenous leukemia. Exp. Hematol. *19*, 1017–1024.

Emanuel, P.D., Bates, L.J., Castleberry, R.P., Gualtieri, R.J., and Zuckerman, K.S. (1991b). Seletive hypersensitivity to granulocyte-macrophage colony stimulating factor by juvenile chronic myeloid leukemia hematopoietic progenitors. Blood *77*, 925–929.

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

Estrov, Z., Grunberger, T., Chan, H.S., and Freedman, M.H. (1986). Juvenile chronic myelogenous leukemia: characterization of the disease using cell cultures. Blood *67*, 1382–1387.

Freedman, M.H., Cohen, A., Grunberger, T., Bunin, N., Luddy, R.E., Saunders, E.F., Shahidi, N., Lau, A., and Estrov, Z. (1992). Central role of tumor necrosis factor, GM-CSF, and interleukin 1 in the pathogenesis of juvenile chronic myelogenous leukemia. Br. J. Haematol. *80*, 40–48.

Gonda, T.J., Buckmaster, C., and Ramsay, R.G. (1989a). Activation of c-myb by carboxy-terminal truncation: relationship to transformation of murine hematopoietic cells in vitro. EMBO J. *8*, 1777–1783.

Gonda, T.J., Ramsay, R.G., and Johnson, G.R. (1989b). Murine myeloid cell lines derived by in vitro infection with recombinant c-myb retroviruses express myb from rearranged vector proviruses. EMBO J. *8*, 1767–1775.

Gonda, T.J., Macmillan, E.M., Townsend, P.V., and Hapel, A.J. (1993). Differentiation state and responses to hematopoietic growth factors of murine myeloid cells transformed by myb. Blood *82*, 2813–2822.

Gualtieri, R.J., Emanuel, P.D., Zuckerman, K.S., Martin, G., Clark, S.C., Shadduck, R.K., Dracker, R.A., Akabutu, J., Nitschke, R., Hetherington, M.L., et al. (1989). Granulocyte-macrophage colony-stimulating factor is an endogenous regulator of cell proliferation in juvenile chronic myelogenous leukemia. Blood *74*, 2360–2367.

Holmstrom, T.H., Chow, S.C., Elo, I., Coffey, E.T., Orrenius, S., Sistonen, L., and Eriksson, J.E. (1998). Suppression of Fas/APO-1-mediated apoptosis by mitogen-activated kinase signaling. J. Immunol. *160*, 2626–2636.

Hsu, J.C., Cressman, D.E., and Taub, R. (1993). Promoter-specific transactivation and inhibition mediated by JunB. Cancer Res. *53*, 3789–3794.

Huber, M., Helgason, C.D., Scheid, M.P., Duronio, V., Humphries, R.K., and Krystal, G. (1998). Targeted disruption of SHIP leads to Steel factor-induced degranulation of mast cells. EMBO J. *17*, 7311–7319.

Ingram, D.A., Yang, F.C., Travers, J.B., Wenning, M.J., Hiatt, K., New, S., Hood, A., Shannon, K., Williams, D.A., and Clapp, D.W. (2000). Genetic and biochemical evidence that haploinsufficiency of the Nf1 tumor suppressor gene modulates melanocyte and mast cell fates In vivo. J. Exp. Med. *191*, 181–188.

Jacks, T., Shih, S., Schmitt, E.M., Bronson, R.T., Bernards, A., and Weinberg, R.A. (1994). Tumorigenic and developmental consequences of a targeted *Nf1* mutation in the mouse. Nat. Genet. *7*, 353–361.

Klesse, L.J., and Parada, L.F. (1998). p21 ras and phosphatidylinositol-3 kinase are required for survival of wild-type and NF1 mutant sensory neurons. J. Neurosci. *18*, 10420–10428.

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.

Li, H., Velasco-Miguel, S., Vass, W.C., Parada, L.F., and DeClue, J.E. (2002). Epidermal growth factor receptor signaling pathways are associated with tumorigenesis in the Nf1:p53 mouse tumor model. Cancer Res. *62*, 4507–4513.

Liu, Q., Sasaki, T., Kozieradzki, I., Wakeham, A., Itie, A., Dumont, D.J., and Penninger, J.M. (1999). SHIP is a negative regulator of growth factor receptor-mediated PKB/Akt activation and myeloid cell survival. Genes Dev. *13*, 786–791.

O'Garra, A., Barbis, D., Wu, J., Hodgkin, P.D., Abrams, J., and Howard, M. (1989). The BCL1 B lymphoma responds to IL-4, IL-5, and GM-CSF. Cell. Immunol. *123*, 189–200.

Passegue, E., Jochum, W., Schorpp-Kistner, M., Mohle-Steinlein, U., and Wagner, E.F. (2001). Chronic myeloid leukemia with Increased granulocyte progenitors in mice lacking JunB expression in the myeloid lineage. Cell *104*, 21–32.

Riccardi, V.M., and Eichner, J.E. (1986). Neurofibromatosis (Baltimore, MD: Johns Hopkins University Press).

Schiro, R., Longoni, D., Rossi, V., Maglia, O., Doni, A., Arsura, M., Carrara, G., Masera, G., Vannier, E., Dinarello, C.A., et al. (1994). Suppression of juvenile chronic myelogenous leukemia colony growth by interleukin-1 receptor antagonist. Blood *83*, 460–465.

Schorpp-Kistner, M., Wang, Z.Q., Angel, P., and Wagner, E.F. (1999). JunB is essential for mammalian placentation. EMBO J. *18*, 934–948.

Shannon, K.M., Watterson, J., Johnson, P., O'Connell, P., Lange, B., Shah, N., Kan, Y.W., and Priest, J.R. (1992). Monosomy 7 myeloproliferative disease in children with neurofibromatosis, type 1: epidemiology and molecular analysis. Blood *79*, 1311–1318.

Side, L.E., and Shannon, K.M. (1998). The NF1 gene as a tumor suppressor. In Neurofibromatosis Type *1*, M. Upashyaya and D.N. Cooper, eds. (Oxford, UK: Bios Scientific Publishers), pp. 133–152.

Stiller, C.A., Chessells, J.M., and Fitchett, M. (1994). Neurofibromatosis and childhood leukemia/lymphoma: A population-based UKCCSG study. Br. J. Cancer *70*, 969–972.

Szabowski, A., Maas-Szabowski, N., Andrecht, S., Kolbus, A., Schorpp-Kistner, M., Fusenig, N.E., and Angel, P. (2000). c-Jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. Cell *103*, 745–755.

Taylor, S.J., and Shalloway, D. (1996). Cell cycle-dependent activation of Ras. Curr. Biol. *6*, 1621–1627.

Vogel, K.S., Brannan, C.I., Jenkins, N.A., Copeland, N.G., and Parada, L. (1995). Loss of neurofibromin results in neurotropin-independent survival of embryonic sensory and sympathetic neurons. Cell *82*, 733–742.

Wang, C.Y., Bassuk, A.G., Boise, L.H., Thompson, C.B., Bravo, R., and Leiden, J.M. (1994). Activation of the granulocyte-macrophage colony-stimulating factor promoter in T cells requires cooperative binding of Elf-1 and AP-1 transcription factors. Mol. Cell. Biol. *14*, 1153–1159.

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.