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Biochemical and Functional Analysis of Ras Pathway Mutations in Myeloid Leukemia and Developmental Disorders

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Biochemical and Functional Analysis of Ras Pathway Mutations in Myeloid Leukemia and Developmental Disorders

by

Suzanne Schubbert

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
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by

Suzanne Schubbert
This dissertation is dedicated to my mother and father
Acknowledgements

My graduate student years at UCSF have been a very positive experience both professionally and personally. I have had a valuable training experience and have enjoyed working with many talented, knowledgeable, and insightful colleagues, collaborators, and friends. It is difficult to adequately acknowledge the contributions that everyone has made toward my graduate career and development, but I’ll do my best in these next few paragraphs.

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Contributions to presented work

Chapter 2 of this dissertation contains previously published material:

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Cheng, J.W., Lee, C.M., Stokoe, D., Bonifas, J.M., Curtiss, N.P., Gotlib, J., Meshinchi,
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Mignon Loh performed the studies described in Chapter 2 under the guidance of
Kevin Shannon. Dr. Loh supervised the screening of a panel of JMML specimens for
PTPN11 mutations. She also directed the screening of CMML, CML, AML, and MDS
specimens for PTPN11 mutations. PCR and denaturing high-performance liquid
chromatography were used for mutation detection. Shashaank Vattikuti, Melissa
Reynolds, Jennifer Cheng, and Connie Lee provided technical assistance with these
screening efforts. Jeannette Bonifas and Kenneth Lieuw provided assistance with Ptpn11
cloning. David Stokoe assisted with modeling the mutations and understanding the
effects of the mutations on SHP-2 structure. I generated the Ptpn11 expression constructs
and designed and performed the functional and biochemical studies with guidance from
Mignon Loh and Kevin Shannon.

Chapter 3 of this dissertation contains previously published material:

Schubbert, S., Liew, K., Rowe, S.L., Lee, C.M., Li, X., Loh, M.L., Clapp, D.W.,
The experiments in this study were designed and performed by myself under the guidance of Kevin Shannon with a collaboration with XiaXin Li under the supervision of D. Wade Clapp. XiaXin Li performed the HPP-CFC and LPP-CFC assays shown in Figure 3. Sara Rowe assisted with harvesting fetal liver and bone marrow cells from mice and cell culture.

Chapter 4 of this dissertation contains previously published material:

Christian Kratz screened Noonan syndrome patient samples for RAS mutations and discovered the de novo germline KRAS mutations. These screening efforts were performed in collaboration with Martin Zenker, who provided clinical expertise, Charlotte Niemeyer, and other clinical collaborators. The functional studies were performed by myself under the supervision of Kevin Shannon in collaboration with Gideon Bollag. Gideon Bollag introduced the GTP hydrolysis and GAP assays to our laboratory and provided invaluable biochemistry expertise. Hoa Nguyen, Brian West, and Gideon Bollag provided technical assistance with generating recombinant K-Ras and GRD proteins in E. coli.

Chapter 5 of this dissertation contains material that has been submitted to Molecular and Cellular Biology. Coauthors include Gideon Bollag, Natalya Lyubynska, Hoa Nguyen,
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Abstract

Biochemical and Functional Analysis of Ras Pathway Mutations in Myeloid Leukemia and Developmental Disorders

Suzanne Schubbert

Noonan syndrome is a common dominant disorder characterized by short stature, facial dysmorphism, cardiac defects, and a predisposition to juvenile myelomonocytic leukemia (JMML). Germline \textit{PTPN11} mutations cause ~50% of Noonan syndrome. \textit{PTPN11} encodes SHP-2, a protein tyrosine phosphatase that relays signals from activated receptor complexes to Ras and other effectors. Studies of patient samples and in mouse models have demonstrated that hyperactive Ras signaling plays a central role in JMML. Based on the association of JMML with Noonan syndrome and the known role of SHP-2 in Ras signaling, our laboratory and others screened the \textit{PTPN11} gene in JMML, and discovered somatic mutations in ~35% of cases.

I performed functional studies of mutant SHP-2 proteins associated with JMML and Noonan syndrome. I used primary murine hematopoietic cells to investigate the effects of mutant SHP-2 on proliferation, survival, and differentiation. The most common leukemia-associated amino acid substitution (E76K) induced a hypersensitive pattern of myeloid progenitor colony growth in response to granulocyte-macrophage colony-stimulating factor and interleukin 3 that was dependent on SHP-2 catalytic activity. E76K SHP-2 expression also enhanced the growth of immature progenitor cells, perturbed erythroid growth, and impaired normal differentiation. In addition, leukemia-associated SHP-2 mutations conferred stronger phenotypes in primary hematopoietic
progenitors than a germline mutation found in individuals with Noonan syndrome. Although *PTPN11* mutations account for 50% of Noonan syndrome, the genetic lesions in the remaining individuals were unknown. Our collaborators discovered *de novo* germline *KRAS* mutations that introduce V14I, T58I, or D153V amino acid substitutions in individuals with Noonan syndrome and P34R and F156L alterations in individuals with cardio-facio-cutaneous syndrome, which has overlapping phenotypic features with Noonan syndrome. I performed biochemical and functional analysis of these novel syndrome-associated K-Ras proteins. Mutant K-Ras proteins demonstrate a range of gain-of-function effects in different cell types, and biochemical analysis supports the idea that the intrinsic Ras guanosine nucleotide triphosphatase (GTPase) activity, the responsiveness of these proteins to GTPase activating proteins, and guanine nucleotide exchange all regulate developmental programs *in vivo.*
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Chapter 1

Introduction
Ras proteins are signal switch molecules that regulate cell fates by cycling between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound conformations (Ras-GTP and Ras-GDP) (reviewed in\textsuperscript{1,2}). The three mammalian \textit{RAS} genes encode four highly homologous 21 kD proteins – H-Ras, N-Ras, K-Ras4A and K-Ras4B; the latter two isoforms result from alternative splicing at the C-terminus. Figure 1 presents a simplified overview of Ras signaling. The competing activities of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) control Ras-GTP levels \textit{in vivo} (reviewed in\textsuperscript{2,3}). Ras proteins regulate cellular responses to many extracellular stimuli, including soluble growth factors. Growth factor binding to cell surface receptors creates docking sites for adapter molecules and signal relay proteins that recruit and activate GEFs such as SOS1. GEFs displace guanine nucleotides from Ras and permit passive binding to GTP, which is abundant in the cytosol.

When GTP-bound, Ras modulates cell behavior by binding to and activating distinct classes of effector molecules. The Raf/MEK/ERK cascade is the best characterized Ras effector pathway\textsuperscript{4}. There are three Raf serine/threonine kinases (A-Raf, B-Raf, and Raf-1) that activate the MEK/ERK kinase cascade. ERK kinases can phosphorylate both cytosolic and nuclear substrates, which include transcription factors such as \textit{c-jun} and ELK1, the latter an E26 transformation-specific sequence (ETS) family member that forms part of the serum response factor that regulates \textit{FOS} expression\textsuperscript{5}. Jun and Fos proteins form the activator protein-1 (AP-1) transcription factor. Activation of these transcriptional regulators can lead to the expression of proteins that control cell cycle progression, such as cyclin D\textsuperscript{6}. Ras-GTP also binds the catalytic subunit of type I
phosphatidylinositol 3-kinases (PI3K)s\textsuperscript{7,8}. This binding results in the translocation of PI3K to the plasma membrane and subsequent activation. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate, which activates downstream kinases such as 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Akt\textsuperscript{9}. Akt is a kinase that promotes survival in many cell types. Akt phosphorylates and inactivates several proapoptotic proteins including BAD and Forkhead (FKHR) transcription factors\textsuperscript{10,11}. PI3K also activates Rac, a Rho family GTPase, which has been shown to be important for transformation by oncogenic Ras in some cellular contexts\textsuperscript{12}. Ras can also activate a family of exchange factors for the Ral small GTPases, which includes RalGDS, RalGDS-like gene (RGL), and RGL\textsuperscript{213}. These exchange factors activate RalA/B, which can stimulate phospholipase D. Signaling from Ras-GTP is terminated by intrinsic Ras GTPase activity. This slow “off” reaction is greatly augmented by GAPs, which bind to the effector domain of Ras-GTP and accelerate hydrolysis by stabilizing a high-energy transition state that occurs during the Ras-GTP hydrolysis reaction.

Activating RAS mutations occur in ~30% of human cancers. Specific RAS genes are mutated in different malignancies: KRAS mutations are prevalent in pancreatic, colorectal, endometrial, biliary tract, lung, and cervical cancers; KRAS and NRAS mutations are found in myeloid malignancies; and NRAS and HRAS mutations predominate in melanoma and bladder cancer, respectively\textsuperscript{14,15}. In most cases, the somatic missense RAS mutations found in cancer cells introduce amino acid substitutions at positions 12, 13, and 61. These changes impair the intrinsic GTPase activity and confer resistance to GAPs, thereby causing cancer-associated mutant Ras proteins to
accumulate in the active, GTP-bound conformation\textsuperscript{16}. The three-dimensional structure of H-Ras bound to the catalytic domain of p120 GAP, known as the GAP-related domain (GRD), has been solved and provides a structural understanding of the biochemical activation of oncogenic Ras proteins\textsuperscript{17} (Figure 2). GAP proteins have a highly conserved arginine finger that interacts with the phosphate binding loop (P loop) of Ras. This interaction stimulates catalysis by stabilizing a transition state of the Ras-GTP hydrolysis reaction. Glutamine-61 is essential for GTP hydrolysis, and substituting any amino acid at this position except glutamic acid blocks hydrolysis\textsuperscript{18}. In the Ras-GAP complex, glutamine-61 points toward the phosphate chain of bound guanine nucleotide and forms a hydrogen bond with the backbone carbonyl group of the catalytic arginine residue, which thereby contributes to the stability of the GTP to GDP transition state. Replacing glycine-12 of Ras with any other amino acid except proline also activates Ras biochemically. These substitutions are thought to be unfavorable in the GTP to GDP transition state because of steric clash of side chains with the arginine finger and with the side chain of glutamine-61. Substituting proline for glycine-12 renders Ras resistant to GAPs, but this protein has increased intrinsic GTP hydrolysis\textsuperscript{19,20}. Importantly, this mutant does not transform cells, which suggests that levels of intrinsic GTPase activity are biologically relevant even though GAPs accelerate GTP hydrolysis thousands of fold. Consistent with this idea, the transforming potential of H-Ras proteins with different codon 61 substitutions is inversely related to intrinsic GTPase activities\textsuperscript{2}.

Juvenile myelomonocytic leukemia (JMML) is a relentless myeloproliferative disorder of early childhood characterized by an excessive proliferation of myeloid lineage cells that infiltrate hematopoietic and non-hematopoietic tissues\textsuperscript{21,22}. A biologic hallmark
of JMML is a hypersensitive pattern of colony forming unit granulocyte-macrophage (CFU-GM) progenitor growth in methylcellulose cultures stimulated with granulocyte-macrophage colony stimulating factor (GM-CSF)\textsuperscript{23}. Studies of human samples and in mouse models strongly implicate hyperactive Ras signaling in the pathogenesis of JMML\textsuperscript{24-27}. Approximately 25\% of JMML bone marrows show oncogenic \textit{RAS} mutations\textsuperscript{28,29}. In addition, the incidence of JMML is increased by >200 fold in children with neurofibromatosis type 1 (NF1), a common familial disorder that is associated with neurocutaneous abnormalities, learning disabilities, and a predisposition to tumors\textsuperscript{30-32}. NF1 is caused by germline deletions or loss of function mutations in the \textit{NF1} gene. \textit{NF1} encodes neurofibromin, a GAP that negatively regulates Ras output by accelerating GTP hydrolysis\textsuperscript{1,2}. Genetic and biochemical analysis of JMML samples have shown that \textit{NF1} functions as a tumor suppressor by negatively regulating Ras\textsuperscript{24,33,34}. Taken together, oncogenic \textit{RAS} mutations or inactivation of \textit{NF1} are found in ~50\% of JMML samples, and are largely mutually exclusive\textsuperscript{29,34,35}.

In addition to NF1, JMML was reported in a few patients with Noonan syndrome\textsuperscript{36-38}, an autosomal dominant disorder characterized by facial dysmorphism, short stature, skeletal malformations, and cardiac defects (reviewed in\textsuperscript{39}). Children with Noonan syndrome show a spectrum of hematologic abnormalities including isolated monocytosis, myeloid disorders with features of chronic myelomonocytic leukemia (CMML) that remit spontaneously and, rarely, JMML\textsuperscript{36-38}. Bone marrow cells from Noonan syndrome patients with JMML show characteristic GM-CSF hypersensitivity in methylcellulose cultures\textsuperscript{38}.
Missense mutations in *PTPN11* cause approximately 50% of cases of Noonan syndrome\textsuperscript{40,41}. *PTPN11* encodes SHP-2, a non-receptor protein tyrosine phosphatase (PTPase) that relays signals from activated receptor complexes to Ras, Src family kinases, and other signaling molecules\textsuperscript{42,43}. SHP-2 contains two src homology 2 (SH2) domains and a catalytic PTPase domain (Figure 3A). The SHP-2 PTPase is activated by binding to phosphotyrosyl peptides through its N-SH2 domain\textsuperscript{42,44}. The SHP-2 crystal structure predicts that these interactions induce a conformational shift that relieves inhibition of the PTPase by the N-SH2 domain\textsuperscript{45}. Most of the mutations reported in Noonan syndrome kindreds are found in exons 3 and 8, which encode segments of the N-SH2 and PTPase domains, respectively (Figure 3B). Molecular modeling suggests that almost all of the exon 3 mutations activate phosphatase activity by altering N-SH2 amino acids that interact with the PTPase domain\textsuperscript{40,41}. More recent biochemical analysis has demonstrated that Noonan syndrome-associated *PTPN11* mutations encode gain-of-function SHP-2 proteins that variably deregulate phosphatase activity, the affinity of the SH2 domains for phosphotyrosyl ligands, and/or substrate specificities\textsuperscript{46}.

SHP-2 participates in signal transduction downstream of cytokine and growth factor receptors to regulate multiple cellular responses including proliferation, differentiation, and migration\textsuperscript{42,43,47,48}. The protein is expressed at high levels in hematopoietic cells and undergoes rapid tyrosine phosphorylation upon activation of the c-KIT, interleukin 3 (IL-3), GM-CSF, and erythropoietin receptors\textsuperscript{47,49,50}. Upon growth factor or cytokine binding, SHP-2 can directly interact with some receptors, including the PDGF receptor and β–common (βc) subunit of the GM-CSF receptor, and can associate with various adapter proteins such as Grb2, FRS-2, IRS-1, Gab1, and Gab2\textsuperscript{48,51} (Figures 1
SHP-2 most often plays a positive role in transducing signals, which is mediated, at least in part, through the Ras/Raf/MEK/ERK cascade in hematopoietic and non-hematopoietic cells. Many studies demonstrate that SHP-2 phosphatase activity is required for its biological functions, however some evidence supports PTPase-independent roles of SHP-2. Genetic and biochemical studies of Corkscrew (Csw), the Drosophila homolog of SHP-2, have revealed a role for Csw in the positive regulation of signal strength from the Torso receptor tyrosine kinase that contributes to terminal structure development in Drosophila. Csw dephosphorylates the Torso receptor, which prevents the docking of Ras GAP, a negative regulator of Ras, to the receptor. In addition, Csw can function as an adapter to recruit Drk, the Drosophila Grb2 homolog, to the activated receptor, which physically links the receptor to Ras activation. While extensive data from Drosophila, Xenopus, and mammalian cells place SHP-2 upstream of Ras, other observations support a more complex role that includes functions either parallel to or downstream of Ras.

Homozygous mouse Ptpn11 mutant embryos fail around E9.5 with multiple defects in gastrulation and mesodermal patterning. Loss of Ptpn11 also has profound effects on the developing hematopoietic system. Ptpn11-deficient yolk sacs contain markedly reduced numbers of hematopoietic colony forming cells, and mutant embryonic stem cells do not contribute to hematopoiesis in chimeras. These and other studies implicate SHP-2 as a crucial mediator of hematopoietic cell fates that modulates signaling from activated receptor complexes.

Together, reports of JMML in children with Noonan syndrome, previous data implicating hyperactive Ras in JMML, the known role of SHP-2 as a positive regulator of
Ras/Raf/MEK/ERK signaling, and the essential role of SHP-2 in myeloid cell
development suggested that mutations of PTPN11 might contribute to JMML and other
myeloid malignancies. To test this hypothesis, our laboratory screened specimens from
patients with JMML and other myeloid malignancies for mutations in PTPN11. Multiple
independent missense PTPN11 mutations were detected in patients with JMML and
CMML. JMML and CMML are both classified as myeloproliferative disorders (MPD)
which are characterized by clonal proliferation of myeloid lineage cells that retain the
capacity to differentiate. We have detected somatic PTPN11 mutations in ~35% of
JMML patients, which are exclusive from RAS mutations and NFI loss. Interestingly,
almost all of these leukemia-associated mutations introduce amino acid substitutions that
are largely distinct from those found in Noonan syndrome\textsuperscript{64-68} (Figure 3B). These
mutations alter the N-SH2 domain of SHP-2 and are predicted to disrupt the inhibitory
interaction between the N-SH2 and PTPase domains of the protein, thereby favoring the
active conformation of the molecule\textsuperscript{45}. The first goal of my thesis work was to determine
the cellular and biochemical consequences of expressing SHP-2 proteins encoded by
leukemia-associated mutant PTPN11 alleles in cultured cell lines. This work is detailed
in Chapter 2 of this dissertation.

The E76K and D61Y substitutions are the most common somatic PTPN11
mutations identified in JMML\textsuperscript{64-67}. Glutamate 76 and Aspartate 61 lie within the N-SH2
domain, and form key contacts with the PTPase domain that are thought to inhibit its
catalytic activity\textsuperscript{45}. E76A and D61A mutant SHP-2 proteins display elevated
phosphatase activity and exhibit gain-of-function activity in a Xenopus animal cap
assay\textsuperscript{57}. The leukemia-associated E76K SHP-2 protein shows elevated phosphatase
activity when expressed in COS-7 cells\textsuperscript{64} and enhances IL-3-independent survival of transduced Ba/F3 cells\textsuperscript{65}.

In order to understand how mutations in \textit{PTPN11} perturb hematopoietic growth and to determine if leukemia-associated \textit{PTPN11} alleles can induce a myeloproliferative disorder (MPD) or leukemia in mice, it is essential to perform functional studies in primary hematopoietic cells. In the second part of my thesis work, I utilized murine bone marrow and fetal liver cells to investigate the effects of Noonan syndrome and JMML-associated mutant SHP-2 proteins on proliferation, survival, and differentiation. In work detailed in Chapter 3 of this dissertation, I found that E76K mutant SHP-2 confers hypersensitivity to GM-CSF and IL-3 in immature and committed myeloid progenitors that is dependent on SHP-2 PTPase activity. Fetal liver cells that were transduced with E76K SHP-2 also formed many more burst forming unit-erythroid (BFU-E) colonies. I also found that expressing E76K SHP-2 results in more pronounced growth factor hypersensitivity than the leukemia-associated D61Y mutant SHP-2, and that both of these mutations confer stronger hematopoietic phenotypes than a N308S substitution that causes Noonan syndrome. E76K SHP-2 expression also induced prolonged progenitor survival, aberrant differentiation, and increased proliferation in liquid cultures. Together, these studies and reports from two other groups\textsuperscript{69,70} provide extensive data regarding the \textit{in vitro} and \textit{in vivo} effects of leukemia-associated \textit{PTPN11} mutations in primary hematopoietic cells.

Noonan syndrome overlaps phenotypically with other several other developmental disorders, including Costello and cardio-facio-cutaneous (CFC) syndromes, which are also characterized by facial dysmorphism, heart abnormalities,
short stature, and mental retardation (Table 1). Patients with Costello syndrome are predisposed to specific cancers, including rhabdomyosarcoma, ganglioneuroblastoma, and bladder cancer (Table 1). These individuals do not have mutations in PTPN11, and Aoki and colleagues\textsuperscript{71} hypothesized that Costello syndrome may be caused by mutations in genes encoding proteins involved in SHP-2 signaling pathways. They identified \textit{de novo} germline mutations in HRAS in 12 of 13 individuals with Costello syndrome, which was corroborated by other groups\textsuperscript{72,73}. Remarkably, the HRAS alleles identified in Costello syndrome introduce amino acid substitutions at codons 12 and 13, such as G12S and G12A that also occur as somatic mutations in tumors. Cultured fibroblasts from affected individuals demonstrate increased proliferation\textsuperscript{71}, and genetic analysis of tumors from two patients revealed loss or silencing of the normal HRAS allele\textsuperscript{71,72}.

\textit{RAS} genes were also considered good candidate genes in the \~50\% of Noonan syndrome individuals without PTPN11 mutations and individuals with CFC syndrome. Our collaborators discovered \textit{de novo} germline KRAS mutations that introduce V14I, T58I, or D153V amino acid substitutions in individuals with Noonan syndrome and P34R or F156L alterations in individuals with CFC syndrome. In the third portion of my thesis work, I investigated the biochemical and functional properties of these novel K-Ras proteins. Chapter 4 of this dissertation describes detailed studies of two Noonan syndrome-associated mutant K-Ras proteins, V14I and T58I, which have not previously been reported in cancer or any human disease. The V14I substitution is located in the P loop of the K-Ras protein and the T58I substitution is near the Switch II domain. The infant with the T58I substitution presented with a JMML-like myeloproliferative disease. Recombinant V14I and T58I K-Ras proteins displayed impaired intrinsic GTP hydrolysis.
and responsiveness to GTPase-activating proteins (GAPs) in comparison to wild-type K-Ras, but were not as defective as oncogenic G12D K-Ras. T58I and V14I K-Ras rendered primary hematopoietic progenitors hypersensitive to growth factors and deregulated signal transduction in a cell-lineage specific manner.

My most recent studies have focused on the P34R, D153V, and F156L K-Ras mutant proteins. The P34R substitution is located in the Switch I domain, which directly binds Ras effectors. The P34R mutation, which was previously identified in a screen for activating mutations in the effector domain of H-Ras, transforms rodent fibroblasts, accumulates in the GTP-bound state, and can activate Raf-1. The D153V and F156L substitutions are located in the α5-helix of K-Ras4B. The D153V substitution has also been reported in CFC and severe Noonan syndrome. Structural modeling in one study suggested that aspartate-153 participates in interactions that contribute to guanine nucleotide binding. These authors predicted that D153V destabilizes guanine nucleotide binding and leads to increased GDP and GTP dissociation. Since GTP is approximately 25 times more abundant in cells, this would result in more active, GTP bound Ras in vivo. Phenylalanine-156 is conserved in all members of the Ras superfamily of proteins and in vitro analysis of the F156L substitution in H-Ras demonstrated that this mutant displays a rapid rate of guanine nucleotide dissociation and transforms NIH3T3 cells. Chapter 5 of this dissertation describes the studies I have performed to examine the biochemical and functional properties of the syndrome-associated P34R, D153V, and F156L K-Ras proteins. Both P34R and D153V K-Ras display normal levels of intrinsic GTP hydrolysis, whereas F156L K-Ras appears as defective intrinsically as oncogenic G12D K-Ras. P34R K-Ras is completely resistant to
both neurofibromin and p120 GAP, which is intriguing as these data suggest that the ability of GAPs to down-regulate Ras-GTP levels is dispensable for development. In contrast, D153V K-Ras has a normal response to GAPs, whereas F156L K-Ras displays a partial response. Interestingly, the F156L K-Ras mutant displays a markedly increased guanine nucleotide exchange rate in vitro. P34R, D153V, and F156L K-Ras show a range of gain-of-function effects in cells, but are less potent than oncogenic G12D K-Ras. Notably, these mutant K-Ras proteins induce growth factor hypersensitivity in primary hematopoietic progenitors and aberrantly activate Ras signaling pathways. Together, these data show that deregulated K-Ras signaling causes Noonan and CFC syndromes, and underscore the importance of K-Ras in development. The range of biochemical and functional properties displayed by mutant K-Ras proteins supports the idea that intrinsic GTPase activity, responsiveness to GAPs, and guanine nucleotide exchange all regulate Ras output in vivo.
Table 1

Developmental disorders associated with aberrant Ras signaling

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Causative Gene(s)</th>
<th>Associated tumors and cancers</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>Neurofibromatosis type 1</td>
<td>NF1</td>
<td>Neurofibromas, Astrocytoma, Pheochromocytoma, JMML, Malignant peripheral nerve sheath tumors</td>
<td>Familial cancer syndrome caused by loss-of-function mutations affecting neurofibromin. Hallmark features include hyperpigmented skin lesions and benign neurofibromas. Learning disabilities and vascular abnormalities common. Malignancies frequently show loss of normal NF1 allele.</td>
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<tr>
<td>Noonan syndrome</td>
<td>PTPN11, KRAS, SOS1</td>
<td>JMML</td>
<td>Mutations encode gain-of-function proteins in upstream components of the Ras/Raf/MEK/ERK pathway. Clinical features include short stature, facial dysmorphism, skeletal abnormalities, cardiac defects, and learning disabilities.</td>
</tr>
<tr>
<td>LEOPARD syndrome</td>
<td>PTPN11</td>
<td>Neuroblastoma, Myeloid leukemia</td>
<td>Mutations encode SHP-2 proteins with defective phosphatase activity and dominant negative properties. Clinical features include short stature, cardiac abnormalities, lentigines, and deafness.</td>
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<tr>
<td>Costello syndrome</td>
<td>HRAS</td>
<td>Rhabdomyosarcoma, Neuroblastoma, Ganglioneuroblastoma, Bladder cancer</td>
<td>Mutations encode strong gain-of-function proteins and overlap with somatic mutations found in cancer. Loss of wild-type HRAS allele reported in malignancies of Costello syndrome individuals.</td>
</tr>
<tr>
<td>Cardio-facio-cutaneous syndrome</td>
<td>KRAS, BRAF, MEK1, MEK2</td>
<td>none</td>
<td>Mutations occur in downstream components of the Ras/Raf/MEK/ERK pathway. Most mutant proteins show biochemical gain-of-function.</td>
</tr>
</tbody>
</table>
**Figure 1**

**The Ras signaling pathway.**

This figure highlights proteins affected by mutations in developmental disorders and cancer. Growth factor binding to cell surface receptors results in activated receptor complexes, which contain adapters such as SHC (SH2-containing protein) and GRB2 (growth factor receptor bound protein 2). These proteins recruit SHP-2 and SOS1, the latter increasing Ras-GTP levels by catalyzing nucleotide exchange on Ras. The GTPase-activating protein neurofibromin (NF1) binds to Ras-GTP and accelerates the conversion of Ras-GTP to Ras-GDP, which terminates signaling. Multiple Ras-GTP effector pathways have been described, and some of the key effectors are depicted here. The B-RAF/MEK/ERK kinase cascade often determines proliferation and becomes dysregulated in certain cancers and in developmental disorders such as cardio-facio-cutaneous syndrome. Ras also activates the PI3K/PDK1/Akt pathway that frequently determines cellular survival. RalGDS and Tiam1 are exchange factors of Ral and Rac, respectively. Among the effectors of Ral is phospholipase D (PLD) an enzyme that regulates vesicle trafficking. Rac regulates actin dynamics and, therefore, the cytoskeleton. Ras also binds and activates the enzyme phospholipase C epsilon (PLCε), whose hydrolytic products regulate calcium signaling and the protein kinase C (PKC) family. Figure adapted from Schubbert et al. 79.
Figure 1
Figure 2

The structure of Ras.

Three dimensional structure of Ras-GTP, highlighting key residues involved in disease. Amino acids that are altered by somatic mutations in human tumors are represented using yellow carbons (glycine-12, glycine-13, and glutamine-61). The residues affected by germline mutations found in individuals with Noonan and CFC syndromes are represented using white carbons (valine-14, glutamine-22, proline-34, isoleucine-36, threonine-58, valine-152, glutamate-153, and phenylalanine-156). The loops that are important in binding to effectors, switch I and switch II, are shown as red ropes. Also shown is the arginine finger of GAP proteins, which stimulates the GTP hydrolysis reaction (shown as a blue rope). Arginine-789 from the proximal loop of the p120 GAP arginine finger is represented using blue carbons. The guanine nucleotide is shown as a stick representation colored in black. Figure courtesy of K. Zhang.
Figure 3

SHP-2 domain structure and Noonan syndrome and JMML-associated amino acid substitutions.

(A) Schematic diagram of the PTPN11 gene and functional domains of the encoded SHP-2 protein. (B) Somatic PTPN11 mutations in JMML are largely distinct from the germline mutations that cause Noonan syndrome (NS). Residues located within the N-SH2 domain encoded by exon 3 and residues located within a portion of the catalytic phosphatase domain encoded by exon 13 are shown. Amino acid substitutions found in JMML are shown in red, substitutions reported in individuals with NS with features of JMML are shown in blue, and substitutions found in NS are shown in green. Most all mutations reported in JMML and NS/JMML are located in these depicted regions, whereas mutations associated with NS alone alter residues of SHP-2 outside of these regions in ~50% of cases. Panel B adapted from Kratz et al.68.
Figure 3

(A)

(B)

N-SH2 domain

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PTPase domain

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<td>Ser502</td>
<td>Gly503</td>
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<td>Thr</td>
<td>Arg</td>
<td>Val</td>
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</tbody>
</table>
Figure 4.

Role of SHP-2 in GM-CSF mediated signal transduction in hematopoietic cells.

In hematopoietic cells stimulated with GM-CSF, SHP-2 is tyrosine phosphorylated and coimmunoprecipitates with the βc subunit of the GM-CSF receptor and the adapter protein Grb2\textsuperscript{80}. Upon receptor activation, SHP-2 associates with phosphorylated tyrosines 577, 612, and 695 of βc and can bind to at least one site (Y612) directly\textsuperscript{49,50,80}. Upon cytokine stimulation, SHP-2 also associates with the adapter protein Gab2, which is constitutively bound to Grb2\textsuperscript{58}. Another adapter, Shc, directly binds to phosphorylated Y577 of activated βc and becomes tyrosine phosphorylated\textsuperscript{50,80,81}. Phosphorylated Shc recruits the Grb2/Gab2 complex, which further recruits SHP-2. Thus SHP-2 may mediate signaling from activated βc by directly binding to the receptor and/or by associating with adapter proteins that are recruited to the receptor. Interestingly, among the eight tyrosines of βc, the three tyrosines Y577, Y612, and Y695 are individually sufficient to induce SHP-2 phosphorylation, its association with Grb2, and activation of Raf and ERK\textsuperscript{49}. 
Figure 4
References

Chapter 2

Somatic mutations in *PTPN11* implicate the protein tyrosine phosphatase SHP-2 in leukemogenesis
Abstract

The *PTPN11* gene encodes SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase), a non-receptor protein tyrosine phosphatase (PTPase) that relays signals from activated growth factor receptors to Ras and other signaling molecules. Mutations in *PTPN11* cause Noonan syndrome, a developmental disorder characterized by cardiac and skeletal defects. Noonan syndrome is also associated with a spectrum of hematologic disorders including juvenile myelomonocytic leukemia (JMML). To test the hypothesis that *PTPN11* mutations might contribute to myeloid leukemia, our laboratory screened the entire coding region for mutations in 51 JMML specimens and in selected exons from 60 patients with other myeloid malignancies. Missense mutations in *PTPN11* were detected in 16 of 49 JMML specimens from patients without Noonan syndrome, but they were less common in other myeloid malignancies. *RAS, NF1,* and *PTPN11* mutations are largely mutually exclusive in JMML, which suggests that mutant SHP-2 proteins deregulate myeloid growth through Ras. However, although Ba/F3 cells engineered to express leukemia-associated SHP-2 proteins showed enhanced growth factor-independent survival, biochemical analysis failed to demonstrate hyperactivation of the Ras effectors extracellular–regulated kinase (ERK) or Akt. We conclude that SHP-2 is an important cellular PTPase that is mutated in myeloid malignancies. Further investigation is required to clarify how these mutant proteins interact with Ras and other effectors to deregulate myeloid growth.
Introduction

Juvenile myelomonocytic leukemia (JMML) is a relentless myeloproliferative disorder of young children characterized by over-production of myeloid cells that infiltrate hematopoietic and non-hematopoietic tissues\(^1,2\). A hallmark of JMML is a hypersensitive pattern of colony forming unit granulocyte-macrophage (CFU-GM) progenitor growth in cultures stimulated with granulocyte-macrophage colony stimulating factor (GM-CSF)\(^3\). Studies of human samples and mouse models strongly implicate hyperactive Ras in the pathogenesis of JMML. Approximately 25% of JMML bone marrows show oncogenic RAS mutations\(^4,5\). In addition, the incidence of JMML is increased by >200 fold in children with neurofibromatosis type 1 (NF1)\(^6,7\). The NF1 gene encodes neurofibromin, a GTPase activating protein (GAP) that negatively regulates Ras output by accelerating GTP hydrolysis\(^8,9\). Genetic and biochemical analysis of JMML samples have shown that NF1 functions as a tumor suppressor by negatively regulating Ras\(^10-12\). Taken together, oncogenic RAS mutations or inactivation of NF1 are found in \(~50\%\) of JMML samples, and appear to be mutually exclusive\(^5,12,13\).

Missense mutations in PTPN11 were shown to cause Noonan syndrome, a developmental disorder characterized by cardiac defects, facial dysmorphism, and skeletal malformations\(^14,15\). SHP-2, the non-receptor tyrosine PTPase encoded by PTPN11, contains two src homology 2 (SH2) domains and a catalytic PTPase domain. The SHP-2 PTPase is activated by binding to phosphotyrosyl peptides through its N-SH2 domain\(^16,17\). The SHP-2 crystal structure predicts that these interactions induce a conformational shift that relieves inhibition of the PTPase by the N-SH2 domain\(^18\). Most of the mutations reported in Noonan syndrome kindreds are found in exons 3 and 8,
which encode segments of the N-SH2 and PTPase domains, respectively. Molecular
modeling suggests that almost all of these exon 3 mutations activate phosphatase activity
by altering N-SH2 amino acids that interact with the PTPase domain\textsuperscript{14,15}.

SHP-2 participates in signal transduction downstream of growth factor receptors
to regulate multiple responses including proliferation, differentiation, and migration\textsuperscript{19,20}. The protein is expressed at high levels in hematopoietic cells and undergoes rapid
tyrosine phosphorylation upon activation of the c-KIT, interleukin 3 (IL-3), GM-CSF,
and erythropoietin receptors\textsuperscript{19,21,22}. Gab2, which exists in a complex with Grb-2 in
unstimulated Ba/F3 cells, becomes phosphorylated and associates with SHP-2 upon
growth factor stimulation\textsuperscript{23,24}. SHP-2 most often plays a positive role in transducing
signals, which is mediated, at least in part, through the Ras/Raf/ERK cascade in
hematopoietic and non-hematopoietic cells\textsuperscript{19,20,25}. Homozygous \textit{Ptpn11} mutant mouse
embryos fail around E9.5 with multiple defects in mesodermal patterning and body
organization\textsuperscript{26,27}. Loss of \textit{Ptpn11} function also has profound effects on the developing
hematopoietic system\textsuperscript{28-31}. \textit{Ptpn11}-deficient yolk sacs contain markedly reduced numbers
of hematopoietic colony forming cells, and mutant embryonic stem cells do not
contribute to hematopoiesis in chimeras\textsuperscript{29}. These and other studies implicate SHP-2 as a
crucial effector of hematopoietic cell fates that modulates signaling from activated
growth factor receptors.

Children with Noonan syndrome show a spectrum of hematologic abnormalities
including isolated monocytosis, myeloid disorders with features of chronic
myelomonocytic leukemia (CMML) that remit spontaneously and, rarely, JMML\textsuperscript{32-34}.
Bone marrow cells from Noonan syndrome patients with JMML show characteristic GM-
CSF hypersensitivity in methylcellulose cultures. These observations, data implicating hyperactive Ras in the pathogenesis of JMML, and the role of SHP-2 in relaying signals from hematopoietic growth factor receptors to Ras identify \textit{PTPN11} as an excellent candidate gene that might be mutated in cases of JMML without abnormalities in \textit{RAS} or \textit{NF1}. Our laboratory screened a well-characterized panel of JMML specimens for \textit{PTPN11} mutations. This study, led by Mignon Loh, identified somatic mutations in \textit{PTPN11} in \(\sim35\)% of cases. These data are consistent with studies performed independently by Tartaglia and colleagues. Genetic evidence strongly implicates these mutations as conferring a growth advantage by deregulating Ras. Although we did not detect hyperactive Ras signaling in Ba/F3 cells engineered to express leukemia-associated SHP-2 mutant proteins, transduced cells consistently show reduced cell death upon growth factor withdrawal.
Results

Missense \textit{PTPN11} mutations in JMML Specimens. On the basis of case reports of JMML in Noonan syndrome patients, our laboratory first investigated 51 JMML specimens for \textit{PTPN11} mutations including two from children with a clinical diagnosis of Noonan syndrome. These studies uncovered missense mutations in 16 of 49 JMML samples from patients without Noonan syndrome, which are summarized in Table 1. \textit{PTPN11} mutations were also identified in both patients with Noonan syndrome. Fifteen of 16 \textit{PTPN11} mutations detected in sporadic cases of JMML occurred in exon 3, which encodes a segment of the N-SH2 domain (Table 1). The only exception was an exon 4 mutation that we identified in a specimen from a 2-month infant. This mutation has also been reported in Noonan syndrome\textsuperscript{15}. While the nature of this \textit{PTPN11} mutation and the early age of diagnosis are suggestive of Noonan syndrome, detailed clinical information was not available in this case. Both of the JMML specimens from children known to have Noonan syndrome demonstrated different \textit{PTPN11} mutations from the patients without Noonan syndrome, including one substitution in exon 13 (Table 1).

\textit{Distribution of PTPN11, RAS, and NF1 Mutations in JMML.} If SHP-2 functions in a growth control signaling pathway that includes the GM-CSF receptor, Ras, and neurofibromin in myeloid cells, \textit{PTPN11} mutations might be restricted to leukemia samples without \textit{RAS} or \textit{NF1} mutations. This hypothesis is based on the idea that mutating any component would deregulate the entire cascade and that another mutation would confer little, if any, additional selective advantage. Indeed, previous studies that included most of the JMML specimens investigated in this report revealed \textit{RAS} and \textit{NF1}
mutations in mutually exclusive subsets\(^5,10,12,13\). Similarly, *BRAF* mutations are largely restricted to melanomas without *RAS* mutations\(^36\). The 49 JMML specimens from patients without Noonan syndrome were divided into 3 groups: (1) samples from patients with a clinical diagnosis of NF1 or an *NF1* mutation, (2) samples with *RAS* (*KRAS* or *NRAS* mutations), or (3) all other JMML samples. When these groups were compared, there was a statistically significant difference in the frequency of *PTPN11* mutations in group 3 versus groups 1 and 2 (Table 2). These data provide genetic evidence that mutant SHP-2 proteins contribute to leukemogenesis through a Ras-dependent mechanism. Two specimens that were assigned to the NF1 group showed *PTPN11* mutations. In one patient with clinical evidence of NF1, extensive molecular analysis did not disclose either loss of the normal *NF1* allele or a truncating mutation in the coding region in the leukemic clone. Because some patients with Noonan syndrome show clinical features of NF1\(^37,38\), it is possible that this child was misdiagnosed. The other sample was from a 3 year old patient without clinical evidence of NF1 that was included in a series of JMML cases studied for *NF1* mutations\(^13\). Molecular analysis of the bone marrow demonstrated a nonsense mutation (G4614A) in exon 27a of *NF1*, but both *NF1* alleles were retained. It is formally possible that this mutant allele retains some GAP activity, or that this represents an instance in which leukemic outgrowth in an NF1 patient resulted from a somatic *PTPN11* mutation rather than loss of the normal *NF1* allele.

**PTPN11 Mutations in Other Hematopoietic Malignancies.** The prevalence of *PTPN11* mutations in JMML suggested that somatic mutations might also exist in other myeloid malignancies. To address this question, our laboratory analyzed 60 specimens from
children and adults with CMML, chronic myeloid leukemia (CML), acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and therapy-related MDS/AML (t-MDS/AML). \textit{PTPN11} mutations were detected in 7 of these samples (11%), including 1 of 4 from patients with CMML, 0 of 11 with CML, 3 of 28 with AML, 2 of 7 with MDS, and in 1 of 10 with t-MDS/AML (Table 1). Based on the results of these studies, a focused analysis of exons 3, 4, 5, 8, and 13 was performed in a cohort of 95 pediatric AML specimens and 2 mutations were detected in exon 3. The absence of \textit{PTPN11} mutations in CML is consistent with genetic and biochemical data implicating Ras as a downstream target of Bcr-Abl in myeloid leukemogenesis\textsuperscript{39,40}. Five of the 9 samples with \textit{PTPN11} mutations also had chromosome 7 abnormalities (monosomy 7).

Overall, 25 of the 27 \textit{PTPN11} mutations identified in hematopoietic malignances change amino acids within the N-SH2 domain (exon 3), with codons 61, 72, and 76 affected in 4, 6, and 9 cases, respectively (Table 1). By contrast, although exon 3 is also commonly involved in Noonan syndrome, the overall spectrum is much broader with mutations also seen in exons 2, 4, 7, 8, and 13\textsuperscript{14,15}. Moreover, only two of the leukemia-associated amino acid substitutions identified within exon 3 have been reported in Noonan syndrome\textsuperscript{14,15}. The JMML specimens analyzed by Tartaglia and colleagues\textsuperscript{35} displayed a similar pattern of \textit{PTPN11} mutations. Based on the SHP-2 crystal structure, each \textit{PTPN11} mutation is predicted to disrupt the inhibitory interaction of the N-SH2 domain with the PTPase domain (Figs. 1A and B).

**Functional and Biochemical Studies in Ba/F3 Cells.** Ba/F3 cells have been used to investigate SHP-2 activation in hematopoietic cells\textsuperscript{41-43} and to interrogate the functional
consequences of leukemia-associated mutant proteins\textsuperscript{44-48}. I exploited this pro-B cell line, which is dependent upon IL-3 for survival and proliferation, to express the mutant D61Y and E76K SHP-2 proteins identified in JMML patient samples. Ba/F3 cells were infected with retroviral vectors engineered to co-express wild-type or mutant SHP-2 with a puromycin resistance gene, then cultured with IL-3 and puromycin. This infection-selection procedure was performed on three independent occasions to insure that the biologic effects of expressing mutant SHP-2 proteins were reproducible. After 4 days, the transduced Ba/F3 cells were transferred to medium without IL-3 to assess the effects of expressing wild-type or mutant SHP-2 on survival and proliferation. At this time, cells were also collected to measure SHP-2 expression and to interrogate Ras effector cascades. Wild-type and mutant SHP-2 proteins were expressed at similar levels that were higher than in parental Ba/F3 cells (Fig. 2A, bottom panel). We investigated ERK and Akt phosphorylation in cells that were deprived of serum and IL-3 for 6 hours, then stimulated with IL-3. Surprisingly, I did not observe increased levels of phosphorylated ERK or Akt in resting or IL-3-stimulated Ba/F3 cells that expressed either mutant SHP-2 protein (Fig. 2A). Signal transduction experiments were performed under a variety of experimental conditions that included varying the concentration of IL-3 and the time course with similar results (data not shown). We also assessed the effects of wild-type and mutant SHP-2 proteins on the survival and growth of transduced Ba/F3 cells after IL-3 withdrawal. The cells were cultured in triplicate at 1 x 10\textsuperscript{6} cells per plate and were counted every other day beginning on day 8. Under these conditions, expression of the E76K mutant consistently enhanced the survival of Ba/F3 cells (Fig. 2B). We also observed subtle, but reproducible, effects of the D61Y SHP-2 protein (Fig.
Importantly, Ba/F3 cells transduced with mutant SHP-2 proteins did not expand during the 2-3 week culture period, but sustained higher numbers of viable cells. However, Ba/F3 cells that expressed either mutant SHP-2 protein frequently demonstrated growth factor-independent proliferation after prolonged time in culture. This was never observed in cells that had been transduced with either empty vector or with the wild-type SHP-2 virus.

Discussion

We find that missense mutations in PTPN11 are common in JMML and exist in other myeloid malignancies. Based on a number of considerations, these amino acid substitutions are almost certain to represent pathologic mutations. First, we did not detect any of these leukemia-associated mutations in 22 normal bone marrow specimens, and they were not identified in more than 100 control subjects screened by Tartaglia et al. Second, as would be expected if PTPN11 mutations result in a gain-of-function, we have not identified deletions, insertions, or substitutions leading to premature termination of protein translation. Third, the data shown in Table 2 argues strongly that these alterations are not random, but are functionally equivalent to oncogenic mutations in RAS or inactivation of NF1. Fourth, based on the crystal structure of SHP-2, each of these mutations is predicted to disrupt the inhibitory interaction of the N-SH2 domain with the PTPase domain (Figs. 1A and B). Indeed, in an elegant series of experiments in the Xenopus animal cap assay, O’Reilly and coworkers generated alanine substitutions corresponding to D61 and E76. These investigators showed that mutant SHP-2 proteins exhibited elevated PTPase activity and conferred a gain-of-function elongation phenotype
which is known to involve activation of Ras/ERK signaling downstream of the fibroblast growth factor (FGF) receptor. Similarly, our data in the Ba/F3 cell line demonstrate that these mutations have phenotypic consequences in hematopoietic cells. Finally, Tartaglia and coworkers independently found a similar incidence and spectrum of somatic PTPN11 mutations in a different series of JMML samples.

Although many of the somatic PTPN11 mutations identified in leukemia specimens alter the same codons as in Noonan syndrome, the spectrum is distinct with respect to the pattern of amino acid substitutions and specificity for exon 3. Furthermore, the two mutations that we detected in JMML specimens from children with Noonan syndrome are uncommon, with only one being previously reported. The distribution of PTPN11 mutations found in JMML suggests that these alleles might be deleterious in embryonic life. Consistent with this idea, the D61Y and E76K mutant proteins show higher phosphatase activities than the most common substitution found in children with Noonan syndrome (N308D).

Expressing the D61Y and E76K mutations enhanced the survival of transduced Ba/F3 cells that were deprived of IL-3. Interestingly, the E76K mutation, which shows higher PTPase activity, is more potent in this assay. While mutant SHP-2 proteins did not acutely induce IL-3-independent proliferation, there was a lower rate of attrition than in control cells expressing either empty vector or wild-type SHP-2. This is reminiscent of the effects of the E2A-HLF fusion protein in Ba/F3 cells. Despite compelling genetic evidence that the PTPN11 mutations found in JMML deregulate growth through a Ras-dependent mechanism, we did not detect aberrant activation of ERK or Akt in transduced Ba/F3 cells. Substitutions at the D61 and E76 positions of SHP-2 perturb Ras signaling
in other systems\textsuperscript{35,49}. The discrepancies between these studies and our data in Ba/F3 cells might be due to differences in the expression levels of mutant proteins and/or the cellular context. It is interesting that Tartaglia et al.\textsuperscript{35} reported relatively modest levels of ERK activation in COS-7 cells with basal and serum-stimulated kinase levels that were equivalent to wild-type, but prolonged activation in cells expressing mutant SHP-2 proteins. Furthermore, although these authors observed increased proliferation in COS-7 cells, our data in hematopoietic cells support the idea that the predominant effect of mutant SHP-2 proteins is to reduce the requirement for growth factors in cell survival. This model is consistent with data from cultured \textit{Nf1} deficient myeloid cells, which also survive in the absence of exogenous growth factors\textsuperscript{50}.

Our data raise the possibility that \textit{PTPN11} mutations cooperate with other genetic lesions to induce JMML. This idea is consistent with the clinical observation that hematologic abnormalities, including some JMML-like myeloid disorders, may remit spontaneously in children with Noonan syndrome. Along these lines, it is interesting that we detected loss of the normal \textit{PTPN11} allele in one JMML and a second sample showed both heterozygous inactivation of \textit{NF1} and a \textit{PTPN11} mutation. Our studies to date also suggest that \textit{PTPN11} mutations are relatively common in myeloid malignancies with monosomy 7. In previous reports, monosomy 7 has been associated with \textit{RAS} mutations and with \textit{NF1}\textsuperscript{51-55}. The finding of \textit{PTPN11} mutations in myeloid malignancies with monosomy 7 lends further support to the idea that hyperactive Ras cooperates with loss of a critical gene (or genes) on the long arm of chromosome 7 in leukemogenesis. Expressing leukemia-associated \textit{PTPN11} alleles in primary murine hematopoietic cells
will help to elucidate the cellular, biochemical, and phenotypic consequences of these mutations and the requirement for cooperating events.

The high prevalence of *PTPN11* mutations in JMML is intriguing and may indicate a specific role for SHP-2 in regulating GM-CSF signaling, perhaps in the context of fetal and neonatal hematopoiesis. Phosphorylation of Tyr-577 on the bc chain of the activated GM-CSF receptor provides a docking site for Shc, which recruits Grb2, Gab2, SHP-2 and the p85 subunit of PI3K, and induces downstream activation of Akt. Interestingly, SHP-2 also interacts directly with βc at Tyr-612 and at Tyr-695. While the functional importance of these sites is uncertain, Tyr-612 can induce Gab-2 phosphorylation independent of Tyr-577. The general idea that mutant SHP-2 molecules might deregulate Ras by aberrantly amplifying signals from activated growth factor receptors is consistent with data from *Xenopus*. In this system, mutant SHP-2 proteins showed elevated PTPase activities and promoted elongation in the absence of FGF. Interestingly, ectopic expression of the D61A and E76A mutants was insufficient to induce mesoderm induction, but reduced the requirement for FGF to complete this process. By analogy, the *PTPN11* mutations found in JMML might contribute to leukemogenesis by hyperactivating Ras signaling at physiologic levels of GM-CSF. This idea is consistent with the hypersensitive pattern of CFU-GM colony growth observed when JMML bone marrows or *Nf1* mutant hematopoietic cells are cultured in methylcellulose, and with the profound attenuation of the murine JMML-like myeloproliferative disease with *Gmcsf* ablation. Elucidating how mutant SHP-2 proteins interact biochemically with the GM-CSF receptor, with adapter molecules, and with other phosphotyrosyl substrates in primary myeloid cells will extend our knowledge.
of normal and leukemic signal transduction. The D61A and E76A mutants created by O’Reilly and coworkers\textsuperscript{49} retain the capacity to bind phosphotyrosyl substrates, and it will be important to confirm that this is also true of leukemia-associated mutant SHP-2 molecules.

In 1994, Sawyers and Denny\textsuperscript{39} pointed out that Ras signaling is perturbed in myeloid malignancies by distinct genetic mechanisms such as oncogenic \textit{RAS} point mutations, the \textit{BCR-ABL} translocation, and \textit{NFI} inactivation. Since then, the FLT3 and c-KIT receptors have joined the list of mutant proteins that appear to contribute to myeloid leukemogenesis, at least in part, through hyperactive Ras. SHP-2 represents the first tyrosine phosphatase that functions as an oncogene in human cancer and genetic data support the hypothesis that these mutations deregulate myeloid growth through a Ras-dependent mechanism. Fully characterizing how SHP-2 and other molecules that relay signals from activated growth factor receptors to Ras contribute to tumorigenesis may uncover novel therapeutic targets.
Materials and Methods

Leukemia Samples. Archived bone marrow or peripheral blood specimens from patients with hematologic malignancies were collected by our laboratory or accrued by the Hematopoietic Tissue Cell Bank of the UCSF Comprehensive Cancer Center. An additional 95 DNA samples were analyzed from a national pediatric AML trial conducted by the Children’s Cancer Group between 1996-2002. Approval for this study was obtained from the UCSF Committee on Human Research.

Mutation Detection. PCR for exons 1-6 and 8-15 were performed according to previously published methods\(^\text{15}\). Exon 7 was amplified using forward primer 5’GAAGTAATGCTGATCCAGGC3’, reverse primer 5’AAGAGCACACGACCCTGAGG3’, and Accuprime Taq (Invitrogen). Fifty-100 nanograms of DNA were used for each 50 µl reaction. PCR products were visualized on agarose gels prior to DHPLC analysis and concentrations of primers (Integrated DNA Technologies), dNTPs (Roche), Mg\(^{++}\) (Applied Biosystems), and Amplitaq Gold (Applied Biosystems), as well as PCR conditions, were optimized. DHPLC was conducted on a Helix HPLC (Varian) using a DNASep column (Transgenomics), and analyzed according to methods previously published\(^\text{14,15}\). Abnormal spectrographs were enzyme purified using 1 unit of alkaline shrimp phosphatase (Roche) and 1 unit exonuclease I (USB), incubated for 1 hour at 37\(^\circ\)C, and heat inactivated for 15 minutes at 95\(^\circ\)C. Purified products were subsequently sequenced via a Prism 3700 Sequencer (ABI). The procedures for analyzing \textit{RAS} and \textit{NF1} for mutations have been described\(^\text{5,10,12}\).
**SHP-2 Expression Constructs.** Oligonucleotide primers containing attB sites for use with Gateway cloning technology (Life Technologies) as well as murine Ptpn11 gene specific sequences were used to amplify cDNA sequences from the ATG start codon to nucleotide 1862. Kozak sequences were incorporated into the primer sequence to allow efficient translation. Then, 30 cycles of amplification using Elongase polymerase (Invitrogen) was used to generate a PCR product for use in the BP reaction to generate an entry clone. Next, using the LR enzyme mix from Gateway technology, Ptpn11 was cloned into a MSCV-based retroviral vector containing a puromycin resistance cassette that we modified for use with the Gateway system. The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to generate point mutations into the Ptpn11 expression constructs, which were confirmed by sequencing.

**Analysis of Ba/F3 Cells.** MSCV-puro plasmids engineered to express wild-type SHP-2, or the D61Y, or E76K mutant proteins were cotransfected with plasmids encoding retroviral gag-pol and env proteins into Phoenix cells (a generous gift of Gary Nolan, Stanford University) using Lipofectamine2000 (Invitrogen). Supernatants from transfected cells were used to transduce Ba/F3 cells. Transduced cells were selected for growth in 2.5 mg/mL of puromycin for 4 days and expression of wild-type and mutant SHP-2 was confirmed by Western blot. Ba/F3 parental cells, cells transduced with a control MSCV-puro vector, and lines expressing wild-type or mutant SHP-2 proteins were cultured in RPMI 1640 supplemented with 10% FBS and 1 ng/mL recombinant murine IL-3 (Peprotech). After puromycin selection, transduced cells were washed twice in RPMI/10%FBS. A total of 1 x 10⁶ cells were seeded into 10 cm dishes and viable cells
were counted by trypan blue exclusion for 2-3 weeks. To assess signal transduction, Ba/F3 cells were deprived of IL-3 for 6 hours and then stimulated with 10 ng/mL of IL-3 for 10 min. Cell lysis and immunoblotting for ERK and Akt were performed as previously described (Donovan et al., 2002). A monoclonal antibody from Transduction Laboratories, Becton Dickinson Biosciences (La Jolla, CA, Cat. No. 610621) was used to assess SHP-2 expression.
Acknowledgements

We are grateful to Dr. Gary Gilliland for providing Ba/F3 cells and for helpful comments. We thank Dr. Robert Hawley for the MSCV vector, and Dr. Gary Nolan for Phoenix packaging cells. We also acknowledge all of the families and physicians who generously provided blood or bone marrow for analysis. MLL is supported by NIH grants K23 CA80915 and CHRC HD28825. This work was also supported by NIH grant P01 CA40046 (MML and KMS), CA80916 (PDE), and HL04409 (JG), by Cancer Center Core Grant P30 CA82103 (UCSF Tissue Cell Bank), by an award from the U.S. Army Chronic Myelogenous Leukemia Program (Project CM020058) and by the Frank Campini Foundation (MLL and KMS). KHL is the Hammond Research Fellow of the National Childhood Cancer Foundation.
Table 1

*PTPN11* mutations detected in myeloid malignancies

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<tr>
<th>Nucleotide</th>
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<th>No. of Cases</th>
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<td>JMML</td>
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<tr>
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Table 2

Incidence of somatic *PTPN11* mutations in subsets of JMML patients (n = 49)

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<th>Patient Group</th>
<th>No. of Cases</th>
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*Refers to difference between “JMML Only” and other groups (by chi square test).
Figure 1

Sites of exon 3 mutations and predicted effects on SHP-2 structure.

(A) Schematic diagram of the *PTPN11* gene with functional domains. The amino acid sequence of the N-SH2 domain is highlighted below. The interaction sites between the N-SH2 and PTP domains are indicated in red. The sites of the exon 3 mutations reported here are indicated by the arrowheads. (B) The catalytic cysteine, Cys459 is shown (green dots) as are two of the residues mutated in leukemia samples, D61 and E76 (red dots). These residues make critical contacts with the catalytic domain, and mutation of these is predicted to disrupt the inhibition of the catalytic domain by the amino-terminal SH2 domain. The N-terminal SH2 domain is shown in blue, the C-terminal SH2 domain in yellow, and the catalytic domain in pink. The figure was generated using Swiss-pdb-viewer.
Figure 1

A

B
Figure 2

Effects of expressing wild-type and mutant SHP-2 proteins on ERK and Akt activation and survival in Ba/F3 cells.

(A) Duplicate aliquots of Ba/F3 cells transduced with retroviruses encoding various SHP-2 constructs (wild-type (WT), D61Y, or E76) were collected after 4 days of growth and selection in medium containing IL-3 and puromycin. Parental Ba/F3 cells are labeled P. The cells were starved for 6 hours and lysed without stimulation (-) or after exposure to 10 ng/mL of IL-3 for 10 min (+). The bottom panel shows SHP-2 expression, which was equivalent in cells transduced with the WT, D61Y, or E76K vectors and elevated above the levels in parental Ba/F3 cells or in cells infected with the empty vector (not shown). Ba/F3 cells expressing all of the SHP-2 constructs showed low levels of phosphorylated ERK (p-ERK) and Akt (p-Akt) after starvation, with robust and equivalent activation in response to IL-3. Parental and transduced Ba/F3 cells expressed similar total levels of ERK2 and Akt. (B) Ba/F3 cell counts after IL-3 withdrawal. Ba/F3 cells infected with the MSCV-puro vector (closed squares), and cells expressing either WT SHP-2 (closed diamonds), the D61Y SHP-2 mutant protein (open triangles), or the E76K SHP-2 mutant protein (open circles) were plated in triplicate at 1 x 10^6 cells/plate in the absence of IL-3. Cells were counted starting on day 8.
Figure 2

A

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</table>

B

![Graph showing cell number over days](image)

Cell number (x10^5)

Day
References


Chapter 3

Functional analysis of leukemia-associated

*PTPN11* mutations in primary

hematopoietic cells
Abstract

PTPN11 encodes the protein tyrosine phosphatase SHP-2, which relays signals from growth factor receptors to Ras and other effectors. Germline PTPN11 mutations underlie ~50% of Noonan syndrome, a developmental disorder that is associated with an elevated risk of juvenile myelomonocytic leukemia (JMML). Somatic PTPN11 mutations were recently identified in ~35% of JMML patients; these mutations introduce amino acid substitutions that are largely distinct from those found in Noonan syndrome. We assessed the functional consequences of leukemia-associated PTPN11 mutations in murine hematopoietic cells. Expressing an E76K SHP-2 protein induced a hypersensitive pattern of colony-forming unit granulocyte-macrophage (CFU-GM) colony growth in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3) that was dependent on SHP-2 catalytic activity. E76K SHP-2 expression also enhanced the growth of immature progenitor cells with high replating potential, perturbed erythroid growth, and impaired normal differentiation in liquid cultures. In addition, leukemia-associated SHP-2 mutations conferred a stronger phenotype than a germline mutation found in patients with Noonan syndrome. Mutant SHP-2 proteins induce aberrant growth in multiple hematopoietic compartments, which supports a primary role of hyperactive Ras in the pathogenesis of JMML.
Introduction

The PTPN11 gene encodes SHP-2, a non-receptor tyrosine phosphatase (PTPase) that relays signals from activated growth factor receptors to p21^ras (Ras), Src family kinases, and other signaling molecules (reviewed in^1,2). SHP-2 contains two src homology 2 (SH2) domains and a catalytic PTPase domain. The SHP-2 crystal structure predicts that binding of the N-SH2 domain to phosphotyrosyl peptides results in a conformational shift that relieves inhibition of the PTPase and activates SHP-2 function^3.

Missense mutations in PTPN11 underlie ~50% of cases of Noonan syndrome, a developmental disorder characterized by cardiac defects, facial dysmorphism, and skeletal malformations^4. Most of the PTPN11 mutations found in Noonan syndrome introduce amino acid substitutions within the N-SH2 and PTPase domains^4,5. Molecular modeling and biochemical data suggest that exon 3 mutations dominantly activate SHP-2 phosphatase activity by altering critical N-SH2 amino acids that lie on the interface with the PTPase domain^4,5.

Infants with Noonan syndrome show a spectrum of hematologic abnormalities that includes isolated monocytosis as well as myeloid disorders with features of chronic myelomonocytic leukemia (CMML) that may remit spontaneously^6-8. Noonan syndrome patients are also predisposed to juvenile myelomonocytic leukemia (JMML), an aggressive myeloproliferative disorder (MPD) characterized by leukocytosis, tissue infiltration, and hypersensitivity to granulocyte-macrophage colony stimulating factor (GM-CSF)^9,10. Studies of JMML specimens and experiments in mutant strains of mice strongly implicate aberrant Ras signaling in response to GM-CSF and other hematopoietic growth factors in the pathogenesis of the MPD^11-16. Approximately 50%
of JMML bone marrows demonstrate a *RAS* gene mutation or inactivation of the *NF1* tumor suppressor, which encodes a GTPase activating protein that negatively regulates Ras output\textsuperscript{17-20}. The association of Noonan syndrome with JMML and the known role of SHP-2 as a positive effector of Ras signaling in many systems\textsuperscript{2,21} suggested that *PTPN11* mutations might contribute to leukemogenesis. Indeed, somatic *PTPN11* mutations occur in \~35\% of JMML samples from children without Noonan syndrome and have been detected at a lower frequency in other lymphoid and myeloid malignancies\textsuperscript{22-25}. Interestingly, almost all of these leukemia-associated mutations introduce amino acid substitutions within the N-SH2 domain that are largely distinct from those found in Noonan syndrome\textsuperscript{22-25}. Araki and colleagues\textsuperscript{26} recently generated a mouse model of Noonan syndrome by constitutively expressing an amino acid substitution (D61G) identified in human patients from the endogenous murine *Ptpn11* locus. Heterozygous *Ptpn11\textsuperscript{D61G}* mutant mice show cardiac and skeletal defects and develop a subacute MPD that models some aspects of JMML\textsuperscript{26}.

The E76K substitution is the most common somatic *PTPN11* mutation identified in JMML\textsuperscript{22-25}. Glutamate 76 lies within the N-SH2 domain, and forms key contacts with the PTPase domain that are thought to inhibit its catalytic activity\textsuperscript{3}. An E76A mutant SHP-2 protein displays elevated phosphatase activity and exhibits gain of function activity in a *Xenopus* animal cap assay\textsuperscript{21}. The leukemia-associated E76K SHP-2 protein shows elevated phosphatase activity when expressed in COS-7 cells\textsuperscript{22} and enhances interleukin 3 (IL-3)-independent survival of transduced Ba/F3 cells\textsuperscript{23}. We have investigated the effects of E76K SHP-2 expression in primary fetal liver and bone marrow cells. We find that this mutant SHP-2 protein confers hypersensitivity to GM-
CSF and IL-3 in immature and committed progenitors that is dependent on SHP-2 PTPase activity. Fetal liver cells that were transduced with E76K SHP-2 also formed many more burst forming unit-erythroid (BFU-E) colonies. We also find that expressing E76K SHP-2 results in more pronounced growth factor hypersensitivity than another leukemia-associated SHP-2 mutation (D61Y), and that both of these mutations confer stronger hematopoietic phenotypes than a N308S substitution that occurs in patients with Noonan syndrome. Cells that were transduced with E76K SHP-2 also showed persistent progenitor activity, aberrant differentiation, and increased proliferation in liquid cultures. Together, these studies and two recent articles\textsuperscript{27,28} provide extensive data regarding the \textit{in vitro} and \textit{in vivo} effects of leukemia-associated \textit{PTPN11} mutations in primary hematopoietic cells.
Results

GFP-positive fetal liver cells that were infected with wild-type (WT) or E76K virus expressed similar amounts of SHP-2 protein, which were approximately 2-3 fold above endogenous levels (Fig. 1A). E76K SHP-2 induced a hypersensitive pattern of CFU-GM growth in fetal liver cells, which was manifest by the appearance of colonies in the absence of GM-CSF and enhanced myeloid colony formation at low concentrations of growth factor (Fig. 1B). Furthermore, E76K SHP-2-expressing cells formed significantly larger CFU-GM colonies than cells transduced with the WT virus, which showed the spreading morphology of large macrophage colonies (Fig. 1C). Consistent with this, Wright-Giemsa staining revealed a high percentage of monocytes and macrophages and relatively few neutrophils in E76K SHP-2 cultures relative to WT colonies (data not shown). E76K SHP-2-expressing fetal liver cells are also hypersensitive to IL-3 and show aberrant morphology (Figs. 1D and 1E). Transduced adult murine bone marrow demonstrated a similar pattern of progenitor colony growth as fetal liver cells (data not shown). The addition of a non-saturating dose of stem cell factor (SCF; 10 ng per mL) to methylcellulose medium with GM-CSF or IL-3 did not enhance the hypersensitive pattern of CFU-GM formation of E76K expressing cells, but induced a modest increase in the number of colonies formed at low and intermediate growth factor concentrations in WT SHP-2-expressing cells (data not shown).

The SHP-2 PTPase is activated by binding to phosphotyrosyl residues on target proteins, where it may also serve as an adapter to recruit other signaling molecules\(^\text{2}\). Whereas catalytic activity is essential for relaying signals to downstream effectors and for most of the known biologic effects of SHP-2, some evidence also supports PTPase-
independent roles of SHP-2\textsuperscript{29-32}. In human SHP-2, cysteine 459 is required for catalytic activity. As C459 corresponds to C463 in murine SHP-2, we expressed a doubly mutant SHP-2 protein with E76K and C463S substitutions in fetal liver cells, and assessed myeloid progenitor colony formation in response to GM-CSF. Ablating PTPase activity abolished hypersensitive CFU-GM growth (Fig. 2A). Importantly, cells expressing WT SHP-2, the PTPase C463S mutant alone, or the E76K-C463S double mutation all demonstrated a normal pattern of myeloid progenitor colony growth (Fig. 2A). Thus, C463S and E76K-C463S SHP-2 proteins do not exhibit dominant negative activity in this system.

The GM-CSF, IL-3, and IL-5 receptors share a common β subunit (β\textsubscript{c}) that associates with unique α chains to mediate biological responses to these cytokines\textsuperscript{33}. In mice, a second gene called β\textsubscript{IL-3} encodes a β subunit that can only associate with the IL-3α chain. To determine if hypersensitivity to IL-3 requires β\textsubscript{c}, we expressed E76K SHP-2 in WT and β\textsubscript{c}-deficient bone marrow cells and compared CFU-GM colony growth over a range of IL-3 concentrations. These studies showed that E76K SHP-2 does not require β\textsubscript{c} to induce hypersensitivity to IL-3 (Fig. 2B).

CFU-GM comprise a relatively differentiated, lineage-restricted myeloid progenitor population. To investigate the effects of E76K SHP-2 expression in other compartments, we assayed the growth of HPP-CFC, a primitive myeloid progenitor with extensive replating potential, and LPP-CFC from c-kit-positive bone marrow cells that were infected with E76K SHP-2 or WT SHP-2 virus. Transduced cells were isolated by sorting and cultured in methylcellulose medium supplemented with saturating concentrations of GM-CSF or IL-3. Under these conditions, E76K SHP-2-expressing
cells formed significantly more HPP-CFC and LPP-CFC colonies than cells transduced with WT SHP-2 (Fig. 3). In addition, the individual HPP-CFC and LPP-CFC colonies formed from E76K SHP-2 cells were abnormally large. These data demonstrate that E76K SHP-2 perturbs the growth of both lineage restricted and multi-lineage hematopoietic progenitors.

To compare the effects of expressing E76K or WT SHP-2 in bulk populations of hematopoietic cells, freshly transduced GFP-positive fetal liver cells were placed in liquid cultures that were supplemented with a saturating concentration of GM-CSF to promote the survival of myelomonocytic cells. These cells were washed after 48 hours, transferred to medium without added cytokines, and counted every 2 days. E76K SHP-2-expressing cultures demonstrated significantly higher cell counts (Fig. 4A), and a distinctive pattern of differentiation characterized by an abundance of monocytes and macrophages with a subpopulation of cells with blast-like morphology (Fig 4B). By contrast, most of the cells that were transduced with WT SHP-2 differentiated into mature neutrophils (Fig. 4B). E76K SHP-2 cultures also retained significant numbers of BFU-E and CFU-GM even after 1 week (Fig. 4C). To assess the proliferative potential of myeloid progenitors in E76K SHP-2 and WT cultures, we isolated GFP-positive fetal liver cells as above, maintained them in GM-CSF, and measured BrdU incorporation over time. The number of proliferating cells was increased in E76K SHP-2 cultures when assayed after 4-7 days (Figs. 4D, 4E). Interestingly, despite the persistence of greater numbers of live cells that included viable progenitors, E76K expression did not immortalize primary fetal liver cells.
BFU-E colony formation normally requires both EPO and a source of burst promoting activity such as GM-CSF or IL-3. Interestingly, GFP-positive fetal liver cells that were infected with the E76K SHP-2 virus formed large numbers of BFU-E colonies in methylcellulose cultures supplemented with a saturating concentration of recombinant EPO alone (Fig 5A). E76K SHP-2-expressing cells also generated EPO-independent BFU-E. We observed increases in both the number of colonies and the size of the individual BFU-E colonies at higher concentrations of EPO (Fig. 5B). By contrast, cells expressing WT SHP-2 formed rare BFU-E colonies at saturating doses of EPO that were much smaller than the corresponding E76K SHP-2 erythroid colonies (Fig. 5A).

A G-T transversion at nucleotide position 181, which results in a D61Y amino acid substitution in the N-SH2 domain, is another common somatic PTPN11 mutation found in JMML specimens. Like glutamic acid 76, aspartic acid 61 mediates contacts between the N-SH2 and PTP domains. Interestingly, while the D61Y mutation has elevated phosphatase activity in COS-7 cells, it is less active than E76K, and this mutant has less potent pro-survival effects in Ba/F3 cells. We therefore compared the effects of the D61Y SHP-2 and E76K SHP-2 proteins on CFU-GM growth. Like E76K SHP-2, fetal liver cells transduced in parallel with D61Y SHP-2 exhibited a hypersensitive pattern of myeloid progenitor growth in response to GM-CSF and IL-3, and formed colonies in the absence of exogenous cytokine (Fig. 6A). However, D61Y-expressing cells consistently were less hypersensitive than the fetal liver cells that were transduced in parallel with the E76K virus. Asparagine 308 is the most commonly mutated amino acid in individuals with Noonan syndrome. This amino acid resides in the PTPase domain of SHP-2 and mutations at this codon have not been identified in
JMML specimens. We expressed a N308S mutation found in Noonan syndrome individuals in fetal liver cells and investigated the ability of these cells to form CFU-GM colonies. Whereas N308S SHP-2-expressing fetal liver cells also displayed a hypersensitive pattern of CFU-GM colony growth in response to GM-CSF, this mutant protein is markedly less potent \textit{in vitro} than E76K (Fig 6B). Moreover, cells engineered to express N308S SHP-2 never formed colonies in the absence of added cytokine.

To investigate the consequences of mutant SHP-2 expression \textit{in vivo}, primary fetal liver and bone marrow cells from C57Bl/6 donors were transduced with retroviruses encoding E76K, D61Y, or WT SHP-2 proteins and then transplanted into lethally irradiated congeneric recipients. These mice were monitored for signs of MPD, which included obtaining monthly complete blood counts. The percentage of transduced GFP-positive donor-derived cells was assessed by flow cytometry. Unfortunately, many mice died between 2 and 5 months after transplant for unknown reasons. Excess mortality was not due to overt MPD and mice that received cells transduced with empty vector, WT SHP-2, or mutant SHP-2 displayed similar survivals. Moreover, the blood leukocyte counts of mice that received E76K or D61Y-transduced bone marrow or fetal liver cells were not consistently higher 1-5 months after adoptive transfer than the counts of mice transplanted with cells infected with either WT SHP-2 or empty vector constructs. Although the transduced cells that we injected were consistently 20-40% GFP-positive, these levels varied widely in recipient animals and were frequently <10% (data not shown). Surviving recipients were sacrificed for analysis ~5 months post transplant. Interestingly, whereas splenocytes harvested from several mice transplanted with cells engineered to express E76K or D61Y SHP-2 formed CFU-GM colonies in response to
GM-CSF, myeloid colonies were never detected in the spleens of mice transplanted with cells transduced with WT SHP-2 or the empty vector. Some of the mice with abnormal splenic CFU-GM colony growth were anemic (hemoglobin 3.3 – 6.1 g/dL), and also showed disrupted splenic architecture with effacement of germinal centers and myeloid infiltration (data not shown). These hematopoietic abnormalities correlated with relatively high levels of GFP-positive cells in blood, marrow, and spleen (30 - 60%).

**Discussion**

Hypersensitivity to GM-CSF is an *in vitro* hallmark of JMML. The observation that hematopoietic cells from *Nf1* and *Kras* mutant mice also demonstrate a hypersensitive pattern of CFU-GM colony growth in methylcellulose links aberrant Ras activation to this cellular phenotype. In work performed to date, *KRAS2, NRAS, NF1*, and *PTPN11* mutations have largely been identified in mutually exclusive subsets of JMML patients, which infers that the encoded proteins are components of the same growth control network. We find that expressing leukemia-associated SHP-2 proteins in primary hematopoietic cells induces hypersensitivity of myeloid progenitors to GM-CSF and IL-3. Like CFU-GM colonies grown from *Nf1* and *Kras* mutant mice, colonies generated from E76K SHP-2-expressing fetal liver cells contain a high percentage of monocytes and macrophages. These data, which are in agreement with two recent reports, infer that hyperactive Ras perturbs myeloid differentiation by actively driving cells toward a monocyte-macrophage fate or by interfering with granulocytic maturation. Similarly, regulated expression of mutant H-Ras in human myeloid progenitors promotes monocytic differentiation, and monocytosis is a major diagnostic criterion for JMML.
The overall pattern of CFU-GM growth induced by expressing leukemia-associated SHP-2 proteins is similar to that of Kras mutant bone marrow cells, which form numerous CFU-GM colonies in the absence of any exogenous cytokine and are hypersensitive to IL-3\textsuperscript{15,16}. By contrast, Nf1-deficient cells, which do not form abnormal numbers of CFU-GM colonies in the presence of low doses of IL-3 and are less hypersensitive to GM-CSF\textsuperscript{11,12,14} most closely reproduce the \textit{in vitro} growth of human JMML cells. The differential effects of expressing mutant SHP-2 versus loss of Nf1 on CFU-GM growth are unlikely to be explained by the use of retroviral-mediated gene transfer as Araki and colleagues\textsuperscript{26} also found that myeloid progenitors from Ptpn11 D61G mice are hypersensitive to both GM-CSF and IL-3. Our studies further show that the effects of E76K SHP-2 are not restricted to CFU-GM, but that this mutation also perturbs the growth of immature myeloid progenitors (HPP-CFC and LPP-CFC). Similarly, the bone marrows of mice that had been reconstituted with Nf1\textsuperscript{-/-} fetal liver cells also contain increased numbers of HPP-CFC and LPP-CFC\textsuperscript{13}.

Adoptive transfer of bone marrow cells transduced with E76K SHP2 or D61Y SHP2 did not consistently induce MPD, although some recipient mice developed anemia in association with splenic infiltration by myeloid and erythroid cells. By contrast, Mohi \textit{et al.}\textsuperscript{28} observed MPD and T-cell leukemias in mice that were transplanted with bone marrow cells engineered to express E76K or D61Y SHP-2. Importantly, we used the same MSCV retroviral backbone as these investigators, transduced a similar percentage of bone marrow cells (and a substantially higher proportion of fetal liver cells), and found identical effects on CFU-GM progenitor colony growth in methylcellulose. However, we expressed SHP-2 proteins in C57Bl/6 hematopoietic cells, while Mohi and colleagues
used Balb/c mice. Interestingly, the ability of \textit{BCR-ABL} to induce MPD is highly dependent on the background strain, with Balb/c bone marrow sensitive to transformation and C57Bl/6 marrow resistant\textsuperscript{35}. Our data therefore suggest that expressing leukemia-associated SHP-2 proteins from retroviral vectors requires one or more modifying genes that are present in the Balb/c strain, but are absent in C57Bl/6 mice, to efficiently induce MPD or T cell leukemia \textit{in vivo}.

In addition to myelomonocytic proliferation, JMML is characterized by anemia, ineffective erythropoiesis, splenic infiltration by erythroid cells, and elevated fetal hemoglobin levels\textsuperscript{9,10}. Increased numbers of BFU-E are present in the blood and bone marrows of JMML patients, some of which form EPO-independent colonies in methylcellulose\textsuperscript{36}. Similarly, we found that expressing E76K SHP-2 in bone marrow and fetal liver cells generated BFU-E colonies in the absence of EPO or a source of burst promoting activity beyond the amount available in normal serum. Adding exogenous EPO further augmented BFU-E colony formation. We also observed anemia and splenic erythroid infiltration in some of the irradiated mice that were transplanted with these cells. Interestingly, somatic activation of oncogenic \textit{Kras} results in anemia and splenomegaly with erythroid infiltration\textsuperscript{15,16}. Furthermore, fetal liver cells transduced with oncogenic \textit{Hras} display a block in terminal erythroid differentiation that can be reversed with a MEK inhibitor\textsuperscript{37,38}. Our data provide further evidence that hyperactive Ras contributes to aberrant erythropoiesis in JMML.

The germline \textit{PTPN11} mutations found in patients with Noonan syndrome are largely distinct from the somatic mutations identified in JMML specimens, which suggests that leukemia-associated mutations encode more severe gain-of-function alleles
that might not be compatible with normal development. The observation that E76K SHP-2 confers profound in vitro CFU-GM hypersensitivity extend previous studies showing that this mutation has relatively high phosphatase activity and promotes Ba/F3 cell survival\textsuperscript{22,23}. Our data showing that the leukemia-associated D61Y mutation has less potent effects on progenitor colony growth, and that the Noonan syndrome-associated N308S mutation had markedly reduced activity, are also consistent with biochemical analysis of these mutant proteins in cell lines\textsuperscript{22}. Two recent papers also compared the effects of expressing E76K and D61Y SHP-2 on myeloid progenitor colony growth\textsuperscript{27,28}. One group described no differences between these mutant proteins; however, their data showed that D61Y SHP-2 induced a lower percentage of maximal CFU-GM colony formation than E76K SHP-2 at GM-CSF concentrations between 0 and 0.1 ng/mL\textsuperscript{27}. Moreover, Mohi and coworkers\textsuperscript{28} found that bone marrow cells transduced with the E76K SHP-2 formed more cytokine-independent CFU-GM colonies than cells expressing the D61Y mutant protein. Our observation that the N308S mutation is remarkably less potent in inducing hypersensitive CFU-GM colony growth over a range of GM-CSF concentrations is also in agreement with their finding that other Noonan syndrome mutations that are not associated with JMML caused little or no cytokine-independent colony formation\textsuperscript{28}. At the time Araki \textit{et al.}\textsuperscript{26} reported D61G SHP-2 mutant mice, this mutation had not been associated with JMML. Interestingly, recent data indicate that D61G is the only \textit{PTPN11} mutation that is detected in Noonan syndrome patients with and without leukemia as well as in sporadic JMML (C. Kratz, C. Niemeyer, M. Tartaglia, and M.L.L.; unpublished data). D61G SHP-2 has lower phosphatase activity than the E76K SHP-2 and it is of interest that CFU-GM from these animals are less hypersensitive
to GM-CSF and IL-3 than cells transduced with E76K SHP-2\textsuperscript{26}. Taken together, the existing data argue in favor of biologic differences between leukemia-associated mutant proteins that correlate with levels of phosphatase activity and, more importantly, demonstrate that these SHP-2 proteins profoundly alter the growth of primary myeloid cells whereas common germline \textit{PTPN11} mutations found in Noonan syndrome patients encode SHP-2 proteins that have relatively subtle effects.

\textit{In vitro} data suggest a catalytic-independent role of SHP-2 in IL-3 and platelet-derived growth factor signaling\textsuperscript{30-32}. By contrast, we found that expressing WT SHP-2 did not alter CFU-GM colony growth and that ablating SHP-2 phosphatase activity in the context of the E76K mutation abolished GM-CSF hypersensitivity. These data directly linking a JMML-associated cellular phenotype to SHP-2 catalytic activity are consistent with extensive structure-function studies performed by Mohi \textit{et al.}\textsuperscript{28}, which also defined essential roles of phosphotyrosyl binding by the SH2 domain and of tyrosine residues that link SHP-2 to Grb2 in cytokine hypersensitivity.

Clonogenic assays measure the growth of rare progenitors in methylcellulose medium supplemented with serum and growth factors. We therefore established liquid cultures to assess the effects of expressing E76K SHP-2 in a mixed population of primary hematopoietic cells. These studies uncovered increased numbers of myeloid and erythroid progenitors at early time points, higher cell numbers at later time points, and aberrant differentiation. Cultured E76K SHP-2-expressing cells that were maintained in GM-CSF also demonstrated an elevated rate of proliferation. These data provide direct evidence that leukemia-associated SHP-2 proteins perturb terminal differentiation programs while also driving the proliferation of myelomonocytic cells. Similarly, Chan \textit{et al.}\textsuperscript{28}...
reported that macrophage progenitors expressing leukemia-associated SHP-2 proteins induced to differentiate \textit{in vitro} showed lower levels of F4/80 expression, a surface marker specific for macrophages, suggesting these cells do not efficiently mature within the macrophage lineage. These authors also observed hyperproliferation of macrophage progenitors expressing leukemia-associated mutant SHP-2 proteins in response to GM-CSF. The \textit{in vitro} phenotypes of primary cells engineered to express mutant SHP-2 proteins are consistent with clinical pathologic features of JMML, which is characterized by both prominent myeloproliferation and by trilineage dysplasia with anemia and thrombocytopenia\textsuperscript{9,10}. Importantly, E76K SHP-2-expressing fetal liver and bone marrow cells eventually die in liquid cultures, which argues that mutant SHP-2 proteins do not immortalize cells or enhance self-renewal capacity. This idea is consistent with data from $K_{ras}^{G12D}$ bone marrow cells, which are profoundly hypersensitive to growth factors but show normal replating efficiency\textsuperscript{15,16}. We have reported that E76K SHP-2 enhances the IL-3-independent survival of Ba/F3 cells\textsuperscript{23}. We found no differences in the rate of annexin V staining (a measure of apoptosis) between WT and E76K SHP-2-expressing GFP-positive fetal liver cells that were cultured without growth factors for 2-24 hours after undergoing transduction (data not shown). However, these data do not exclude the possibility that leukemia-associated SHP-2 proteins enhance the survival of specific subpopulations of hematopoietic cells, an idea that is consistent with the persistence of progenitor activity in E76K SHP-2 liquid cultures after growth factor withdrawal.

We have shown that expressing E76K SHP-2 in primary hematopoietic cells perturbs the growth of immature (LPP-CFC and HPP-CFC) and committed (CFU-GM
and BFU-E) progenitors. These effects of mutant SHP-2 are reminiscent of the phenotypes seen in murine Nf1 and Kras mutant hematopoietic cells and in JMML patient samples, and suggest that hyperactive Ras contributes to leukemogenesis. We were therefore surprised to discover that expressing D61Y or E76K SHP-2 did not deregulate signaling through Ras effectors in Ba/F3 cells. Mohi et al. confirmed these observations, and went on to investigate bone marrow cells from transplanted mice that had been cultured with IL-3 to generate bone marrow mast cells. Interestingly, cells expressing E76K or D61Y SHP-2 proliferated extensively under these conditions, which is also characteristic of Kras mutant cells. Biochemical analyses revealed elevated levels of phosphorylated ERK, Akt, and STAT5 in cells from mice that had been transplanted with cells expressing E76K or D61Y SHP-2. Chan and coworkers generated macrophage progenitors from retrovirally infected bone marrow and found that E76K, D61Y, or D61V SHP-2 induced elevated basal levels of phosphorylated ERK with prolonged activation in response to GM-CSF, but not M-CSF. This pattern of ERK activation in cells stimulated with GM-CSF is similar to our observations in Mac1+ bone marrow cells from Nf1 mutant mice. Together, the existing biochemical data suggest that the effects of mutant SHP-2 proteins on Ras signaling are strongly modulated by both the responding cell type and the stimulus. Understanding the basis of this specificity and identifying critical effectors that are amenable to therapeutic intervention is a rational strategy for improving the outcome of patients with JMML.
Materials and Methods

**SHP-2 Expression Constructs.** Wild-type and mutant \( Ptpn11 \) cDNAs were cloned into a vector derived from the murine stem cell virus (MSCV) backbone\(^{39} \) as described elsewhere\(^{23} \). These plasmids also contain a green fluorescent protein (GFP) cassette driven by an internal ribosomal entry site (IRES) downstream of the \( Ptpn11 \) sequence. Each construct was verified by sequencing.

**Hematopoietic Cell Isolation and Retroviral Transduction.** All experimental procedures involving mice were reviewed and approved by the UCSF Committee on Animal Research. Pregnant wild-type C57Bl/6 females were killed by \( \text{CO}_2 \) inhalation at E14.5 and fetal liver cells were isolated and prepared as described\(^{40} \). Bone marrow cells were collected by killing C57Bl/6 mice by \( \text{CO}_2 \) inhalation that had previously been injected with a single dose of 5-fluorouracil (150 mg/kg), and flushing out marrow from the tibias with Iscove’s Modified Dulbecco’s Medium (IMDM) (GIBCO-BRL, Gaithersburg, MD) supplemented with 20% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT). Fetal liver cells were cultured in a stimulation medium containing StemSpan SFEM (StemCell Technologies, Vancouver, BC), 15% FBS, 100 ng/mL stem cell factor (SCF) (Peprotech, Rocky Hill, NJ), 50 ng/mL FLT-3 ligand (Peprotech), and 100 ng/mL interleukin 11 (R&D Technologies, Minneapolis, MN). Bone marrow cells were cultured in a stimulation medium containing StemSpan SFEM,15% FBS, 100 ng/mL SCF, 50 ng/mL interleukin 6, and 10 ng/mL IL-3 (both from Peprotech). MSCV-\( Ptpn11 \)-IRES-GFP plasmids engineered to express wild-type or mutant SHP-2 proteins were co-transfected with plasmids encoding retroviral gag-pol and env proteins into Phoenix cells.
using Lipofectamine2000 (Invitrogen, Carlsbad, CA). Supernatants from transfected cells were used to transduce fetal liver or bone marrow cells 24 – 72 hours after harvest. Expression levels of wild-type and mutant SHP-2 proteins were evaluated by Western blot as described previously\textsuperscript{23}.

**Colony Assays.** After transduction, GFP-positive fetal liver and bone marrow cells were sorted using a FACS Vantage SE flow cytometer. GFP-positive fetal liver and bone marrow mononuclear cells were seeded in methylcellulose medium (M3231, Stem Cell Technologies) containing recombinant murine GM-CSF or IL-3 (Peprotech). CFU-GM colonies were counted on day 8 by indirect microscopy. For erythroid progenitor assays, cells were seeded in methylcellulose medium containing a saturating concentration of human erythropoietin (EPO) (M3334, Stem Cell Technologies) or in medium that contained varying amounts of recombinant murine EPO (R&D Technologies). BFU-E colonies were enumerated on day 7. Cellular content was evaluated by harvesting colonies into PBS followed by counting, cytospin preparations, and Wright-Giemsa staining. The growth of low proliferating potential colony forming cells (LPP-CFC) and high proliferating potential colony forming cells (HPP-CFC) from c-kit positive bone marrow cells was assessed as described elsewhere\textsuperscript{41}. Briefly, growth factors used for culture of HPP-CFC and LPP-CFC included SCF, IL-1, CSF-1, and GM-CSF or IL-3. Cultures for growth of LPP-CFC were cultured in 8% CO\textsubscript{2}, 5% O\textsubscript{2}, and were scored on day 7 of culture, whereas those for HPP-CFC were scored on day 14 of culture. Colonies were scored by indirect microscopy.
**Liquid Cultures.** After transduction with WT or E76K SHP-2 vectors, sorted GFP-positive fetal liver cells were seeded into 12 well dishes in IMDM with 15% FBS and 2 ng/ml GM-CSF (Peprotech) at $1.2 \times 10^6$ cells per well. After 48 hours, cells were washed in IMDM with 15% FBS and subsequently cultured in medium without GM-CSF. Viable cells were counted by trypan blue exclusion. Cytospins were stained with Wright-Giemsa (Sigma, St. Louis, MO). To assess progenitor activity in liquid cultures, fetal liver cells were removed at various time points and plated in methycellulose medium supplemented with saturating doses of IL-3, IL-6, SCF, and EPO (M3434, Stem Cell Technologies). Progenitor colonies were counted on days 7 and 8. The incorporation of 5-bromo-2-deoxyuridine (BrdU) was measured by incubating cells with 50 µM BrdU (Sigma) for 2-4 hours. The cells were then fixed in 70% ethanol, treated with 2N HCl/Triton X-100, and stained with FITC conjugated mouse anti-BrdU antibody and 7-AAD (both from BD Pharmingen, Mountain View, CA).

**Adoptive Transfer.** Wild-type C57Bl/6 recipient mice that received a single dose of 900 cGy were injected with transduced bone marrow or fetal liver cells immediately after radiation via the dorsal tail vein. Recipients received prophylactic antibiotics consisting of polymixin sulfate and neomycin sulfate for two weeks after radiation.
Acknowledgements

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Figure 1

E76K SHP-2 expression results in a hypersensitive pattern of CFU-GM colony growth.

(A) SHP-2 expression levels in fetal liver cells transduced with retroviruses encoding wild-type (WT), D61Y, or E76K SHP-2 proteins. The blot was also probed with an anti-ERK2 antibody to confirm equal protein loading. (B, D) CFU-GM colony growth of cells expressing either WT or E76K SHP-2 over a range of GM-CSF (B) or IL-3 (D) concentrations. Graphs display the average growth of three independent experiments of cells plated in duplicate. (C, E) Morphology of representative CFU-GM colonies grown in saturating concentrations of GM-CSF (C) or IL-3 (E) from fetal liver cells expressing WT or E76K SHP-2 proteins (40x magnification).
Figure 1

A

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B

% maximal colony growth vs. GM-CSF (ng/ml)

C

WT

E76K

D

% maximal colony growth vs. IL-3 (ng/ml)

E

WT

E76K
Figure 2

Hypersensitive CFU-GM colony growth induced by E76K SHP-2 is dependent on PTPase activity, but does not require the βc subunit.

(A) CFU-GM colony growth of fetal liver cells expressing WT SHP-2, WT-C463S (catalytically inactive WT SHP-2), E76K SHP-2, or E76K-C463S (a catalytically inactive E76K mutant SHP-2) over a range of GM-CSF concentrations. Cells expressing WT, WT-C463S, and E76K-C463S SHP-2 proteins all demonstrate a normal pattern of CFU-GM colony growth. (B) CFU-GM colony growth of WT and βc-deficient (βc-/−) bone marrow cells transduced with WT or E76K SHP-2 vectors over a range of IL-3 concentrations. βc-/− bone marrow cells expressing E76K SHP-2 demonstrate a hypersensitive response that is similar to WT bone marrow cells.
Figure 2

A

![Graph A](image)

B

![Graph B](image)
Figure 3

E76K SHP-2 expression increases HPP-CFC and LPP-CFC growth.

C-kit positive bone marrow cells expressing WT or E76K SHP-2 were cultured with saturating concentrations of SCF, M-CSF, IL-1, and either GM-CSF (A) or IL-3 (B). The number of HPP-CFC and LPP-CFC colonies are plotted per 2,000 cells. Asterisks indicate significant differences between cells transduced with E76K versus WT SHP-2 retroviral constructs (p<0.05).
Figure 3

- Graph shows the comparison of colony number/2,000 cells for HPP-CFC and LPP-CFC under different conditions:
  - WT and E76K conditions are represented.
  - Variables include GM-CSF and IL-3.

For HPP-CFC:
- GM-CSF: WT vs E76K
- IL-3: WT vs E76K

For LPP-CFC:
- GM-CSF: WT vs E76K
- IL-3: WT vs E76K

Significance indicated by *.
Figure 4

E76K SHP-2 expression enhances proliferation and perturbs differentiation of fetal liver cell growth in liquid cultures.

(A) GFP-positive cells that had been transduced with WT or E76K SHP-2 were isolated by sorting, and plated in quadruplicate at $1.2 \times 10^6$ cells per well in medium containing 15% FBS and 2 ng/ml of GM-CSF. GM-CSF was removed from the culture medium after 48 hours (on day 3), and live cells were counted every other day. (B) Cytospin preparations of cells removed after 5 days in culture (200x original magnification). Whereas most of the cells in WT cultures are mature neutrophils, E76K SHP-2 cultures show a predominance of monocyte-macrophage cells with some blast like elements. (C) Progenitor colony growth of cells isolated from WT and E76K SHP-2 liquid cultures in methylcellulose medium supplemented with IL-3, IL-6, SCF, and EPO. (D) BrdU incorporation by liquid culture cells maintained in GM-CSF over time. Data shown are for 2 hours of labeling. Similar differences were observed in WT and E76K SHP-2 cultures that were labeled for 4 hours. (E) Flow cytometry analysis of BrdU incorporation by WT and E76K SHP-2 liquid cultures on day 6. All of the data shown in panels A-E are representative of 3 independent experiments.
Figure 4

A. Graph showing the number of cells vs. days in culture for WT and E76K.

B. Images comparing WT and E76K samples.

C. Bar chart showing the number of colonies per 10^5 cells for WT and E76K on different days.

D. Bar chart showing the percentage of BrdU-positive cells for WT and E76K on different days.

E. Flow cytometry plots comparing 7AAD and BrdU for WT and E76K, showing 22.0% and 41.6% respectively.
**Figure 5**

**Effects of E76K SHP-2 expression on erythroid progenitor growth.**

(A) Fetal liver (FL) and bone marrow (BM) cells transduced with E76K SHP-2 form large numbers of burst-forming unit erythroid (BFU-E) colonies in methylcellulose medium supplemented with a saturating dose of erythropoietin (EPO). In contrast, cells expressing WT SHP-2 form few colonies. (B) E76K SHP-2-expressing fetal liver cells show EPO-independent BFU-E colony growth. The addition of EPO increases both the number and the size of the BFU-E colonies.
Figure 5

A

B

number of colonies/10,000 cells

WT FL  E76K FL  WT BM  E76K BM

number of colonies/200,000 cells

EPO (ng/ml)
Figure 6

Comparative effects of E76K, D61Y, and N308S SHP-2 expression on CFU-GM colony growth.

(A) Fetal liver cells engineered to express D61Y SHP-2 show cytokine-independent CFU-GM colony formation, but are less hypersensitive to GM-CSF than cells expressing E76K SHP-2. (B) Fetal liver cells expressing N308S SHP-2 do not demonstrate GM-CSF-independent colony formation and display modest hypersensitivity.
Figure 6

A

B
References

Chapter 4

Germline *KRAS* mutations cause

Noonan syndrome
Abstract

Noonan syndrome (NS; MIM 163950) is characterized by short stature, facial dysmorphism, and cardiac defects\(^1\). Heterozygous mutations in \textit{PTPN11}, which encodes SHP-2, cause \(~50\%\) of Noonan syndrome\(^1,2\). The SHP-2 phosphatase relays signals from activated receptor complexes to downstream effectors, including Ras\(^3\). We discovered novel \textit{de novo} germline \textit{KRAS} mutations that introduce V14I, T58I, or D153V amino acid substitutions in 5 individuals with Noonan syndrome and a P34R alteration in a patient with cardiofaciocutaneous (CFC) syndrome (MIM 115150), which has overlapping features with Noonan syndrome\(^1,4\). Recombinant V14I and T58I K-Ras proteins display defective intrinsic GTP hydrolysis and impaired responsiveness to GTPase activating proteins, render primary hematopoietic progenitors hypersensitive to growth factors, and deregulate signal transduction in a cell lineage specific manner. These studies establish germline \textit{KRAS} mutations as a cause of human disease and infer that the constellation of developmental abnormalities seen in Noonan syndrome spectrum is due, in large part, to hyperactive Ras.
**Introduction**

Ras proteins regulate cell fates by cycling between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound conformations (Ras-GTP and Ras-GDP) (reviewed in\(^5,6\)). The competing activities of guanosine nucleotide exchange factors (GNEFs) and GTPase activating proteins (GAPs) regulate Ras-GTP levels. Activated growth factor receptors recruit signal relay proteins that stimulate GNEFs, which displace guanine nucleotides from Ras and permit passive binding to GTP. Ras-GTP interacts productively with Raf-1, phosphatidylinositol 3-kinase, Ral-GDS, and other effectors. The intrinsic Ras GTPase terminates signaling by hydrolyzing Ras-GTP to Ras-GDP. This slow “off” reaction is greatly augmented by GAPs\(^5,6\). The somatic \(KRAS\) and \(NRAS\) mutations found in myeloid malignancies and other cancers, which introduce amino acid substitutions at codons 12, 13, and 61, encode proteins that accumulate in the GTP-bound conformation due to defective intrinsic GTPase activity and resistance to GAPs\(^5-7\).

Juvenile myelomonocytic leukemia (JMML) is a myeloproliferative disorder (MPD) characterized by leukocytosis with tissue infiltration and *in vitro* hypersensitivity of myeloid progenitors to granulocyte-macrophage colony stimulating factor (GM-CSF) (reviewed in\(^8\)). The incidence of JMML is increased 200-500 fold in children with neurofibromatosis type 1 (NF1), a familial cancer syndrome caused by inactivating mutations in the \(NF1\) tumor suppressor. Neurofibromin, the \(NF1\) gene product, is a GAP for Ras (reviewed in\(^6,8\)). JMML cells from children with NF1 show biallelic \(NF1\) inactivation and elevated Ras-GTP levels\(^9,10\). Approximately 25% of JMMLs demonstrate somatic \(NRAS\) or \(KRAS\) mutations, which are restricted to patients who do not have NF1\(^8\).
The discovery of heterozygous *PTPN11* mutations as a major cause of Noonan syndrome revealed a novel molecular lesion in JMML cases without *RAS* or *NF1* mutations. SHP-2, the *PTPN11* gene product, is a non-receptor protein tyrosine phosphatase that plays a positive role in transducing signals to Ras-GTP and other effectors (reviewed in ). The *PTPN11* mutations identified in Noonan syndrome are gain-of-function alleles, which encode SHP-2 proteins that variably deregulate phosphatase activity, the affinity of the SH2 domains for phosphotyrosyl ligands, and/or substrate specificities. Infants with Noonan syndrome show a spectrum of hematologic abnormalities and are predisposed to JMML. Specific germline *PTPN11* mutations are associated with JMML, and novel somatic mutations were unexpectedly identified in ~35% of *de novo* JMMLs. Leukemia-associated *PTPN11* alleles encode strong gain-of-function SHP-2 proteins that deregulate Ras signaling and cause aberrant myeloid proliferation in vivo and in vitro. Here we show that germline *KRAS* mutations are a cause of both Noonan syndrome and cardiofaciocutaneous (CFC) syndrome and describe novel biochemical and functional properties of two mutant K-Ras proteins.
Results

We found several novel KRAS mutations in individuals with Noonan and CFC syndromes. A three month old female with Noonan syndrome and a severe clinical phenotype presented with a JMML-like MPD (patient 1, Table 1). We did not identify a PTPN11 mutation in her leukocyte DNA, but instead found a novel heterozygous C→T transition at position 173 of KRAS, predicting a T58I substitution (Fig. 1). This mutation was also present in her buccal cells, but absent in parental DNA. Analysis of 124 Noonan syndrome patients without PTPN11 mutations revealed a novel heterozygous G→A transition at nucleotide 40 of KRAS predicting a V14I substitution in three unrelated individuals (patients 2–4 in Table 1). Each individual showed a milder clinical phenotype than patient 1, and none have a history of MPD or cancer (Table 1). Based on these initial findings, we analyzed 50 additional Noonan syndrome patients and 12 with CFC syndrome and identified novel KRAS mutations in one patient with Noonan syndrome and in one individual with CFC syndrome (patients 5–6 in Table 1). All sequence changes occurred de novo, and these KRAS alterations were not found in 200 normal Europeans. All of the predicted amino acid exchanges affect highly conserved residues (Supplementary Fig. 1), and none appear in the National Center for Biotechnology Information database as a single nucleotide polymorphism.

To investigate the biochemical properties of V14I and T58I K-Ras, we produced N-terminal glutathione S-transferase (GST) fusions encoding amino acids 1-166 of each protein as well as wild-type (WT) and oncogenic G12D K-Ras. Both Noonan syndrome-associated mutant proteins had reduced intrinsic GTPase activities relative to WT K-Ras, but hydrolyzed GTP more efficiently than G12D K-Ras (Fig. 2a). The intrinsic GTPase
activity of T58I K-Ras was lower than V14I K-Ras (Fig. 2a). We next assayed the ability
of the GAP related domains (GRDs) of p120 GAP and neurofibromin to stimulate GTP
hydrolysis by WT and mutant K-Ras proteins. As expected, both GRDs markedly
enhanced the GTPase activity of WT K-Ras, whereas G12D K-Ras was highly resistant
(Fig. 2b, c). V14I K-Ras was impaired relative to the WT protein, but more responsive
than G12D K-Ras, to p120 GAP and neurofibromin (Fig. 2, c). Despite lower intrinsic
GTPase activity, T58I K-Ras was activated to the same degree as the V14I protein by
neurofibromin (Fig. 2b). Interestingly, T58I K-Ras was more responsive to p120 GAP
than to neurofibromin, though less responsive to both GAPs than WT K-Ras (Fig. 2c).
Val14 and Thr58 are near the active site and the Ras/GAP interface (Supplementary Fig.
2). Since both side chains point away from the interface, it is likely that the effects of
mutating these residues to isoleucine on GTP hydrolysis are mediated by subtle
conformational changes (Supplementary Fig. 2).

To assess the effects of V14I and T58I K-Ras on hematopoietic progenitor colony
growth, we infected mouse fetal liver cells with murine stem cell virus (MSCV) vectors
encoding full length WT or mutant K-Ras proteins with a green fluorescent protein (GFP)
gene downstream of an internal ribosomal entry site. GFP-positive cells were isolated
by cell sorting and plated in methylcellulose medium over a range of GM-CSF
concentrations to enumerate CFU-GM colonies. As expected from previous data, G12D K-Ras and E76K SHP-2 had strong phenotypic effects in this assay with cytokine-
independent CFU-GM colony formation and a hypersensitive pattern of growth at low
concentrations of GM-CSF (Fig. 3a and data not shown). Expression of T58I K-Ras
induced a hypersensitive pattern of CFU-GM growth in response to GM-CSF that was
less pronounced than G12D K-Ras (Fig. 3a). Although subtle, V14I K-Ras also reproducibly caused GM-CSF hypersensitivity (Fig. 3a). Fetal liver cells expressing mutant K-Ras proteins also formed more burst forming unit-erythroid (BFU-E) colonies in response to erythropoietin than WT cells (Fig. 3b). As in myeloid progenitors, the T58I mutant protein was more potent than V14I K-Ras, but attenuated relative to G12D K-Ras.

We expanded macrophage progenitors in macrophage colony stimulating factor (M-CSF) to investigate the biochemical properties of V14I and T58I K-Ras in myeloid cells. Macrophage progenitors expressing either T58I or G12D K-Ras grew more rapidly (data not shown) and showed higher Ras protein levels than cells expressing WT or V14I K-Ras (Fig 3c). Ras activation was assayed in macrophage progenitors that were deprived of M-CSF and serum for 24 hours, then stimulated with GM-CSF. Cells expressing WT or V14I K-Ras had low levels of Ras-GTP after starvation that increased markedly 10 minutes after GM-CSF stimulation and declined by 60 minutes (Fig. 3c). By contrast, macrophage progenitors expressing either T58I or G12D K-Ras showed elevated levels of Ras-GTP in the starved state with robust and prolonged activation of Ras-GTP in response to GM-CSF (Fig. 3c). The levels of Ras-GTP observed in macrophage progenitors correlated with the amounts of phosphorylated MEK and Akt (Fig. 3c). We also investigated Ras signaling in COS-7 monkey kidney cells. Surprisingly, COS-7 cells expressing V14I K-Ras demonstrated higher basal and serum-starved Ras-GTP levels than cells expressing T58I K-Ras (Fig. 3d). In murine embryonic fibroblasts (MEFs), WT K-Ras did not cause focus formation and G12D K-Ras expression resulted in morphologic senescence, as expected from previous data.
Interestingly, V14I and T58I K-Ras induced focus formation with greater numbers seen in MEFs expressing the V14I mutant protein (data not shown).

**Discussion**

Aoki and coworkers\(^{20}\) recently showed that germline *HRAS* mutations cause Costello syndrome, which shares some clinical features with Noonan syndrome. These *HRAS* alleles introduce amino acid substitutions at codons 12 and 13 such as G12D and G12V that also occur as somatic mutations in cancer and have been characterized extensively\(^{5-7}\). By contrast, the *KRAS* mutations that we discovered in children with Noonan syndrome are unprecedented and encode proteins with novel biochemical and functional properties. These data, which suggest that H-Ras and K-Ras have specific roles in development that overlap in some organs, also support the idea that strong *KRAS* gain-of-function alleles are likely to be incompatible with life. Widespread expression of *Kras*\(^{G12D}\) from its endogenous promoter\(^{21}\) or homozygous *Kras* inactivation\(^{22}\) is lethal during embryogenesis whereas *Hras* and *Nras* knock out mice are viable. These reports and our data infer that the degree and duration of K-Ras signaling plays a critical role in regulating mammalian developmental programs, which is consistent with studies showing that modulating Ras/ERK activation downstream of tyrosine kinase receptors has dramatic phenotypic effects in cultured cells (reviewed in\(^{23}\)). The importance of K-Ras signaling in regulating embryonic cell fates may also partially explain why *KRAS* is mutated far more frequently than *HRAS* or *NRAS* in human cancers.

The patients with V14I substitutions had a milder clinical phenotype than the infant with the T58I mutation. While we are unable to draw conclusions regarding
genotype/phenotype correlations from this small series of patients, it is intriguing that T58I K-Ras has lower intrinsic GTPase activity than V14I K-Ras, but responds similarly to neurofibromin and is more strongly activated by p120 GAP. Because T58I K-Ras responds as well or better than V14I K-Ras to GAPs, the impaired intrinsic K-Ras GTPase activity might underlie the severe clinical phenotype in patient 1. Although GAPs accelerate GTP hydrolysis thousands of fold, the idea that variable levels of intrinsic GTPase activity are biologically relevant is consistent with studies in which the transforming potential of H-Ras proteins with different codon 61 substitutions is inversely related to intrinsic GTPase activities. Furthermore, substituting Gly12 to proline results in a Ras protein that is resistant to GAPs, but retains near normal intrinsic GTPase and GDP dissociation rates. This protein does not transform cultured cells.

A provocative implication of our data is that cell context strongly influences how hyperactive Ras perturbs signaling pathways and cellular phenotypes. Whereas T58I K-Ras was more potent in hematopoietic progenitors, V14I K-Ras had more pronounced effects in COS-7 cells and MEFs. Observations in children with NF1 who develop JMML and insights from Nf1 mutant mice reveal a non-redundant role of neurofibromin in regulating Ras signaling in myeloid lineage cells. The neurofibromin GRD, but not the p120 GAP GRD, corrects the growth properties of cultured Nf1 deficient hematopoietic cells. Since T58I K-Ras GTPase is highly responsive to p120 GAP, the effects of this mutant protein may be attenuated in cell types in which p120 GAP activity predominates. We have not yet characterized either D153V or P34R K-Ras. However, Stone and coworkers isolated P34R in a screen for activating substitutions in the c-H-Ras effector domain and found that P34R H-Ras was insensitive to GAPs, but had
comparable intrinsic GTPase activity to WT H-Ras\textsuperscript{27}. Additional investigation of these proteins will provide further insights regarding the properties of germline \textit{KRAS} mutations that result in human disease.

E76K SHP-2 and G12D K-Ras are strong gain-of-function mutant proteins that only result from cancer-associated somatic mutations. A D61G SHP-2 alteration that occurs both in the germline and as a somatic mutation in leukemia shows intermediate potency in hematopoietic progenitors, causes decreased viability in knock in mice due to cardiac malformations, and induces Noonan syndrome-like facial abnormalities and an indolent MPD in surviving animals\textsuperscript{28}. These findings are reminiscent of our patient with the T58I K-Ras substitution. Importantly, D61G mutant mice demonstrated elevated levels of phosphorylated ERK that was restricted to specific tissues\textsuperscript{28}. By contrast, N308S and N308D are common Noonan syndrome-associated SHP-2 substitutions that are not associated with JMML\textsuperscript{2,29}. These proteins have modest effects on CFU-GM colony growth, which are similar to V14I K-Ras\textsuperscript{14,16}. We are exploiting a conditional knock in strategy to interrogate the developmental and tumorigenic consequences of V14I and T58I K-Ras (D.A. Tuveson and K.S.; unpublished data).

Our data showing that the V14I and T58I K-Ras substitutions confer similar biochemical and cellular phenotypes as NS-associated SHP-2 mutations strongly implicates hyperactive Ras as a critical biochemical lesion in Noonan syndrome. Consequently, we speculate that germline mutations in other genes that deregulate K-Ras signaling will be identified in the \textasciitilde45\% of Noonan syndrome patients without \textit{PTPN11} or \textit{KRAS} mutations.
Materials and Methods

**Patients.** We obtained DNA samples from patients with Noonan syndrome who were ascertained and followed at three large clinical centers. Noonan syndrome was diagnosed on the basis of standardized clinical criteria assessed by experienced clinical geneticists. Informed consent was obtained from all subjects and/or their parents. The experimental procedures were approved by the Committees for Human Research at Erlangen and Freiburg.

**Mutation Detection.** We isolated genomic DNA from blood lymphocytes using standard procedures. Mutational screening of the *PTPN11* gene was carried as previously described\(^2,11,29\), and was negative in all patients. Mutational screening of *NRAS*, *HRAS*, and *KRAS* (isoforms 4a and 4b) was carried out by direct, bidirectional sequencing of purified polymerase chain reaction (PCR) products using the ABI BigDye Termintor Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI3100 Capillary Array Sequencer (Applied Biosystems) or by means of denaturing high-performance liquid chromatography (DHPLC), through use of the Wave DNA Fragment Analysis System (Transgenomics). PCR products showing an abnormal DHPLC profile were re-amplified from genomic DNA for confirmation on a second screen before sequencing.

**KRAS Expression Constructs.** Wild-type (WT) *KRAS* cDNA was cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA). The QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce point mutations, which were verified by sequencing. Gateway technology (Invitrogen) was used to clone WT
and mutant KRAS cDNAs into the pDEST12.2 vector (Invitrogen) and into the MSCV backbone containing a green fluorescent protein (GFP) cassette driven by an internal ribosomal entry site (IRES) downstream of the KRAS sequence. In addition, KRAS cDNA encoding the first 166 amino acids of WT and mutant proteins was cloned into the pGEX-4T-2 vector (Amersham, Piscataway, NJ) to generate recombinant N-terminal GST fusion proteins.

**Intrinsic and GAP-Stimulated GTP Hydrolysis.** These assays have been described elsewhere\(^9,18\). Briefly, recombinant GRD proteins from p120 GAP and neurofibromin were produced in *E. coli*. We incubated 2 nM of each recombinant GST-K-Ras fusion protein that had been preloaded with (\(\gamma\)-\(^{32}\)P)GTP without (intrinsic GTPase activity assay) or with GRD proteins (GAP assays) at ambient temperature. The reactions were terminated by quenching with acid. The free phosphate was then extracted from the aqueous layer and quantified in a scintillation counter. Intrinsic GTP hydrolysis was measured over a time course from 10-80 minutes whereas GAP activity was assessed at 8 minutes. All assays were performed in duplicate.

**Retroviral Transduction and Hematopoietic Progenitor Assays.** All experimental procedures involving mice were reviewed and approved by the UCSF Committee on Animal Research. Pregnant WT C57B1/6 females were humanely killed by CO\(_2\) inhalation at E14.5 and fetal liver cells were isolated and prepared as described\(^{16}\). Fetal liver cells were cultured in a stimulation medium containing StemSpan SFEM (StemCell Technologies, Vancouver, BC, Canada), 15% FBS, 100 ng/mL stem cell factor (SCF;
Peprotech, Rocky Hill, NJ), 50 ng/mL FLT-3 ligand (Peprotech), and 100 ng/mL IL-11 (R&D Systems, Minneapolis, MN). MSCV-KRAS-IRE5-GFP plasmids engineered to express WT or mutant K-Ras proteins were cotransfected with plasmids encoding retroviral gag-pol and env proteins into Phoenix cells using Lipofectamine2000 (Invitrogen). Supernatants from transfected cells were used to transduce fetal liver cells 24 to 72 hours after harvest. After transduction, GFP-positive fetal liver cells were sorted on a FACSVantage SE or a FACSARia (both from BD Biosciences, San Jose, CA). To enumerate CFU-GM colonies, GFP-positive fetal liver cells were seeded in methylcellulose medium (M3231; StemCell Technologies) containing recombinant murine GM-CSF (Peprotech). Colonies were counted on day 8 by indirect microscopy. BFU-E colonies were grown in methylcellulose medium (M3234; StemCell Technologies) containing a range of murine recombinant erythropoietin concentrations (R&D Technologies) and counted on day 7. Cellular content was evaluated by harvesting colonies into phosphate-buffered saline followed by counting, cytopsin preparations, and Wright-Giemsa staining. Macrophage progenitors were grown by culturing transduced, GFP-positive fetal liver cells in 50 ng/mL M-CSF (Peprotech) as described by Chan et al.15

**Ras-GTP Assay and Western Blot Analysis.** To assess Ras activation in macrophage progenitor cultures, cells were starved in Iscove’s modified Dulbecco’s medium (IMDM; Gibco-BRL, Gaithersburg, MD) for 24 hours and then stimulated with 10 ng/mL recombinant murine GM-CSF (Peprotech) for 10 or 60 minutes. The cells were collected using cell dissociation buffer (Invitrogen). COS-7 cells were transiently transfected using
Lipofectamine2000 (Invitrogen) with pDEST12.2 vectors encoding WT, V14I, T58I, and G12D K-Ras mutant proteins. The medium was changed on cells 24 hours after transfection to DME-H21 containing 0.1% FBS and cells were collected after 6 and 12.5 hours. Macrophage progenitors and COS-7 cells were lysed and Ras-GTP levels were measured as described previously\textsuperscript{30} using Raf-1 RBD agarose (Upstate, Lake Placid, NY). The antibodies used for immunoblotting included anti-Pan-Ras (Ab-3) (Calbiochem, San Diego, CA), anti-phospho-MEK1/2 (Ser217/221), anti-MEK1/2, anti-\(\beta\)-actin (all from Cell Signaling, Beverly, MA), and anti-phospho-AKT (a generous gift of David Stokoe, UCSF).
Acknowledgements

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Table 1

Clinical features of patients with Noonan syndrome and CFC syndrome and germline \textit{KRAS} mutations

<table>
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<td>V14I</td>
<td>V14I</td>
<td>P34R</td>
<td>D153V</td>
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<td>PS, left ventricle hypertrophy</td>
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<td>3rd centile</td>
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<td>NS</td>
<td>NS</td>
<td>CFC</td>
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* received growth hormone treatment from the age of 5 years

\textbf{Legend.} Patient 5, who was diagnosed with CFC on the basis of standard criteria, had a Kavamura index of 12.1. HOCM: hypertrophic obstructive cardiomyopathy; HCM:
hypertrophic cardiomyopathy; ASD: atrial septal defect; VSD: ventricular septal defect; PS: pulmonary stenosis; NS: Noonan syndrome; CFC: cardiofaciocutaneous syndrome
Figure 1

Clinical phenotypes and KRAS mutations in patients with Noonan and CFC syndromes. (a, b) Clinical appearance of patient 1 at age 14 months and of patient 3 at age 10 years. (c) Exon-intron structure of the human KRAS gene showing non-coding exons as blue bars and protein coding exons as black or purple bars. Alternative splicing results in two K-Ras isoforms (“4a” and “4b”) that differ at the C terminus. K-Ras4a includes exon 4a (purple bar), which contains a “stop” codon, whereas the C terminal amino acids of K-Ras4b are encoded by exon 4b. (d) Structure of the K-Ras4b protein showing the P loop (P-L), Switch I (Sw I), and Switch II (Sw II) domains. (e) Amino acid substitutions found in patients with Noonan and CFC syndromes with sequence alignments to the normal H-, N-, and K-Ras proteins. Val14 is located in the P loop, Pro34 is in Sw I, Thr58 is near the NH₂ boundary of Sw II, and Asp153 is in the α5 helix. The three amino acids (G12, G13, and Q61) that are altered by cancer-associated somatic KRAS, NRAS, and HRAS mutations are marked with asterisks.
Figure 1
Figure 2

Intrinsic GTP hydrolysis of WT and mutant K-Ras proteins and responses to GAPs.

(a) Intrinsic GTP hydrolysis measured as the number of counts per minute (cpm) released over time. (b, c) Phosphate release by WT and mutant K-Ras proteins (cpm) in response to 2.5 – 40 nM of neurofibromin or p120 GAP GRD. Note the similar response of V14I K-Ras to both GAPs. By contrast, p120 GAP accelerates GTP hydrolysis by T58I K-Ras to a much greater extent than neurofibromin.
Figure 2

a

![Graph showing GTP hydrolysis over time for different proteins (WT, T58I, V14I, G12D).](image)

b

![Graph showing GTP hydrolysis vs. [NF1-GRD] concentration for different proteins (WT, T58I, V14I, G12D).](image)

c

![Graph showing GTP hydrolysis vs. [p120 GAP-GRD] concentration for different proteins (WT, T58I, V14I, G12D).](image)
Figure 3

Functional and biochemical characteristics of V14I and T58I K-Ras proteins.

(a) CFU-GM colony growth of fetal liver cells expressing WT, V14I, T58I, or G12D K-Ras over a range of GM-CSF concentrations. G12D K-Ras colonies were large and monocytic whereas T58I and V14I colonies resembled WT CFU-GM colonies with a normal mixture of neutrophils and monocytes. (b) BFU-E colony formation from fetal liver cells expressing WT, V14I, T58I, or G12D K-Ras. Data show number of colonies per 150,000 GFP-positive cells. (c) Activation of Ras and downstream effectors in macrophage progenitor cultures expressing WT and mutant K-Ras proteins in response to 10 ng/mL of GM-CSF. (d) Ras signaling in transiently transfected COS-7 cells under basal conditions (cells growing in serum), or after 6 and 12.5 hours in 0.1% fetal bovine serum.
Figure 3

a

![Graph depicting % maximal colony growth vs. GM-CSF (ng/ml)]

b

![Graph depicting number of BFU-E colonies vs. EPO (ng/ml)]

c

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d

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Supplementary Figure 1

Sequence alignments of two regions of human K-Ras isoforms with their orthologs in different species.

Black and grey shadings indicate identical and similar residues, respectively. The highly conserved residues mutated in patients with Noonan and CFC syndromes are marked in red and green, respectively. The amino acid residue at position 153 varies between the similar residues Asp and Glu, but no other residue is tolerated throughout evolution. Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Dr, *Danio rerio*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Ca, *Candida albicans*. 
Supplementary Figure 1

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References from the public database:
NP_203524.1: c-K-ras2 protein isoform a [Homo sapiens]
NP_004976.2: c-K-ras2 protein isoform b [Homo sapiens]
P3285: RASS, MOUSE GTPase K-Ras (K-Ras 2) (K-Ras) (c-K-ras) [Mus musculus]
NP_067295.2: c-K-ras2 protein [Mus musculus]
CAAG72253.1: proto-oncogene K-Ras2A [Xenopus laevis]
CAAG72256.1: K ras [Xenopus laevis]
AAH43815.1: Similar to Kirsten rat sarcoma oncogene 2, expressed [Danio rerio]
AABF12514.1: Ras1 [Drosophila melanogaster]
P3291: LET500 CAEEL Ras protein let-50 (Lethal protein 50) [Caenorhabditis elegans]
LAL50825.1: Ras GTPase [Dactylostelium discoideum]
JCS528: Ras2 protein - slime mold [Dactylostelium discoideum]
AALF035265.1: Ras homolog type A [Candida albicans]
AALF035267.1: Ras homolog type B [Candida albicans]
Supplementary Figure 2

Locations of valine-14 and threonine-58 in the Ras/p120 GAP co-crystal structure. (a) shows the full complex and (b) zooms in on the active site. The secondary structure of the Ras molecule is colored cyan and the p120 GAP molecule is colored blue. Val14 and Thr58 in the Ras molecule are shown in yellow, GDP is colored by atom type, and Arg789 from the p120 GAP molecule (the "arginine finger") is also highlighted. Several loops of the Ras molecule are also differentiated: switch I and switch II, which form part of the interaction with p120 GAP, are colored red, while the active site "P-loop" is colored green. Note that a magnesium and an aluminum fluoride that stabilize the Ras/GAP complex in the crystal structure are shown in purple.
Supplementary Figure 2

(a)

(b)
References

Chapter 5

Biochemical and functional characterization of germline *KRAS* mutations
Abstract

Germline missense mutations in *HRAS* and *KRAS* and in genes encoding molecules that function up or downstream of Ras in cellular signaling networks cause a group of related developmental disorders that includes Costello syndrome, Noonan syndrome, and cardio-facio-cutaneous syndrome. We performed detailed biochemical and functional studies of three mutant K-Ras proteins (P34R, D153V, and F156L) found in individuals with Noonan and cardio-facio-cutaneous syndromes. Mutant K-Ras proteins demonstrate a range of gain-of-function effects in different cell types, and biochemical analysis supports the idea that the intrinsic Ras guanosine nucleotide triphosphatase (GTPase) activity, the responsiveness of these proteins to GTPase activating proteins, and guanine nucleotide binding/dissociation all regulate developmental programs *in vivo.*
Introduction

Ras proteins are signal switch molecules that cycle between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound conformations (Ras-GTP and Ras-GDP) (reviewed in\textsuperscript{1,2}). The counterbalancing activities of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) control Ras-GTP levels \textit{in vivo} (reviewed in\textsuperscript{2}). SOS1, the major GEF in many mammalian cells, is recruited to protein complexes that assemble on activated growth factor receptors. SOS1 binds to Ras to displace bound guanine nucleotides, and Ras then passively rebinds to either GDP or GTP. Because GTP is much more abundant in the cytosol\textsuperscript{3}, nucleotide exchange increases intracellular Ras-GTP levels. GTP binding induces a conformational shift in the Switch I and II domains of Ras that allows Ras-GTP to interact productively with effectors such as Raf family members, phosphatidylinositol 3 (PI3) kinase, and Ral-GDS. Signaling is terminated when Ras-GTP is hydrolyzed to Ras-GDP. This reaction is catalyzed by an inefficient intrinsic Ras GTPase activity that is markedly accelerated by binding to GAPs. Neurofibromin and p120 GAP are the predominant GAPs in most mammalian cells\textsuperscript{2,4,5}.

Somatic missense \textit{KRAS} mutations that introduce amino acid substitutions at positions 12, 13, and 61 are among the most common molecular lesions found in human cancer. Oncogenic K-Ras proteins accumulate in the GTP-bound conformation due to defective intrinsic GTPase activity and resistance to GAPs\textsuperscript{6}. Surprisingly, germline \textit{KRAS} mutations that encode novel amino acid substitutions not found in cancer were recently discovered in 2-4\% of individuals with Noonan syndrome as well as in some persons with cardio-facio-cutaneous (CFC) syndrome\textsuperscript{7-10}. We found that two Noonan
syndrome-associated K-Ras proteins (V14I and T58I K-Ras) are gain-of-function alleles that are less activated than oncogenic G12D K-Ras by a variety of biochemical and functional criteria\(^9\). V14 is located within the K-Ras phosphate-binding (P) loop whereas T58 is near the Switch II domain. Figure 1A shows the locations of amino acid substitutions found in persons with Noonan and CFC syndromes, which include alterations within the K-Ras Switch I domain (P34L, P34Q, P34R, and I36M) and in the \(\alpha\)-5 helix of the 4B isoform (V152G, D153V, F156I, and F156L)\(^7\)–\(^10\). Here we describe a comprehensive biochemical and functional analysis of three mutant K-Ras proteins that cause Noonan and CFC syndromes: P34R, D153V, and F156L K-Ras. The phenotypic features of persons with each mutation have been described\(^7\)–\(^9\), including an individual with the F156L substitution who is one of two siblings with independent germline \(RAS\) gene mutations\(^10\).
Results and Discussion

We first loaded N-terminal GST fusion proteins encoding amino acids 1-166 of wild-type (WT), G12D, P34R, D153V, and F156L K-Ras with \( \gamma^{-32}P \)GTP and measured intrinsic GTPase activities. P34R and D153V K-Ras both displayed normal intrinsic rates of GTP hydrolysis (Fig. 1B). In contrast, the F156L mutant protein demonstrated impaired intrinsic GTPase activity that was similar to oncogenic G12D K-Ras (Fig. 1B). We next assayed the ability of the GAP-related domains (GRDs) of neurofibromin and p120 GAP to stimulate K-Ras GTPase activity. As expected\textsuperscript{11,12}, both GAPs markedly enhanced the GTPase activity of WT K-Ras, whereas G12D K-Ras was resistant (Figs. 1C, 1D). Remarkably, the P34R K-Ras GTPase was insensitive to both GAPs. F156L K-Ras showed an intermediate level of responsiveness, whereas D153V showed equivalent GTP hydrolysis to WT K-Ras (Figs. 1C, 1D).

Based on structural modeling, Carta and colleagues\textsuperscript{7} proposed that the D153V substitution destabilizes regions of K-Ras that contribute to guanine nucleotide binding, and they predicted that this destabilization would result in increased guanine nucleotide dissociation. To test this hypothesis directly, we assayed guanine nucleotide dissociation from recombinant K-Ras proteins. D153V K-Ras showed a normal rate of guanine nucleotide dissociation over a 2 – 120 minute time course (Fig. 1E and data not shown). By contrast, F156L K-Ras displayed a markedly increased rate of guanine nucleotide dissociation, which is consistent with a previous study of F156L H-Ras\textsuperscript{13}.

We next expressed mutant K-Ras proteins in COS-7 monkey kidney cells and measured Ras-GTP levels and the phosphorylation of downstream effectors. Cells expressing P34R K-Ras displayed elevated levels of Ras-GTP under basal growth
conditions in 10% fetal calf serum and after serum deprivation (Fig. 2A). Ras-GTP levels were modestly elevated in D153V-expressing cells in basal growth conditions, but similar to WT-K-Ras after 6 and 12 hours of serum deprivation (Fig. 2A and data not shown). P34R and D153V K-Ras expression also consistently induced higher levels of phosphorylated MEK and ERK (pMEK and pERK) in COS-7 cells (Fig. 2A). Cells expressing F156L K-Ras displayed elevated levels of Ras-GTP and pMEK that were similar to what we observed with the P34R mutant protein. 10

Infants with Noonan syndrome show a spectrum of hematologic abnormalities and are predisposed to juvenile myelomonocytic leukemia (JMML). 14 We have previously shown that the mutant SHP-2 and K-Ras proteins identified in Noonan and JMML have gain-of-function effects in primary hematopoietic progenitors, which correlate with the degree of biochemical activation. 9,15 We therefore infected mouse fetal liver cells with murine stem cell virus (MSCV) vectors encoding full length WT or mutant K-Ras proteins and a green fluorescent protein (GFP) gene downstream of an internal ribosomal entry site (IRES). GFP-positive cells were isolated by cell sorting and were plated in methylcellulose medium over a range of granulocyte/macrophage colony-stimulating factor (GM-CSF) concentrations to enumerate colony-forming unit-granulocyte/macrophage (CFU-GM) colonies. P34R, D153V, and F156L K-Ras all induced a hypersensitive pattern of CFU-GM colony formation that was most pronounced in cells expressing P34R K-Ras (Fig. 2B and Supplementary Fig. 1). The D153V mutant protein displayed the weakest effects with respect to both the number of colonies and their size and morphology. Importantly, only oncogenic G12D K-Ras induced cytokine-independent CFU-GM colony growth (Fig. 2B). We also investigated
the effects of this K-Ras allele series on burst forming unit-erythroid (BFU-E) colony formation. As in myeloid progenitors, P34R, D153V, F156L K-Ras all potentiated BFU-E colony growth with the same relative potency as in the CFU-GM progenitor assay (Fig. 2C and Supplementary Fig. 2).

To investigate Ras signaling in a disease-relevant primary cell type, we infected fetal liver cells with MSCV-KRAS-IRES-GFP viruses and cultured them in macrophage colony stimulating factor (M-CSF) to generate macrophage progenitors. Cells expressing mutant K-Ras proteins grew more rapidly (data not shown) and showed variable levels of pMEK, pERK, pAkt, and pS6 that correlated with their effects on CFU-GM and BFU-E colony growth (Fig. 2B, 2C). Notably, macrophage progenitors expressing P34R or G12D K-Ras showed markedly increased levels of pMEK, pERK, pAkt, and pS6 (Fig. 2D). F156L K-Ras induced a modest increase in both pAkt and pS6, but pMEK and pERK levels were normal. As in the colony assays, D153V K-Ras was the least potent allele in macrophage progenitors with cells expressing this mutant protein only showing elevated levels of pS6 (Fig. 2D).

The patterns of somatic and germline KRAS, HRAS, and NRAS mutations observed in cancer and developmental disorders infer distinct biologic functions for specific Ras isoforms. Germline NRAS mutations have not been reported to date and almost all of the germline HRAS mutations found in Costello syndrome introduce amino acid substitutions at positions that are also commonly altered in cancer (codons 12 and 13). By contrast, the KRAS mutations discovered in persons with Noonan and CFC syndromes are not found in cancer. Kras is the only Ras gene that is essential for murine
embryogenesis\textsuperscript{16,17}, and \textit{KRAS} is by far the most commonly mutated \textit{RAS} gene in human tumors\textsuperscript{18}. Thus, fully characterizing these novel K-Ras mutant proteins \textit{in vitro} and in cells is an important priority. We find that they display a complex pattern of intrinsic biochemical properties (Table 1) and have variable effects on cellular signaling and hematopoietic progenitor colony growth (Fig. 2).

P34R and G12D K-Ras are insensitive to p120 GAP and neurofibromin; however, P34R K-Ras retains normal intrinsic GTPase activity. P34R K-Ras is more potent than other proteins encoded by germline \textit{KRAS} mutations by many criteria; it accumulates in the GTP-bound conformation and induces high levels of pERK, pMEK, pAkt, and pS6 in COS-7 cells and primary macrophages, and has dramatic effects on hematopoietic progenitor colony growth. These data as well as the disease phenotypes observed in individuals with NF1, Noonan, and CFC syndrome support an essential role of GAPs as negative regulators of Ras signaling in normal development. It is surprising that the P34R mutation is highly resistant to GAPs because homozygous inactivation of either \textit{Gap} or \textit{Nf1} is lethal in mouse embryos\textsuperscript{19-21}. Possible explanations for this apparent paradox include: (1) differential requirements for Ras signaling in murine and human development; (2) the existence of essential Ras or GAP-independent functions of neurofibromin and p120 GAP; (3) compromised affinity of P34R K-Ras for effectors; and, (4) the expression of normal Ras proteins in the tissues of individuals with germline \textit{KRAS} mutations, which can be regulated by GAPs. Our studies of P34R K-Ras also suggest that levels of intrinsic K-Ras GTPase activity modulates cell fates in specific tissues as only G12D K-Ras induces cytokine-independent CFU-GM and BFU-E colony growth. In addition, G12D K-Ras induces more robust hematopoietic colony growth.
compared to P34R K-Ras, suggesting the intrinsic K-Ras GTPase activity may also modulate sensitivity of progenitors to growth factors.

The strong effects of P34R K-Ras that we observed in various cell types could be explained, in part, by over-expression, which might have overcome reduced affinity for effectors that could be relevant when the protein is expressed at endogenous levels. We are generating K\textit{ras}\textsuperscript{P34R} “knock in” mice to address this question and to determine the developmental consequences of expressing P34R K-Ras from the \textit{Kras} locus (D.A. Tuveson and KMS; unpublished data). Interestingly, Stone and coworkers\textsuperscript{22} isolated P34R in a screen for activating substitutions in the c-H-Ras effector domain that could transform fibroblasts. They found that P34R H-Ras was insensitive to GAPs and a competitive binding assay suggested that this resistance might be due to decreased affinity of the mutant protein for p120 GAP.

In contrast to P34R K-Ras, the F156L mutant protein has defective intrinsic GTPase activity, but is partially responsive to GAPs. F156L K-Ras also shows a rapid rate of nucleotide exchange, and accumulates in the GTP-bound conformation in cells. These biochemical data are consistent with a previous study of F156L H-Ras, which also reported that the H-Ras mutant protein showed modest transforming potential in fibroblasts\textsuperscript{13}. We believe the rapid rate of nucleotide dissociation did not significantly affect our ability to assay intrinsic and GAP-stimulated GTP hydrolysis of F156L K-Ras because the experimental conditions allowed for efficient rebinding of labeled nucleotide.

We have not uncovered aberrant biochemical properties of the recombinant D153V mutant protein. D153V K-Ras had the weakest effects on signaling in COS-7 cells and primary macrophages and induced modest, but reproducible, GM-CSF
hypersensitivity in hematopoietic progenitors that is similar to the effects of germline
SHP-2 mutant proteins in these cells\textsuperscript{15,23}. Primary hematopoietic stem/progenitor cells
from \textit{Mx1-Cre; Kras} \textsuperscript{G12D} compound mutant mice show elevated levels of pS6, which are
due to inputs from both the PI3 kinase/Akt and Raf/MEK/ERK cascades\textsuperscript{24}. Similarly,
pS6 showed the greatest difference between WT macrophage progenitors and cells
expressing each germline K-Ras mutant protein, including D153V. It is possible that our
\textit{in vitro} assays are not sufficiently sensitive to detect subtle biochemical alterations in the
recombinant D153V K-Ras protein or that it is deregulated by a novel mechanism such as
increased effector affinity or enhanced sensitivity to SOS1 or another exchange factor.
Further investigation of this mutation, which is associated with a range of clinical
manifestations, may uncover a novel mechanism of regulating Ras output \textit{in vivo}.

Many of the mutations detected in persons with disorders of the Noonan
syndrome spectrum introduce novel amino acid substitutions in SHP-2, SOS1, H-Ras, K-
Ras, B-Raf, MEK1, and MEK2. Characterizing the biochemical and functional
properties of these mutant proteins can provide unexpected insights into mechanisms of
normal growth control. This principle is perhaps best illustrated by in depth studies of
mutant SHP-2 proteins resulting from somatic and germline \textit{PTPN11} mutations\textsuperscript{15,23,25-29}.
Somatic leukemia-associated \textit{PTPN11} alleles encode strong gain-of-function SHP-2
proteins with elevated phosphatase activity, which is essential for aberrant hematopoietic
growth\textsuperscript{15,23,25,28,29}. However, a thorough biochemical analysis of the germline \textit{PTPN11}
mutations found in Noonan syndrome uncovered a more complex picture with mutant
SHP-2 proteins displaying variable effects on phosphatase activity, the affinity of the
SH2 domains for phosphotyrosyl ligands, and substrate specificities\textsuperscript{28}. Most surprisingly,
the ***PTPN11*** mutations that cause LEOPARD syndrome, a disorder that shares some clinical features with Noonan syndrome, abrogate or markedly diminish SHP-2 phosphatase activity\(^{29-31}\).

Our studies of mutant K-Ras proteins found in Noonan and CFC syndromes support the idea that the intrinsic Ras GTPase activity, the responsiveness of these proteins to GAPs, and guanine nucleotide binding/dissociation all regulate developmental programs \textit{in vivo}. Analysis of \(\alpha\)-5 helix mutations highlights the importance of regions outside the P-loop and Switch domains in Ras regulation. As the molecular causes of developmental disorders are discovered, it is worth considering if the terms that are used to describe these diseases should be replaced by a diagnosis that is based on the affected gene. The highly variable and cell context-specific properties of K-Ras mutant proteins found in human developmental disorders argue for establishing a hybrid classification system that includes both the clinical syndrome and the identity of the underlying mutation (e.g. Noonan syndrome due to a K-Ras D153 substitution). This will provide the most accurate information for performing genotype/phenotype correlations. Furthermore, the distinct biochemical properties of mutant proteins identified in patients with the same clinical diagnosis will likely affect responses to molecularly targeted therapeutics.
Materials and Methods

**KRAS Expression Constructs.** Wild-type (WT) KRAS human cDNA was cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA). The QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce point mutations, which were verified by sequencing. Gateway technology (Invitrogen) was used to clone WT and mutant KRAS cDNAs into the pDEST12.2 vector (Invitrogen) and into the MSCV backbone containing a green fluorescent protein (GFP) cassette driven by an internal ribosomal entry site (IRES) downstream of the KRAS sequence. In addition, KRAS cDNA encoding the first 166 amino acids of WT and mutant proteins was cloned into the pGEX-4T-2 vector (Amersham, Piscataway, NJ) to generate recombinant N-terminal GST fusion proteins.

**Biochemical Analysis of Recombinant Proteins.** Intrinsic and GAP-stimulated GTP hydrolysis assays have been described in detail and were performed as described previously. Recombinant GAP-related domain (GRD) proteins from p120 GAP and neurofibromin were produced in *E.coli*. We incubated 200 nM of each recombinant K-Ras protein that had been preloaded with [γ-32P]GTP without (intrinsic GTPase activity assay) or with GRD proteins (GAP assays) at room temperature. To measure GTP dissociation, recombinant K-Ras proteins (500 nM) were labeled with [α-32P]GTP in 5 mM EDTA at room temperature. Aliquots were removed from the loading reaction and placed into 20 mM Hepes pH 7.3, 50 mM NaCl₂, 2 mM MgCl₂, 2 mM DTT, 0.2 mg/ml BSA, and 0.2 mM (excess) unlabeled GTP to allow guanine nucleotide exchange for the designated time points. The amount of labeled guanine nucleotide bound to K-Ras was
determined by vacuum filtration through nitrocellulose filters (0.22 µM pore size) and liquid scintillation counting. Each data point was derived from duplicate samples and the recombinant proteins were investigated in multiple independent experiments.

**Retroviral Transduction and Hematopoietic Progenitor Assays.** All experimental procedures involving mice were reviewed and approved by the UCSF Committee on Animal Research. These assays were performed as described elsewhere on cells that were first infected with MSCV-KRAS-IRES-GFP retroviruses engineered to express WT or mutant K-Ras proteins and then sorted to isolate GFP-positive cells for culture in methylcellulose medium (M3231 for CFU-GM assays and M3234 for BFU-E assays; both from StemCell Technologies, Vancouver BC). CFU-GM and BFU-E colonies were grown over a range of recombinant murine GM-CSF (Peprotech; Rocky Hill NJ) or recombinant murine erythropoietin concentrations (R&D Technologies; Minneapolis MN), respectively, and were counted by indirect microscopy. Macrophage progenitors were grown by culturing transduced, GFP-positive fetal liver cells in 50 ng/mL M-CSF (Peprotech) as described by Chan et al.

**Ras Signaling and Western Blot Analysis.** COS-7 cells were transiently transfected using Lipofectamine2000 (Invitrogen) with pDEST12.2 vectors encoding WT, P34R, D153V, F156L, and G12D K-Ras mutant proteins. The medium was changed on cells 24 hours after transfection to DME-H21 containing 0.1% FBS and cells were collected after 6 hours. COS-7 cells were lysed and Ras-GTP levels were measured as described previously using Raf-1 RBD agarose (Upstate, Lake Placid, NY). Macrophage
progenitors were collected using cell dissociation buffer (Invitrogen) and lysed similarly
to COS-7 cells. The antibodies used for immunoblotting included anti-K-Ras (F234)
(Santa Cruz Biotechnology), and anti-phospho-MEK1/2 (Ser217/221), anti-MEK1/2,
anti-phospho-p44/42 MAPK/ERK1/2 (Thr202/Tyr204), anti-β-actin, anti-phospho-S6
(Ser235/236) (all from Cell Signaling, Beverly, MA), and anti-phospho-Akt (a generous
gift of David Stokoe, UCSF).
Acknowledgements

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Table 1

Summary of biochemical properties of mutant K-Ras proteins

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<th>K-Ras</th>
<th>Clinical phenotype</th>
<th>Intrinsic GTPase Activity</th>
<th>Response to neurofibromin</th>
<th>Response to p120 GAP</th>
<th>Nucleotide exchange</th>
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Legend. Specific characteristics of each mutant are displayed according to a scoring system that relates the biochemical activities of each mutant protein to wild-type (WT) and G12D K-Ras. Abbreviations: CS: Costello syndrome; NS: Noonan syndrome; CFC: cardio-facio-cutaneous syndrome; NS/CFC: overlapping clinical features of NS and CFC syndrome.
Figure 1

Biochemical analysis of wild-type (WT) and mutant K-Ras proteins.

(A) Schematic representation of K-Ras4B showing the distribution of the amino acid substitutions encoded by germline mutations found in developmental disorders (above) and the three amino acids that are commonly altered by cancer-associated somatic mutations (below). The P-loop, Switch I (Sw I), and Switch II (Sw II) domains are conserved among all Ras isoforms (H-Ras, N-Ras, K-Ras4A, and K-Ras4B). These isoforms vary considerably in the hypervariable region. The germline substitutions characterized in this study are shown in red. (B) Intrinsic GTP hydrolysis measured as the number of counts per minute (cpm) released over time. (C, D) GTP hydrolysis stimulated by various concentrations of the GRD of neurofibromin (C) or p120 GAP (D). GTP hydrolysis was measured after 8 min. (E) Dissociation of bound GTP from WT and mutant K-Ras proteins over time.
Figure 1

A

B

C

D
Figure 1

E

![Graph showing percent bound over time for different conditions.](image)
Figure 2


(A) Ras signaling in transiently transfected COS-7 cells under basal growth conditions or after 6 h in 0.1% fetal bovine serum (starvation). (B) CFU-GM colony formation of fetal liver cells expressing WT or mutant K-Ras proteins over a range of GM-CSF concentrations. (C) BFU-E colony formation of fetal liver cells expressing WT and mutant K-Ras proteins over a range of erythropoietin (EPO) concentrations. Data show number of colonies per 200,000 GFP-positive fetal liver cells. (D) Phosphorylation of signaling proteins downstream of Ras in macrophage progenitors expressing WT or mutant K-Ras proteins.
Figure 2

A

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K-Ras-GTP
K-Ras
pMEK
pERK
MEK

B

![Graph showing percentage maximal colony formation vs. GM-CSF (ng/ml)]
Figure 2

C

D

WT  P34R  D153V  F156L  G12D

pMEK
pERK
pAKT
pS6
K-Ras
Actin
Supplementary Figure 1

Morphology of representative CFU-GM progenitor colonies grown in saturating concentrations of GM-CSF (20 ng/ml) from fetal liver cells expressing WT or mutant K-Ras proteins. All images are original magnification x 40.
Supplementary Figure 1

WT

P34R

D153V

F156L

G12D
Supplementary Figure 2

Morphology of representative BFU-E colonies grown in BFU-E colonies grown in 10 ng/ml of erythropoietin from fetal liver cells expressing WT or mutant K-Ras proteins. All images are original magnification x 40.
References


Chapter 6

Conclusions and Discussion
Ras proteins have been among the most intensively studied molecules in biology for over three decades. H-, K-, and N-Ras regulate diverse cellular behaviors through a complex network of effector cascades. RAS genes are the most common targets for dominant gain-of-function mutations in human cancer (reviewed in1). The discoveries of germline mutations in NF1 in neurofibromatosis type I and PTPN11 in Noonan syndrome provided the first indication that aberrant Ras signaling might underlie human developmental disorders (reviewed in2,3). Recently, novel mutations in components of the canonical Ras/Raf/MEK/ERK pathway have been identified in individuals with Noonan, Costello, and cardio-facio-cutaneous syndromes4-7. These developmental disorders share phenotypic features that include facial abnormalities, heart defects, impaired growth and development, and, in some instances, a predisposition to specific cancers3,8. The overall goal of this thesis work has been to investigate the biochemical and functional consequences of Ras pathway mutations associated with developmental disorders of the Noonan syndrome spectrum and myeloid leukemia.

**PTPN11 mutations in juvenile myelomonocytic leukemia and Noonan syndrome.**

Chapter 2 reports the discovery of somatic PTPN11 mutations in ~35% of juvenile myelomonocytic leukemia (JMML) patient specimens, and in a small percentage of other myeloid malignancies. We show that Ba/F3 pro-B cells expressing leukemia-associated mutant SHP-2 proteins display enhanced growth factor-independent survival. This observation is consistent with data from Nf1-deficient myeloid cells, which also survive in the absence of growth factor9. RAS, NF1, and PTPN11 mutations are largely mutually exclusive in JMML, which suggests that mutant SHP-2 proteins deregulate myeloid
growth through Ras. However, biochemical analysis in Ba/F3 cells failed to demonstrate hyperactivation of the Ras effectors ERK or Akt. I also did not detect any Ras signaling abnormalities in other factor-dependent cell lines including FDC-P1 cells, an early myeloid progenitor line. Tartaglia et al. reported modest increases in ERK phosphorylation in COS-7 monkey kidney cells expressing Noonan syndrome and leukemia-associated mutant SHP-2 proteins\textsuperscript{10,11}. The discrepancies between these studies and our data might be due to expression levels of the mutant proteins and/or the cellular context. In addition, and perhaps more importantly, increased ERK phosphorylation was only observed when mutant SHP-2 proteins were introduced in cells overexpressing Gab1, an adapter protein required for EGF-dependent SHP-2 activation\textsuperscript{10,11}. Studies from other investigators that failed to observe aberrant activation of Ras effectors in factor-dependent hematopoietic cell lines expressing mutant SHP-2 proteins are consistent with our data\textsuperscript{12}. Further evidence that the effects of SHP-2 mutant proteins are cell type specific is provided by a knock in mouse model engineered to express endogenous levels of D61G SHP-2, a substitution found in both Noonan syndrome and leukemia\textsuperscript{13}. Interestingly, increased ERK activation was restricted to specific tissues in this model, and mutant mouse embryo fibroblasts displayed normal ERK activation.

In Chapter 3, I describe functional analysis of leukemia-associated mutant SHP-2 proteins in primary murine hematopoietic cells. We found that E76K SHP-2 induced a hypersensitive pattern of CFU-GM colony growth in response to GM-CSF and IL-3 that was dependent on SHP-2 catalytic activity. E76K SHP-2 enhanced growth in multiple hematopoietic compartments, including erythroid and immature progenitor populations, and perturbed normal hematopoietic differentiation. In addition, leukemia-associated
SHP-2 mutations conferred stronger phenotypes than a germline mutation found in individuals with Noonan syndrome.

The observed hypersensitive growth of hematopoietic progenitors expressing leukemia-associated mutant SHP-2 proteins is consistent with the hypersensitivity of JMML cells to GM-CSF, as well as a similar pattern of aberrant myeloid progenitor growth from Nfi-deficient and Kras$^{G12D}$ mutant bone marrow cells$^{14-19}$. CFU-GM colonies grown from Nfi and Kras mutant cells and from cells expressing leukemia-associated mutant SHP-2 proteins are abnormally large and contain many monocytes. Interestingly, peripheral monocytosis is one of the diagnostic criterion for JMML. JMML is also characterized by anemia and ineffective erythropoiesis$^{14,20}$. The effects of leukemia-associated mutant SHP-2 on erythroid progenitors are therefore consistent with clinical and laboratory observations in human JMML and with the block in erythroid differentiation and anemia observed in Kras mutant mice$^{18,21}$.

The somatic $PTPN11$ mutations found in JMML encode amino acid substitutions that are largely distinct from the germline mutations that underlie Noonan syndrome. Disease-associated $PTPN11$ mutations fall into three general groups: germline mutations in individuals with Noonan syndrome, somatic mutations in JMML and other leukemias, and a small group of germline mutations that occur in individuals with Noonan syndrome who develop JMML$^{22}$. We found that leukemia-associated mutant SHP-2 proteins have much stronger gain-of-function effects than a Noonan syndrome-associated mutant protein in progenitor colony assays. This spectrum of mutant SHP-2 potency that I observe in myeloid progenitors is consistent with the data of Mohi et al. who also show intermediate effects of those mutations that occur in individuals with Noonan syndrome.
who develop JMML\textsuperscript{12}. While there are distinct biological, and presumably pathogenic, properties of the three different groups of \textit{PTPN11} mutations, the effects of each mutation on the biochemical properties of SHP-2 appears more complicated. The majority of Noonan syndrome and leukemia-associated mutations affect residues in the N-SH2 or phosphatase domains involved in the autoinhibition of SHP-2. As predicted by structural modeling, most Noonan syndrome or leukemia-associated mutants show elevated basal phosphatase activity and initial studies of mutant SHP-2 proteins proposed that levels of SHP-2 catalytic activity, as measured using recombinant proteins \textit{in vitro}, determined the severity of biological consequences\textsuperscript{10,23-26}. However, more detailed structural and biochemical analyses showed that disease-associated \textit{PTPN11} mutations have variable effects on phosphatase activity, the affinity of the SH2 domains for phosphotyrosyl ligands, and substrate specificities\textsuperscript{24,25}. The ability of SHP-2 to be recruited to upstream signaling molecules, the affinity for substrates, and interactions with effectors likely determine the effects of mutant SHP-2 proteins in specific cellular contexts. Thus, there appears to not be a simple way to predict the pathogenic consequences of \textit{PTPN11} mutations in individuals with Noonan syndrome, which may complicate clinical management under some circumstances.

In our studies, transplantation of bone marrow cells from C57/B6 mice transduced with leukemia-associated SHP-2 mutants did not consistently induce myeloproliferative disease, although some mice developed anemia in association with splenic infiltration by myeloid and erythroid cells. By contrast, Mohi \textit{et al.} showed that transplantation of bone marrow cells from Balb/C strain mice transduced with leukemia-associated SHP-2 mutants caused a fatal myeloproliferative disease and T-cell leukemia\textsuperscript{12}. These data
suggest that one or more strain-specific modifier genes contribute to the disease phenotype in this transplant model. In addition, analysis of retroviral integration sites by Mohi et al. revealed the disease was oligoclonal, suggesting additional events are required for mutant SHP-2 induced myeloid disease\textsuperscript{12}. This hypothesis is consistent with the observation that chromosome 7 deletions are also found in some myeloid malignancies with $PTPN11$\textsuperscript{14,20,27}.

Phosphatase activity is required for most known signaling functions of SHP-2\textsuperscript{28,29}. However, the key substrates that are required to activate the Ras/Raf/MEK/ERK pathway in both normal and disease states remain uncertain\textsuperscript{28,29}. Several substrates have been proposed to mediate Ras pathway activation. One involves a p120 GAP docking site on growth factor receptors or adapters such as Gab proteins; by dephosphorylating such sites, SHP-2 prevents the membrane localization of p120 GAP, thereby preventing the downregulation of Ras\textsuperscript{30-32}. Additionally, SHP-2 has been shown to dephosphorylate the tyrosine phosphorylated feedback inhibitor Sprouty. By inactivating this negative regulator of Ras signaling, SHP-2 would have a generalized positive effect on growth factor dependent signaling\textsuperscript{33,34}. Finally, inactivation of the negative regulatory CSK pathway has also been proposed to mediate SHP-2-dependent Ras activation\textsuperscript{35,36}.

$PTPN11$ mutations also cause multiple lentigines/LEOPARD syndrome, a disorder that shares several clinical features with Noonan syndrome, including facial abnormalities, heart defects, and growth retardation\textsuperscript{3}. However, patients with LEOPARD syndrome manifest pigmentary abnormalities such as lentigines (freckle-like spots), which are uncommon in Noonan syndrome but are reminiscent of some of the cutaneous findings seen in NF1. LEOPARD syndrome-associated $PTPN11$ mutations introduce
amino acid substitutions within the SHP-2 phosphatase domain that are distinct from those found in Noonan syndrome. Interestingly, LEOPARD syndrome-associated mutant SHP-2 proteins have defective phosphatase activity and have dominant negative effects on EGF-stimulated ERK activation\textsuperscript{26,37,38}. It is not known how mutations in \textit{PTPN11} that either impair or activate the SHP-2 phosphatase cause distinct developmental disorders with some similar clinical features. Molecular modeling suggests that LEOPARD syndrome mutations result in open forms of SHP-2\textsuperscript{37} and it is possible that these proteins have gain-of-function effects in the absence of phosphatase activity through an adapter function. SHP-2 has two tyrosine phosphorylation sites near the carboxyl terminus, including a Grb2 binding site, and the role of these tyrosine residues in LEOPARD syndrome remains to be investigated. It is also possible that LEOPARD syndrome-associated mutant proteins affect cell types that are different from those affected in Noonan syndrome during development. In summary, whereas \textit{in vitro} studies suggest that SHP-2 proteins encoded by LEOPARD syndrome-associated \textit{PTPN11} mutations result in loss of function, the complex architecture of Ras signaling networks leaves the possibility open that the phosphatase-defective mutant proteins aberrantly activate Ras.

**Germline RAS mutations in developmental syndromes.**

Noonan syndrome overlaps phenotypically with other disorders, including Costello and CFC syndromes, which are characterized by facial dysmorphism, heart abnormalities, short stature and also by a high incidence of mental retardation. Individuals with Costello syndrome are predisposed to specific cancers, including rhabdomyosarcoma, ganglioneuroblastoma, and bladder cancer. These individuals do not have mutations in
PTPN11, and Aoki and colleagues\textsuperscript{4} identified \textit{de novo} germline HRAS mutations in 12 of 13 individuals with Costello syndrome, which was corroborated by other groups\textsuperscript{39-42}. Remarkably, almost all of the HRAS mutations identified in Costello syndrome introduce amino acid substitutions at codons 12 and 13 that also occur as somatic mutations in tumors. However, the most common substitution identified in Costello syndrome (G12S), is relatively uncommon in cancer. Importantly, biological data indicate that the transforming properties of G12S \textit{H}-Ras are attenuated relative to G12V \textit{H}-Ras, which is the most common cancer-associated substitution\textsuperscript{43}. Germline mutations encoding K117R and A146T \textit{H}-Ras substitutions have also been reported in Costello syndrome\textsuperscript{41,42}. Lysine-117 resides in a conserved guanine nucleotide binding sequence, NKXD (residues 116-119), and substitutions at these positions display increased rates of guanine nucleotide dissociation\textsuperscript{44}. Since the GTP:GDP ratio is ~ 9:1 in cells\textsuperscript{45}, an increased rate of dissociation favors the active, GTP-bound state of Ras. Indeed, K117R \textit{H}-Ras displays an increased rate of guanine nucleotide dissociation and transforms rodent fibroblasts\textsuperscript{44}. Structural analysis of \textit{H}-Ras bound to guanine nucleotides suggests that alanine-146 is involved in binding to the guanine base, and substitution to threonine may similarly increase guanine nucleotide dissociation\textsuperscript{42}. Consistent with the oncogenic potential of the HRAS mutations described above, cultured fibroblasts from individuals with Costello syndrome demonstrate increased proliferation\textsuperscript{4}.

\textit{RAS} genes were also considered good candidate disease genes in the \textasciitilde50\% of individuals with Noonan syndrome without \textit{PTPN11} mutations. As described in Chapter 4, we and our collaborators found several germline \textit{KRAS} mutations in individuals with Noonan and CFC syndromes. Studies from other laboratories have also identified
germline KRAS mutations with an overall prevalence of ~2-4% in Noonan syndrome. Importantly, these mutations introduce novel amino acid substitutions that are not found in cancer. Biochemical and functional analysis of Noonan syndrome-associated K-Ras proteins demonstrates that they are gain-of-function mutants, but are less activated than oncogenic K-Ras proteins. Specifically, the intrinsic GTPase activities of the Noonan syndrome-associated V14I and T58I K-Ras recombinant proteins are lower than wild-type K-Ras, but less impaired than oncogenic G12D K-Ras. Furthermore, both mutant proteins also show intermediate levels of GTP hydrolysis in response to the GAP related domains of p120 GAP and neurofibromin compared to wild-type and G12D K-Ras. A particularly interesting feature of the T58I mutant protein was its differential responsiveness to p120 GAP and neurofibromin. Studies of V14I and T58I K-Ras in hematopoietic progenitors, macrophages, and COS-7 monkey kidney cells showed that these alleles are activated, and support the idea that cell context modulates their functional and biochemical effects.

Amino acid substitutions within the α-5 helix of the K-Ras4B isoform were identified in some patients with Noonan syndrome including V152G, D153V, F156I, and F156L. These residues are located far from the GTP-binding region of the protein. Based on structural modeling, Carta and colleagues hypothesized that the V152G and D153V substitutions destabilize regions of the K-Ras protein that contribute to guanine nucleotide binding, and they predicted that these substitutions increase nucleotide dissociation in a manner similar to K117R H-Ras. We assayed guanine nucleotide dissociation from recombinant D153V K-Ras and observed a normal rate of dissociation over a 2–120 minute time course. By contrast, we found that F156L K-Ras shows a
rapid rate of guanine nucleotide dissociation. Although biochemical analysis of the V152G mutant has not been reported, we speculate that it will have similar properties as D153V K-Ras.

Germline mutations that introduce substitutions at Proline-34 in the Switch I domain of K-Ras have been reported developmental disorders of the Noonan syndrome spectrum\textsuperscript{7,47}. We found that P34R K-Ras has a normal rate of intrinsic GTP hydrolysis, but is completely resistant to stimulation by p120 GAP and neurofibromin. In our functional studies, P34R K-Ras is more potent than other proteins encoded by germline KRAS mutations by many criteria; it accumulates in the GTP-bound conformation and induces high levels of pERK, pMEK, pAkt, and pS6 in COS-7 cells and primary macrophages, and has dramatic effects on hematopoietic progenitor colony growth. These data as well as the disease phenotypes observed in individuals with NF1, Noonan syndrome, and CFC syndrome support an essential role of GAPs as negative regulators of Ras signaling in normal development. Our studies of P34R K-Ras also suggest that levels of intrinsic K-Ras GTPase activity modulate cell fates in specific tissues. For example, expressing G12D K-Ras, but not P34R K-Ras, in hematopoietic progenitors results in cytokine-independent CFU-GM and BFU-E colony growth. Together, the germline KRAS mutations discovered in persons with Noonan and CFC syndromes comprise a unique allele series for assessing the relative importance of the intrinsic K-Ras GTPase, GAPs, and GNEFs in regulating developmental fates.

\textit{RAF} pathway mutations in developmental syndromes.

CFC syndrome is a rare sporadic disorder characterized by distinctive craniofacial
features, heart defects, and mental retardation. As these systems are also affected in Noonan syndrome, it has been uncertain whether Noonan syndrome and CFC are related entities or distinct disorders. Indeed, individuals who demonstrate clinical features of both Noonan and CFC syndromes are sometimes described as having “Noonan syndrome/CFC” or “severe Noonan syndrome”. Based on these clinical observations, it is perhaps not surprising that germline KRAS mutations were identified in some individuals with CFC and Noonan syndrome/CFC. Whether individuals with germline KRAS mutations should be classified clinically as having CFC syndrome, severe Noonan syndrome, or as a distinct pathologic entity, is unclear.

As researchers expanded their candidate gene searches to include other molecular components of Ras effector pathways, two groups independently identified heterozygous missense BRAF mutations as the major cause of CFC syndrome. Germline MEK1 and MEK2 mutations were also discovered in some individuals without BRAF mutations. The finding that germline BRAF mutations underlie most cases of CFC syndrome is of exceptional interest to cancer biologists since, as discussed below, a V600E substitution in B-Raf is one of the most common molecular lesions found in human cancer. The BRAF mutations that occur in CFC syndrome introduce amino acid substitutions that are more widely distributed across the B-Raf protein than substitutions found in cancer, and only a few individuals with CFC syndrome had a substitution that has been reported in tumors. Most CFC-associated B-Raf proteins have increased kinase activity, including some (Q257R, S467A, L485F, and K499E) that are quite strongly activated. However, some mutants show impaired B-Raf kinase activity and did not activate ERK in human 293T kidney epithelial cells. These initial biochemical data argue against a simplistic
hypothesis in which the CFC-associated mutations lead to intermediate strength and/or duration of signaling compared to the robust signaling instigated by oncogenic \textit{BRAF} mutations. However, one potential confounding issue in interpreting these studies is that the levels of oncogenic B-Raf V600E kinase activity relative to wild-type B-Raf that were measured in this report is substantially lower than what others have observed\textsuperscript{53}. This raises the possibility that the germline \textit{BRAF} mutant alleles found in CFC syndrome encode mutant B-Raf proteins with significantly lower kinase activity than B-Raf V600E.

Somatic missense \textit{BRAF} mutations occur in the majority of malignant melanomas. \textit{BRAF} mutations are also found frequently in thyroid (30-50%), colorectal (5-20%), and ovarian cancers (30\%)\textsuperscript{50,52}. Most of the cancer-associated mutations are in the kinase domain, and the V600E substitution accounts for \approx 90\% of mutations\textsuperscript{51,52}. The crystal structure of the B-Raf kinase domain has been solved and has provided an improved understanding of B-Raf regulation\textsuperscript{53}. The majority of cancer-associated \textit{BRAF} mutations encode gain-of-function mutants that constitutively activate the kinase and the MEK/ERK pathway. These mutations appear to disrupt the interaction of the glycine loop and activation segment, which destabilizes the inactive conformation of the protein. The cancer-associated mutants show a wide range of kinase activities, including oncogenic proteins that display impaired kinase activity compared to wild-type B-Raf. Interestingly, these proteins still activate ERK via a mechanism involving Raf-1\textsuperscript{53}, raising an additional mechanistic avenue that might also be exploited by CFC-associated mutations. The V600E mutation is also detected in a large percentage of nevi, which are benign skin lesions of melanocytes that are thought to be senescent\textsuperscript{54,55}. In addition, mice expressing endogenous levels of \textit{B Raf}\textsuperscript{V600E} develop benign lung tumors that rarely
progress to adenocarcinoma and show features of senescence\textsuperscript{56}. These data support that idea that \textit{BRAF} mutations are an early event in tumorigenesis, but are insufficient to cause cancer. Cooperating mutations that enable malignancy are likely to further increase dependence on the B-Raf pathway, since chemical inhibitors of B-Raf signaling selectively block proliferation of tumor cell lines with \textit{BRAF} mutations\textsuperscript{57,58}. Perhaps the absence of cooperating mutations is a primary discriminator between CFC-associated and oncogenic B-Raf pathway activation.

In contrast to \textit{BRAF}, \textit{MEK1} and \textit{MEK2} mutations have not been reported in cancer or in any other human disease. Expressing CFC syndrome-associated MEK mutant proteins in cells resulted in levels of phosphorylated ERK that were elevated compared to cells expressing wild-type MEK, but lower than cells expressing constitutively active MEK mutants\textsuperscript{6}. An important implication of the \textit{RAF} and \textit{MEK} mutations that cause CFC syndrome is that the Raf/MEK/ERK kinase cascade is a critical downstream effector pathway of Ras-GTP in regulating developmental programs. The clinical features of CFC syndrome are generally more severe than other disorders of the Noonan syndrome spectrum. It is intriguing that mutations in the B-Raf/MEK/ERK pathway cause more severe developmental defects, whereas the Noonan syndrome-associated mutations discovered to date, which alter upstream signaling molecules, cause a less profound clinical phenotype even though they could potentially deregulate multiple effector pathways. This implies that the B-Raf pathway also plays a dominant role downstream of Ras-GTP in Noonan syndrome and other developmental disorders, but perhaps some of the additional pathways regulated by Ras-GTP actually serve to mitigate B-Raf/MEK/ERK activation.
**SOS1 mutations in Noonan syndrome.**

Germline mutations in *PTPN11* and *KRAS* cause slightly over half of Noonan syndrome. Based on these data, two groups screened other upstream components of the Ras/Raf/MEK/ERK pathway and uncovered germline missense mutations in *SOS1* in ~10% of Noonan syndrome cases\(^{59,60}\). *SOS1* is a complex, multi-domain protein that is a major Ras guanine nucleotide exchange factor. A tandem Dbl homology and pleckstrin homology (DH and PH) domain serve to mask the catalytic Cdc25 homology domain (also called the Ras-GEF domain). Many of the mutations identified in Noonan syndrome alter amino acids in the DH/PH domain that are thought to contribute to this autoinhibition. Therefore, these mutant *SOS1* proteins are thought to permit increased access of Ras to the catalytic site, thereby increasing the rate of guanine nucleotide exchange\(^{61,62}\). Interestingly, the regulation of *SOS1* is highly sensitized to Ras-GTP levels - a second, allosteric, binding site on *SOS1* allows Ras-GTP to further activate *SOS1* activity through a positive feedback mechanism. Noonan syndrome-associated *SOS1* mutations thus encode proteins that are primed to activate the Ras pathway.

Consistent with this model, expressing mutant *SOS1* proteins in cultured cell lines resulted in sustained activation of Ras and ERK in response to epidermal growth factor. One group reported genotype-phenotype correlations, including an increased incidence of abnormal ectodermal features (facial keratosis pilaris and curly hair), in affected individuals with a *SOS1* mutation compared with the general Noonan syndrome population\(^{60}\). Further investigation is required to firmly establish consistent genotype-phenotype correlations in Noonan syndrome, which may also relate to the biochemical
properties of specific SOS1 mutant proteins.

**Concluding Remarks and Future Directions.**

The germline mutations that cause developmental disorders comprise a novel allele series for furthering our current understanding of how the Ras/Raf/MEK/ERK pathway regulates developmental programs and becomes deregulated in human disease. Remarkably, malignant tumors are relatively uncommon in these disorders, which is in marked contrast to the highly penetrant cancer phenotypes seen in individuals with germline mutations of tumor suppressor genes such as *TP53*, *RB1* (Retinoblastoma 1), *BRCA1* and *BRCA2*. One potential explanation for this observation is that the degree and/or duration of Ras activation are insufficient to initiate tumorigenesis in most tissues. Consistent with this idea, the mutant SHP-2 and K-Ras proteins encoded by germline mutations have attenuated biochemical and cellular phenotypes compared to the corresponding oncoproteins. The example of JMML is particularly intriguing in this respect. Whereas cases that are associated with somatic *PTPN11* or *KRAS* mutations are clinically aggressive, JMML-like myeloproliferative disorders that occur in infants with Noonan syndrome frequently regress without treatment. These data suggest that the strength and duration of hyperactive Ras signaling influences the probability of cancer formation. With the exception of *NF1*, the mutations that cause these developmental disorders are dominant gain-of-function alleles that must be tolerated in the germline. The precedent that widespread *Kras*\(^{G12D}\) expression leads to embryonic lethality in mice\(^63\) suggests that some, if not most, of the dominant gain-of-function mutations found in cancer are incompatible with normal development.
Although the general idea that germline mutant alleles are mild hypermorphs compared to somatic cancer-associated mutations is appealing, the \textit{HRAS} mutations that cause Costello syndrome encode strong gain-of-function proteins and it is therefore not surprising that affected individuals are predisposed to specific benign and malignant tumors. The normal tissues that are perturbed in Costello syndrome (nervous system, musculoskeletal system) overlap with the types of malignancies that are observed (rhabdomyosarcoma, neuroblastoma, ganglioneuroblastoma, and bladder cancer). Perhaps endogenous levels of activated \textit{HRAS} alleles are only transforming in the context of certain tissues or the gene is expressed at low levels in most types of cancer-initiating cells. \textit{HRAS} mutations account for <1% of all cancer-associated \textit{RAS} mutations\textsuperscript{64}. The observation that many individuals with Costello syndrome do not develop cancer provides additional evidence that mutant \textit{HRAS} requires cooperating mutations for tumorigenesis. Interestingly, loss or silencing of the normal \textit{HRAS} allele has been reported in some of the malignancies that arise in the context of Costello syndrome\textsuperscript{4,39}. It is also notable that the kinase activities of some of the mutant B-Raf proteins encoded by CFC syndrome-associated mutations are comparable to B-Raf oncoproteins, yet the germline CFC mutations do not predispose to tumor formation. These observations suggest that the relatively low risk of cancer in many of these developmental syndromes is not entirely due to attenuated biochemical activities of the relevant mutant proteins.

The cancers associated with NF1 and disorders of the Noonan syndrome spectrum arise in specific tissues, suggesting that some cell types are sensitive to changes in Ras activation, while others are not. Myeloid, myogenic, and neural tumors predominate, and it is remarkable that epithelial cancers that show a high incidence of \textit{KRAS} mutations (i.e.
carcinomas of the lung, pancreas, and colon) have not been reported. As Kras\textsuperscript{G12D} efficiently initiates lung and pancreatic cancer in mice\textsuperscript{63,65,66}, the absence of these tumors in individuals with Noonan syndrome and CFC syndrome may be due to the fact that strong gain-of-function KRAS alleles are not tolerated in the germline. This idea is consistent with the observation that Kras, but not Hras or Nras, is essential for murine development\textsuperscript{67-70}. As the aberrant reactivation of developmental programs is thought to be integral to malignant transformation in many tissues, it is perhaps not surprising that KRAS is altered by somatic mutation in cancer cells far more often than either HRAS or NRAS. By contrast, germline oncogenic HRAS mutations and Hras inactivation are compatible with normal development in humans and mice, respectively. The discrete genotype/phenotype associations with respect to the germline RAS mutations found in human developmental disorders – strong HRAS gain-of-function alleles in Costello syndrome, less potent KRAS mutations in Noonan and CFC syndromes, and no alterations in NRAS – certainly underscore the importance of understanding the unique functional role that each Ras isoform plays in development, normal cellular growth, and cancer.

Data from diverse sources such as unbiased genetic screens in yeast, animal models, experiments in which cultured cells or genetically engineered mice were exposed to inhibitors of signaling molecules, and human clinical trials have uncovered unexpected complexity and adaptation in signaling networks\textsuperscript{71}. Indeed, the output of a particular pathway appears to be determined by the continuous, varying contribution of individual proteins rather than one or a few dominant components\textsuperscript{72}. From this vantage point, it is somewhat surprising that mutations of single genes are sufficient to confer overt clinical phenotypes as seen in individuals with germline mutations that perturb Ras signaling. In
considering how and why specific mutations cause disease, it is likely that tissue-specific phenotypes reflect both the primary biochemical properties of a mutant protein and the capacity of the cell to neutralize any deleterious effects by remodeling the pathway. From this perspective, the tissues that are perturbed in NF1 and in Costello, Noonan syndrome, CFC, and LEOPARD syndromes may not only be highly dependent on Ras/Raf/MEK/ERK signaling to specify cell fates, but may also be relatively inflexible with respect to their ability to modulate this pathway. This idea might explain why either decreasing or increasing SHP-2 phosphatase activity has deleterious developmental consequences.

Because the germline mutations that cause NF1 and Costello, Noonan syndrome, CFC, and LEOPARD syndromes are present throughout development, there is a substantial window of time for cells to adapt to them. Moreover, all of the cells in the organism express the same mutant protein, which may activate regulatory feedback loops that involve different cell types. By contrast, cancer-associated \textit{KRAS} and \textit{BRAF} mutations arise somatically in a cell (or in a small field of cells) that is surrounded by normal cells. Given the increasing evidence that cell-cell interactions within tissue microenvironments negatively and positively affect tumor growth, these differences are likely to be important. These recent observations also raise intriguing questions regarding the relationship between the developmental timing of specific mutations and the resultant phenotype(s) in individual cells. The proliferative potential and plasticity of fetal cells may differ substantially from their counterparts in adult tissues. Perhaps the clearest example of how developmental mechanisms that are cell intrinsic influence the potential for malignant growth can be found in the hematopoietic compartment. Although
hematopoiesis is a dynamic and life-long process in which immature stem and progenitor cell populations give rise to differentiated progeny, it is striking that children, but not adults, with NF1 are predisposed to myeloid malignancies. The most straightforward explanation of this clinical observation is that both the “soil” (a susceptible hematopoietic stem cell) and the “seed” (loss of the normal NF1 allele) are essential for leukemogenesis. This general idea is consistent with a provocative study showing that leukemia-associated GATA1 mutations, which cause a transient myeloproliferative disease in infants with Down syndrome, are far more potent in fetal than adult hematopoietic cells. These data infer that phenotypic consequences of specific mutations that perturb Ras signaling are likely to be strongly influenced by the developmental state of the cell in which they occur.

Based on the precedent of KRAS, HRAS, BRAF, and PTPN11, other components of Ras signaling networks that are mutated in developmental disorders are novel candidate oncogenes. It will be of interest to determine if somatic SOS1 mutations occur in cancer and, if so, to compare the spectrum of germline and cancer-associated somatic mutations.

Structural, biochemical, and functional characterization of the mutant proteins encoded by germline PTPN11, HRAS, KRAS, SOS1, BRAF, MEK1 and MEK2 mutations will provide opportunities to better understand the architecture of Ras signaling networks, how cells adapt to hyperactive Ras, and mechanisms of growth control in different cell types. Investigating properties such as intrinsic GTPase activity, responsiveness to GAPs, and guanine nucleotide binding/dissociation in a collection of mutant Ras proteins will further our understanding of the role of specific biochemical characteristics in growth.
control and tumorigenesis. Proteins with unexpected behavior may prove particularly instructive. For example, addressing how excessive SHP-2 PTPase activity causes Noonan syndrome while loss of catalytic activity induces LEOPARD syndrome has implications for understanding the general role of SHP-2 in regulating cell fates. Along these lines, detailed investigation of the biochemical properties of mutant B-Raf proteins found in developmental disorders will likely provide insights into how these molecules are deregulated in cancer. Generating strains of mutant mice that express alleles of interest under control of the endogenous promoters will also be integral for understanding their developmental and tumorigenic consequences in vivo. Together, the discoveries of the past few years open new avenues of research that will enhance our understanding of how Ras signaling networks regulate developmental programs and the pathologic consequences of hyperactive Ras in different cellular contexts. Addressing these questions has important implications for the fields of developmental and cancer biology, and may inform strategies for molecularly targeted therapeutics.
References


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