Members of the Ras superfamily of signaling proteins modulate fundamental cellular processes by cycling between an active GTP-bound conformation and an inactive GDP-bound form. Neurofibromin, the protein product of the \textit{NF1} tumor suppressor gene, and p120GAP are GTPase-activating proteins (GAPs) for p21\textsuperscript{Ras} (Ras) and negatively regulate output by accelerating GTP hydrolysis on Ras. Neurofibromin and p120GAP differ markedly outside of their conserved GAP-related domains (GRDs), and it is therefore unknown if the respective GRDs contribute functional specificity. To address this question, we expressed the GRDs of neurofibromin and p120GAP in primary cells from \textit{NF1} mutant mice in vitro and in vivo. Here we show that expression of neurofibromin GRD, but not the p120GAP GRD, restores normal growth and cytokine signaling in three lineages of primary \textit{NF1}-deficient cells that have been implicated in the pathogenesis of neurofibromatosis type 1 (NF1). Furthermore, utilizing a GAP-inactive mutant \textit{NF1} GRD identified in a family with NF1, we demonstrate that growth restoration is a function of \textit{NF1} GRD GAP activity on p21\textsuperscript{Ras}. Thus, the GRDs of neurofibromin and p120GAP specify nonoverlapping functions in multiple primary cell types.

Mutations in \textit{NF1} cause neurofibromatosis type 1 (NF1),\textsuperscript{1} a common disorder characterized by increased risk of specific benign and malignant tumors that primarily arise from neural crest-derived tissues. Children with NF1 are predisposed to juvenile myelomonoctytic leukemia (JMML) (1, 2), and 10% of heterozygous \textit{NF1} mutant mice also develop a JMML-like myeloproliferative disorder. JMML bone marrows and other NF1-associated tumors frequently show loss of constitutional heterozygosity at \textit{NF1}, consistent with its tumor suppressor function. Although neurofibromin is a large protein, the GRD is the only segment known to function in growth control. Like p120GAP, the GRD of neurofibromin binds Ras with high affinity and induces a 10\textsuperscript{5}-fold increase in the rate of GTP hydrolysis. The high degree of sequence homology within the catalytic domains of p120GAP and neurofibromin led to the hypothesis that these proteins may be functionally interchangeable (3). However, \textit{NF1} and \textit{Gap} mutant mice have distinct phenotypes, and mutations in the human p120GAP catalytic domains are not associated with any disease state. Moreover, neurofibromin and p120GAP differ markedly in their non-GAP domains. p120GAP is a smaller protein that contains a number of modules common to signaling proteins including Src homology-2 and Src homology-3 and pleckstrin homology domains. None of these domains are present in neurofibromin. In fact, neurofibromin is most closely related to products of the yeast \textit{IRA1} and \textit{IRA2} genes, and its GRD can complement the heat shock phenotype of \textit{IRA} mutant strains resulting from hyperactive Ras (4).

\textit{NF1}-deficient hematopoietic cells and fibroblasts provide an excellent system for dissecting the role of neurofibromin in regulating cell growth, because efficient vectors for transducing genes into these cells are available. Furthermore, Ras signaling and cell growth can be assayed in these primary cell populations. A hallmark of both human JMML cells and of murine \textit{NF1}-deficient myeloid progenitor cells is a selective hypersensitivity of cultured FU\textsuperscript{C}-GM progenitors to granulocyte macrophage-colony stimulating factor (GM-CSF) (5). Whereas homozgyous \textit{NF1} mutant (\textit{NF1}−/−) embryos die in utero, adoptive transfer of \textit{NF1}−/− fetal liver cells consistently induces a JMML-like myeloproliferative disorder in irradiated recipients (6, 7). GM-CSF plays a central role in establishing and maintaining this phenotype in vivo (8). Primary \textit{NF1}−/− hematopoietic cells demonstrate constitutive activation of Ras-ERK signaling with hyperactivation in response to GM-CSF and other cytokines (7). Similarly, mast cells and fibroblasts have a hyperproliferative phenotype in response to stem cell factor (SCF) (9) and epidermal growth factor (EGF) (10), respectively.

**EXPERIMENTAL PROCEDURES**

\textbf{Isolation of Fetal Hematopoietic Cells—}\textit{NF1}+/+ mice were mated to produce \textit{NF1}−/− embryos. Pregnant \textit{NF1}+/+ females were sacrificed at day 13.5 of gestation. Individual fetal livers were placed in Iscove’s modified Dulbecco’s medium supplemented with 20% fetal calf serum as described previously (7). A single cell suspension was prepared by passing the hepatic tissues through progressively smaller needles (16–27-gauge).

\textbf{Genotyping Fetal Tissues—}Genomic DNA was isolated from fetal tissues as described previously (7). The targeting vector used to disrupt the murine \textit{NF1} gene truncates exon 31 and inserts a neomycin resist-
ance gene (neo) (11). Previously described primers were employed to distinguish disrupted and wild type genes in an assay based on the polymerase chain reaction (11).

Generation of Recombinant Retroviral Plasmids—Recombinant retroviral constructs were developed using the murine stem cell virus (MSCV) backbone described by Dr. Robert Hawley (12). The internal sequences of these constructs are under the transcriptional control of the myeloproliferative sarcoma retrovirus promoter. The construct also contains a puromycin resistance gene, pac, which is under the transcriptional control of the phosphoglycerate kinase promoter. By using standard cloning techniques, three viruses were developed for use in these experiments as follows: 1) a virus expressing the full-length NF1 GRD (13) and pac (MSCV-NF1 GRD-pac); 2) a construct encoding the p120GAP GRD and pac (13) (MSCV-p120GAP GRD-pac); and 3) a construct encoding the selectable marker gene alone (MSCV-pac). The NF1 GRD and p120GAP GRD constructs both contain a KT3 epitope tag at the 3' end of the GRD sequences. Mutagenesis of NF1 GRD (R1276P) was accomplished utilizing an in vitro site-directed mutagenesis kit (Stratagene).

Transfection of Retrovirus Plasmid into Packaging Cell Lines and Evaluation of Retroviral Titer—Recombinant retrovirus plasmids were transfected as previously described into a GP + E 86 packaging cell line previously developed by Dr. Arthur Banks (14). Evaluation of the retroviral titer of individual clones expressing the NF1 GRD cDNA or p120GRD cDNA was determined using serial dilutions of viral supernatant then selected by adding puromycin (1 μg/ml) (13) to the cultures to select for virus production. 

Expression and Activity of Recombinant Protein in Primary Cells—Murine embryonic fibroblasts were transduced with the respective retroviruses, and expression of NF1 GRD and p120GAP GRD was analyzed by Western blotting using the anti-KT3 antibody (13). Activity of recombinant protein was then determined by incubating immunoprecipitated protein with Ras-GTP and measuring phosphate release in a GAP activity assay as described previously (15).

Retroviral Infection of Hematopoietic Progenitors—The transduction protocol has been previously described (16) and was employed here with minor modifications. Briefly, low density mononuclear cells recovered from genotyped livers were prestimulated for 48 h in liquid cultures of Iscove's modified Dulbecco's medium containing 20% fetal bovine serum (HyClone) supplemented with SCF (100 ng/ml) (PeproTech) and interleukin-6 (200 units/ml) (PeproTech). Cells were transduced on mitomycin C-treated E86 producer cells in the presence of SCF, interleukin-6, and Polybrene (5 μg/ml) for 48 h. Transduced cells were then plated in methylcellulose culture as described below.

Methylcellulose Cultures—Following culture, fetal liver cells were recovered and plated at a concentration of 5 × 10^4 cells/ml in triplicate methylcellulose cultures containing increasing log doses of GM-CSF (0.01–10 ng/ml) (Peprotech) and puromycin (1 μg/ml) to select for transduced progenitors. Peritoneal cells were plated at a concentration of 1 × 10^6 cells/ml in methylcellulose cultures containing 10 units/ml and 100 ng/ml SCF. Cells cultured were maintained at 37 °C in a humidified incubator containing 5% CO_2 and 95% O_2 and colony forming unit-granulocyte macrophage (CFU-GM) and colony forming unit-mast (CFU-Mast) were scored on day 10 of culture.

Growth Kinetics Assays—Murine embryonic fibroblasts (MEFs) from individual NF1 +/+ and NF1 −/− embryonic mice were transduced with viral supernatant then selected by adding puromycin (1 μg/ml) to the cultures. Selected fibroblasts were seeded in a 6-well tissue culture-treated plate at a concentration of 2 × 10^5 cells per well and cultured for 48 h. One μCi of [3H]thymidine was added to all cultures during the last 6 h of culture incubation. Triplicate cultures were collected on glass filters using a cell harvester, and thymidine incorporation was determined by a scillation counter. In vitro proliferation of BMMCs was assayed as previously described (9). BMMCs were deprived of growth factors for 24 h, and 2 × 10^5 cells were plated in triplicate in 24-well dishes in 1 ml of RPMI containing 1% glutamine, 10% fetal bovine serum, and 100 ng/ml SCF in a 37 °C, 5% CO_2 humidified incubator. Viable cells were enumerated by trypan blue exclusion 72 h following culture initiation.

Kinase Assay—Activation of ERK and Akt was determined in primary MEFs and c-Kit+ hematopoietic cells by culturing cells in media containing 1% fetal calf serum, 2% penicillin/streptomycin, and 1% L-glutamine for 24 h to establish quiescence. Cells were then stimulated with 5 ng/ml GM-CSF (c-Kit+ cells) or 100 ng/ml RGF (MEFs) (PeproTech). Cells were lysed in nonionic lysis buffer as described previously (9) and equalized for protein concentration using a Pierce BCA assay.

**Fig. 1. Recombinant proteins.** A, constructs developed to express NF1 GRD, p120GAP GRD, or the selectable marker gene alone are indicated. LTR, long terminal repeat. B, expression of recombinant retroviral proteins in three MEF lines analyzed by Western blotting using a KT3 monoclonal antibody. NF1 GRD is ~55 kDa and p120GAP GRD runs at ~40 kDa. C, GAP activity of recombinant proteins recovered from lysates of transduced MEFs. Purified p120 GAP was used as a positive control for Ras GAP activity. Activities of KT3-immunoprecipitated recombinant proteins and control GAP are expressed as fold GAP activity over GAP activity assessed in pac-transduced MEFs, which have been ascribed a value of 1. NS, not significant; p < 0.15.

(Pierce). ERK2 immunoprecipitations were carried out with an anti-ERK2 (C-14) antibody (Santa Cruz Biotechnology) and protein A-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4 °C. Akt immunoprecipitations were carried out with an anti-Akt antibody (New England Biolabs). To determine activity of the respective kinases, immunobeads were subjected to an in vitro kinase reaction using an Elk-1 expression protein (ERK2) (New England Biolabs) or histone 2B (Akt) (Roche Molecular Biochemicals) as substrate. Briefly, the reactions were carried out in 30 μl containing 20 mM MgCl_2, 0.1 mM sodium vanadate, 1 mM dithiothreitol, 30 mM β-glycerophosphate, 5 mM EGTA, 20 mM MOPS, 1 μM ATP, and 10 μg of substrate protein in the presence of 2.5 μCi of [γ-32P]ATP. Reaction mixtures were incubated at 30 °C for 30 min, and reactions were terminated by the addition of 10 μl of sample buffer. Reaction mixtures were resolved by SDS-10% polyacrylamide gel electrophoresis. Gels were dried and subjected to autoradiography. The relative amounts of incorporated radioactivity were determined by densitometry using NIH free software.

In Vivo Mast Cell Knock-in Model—1 × 10^6 transduced and selected BMMCs were injected into the peritoneum of nine W/W^v mice. 10 weeks following transplantation, peritoneal mast cells were recovered.
Retroviruses encoding NF1 GRD (MSCV-NF1 GRD-pac), p120GAP GRD (MSCV-p120GAP GRD-pac), and the control retrovirus (MSCV-pac) are shown in Fig. 1A. Each retrovirus expresses pac sequences that confer puromycin resistance. Recombinant GRD peptide expression was analyzed by immunoblotting utilizing an antibody specific to a KT3 epitope tag incorporated into both GRD peptides (13). Immunoblot analysis of transduced Nf1−/− fibroblasts shows that NF1 GRD and p120GAP GRD are expressed at similar levels in primary cells (Fig. 1B). Furthermore, the immunoprecipitated GRD peptides had similar GAP activity stimulating equivalent phosphate release from GTP-loaded Ras (Fig. 1C). Together, these data demonstrate that recombinant NF1 and p120GAP GRD peptides are expressed at equivalent levels in fibroblasts and accelerate GTP hydrolysis on Ras.

To examine whether expression of NF1 GRD and/or p120GAP GRD can correct the hypersensitivity of Nf1−/− myeloid progenitors to GM-CSF, CFU-GM colonies were enumerated in methylcellulose cultures of transduced Nf1−/− and wild type fetal liver cells. Colony growth was assayed over a range of GM-CSF concentrations in the presence of puromycin. Nf1−/− progenitors transduced with either the control virus or with the p120GAP GRD virus demonstrate a hypersensitive pattern of CFU-GM colony growth (Fig. 2A). Retroviral mediated expression of full-length p120GAP in Nf1−/− myeloid progenitors also failed to restore normal cytokine responsiveness (data not shown). In contrast, Nf1−/− myeloid progenitors expressing NF1 GRD displayed a normal pattern of CFU-GM colony growth in response to GM-CSF in six independent experiments. Similar data were observed when the proliferative rates of transduced Nf1−/− fetal liver CFU-GM were analyzed following stimulation of c-Kit+ cells with saturating concentrations of SCF, a second cytokine known to induce hyperproliferation of Nf1-deficient progenitors (7, 9) (data not shown). Thus, expression of NF1 GRD alone is sufficient to correct the hyper-responsiveness and aberrant proliferation of Nf1−/− deficient myeloid cells.

To test whether these observations were operative in another lineage, we expressed recombinant GRD sequences in Nf1−/− fibroblasts and measured proliferation. MEFs isolated from Nf1−/− embryos exhibit increased proliferation in culture compared with wild type MEFs (18, 19). Triplicate cultures of transduced fibroblasts from individual embryos were established, and proliferation was assessed by thymidine incorporation 48 h later. Nf1−/− deficient MEFs transduced with the control virus or with the p120GAP GRD virus demonstrated a 4-fold higher rate of thymidine incorporation compared with wild type fibroblasts (Fig. 2B). In contrast, expression of recombinant NF1 GRD restored the rate of DNA synthesis in Nf1−/− deficient MEFs to wild type levels. Similar data were observed when proliferation was evaluated by cell counting (data not shown). Therefore, these data confirm the specificity of the NF1 GRD to reduce the hyperproliferation of another primary cell type.

Studies of leukemic cells from children with NF1 and murine Nf1−/− hematopoietic cells have shown elevated levels of GTP-bound Ras and hyperactivation of ERK in response to hematopoietic cytokines (6, 7, 20). To determine whether expression of GRD sequences could reduce ERK activity to wild type levels in Nf1−/− hematopoietic cells, Nf1−/− c-Kit+ cells were transduced with the respective retroviruses, selected in puromycin, and assayed for ERK kinase activity in response to GM-CSF. Nf1−/− deficient cells transduced with the control virus or with the p120GAP GRD virus had markedly elevated ERK activity in response to GM-CSF compared with wild type c-Kit+ cells (Fig. 3A). In contrast, ERK activation in GM-CSF-stimulated Nf1−/− cells transduced with the NF1 GRD-encoding vector was comparable to wild type cells. A similar pattern of ERK activation was observed when MEFs transduced with the respective retroviruses were stimulated with EGF (Fig. 3B). These biochemical data in Nf1−/− deficient cells transduced with the control virus or with the p120GAP GRD virus had markedly elevated ERK activity in response to GM-CSF compared with wild type c-Kit+ cells (Fig. 3A). In contrast, ERK activation in GM-CSF-stimulated Nf1−/− cells transduced with the NF1 GRD-encoding vector was comparable to wild type cells. A similar pattern of ERK activation was observed when MEFs transduced with the respective retroviruses were stimulated with EGF (Fig. 3B). These biochemical data in Nf1−/− deficient hematopoietic progenitors and fibroblasts show that expression of NF1 GRD selectively reduces hyperactive Ras-ERK signaling previously shown to be important in mitogenesis. In addition, we have observed that NF1 GRD but not p120GAP GRD corrects hyperactivation of Akt, a downstream effector of the Ras-phosphatidylinositol 3-kinase pathway (data not shown). Collectively, these biochemical observations are consistent with the correction of the hyperproliferative phenotype seen in cell culture assays.

Although these data strongly suggest that the NF1 GRD is
sufficient to control cellular proliferation, the most definitive method to test gene function is to introduce recombinant sequences into primary cells lacking the gene of interest and examine cellular function in vivo. We have recently demonstrated that bone marrow-derived mast cells (BMMCs) generated from Nf1+/− mice exhibit increased proliferation to SCF (9). Similarly, we have now observed that Nf1−/− mast cells derived from fetal livers also have increased proliferation in response to SCF compared with wild type mast cells or Nf1−/− mast cells expressing NF1 GRD (data not shown). Given these observations, we next utilized a mast cell “knock-in” model to evaluate the effects of transgene expression in vivo (21). W/W mice are mast cell-deficient secondary to a mutation in the c-Kit receptor. Many groups have recently established that embryonic or adult progenitor mast cells can fully reconstitute W/W mice to wild type levels in all relevant organs (21–23). In addition, one group has recently proposed that in vivo reconstitution of mast cell precursors derived from embryonic stem cells is an approach to evaluate the in vivo function of a range of embryonic lethal mutations (24). Therefore, direct injection of cultured mast cells into the peritoneum of these mice offers a sensitive measure of mast cell function in vivo. Since these mice contain no peritoneal mast cells and cultured mast cells can repopulate the peritoneum of these mice, equivalent numbers of transduced wild type or Nf1-deficient mast cells were injected into the peritoneums of W/W mice, and peritoneal mast cell numbers were examined 10 weeks following injection. In three independent experiments, mice transplanted with Nf1−/− mast cells had a 3-fold increase in peritoneal mast cell numbers compared with mice transplanted with wild type mast cells or with Nf1−/− mast cells expressing NF1 GRD (Fig. 4A). We also assessed the ability of these reconstituted mast cells of the respective experimental groups to form clonal mast cell progenitors in vitro. Mast cell progenitors cultured from the peritoneum of mice transplanted with Nf1−/− cells expressing only reporter sequences had a 4-fold increase in mast cell progenitors compared with wild type cells and Nf1−/− mast cells expressing NF1 GRD (Fig. 4B). Thus, introduction of NF1 GRD sequences is sufficient to restore normal mast cell growth in vitro and in vivo.

Given the specificity of the NF1 GRD in correcting the cellular and biochemical Nf1−/− phenotype, we next tested whether this restoration of normal growth is due to a direct effect on Ras proteins. We generated a GAP-inactive mutant of
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NF1 GRD that harbors a mutation in the arginines finger loop (R1276P). This mutation, identified in NF1-associated malignancies, greatly reduces neurofibromin GAP activity (25, 26). The R1276P mutant recombinant protein had no catalytic activity in a GAP assay (Fig. 5A), and expression failed to restore normal GM-CSF responsiveness to NF1/−/− hematopoietic progenitors (Fig. 5B). Similarly, expression of this mutant GRD failed to correct the characteristic ERK2 hyperactivation observed in NF1/−/− MEFs (Fig. 5C). Thus, NF1 GRD exerts a Ras-specific function as regulation of cellular proliferation is dependent on Ras-GAP activity.

Despite its large size, the GRD encodes the only well established functional domain of neurofibromin. Previous studies in immortalized cell lines indicate that expressing NF1 GRD is sufficient to suppress the transformation capacity of an oncogenic K-Ras in a colon carcinoma cell line (27). However, such data cannot implicate sufficient of NF1 GRD to restore normal cellular proliferation. The large size of neurofibromin has led investigators to search for other functional domains outside of the GRD. Recently, a missense mutation was described in an NF1 patient that disrupts a protein kinase A (PKA) phosphoacceptor consensus sequence (28), and these investigators hypothesize that these sequences may define a second functional domain of neurofibromin. These data are of additional interest because of recent observations in Drosophila that have suggested a link between cAMP-mediated signaling and NF1 (29). Although our construct does not contain this putative PKA consensus site, expression of recombinant NF1 GRD was sufficient to restore normal growth and ameliorate the biochemical deficits observed in three cell types frequently implicated in NF1-associated malignancies. Thus, the role of the hypothesized PKA sequences in the human NF1 gene and its relationship to Drosophila function remain unclear. It may be possible that PKA can modulate GAP activity by an unidentified mechanism. For example, lipid regulation of neurofibromin GAP activity differs between full-length protein and its isolated GRD (30). Perhaps PKA-dependent phosphorylation of the full-length protein could expose a lipid-binding site and result in modulation of activity. Additionally, despite a retention of structural homology, it is possible that species differences between Drosophila and humans result in a divergence of function.

Importantly, correction of the cellular and biochemical deficits of NF1/−/− cells by NF1 GRD is dependent on Ras GAP activity as the R1276P arginine-finger mutant, found in several distinct NF1 malignancies, failed to restore normal proliferation. This suggests that the primary role of these domains in these tested cell types is to modulate Ras activity.

Although expressing p120GAP GRD or full-length p120GAP in primary cells induced similar levels of total GAP activity, this did not alter the cellular or biochemical deficits in NF1/−/− cells. This result is consistent with hyperactive Ras signaling in leukemia cells from patients with NF1 despite near-normal levels of total cellular GAP activity (20). One potential explanation for our observations regarding the differential functions of the two GAPs is that neurofibromin and p120GAP may modulate different Ras isoforms in vivo. Alternatively, our experimental observations might be explained by differences in the catalytic or association kinetics of NF1 GRD and p120GAP GRD with Ras. The crystal structure of the catalytic regions of the two GRDs has been resolved (26, 31), analyzed kinetically (32), and described as retaining high structural homology. Both fragments possess a conserved Ras binding groove, an arginine finger necessary for catalysis, and stabilization components including a phenylalanine-leucine-arginine motif important in positioning of the arginine finger loop. However, differences in amino acid composition within each of these motifs were hypothesized to alter interactions with Ras. Specifically, two residues within the Ras binding groove of the NF1 GRD have been identified that have electrostatic or hydrogen bonding potential with Glu-37 and Glu-63 of Ras. The presence of neutral or oppositely charged residues at these same locations within p120GAP may be responsible, in part, for differential affinities.
of the two GAPs for Ras. Additionally, slight deviations in residues proximal to the arginine finger loop of NF1 GRD are thought to impart more flexibility to this key catalytic motif (26). Overall, these differences may translate into a major alteration in the kinetics of Ras-GTP complex formation with the respective GAPs. In support of these structural data, kinetic data in cell-free systems have demonstrated that the affinity of NF1 for Ras is severalfold higher than that of p120GAP at physiological concentrations of Ras-GTP (33), and the association and dissociation rate constants of p120GAP-Ras complex are faster than that of the neurofibromin-Ras complex (33). Our in vitro and in vivo studies in primary NF1-deficient cells support these structural and in vitro biochemical predictions.

Although the studies presented here suggest distinct roles of NF1 GRD and p120GAP GRD in regulating Ras output in fibroblasts and hematopoietic cells, the contribution of individual residues and localization sequences within these domains to GTP hydrolysis remains unclear. In vitro mutagenesis of p120GAP and neurofibromin, followed by introduction of these sequences into NF1/−/− cells, may help define these functions. In addition, such studies may further define how each of these GAPs coordinates with other signaling proteins and the functional significance of these interactions. Future studies utilizing these techniques will provide a better understanding of the physiological roles of these two GAPs and may further define molecular mechanisms of disease pathogenesis when NF1-specific GAP function is perturbed.

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REFERENCES

1. Brodeur, G. (1994) N. Engl. J. Med. 336, 637–638
2. Side, L., Taylor, B., Cayouette, M., Conner, E., Thompson, P., Luie, M., and Shannon, K. (1997) N. Engl. J. Med. 336, 1713–1720
3. Bollag, G., and McCormick, F. (1992) Nature 356, 663–664
4. Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M., and Collins, F. (1990) Cell 63, 851–859
5. Emanuel, P., Bates, L., Castleberry, R., Gualtieri, R., and Zuckerman, K. (1991) Exp. Hematol. 19, 1017–1024
6. Largaespada, D., Brannan, C., Jenkins, N., and Copeland, N. (1996) Nat. Genet. 12, 137–143
7. Zhang, Y., Vik, T., Ryder, J., Secor, E., Jacks, T., Shannon, K., and Clapp, D. (1998) J. Exp. Med. 187, 1893–1902
8. Birnbaum, R., O’Marcaigh, A., Wardak, Z., Zhang, Y., Drnoff, G., Jacks, T., Clapp, D., and Shannon, K. (2000) Mol. Cell. 5, 189–195
9. Ingram, D., Yang, P., Travers, J., Wensing, M., Hiai, K., New, S., Hood, A., Shannon, K., Williams, D., and Clapp, D. (2000) J. Exp. Med. 191, 181–188
10. Atit, R., Crowe, M., Greenhalgh, D., Wenstrup, R., and Ratner, N. (1999) J. Invest. Dermatol. 112, 825–842
11. Jacks, T., Shih, T., Schmidt, R., Benson, R., Bernards, A., and Weinberg, R. (1994) Nat. Genet. 5, 353–361
12. Hawley, R., Lieu, F., Fong, A., and Hawley, T. (1994) Gene Ther. 1, 136–138
13. Martin, G., Viskochil, D., Bollag, G., McCabe, P., Cressier, W., Haubrich, H., Conroy, L., Clark, R., O’Conell, P., Cawthon, R., Minis, I., and McCormick, F. (1990) Cell 63, 843–849
14. Markowitz, D., Goff, S., and Banks, A. (1998) Virology 167, 400–406
15. Bollag, G., and McCormick, F. (1991) Nature 353, 576–579
16. Clapp, D., Freie, B., Secor, E., Yoder, M., Fortney, K., and Gerson, S. (1995) Exp. Hematol. 23, 630–638
17. Nakano, T., Sonoda, T., Hayashi, C., Yamatodani, A., Kanayama, Y., Yamamura, T., Arai, H., Yonezawa, T., Kitamura, Y., and Galli, S. (1985) J. Exp. Med. 162, 1025–1043
18. Brannan, C., Perkins, A., Vogel, K., Ratner, N., Nordland, M., Reid, S., Buchberg, A., Jenkins, N., Parada, L., and Copeland, N. (1994) Genes Dev. 8, 1019–1029
19. Rosenbaum, T., Boissy, Y., Kombrink, K., Brannan, C., Jenkins, N., Copeland, N., and Ratner, N. (1995) Development 121, 3583–3592
20. Bollag, G., Clarke, D., Shih, S., Adler, F., Zhang, Y., Thompson, P., Lange, B., Freedman, M., McCormick, F., Jacks, T., and Shannon, K. (1996) Nat. Genet. 12, 144–148
21. Rodewald, H., Dessing, M., Dvorak, A., and Galli, S. (1996) Science 271, 818–823
22. Galli, S., Tsai, M., and Weshil, B. (1993) Am. J. Pathol. 142, 965–974
23. Secor, V., Secor, W., Gutekunst, C., and Brown, M. (2000) J. Exp. Med. 191, 123–135
24. Tsai, M., Wedemeyer, J., Ganiatsas, S., Tam, S., Zon, L., and Galli, S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 9186–9190
25. Klse, A., Ahmadian, M., Schuelke, M., Scheffzek, K., Hoffmeyers, S., Greeves, A., Schmitz, F., Kaufmann, D., Peters, H., Wittinghofer, A., and Nurnberg, P. (1998) Hum. Mol. Genet. 7, 1261–1268
26. Scheffzek, K., Ahmadian, M., Wismuller, L., Kaebisch, W., Stege, P., Schmitz, F., and Wittinghofer, A. (1998) EMBO J. 17, 4313–4327
27. Li, Y., and White, R. (1998) Cancer Res. 58, 2872–2876
28. Falsold, R., Hoffmeyer, S., Mischung, C., Gille, C., Ehlers, C., Kuckreylen, N., Abdel-Nour, M., Gewies, A., Peters, H., Kaufmann, D., Buske, A., Tinscher, S., and Nurnberg, P. (2000) Am. J. Hum. Genet. 66, 790–818
29. The, I., Hannigan, E., Cowley, G., Reginald, S., Zhong, Y., Gussela, J., Haririan, I., and Bernards, A. (1997) Science 276, 791–794
30. Bollag, G., McCormick, F., and Clark, R. (1993) EMBO J. 12, 1923–1927
31. Scheffzek, K., Ahmadian, M., Kaebisch, W., Wismuller, L., Lautwein, A., Schmitz, F., and Wittinghofer, A. (1997) Science 277, 333–338
32. Ahmadian, M., Wismuller, L., Lautwein, A., Biachoff, F., and Wittinghofer, A. (1999) J. Biol. Chem. 274, 16409–16415
33. Martin, G., Yatani, A., Clark, R., Conroy, L., Polakis, P., Brown, A., and McCormick, F. (1992) Science 255, 192–194