Somatic activation of oncogenic *Kras* in hematopoietic cells initiates a rapidly fatal myeloproliferative disorder

Benjamin S. Braun*, David A. Tuveson†, Namie Kong*, Doan T. Le*, Scott C. Kogan‡, Jacob Rozmus*, Michelle M. Le Beau§, Tyler E. Jacks¶, and Kevin M. Shannon*‖**

Departments of *Pediatrics and ‡Laboratory Medicine and ‖Comprehensive Cancer Center, University of California, San Francisco, CA 94143; †Abramson Center for Cancer Research, University of Pennsylvania, Philadelphia, PA 19104; §Section of Hematology/Oncology, Department of Medicine and Cancer Research Center, University of Chicago, Chicago, IL 60637; and ¶Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139

RAS mutations are common in myeloid malignancies; however, it is not known whether oncogenic *RAS* can initiate leukemia. We show that expressing mutant K-Ras^{G12D} protein from the endogenous murine locus rapidly induces a fatal myeloproliferative disorder with 100% penetrance characterized by tissue infiltration, hypersensitivity to growth factors, and hyperproliferation. Hematopoietic cells from diseased mice demonstrated increased levels of Ras-GTP, but effector kinases were not constitutively phosphorylated and responded normally to growth factors. Oncogenic *RAS* is sufficient to initiate myeloid leukemogenesis in mice, and this provides an *in vivo* system for biologic and preclinical studies.

Mutations in *KRAS2*, *NRAS*, and *HRAS* are the most common oncogenic alterations found in human cancer (1). *RAS* genes encode a family of 21-kDa proteins that regulate cell fates by cycling between inactive GDP-bound (Ras-GDP) and active GTP-bound (Ras-GTP) conformations (2, 3). Ras-GTP interacts with downstream effectors to elicit diverse cellular responses such as proliferation, differentiation, and survival (2–4). Cancer-associated *RAS* alleles encode proteins that accumulate in the GTP-bound conformation as a result of defects in both intrinsic GTPase activity and resistance to GTPase-activating proteins, which normally accelerate GTP hydrolysis on Ras. Oncogenic *RAS* cooperates with other oncogenes and tumor suppressors to transform cultured cells, which demonstrate elevated Ras-GTP levels and aberrant activation of downstream kinase effector cascades.

Overexpressing mutant Ras in wild-type mouse embryonic fibroblasts (MEFs) causes a growth arrest that is reminiscent of replicative senescence, which can be overcome by mutating *Ink4a* or *Tp53* (5). These data suggest a requirement for cooperating mutations to circumvent Ras-induced senescence and imply that *RAS* mutations might occur late in tumorigenesis, when prior mutations have created a permissive environment. This notion has been challenged by studies in strains of mice engineered to express oncogenic Ras proteins from the endogenous locus. Johnson *et al.* (6) constructed a latent mutant *Kras* allele that was activated *in vivo* by spontaneous homologous recombination. These animals uniformly exhibited aggressive lung tumors, and some also developed lymphomas. This model was refined by inserting a transcriptional repression domain flanked by loxP sites (LSL cassette) into the mutant *Kras* locus (7). Activating this allele by tracheal instillation of an adenovirus encoding Cre recombinase efficiently caused lung cancer. Most recently, a cytomegalovirus (CMV)-Cre transgene was used to induce widespread tissue expression of a different conditional mutant *Kras* allele (8). MEFs from these *Kras*^{V12} mice failed to undergo replicative senescence; however, tumorigenesis was largely restricted to the lung. The authors hypothesized that the oncogenic potential of *Kras* is highly tissue-specific and that many somatic tissues tolerate mutant K-Ras protein expression without adverse phenotypic consequences (8). Although *RAS* mutations are found in advanced tumors from a diverse range of tissues, whether oncogenic *RAS* can initiate tumorigenesis outside the lung is unknown.

NRAS and *KRAS2* mutations occur in ≈30% of acute myeloid leukemias (AML), myeloproliferative disorders (MPD), and myelodysplastic syndromes (MDS) (1). Expansion of myeloid blasts with suppression of normal hematopoiesis is the hallmark of AML, whereas MPD is associated with overproliferation of one or more lineages that retain the capacity to differentiate, and MDS is characterized by low blood cell counts and aberrant bone marrow morphology. Many leukemia-associated genetic lesions such as the *PML-RARA* and *AML1-ETO* fusions are associated with discrete disease phenotypes; by contrast, oncogenic point mutations in the *NRAS* and *KRAS2* genes are found in AML, MPD, and MDS. The existence of *RAS* mutations in diverse myeloid malignancies raises the possibility that they invariably represent secondary events that cooperate with a spectrum of initiating genetic lesions. Consistent with this possibility, some AMLs contain subclones with independent *RAS* mutations, and *RAS* mutations that are detected at diagnosis may disappear over time in patients with persistent or relapsed disease (9–11). The failure of *CMV-Cre, Kras*^{V12} mice to develop myeloid leukemia (8) further suggests that *Ras* mutations do not initiate leukemogenesis.

Attempts to produce a tractable mouse model of myeloid leukemia initiated by oncogenic *RAS* have yielded inconsistent results, with disease arising inefficiently (12) or exclusively in the lymphoid lineage (13, 14). MacKenzie *et al.* (12) found that ≈60% of irradiated recipient mice injected with bone marrow that had been transduced with a retrovirus encoding an oncogenic *Nras* allele developed a spectrum of myeloid malignancies. However, the phenotypic variability, incomplete penetrance, and prolonged latency combined with both impaired *in vitro* proliferation and a high rate of apoptosis in *Nras*-infected cells suggested that cooperating mutations were required (12). The difficulties encountered in engineering a penetrant murine model might indicate that oncogenic *RAS* does not confer a durable proliferative advantage in leukemia-initiating cells or could reflect either absence of Ras expression in a relevant target cell population or failure to modulate transgene expression during hematopoietic differentiation.

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To address this issue directly, we harnessed the IFN-inducible *Mx1-Cre* transgene to direct Cre recombinase expression to hematopoietic cells. Here we show that activating the *Kras^{G12D}* allele engineered by Jackson *et al.* (7) initiates an aggressive MPD with 100% penetrance that is associated with hyperproliferation, aberrant responses to growth factors, and rapid demise.

Materials and Methods

Mouse Strains and Polyinosinic–Polycytidilic Acid (pIpC) Treatment. The procedures were approved by the University of California, San Francisco, Committee on Animal Research. *LSL-Kras^{G12D}* and *Mx1-Cre* (7, 15) pups were injected i.p. with 250 μg of pIpC at 21 days of age (Sigma). *LSL-Kras^{G12D}* genotyping was performed as described (7). Primer sequences and amplification conditions for genotyping *Mx1-Cre* mice are available on request.

Pathologic Examination. Blood smears were stained with Wright–Giemsa (Sigma). Organs were collected in 10% formalin and processed by the Mouse Pathology Shared Resource at the University of California, San Francisco, Comprehensive Cancer Center. Tail vein blood (≈ 50 μl) was obtained in Microvet EDTA tubes (Becton Dickinson) for complete blood counts using a Hemavet 850FS (DREW Scientific). Criteria for killing mice included disheveled appearance, hunching, abnormal gait, and pallor.

Flow Cytometry. Staining with FITC- or phycoerythrin-conjugated antibodies and flow cytometry were performed according to the manufacturer's instructions (Pharmingen). Data were collected by using a FACScan flow cytometer and CELLQUEST software (Becton Dickinson), and analyzed with FLOWJO (TreeStar, Ashland, OR).

Colony Assays. Nucleated bone marrow cells (1×10^5) or splenocytes (2×10^5) were suspended in methylcellulose medium (M3231, Stem Cell Technologies) containing recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF) or IL-3 (PeproTech, Boston) and plated in 1-ml duplicate cultures. The colonies were counted on day 8. Cellular content was evaluated by harvesting colonies into PBS followed by counting, cytospin preparations, and Wright–Giemsa staining. For self-renewal assays, quadruplicate cultures were established in 1 ml of methylcellulose medium supplemented with saturating doses of IL-3, IL-6, stem cell factor, and erythropoietin (M3434, Stem Cell Technologies) (16). The colonies were counted every 7 days, harvested, and replated in fresh medium.

Bone Marrow Mast Cell Cultures. Bone marrow mononuclear cells (5×10^6) were seeded in 10 ml of RPMI medium 1640 (Gibco) with 10% FCS (HyClone), 1 mM glutamine, 50 μM 2-mercaptoethanol, penicillin, streptomycin, and 2.5 ng/ml murine IL-3 (PeproTech). Nonadherent cells were replated in fresh medium at a density of $3–5 \times 10^5$ cells per ml every 2–4 days.

Spectral Karyotype Analysis. Short-term (24-h) cultures of *Mx1-Cre Kras^{G12D}* splenocytes, metaphase cell preparation, and spectral karyotyping were performed as described (17). At least 10 metaphase cells were analyzed per case.

Adoptive Transfer. Recipients received a single fraction of 900 cGy. Sublethal irradiation was administered at a dose of 400 cGy. Eight-week-old recipient mice were injected with donor marrow mononuclear cells immediately after irradiation via the dorsal tail vein. Recipients received prophylactic antibiotics for 2 weeks.

Proliferation Assay. Mice received 150 mg/kg BrdUrd (Sigma) i.p. and, 6 h later, marrow cells and splenocytes were collected and resuspended in Iscove's modified Dulbecco's medium containing 20% FCS. Red cells were lysed, and remaining cells were washed with PBS. Cells were fixed overnight in 1% paraformaldehyde, 0.01% Tween 20 at 4°C, washed twice, and resuspended in DNase (Sigma) for 30 min at 37°C. After two washes, cells were stained with anti-BrdUrd–FITC (Pharmingen) for 20 min. Cells were washed twice, then analyzed by flow cytometry.
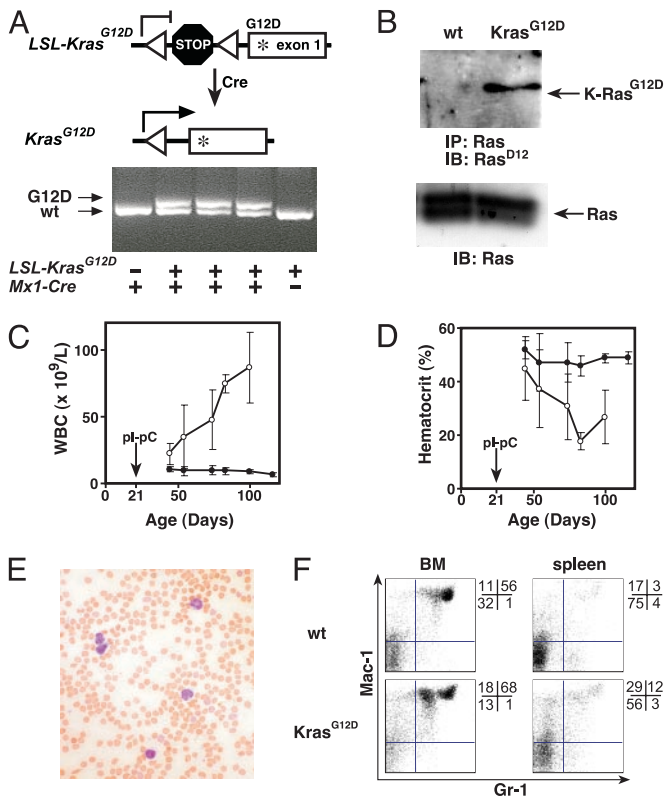
Biochemical Assays. Bone marrow mononuclear cells were harvested in Iscove's modified Dulbecco's medium containing 0.1% FCS, incubated at room temperature for 4 h, then stimulated with GM-CSF for 2–30 min. Cells were then washed with ice-cold PBS containing 1 mM sodium orthovanadate and lysed as described (18). Ras-GTP levels were measured as described elsewhere (18). Western blotting protocols were used to detect mitogen-activated protein (MAP)/extracellular signal-related kinase (ERK) kinase (MEK) (Cell Signaling 9122, 1:1,000), phospho-MEK (Cell Signaling 9121, 1:1,000), Akt (Cell Signaling 9272, 1:1,000) and phospho-Akt (1:5000; gift of D. Stokoe, University of California, San Francisco) by using ECL (Amersham Pharmacia).

Results

LSL-Kras^{G12D} and *Mx1-Cre* mice were mated to generate compound heterozygous animals on a uniform F_1 129Sv/Jae × C57BL/6 background. The *Mx1* promoter can be activated by administering pIpC to stimulate endogenous IFN production (15). *Mx1-Cre LSL-Kras^{G12D}* pups were treated with pIpC to induce Cre recombinase expression, and leukocyte DNA was analyzed 5 weeks later. PCR analysis using allele-specific primers showed that *Mx1-Cre LSL-Kras^{G12D}* mice injected with pIpC uniformly demonstrated the recombined *Kras^{G12D}* allele, which was absent in *LSL-Kras^{G12D}* animals lacking the *Mx1-Cre* transgene (Fig. 1*A*). We refer to animals that were treated with pIpC to excise the LSL element as *Mx1-Cre Kras^{G12D}* mice. Immunoblotting with an antibody specific for the aspartate substitution at codon 12 demonstrated expression of the mutant K-Ras protein in *Mx1-Cre Kras^{G12D}* splenocytes (Fig. 1*B*).

Mx1-Cre Kras^{G12D} mice developed progressive leukocytosis that was already evident in blood specimens collected 3 weeks after plpC injection, which was associated with anemia and normal platelet counts (Fig. 1 *C* and *D*, and data not shown). Blood smears showed myeloid cells at various stages of differentiation (Fig. 1*E*). The predominant population had the morphology of mature monocytes, with some nuclear pleomorphism and cytoplasmic vacuoles. Extensive immunophenotyping of blood leukocytes revealed a major population that was CD45⁺, Mac-1⁺, CD16/32⁺, CD86⁺, CD31⁺, and negative for CD59, CD34, c-kit, Sca-1, Ter119, CD71, CD41, and a panel of B and T cell markers. These findings are consistent with monocytic maturation, although CD59 is usually expressed by murine monocytes. Histopathologic examination of bone marrow from *Mx1-Cre Kras^{G12D}* mice revealed abundant myeloid cells at various stages of differentiation, but a paucity of erythroid precursors. Flow cytometry demonstrated an expanded population of Mac-1⁺, Gr-1ˡᵒ cells; this phenotype is consistent with immature monocytic cells (Fig. 1*F*).

We injected 2×10^6 bone marrow cells from diseased *Mx1-Cre Kras^{G12D}* mice into lethally and sublethally irradiated syngeneic hosts. Four of eight lethally irradiated recipients died 4–5 weeks later without overt signs of MPD. The remaining mice succumbed with MPD 5–7 weeks after adoptive transfer. All eight sublethally irradiated recipients survived for 12 weeks after transplantation with normal blood counts and absence of the recombined *Kras^{G12D}* allele in blood leukocytes. Interestingly, four of these animals died over the next 6 weeks, including two
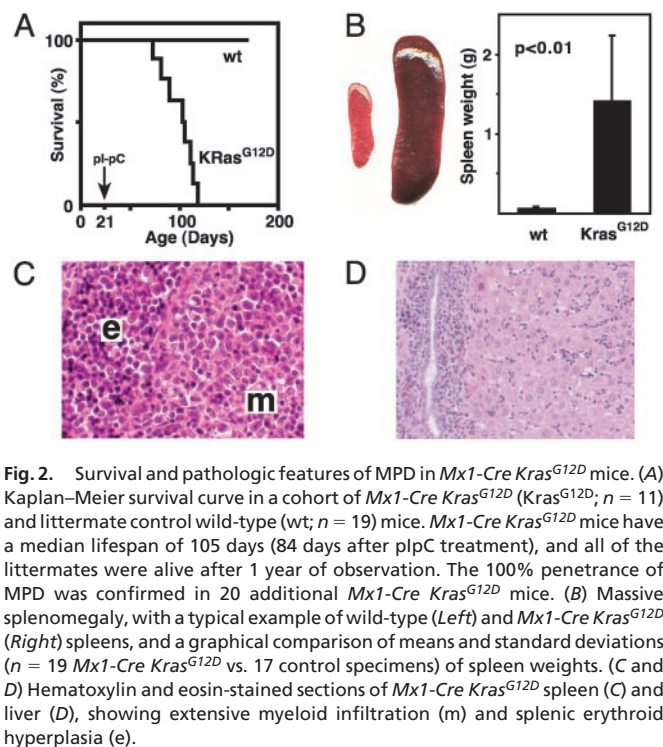
Fig. 1. K-Ras[G12D] expression induces MPD in *Mx1-Cre Kras[G12D]* mice. (*A*) Excision of the stop cassette by Cre recombinase shown in schematic (*Upper*) and by PCR (*Lower*) using primers flanking the loxP sites (triangles). Under these conditions, the wild type (*Kras[WT]*) and mutant (*Kras[G12D]*) give the indicated products, and the unrearranged allele does not amplify. PCR was performed on DNA purified from peripheral blood from mice with the indicated genotypes 3 weeks after injection with pIpC. (*B*) Expression of K-Ras[G12D] protein in *Mx1-Cre Kras[G12D]* splenocytes but not those from a littermate control after treatment with pIpC. Immunoprecipitation of total Ras was followed by immunoblotting with an antibody specific for the K-Ras[G12D] protein (*Upper*). Immunoblot of total cell lysates (*Lower*) demonstrates equal loading. (*C* and *D*) Progressive leukocytosis (*C*) and anemia (*D*) in a cohort of *Mx1-Cre Kras[G12D]* mice (*n* = 11; open circles) and littermate controls (*n* = 19; filled circles) that had serial blood counts performed after pIpC injection at weaning (±SEM). (*E*) Blood smear showing leukocytosis with mature myeloid cells in mice with MPD. (*F*) Flow cytometry of bone marrow and spleen cells from wild-type and *Mx1-Cre Kras[G12D]* mice, 7 weeks after pIpC injection. Proportions of cells within each quadrant are shown to the right of each plot.

that acquired the recombined allele in leukocytes. Whereas these data indicate that the MPD that arises in *Mx1-Cre Kras[G12D]* mice can be transplanted into lethally irradiated recipients, it is uncertain whether it can be transferred into sublethally irradiated mice.

Mx1-Cre Kras[G12D] mice survived for a median of 84 days after pIpC injection (Fig. 2*A*). The spleens of moribund mice were massively enlarged and infiltrated with myeloid and erythroid cells (Fig. 2 *B* and *C*). Consistent with this observation, the Mac-1^{+} and Ter119^{+} populations were markedly expanded, and the Mac-1^{+}, Gr-1^{lo} population that was prominent in the marrow was also readily detected (Fig. 1*F*). A significant fraction of *Mx1-Cre Kras[G12D]* splenocytes expressed c-kit (data not shown), which is found on immature hematopoietic cells and mast cells. Spectral karyotype analysis of *Mx1-Cre Kras[G12D]* splenocytes was normal in all cases (*n* = 7). Mice with MPD also showed moderate hepatomegaly with myeloid infiltration, particularly in periportal areas (Fig. 2*D*). Under guidelines published recently (19), this disease is classified as MPD with excess monocytes.
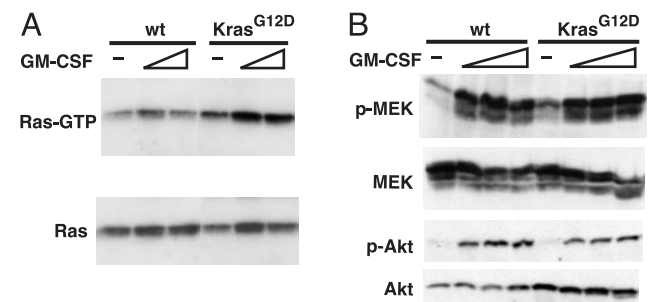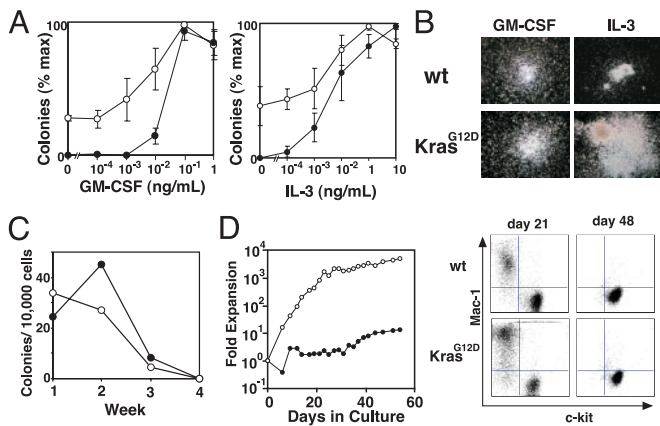

Fig. 2. Survival and pathologic features of MPD in *Mx1-Cre Kras[G12D]* mice. (*A*) Kaplan–Meier survival curve in a cohort of *Mx1-Cre Kras[G12D]* (Kras[G12D]; *n* = 11) and littermate control wild-type (wt; *n* = 19) mice. *Mx1-Cre Kras[G12D]* mice have a median lifespan of 105 days (84 days after pIpC treatment), and all of the littermates were alive after 1 year of observation. The 100% penetrance of MPD was confirmed in 20 additional *Mx1-Cre Kras[G12D]* mice. (*B*) Massive splenomegaly, with a typical example of wild-type (*Left*) and *Mx1-Cre Kras[G12D]* (*Right*) spleens, and a graphical comparison of means and standard deviations (*n* = 19 *Mx1-Cre Kras[G12D]* vs. 17 control specimens) of spleen weights. (*C* and *D*) Hematoxylin and eosin-stained sections of *Mx1-Cre Kras[G12D]* spleen (*C*) and liver (*D*), showing extensive myeloid infiltration (m) and splenic erythroid hyperplasia (e).

Importantly, *Mx1-Cre Kras[G12D]* mice do not develop AML; we did not observe abnormal expansion of immature blast forms in blood or marrow.

We measured the percentage of Ras-GTP and assayed activation of the Ras effectors Akt and MEK in bone marrow mononuclear cells isolated from *Mx1-Cre Kras[G12D]* and control mice. As expected, the mutant cells demonstrated elevated levels of Ras-GTP (Fig. 3*A*); however, this was not associated with constitutive phosphorylation of Akt or MEK. Mutant and control cells showed marked increases in Ras-GTP levels that were associated with equivalent activation of effector kinases in response to growth factor stimulation (Fig. 3*B*). We confirmed these surprising results under multiple experimental conditions in which growth factor concentration and time after stimulation varied widely (data not shown).

Fig. 3. Ras activation and signaling in *Mx1-Cre Kras[G12D]* bone marrow. (*A*) Bone marrow cells from wild-type or *Mx1-Cre Kras[G12D]* mice were starved for 4 h in Iscove's modified Dulbecco's medium with 0.1% FCS, then stimulated for 3 min with 1 or 3 ng/ml GM-CSF. Ras-GTP was affinity-purified with Raf-RBD-agarose and detected by immunoblotting for total Ras (*Upper*). Equal input was demonstrated by immunoblotting (*Lower*). (*B*) Bone marrow was harvested and starved as above, then stimulated with GM-CSF at concentrations of 0, 1, 3, and 10 ng/ml for 5 min and analyzed for the indicated proteins by immunoblotting.

Fig. 4. *In vitro* growth of *Mx1-Cre Kras*[G12D] (open circles) and control hematopoietic cells (filled circles). (*A*) Methylcellulose colony assays using GM-CSF or IL-3 at the indicated concentrations. Experiments were performed by using three mice of each genotype (±SEM). (*B*) Photomicrographs (original magnification ×40) of wild-type (wt) and mutant (Kras[G12D]) myeloid progenitor colonies grown in saturating concentrations of growth factors. (*C*) Colony formation with weekly serial replating of 10,000 unsorted bone marrow cells in methylcellulose. (*D*) Growth kinetics and flow cytometry of bone marrow cells cultured in the presence of IL-3.

The growth of *Kras*[G12D] progenitors was assayed in methylcellulose medium. Bone marrow mononuclear cells from mice with MPD, but not controls, formed significant numbers of colony-forming unit granulocyte-macrophage (CFU-GM) colonies in the absence of exogenous cytokines. Low concentrations of GM-CSF that did not induce colony formation from wild-type cells dramatically increased the size and number of *Mx1-Cre Kras*[G12D] colonies (Fig. 4*A*). CFU-GM colonies grown from *Mx1-Cre Kras*[G12D] bone marrow and spleens in the presence of saturating concentrations of GM-CSF were very large and displayed an abnormal, spreading morphology (Fig. 4*B*). *Mx1-Cre Kras*[G12D] CFU-GM also showed a dramatic proliferative response to IL-3. We also counted the total number of cells present in methylcellulose cultures containing saturating levels of GM-CSF or IL-3. In wild-type cultures, the total number of nucleated cells increased 4-fold in response to GM-CSF and 20-fold with IL-3. *Mx1-Cre Kras*[G12D] cultures showed greatly enhanced proliferation, with a 9-fold increase in cell numbers without added cytokines, a 19-fold increase with GM-CSF, and a 37-fold increase with IL-3.

To compare the self-renewal of *Mx1-Cre Kras*[G12D] and wild-type progenitors, we seeded 1×10^4 marrow cells in methylcellulose medium containing saturating concentrations of growth factors and counted colonies after 7 days. The cells were then collected and washed, and 1×10^4 cells were transferred into new methylcellulose each week. *Mx1-Cre Kras*[G12D] bone marrow cells showed normal replating efficiency in this assay (Fig. 4*C*). Bone marrow mast cell cultures established from *Mx1-Cre Kras*[G12D] mice demonstrated enormous proliferation of Mac1^{+} cells for

the first few weeks, but reverted to wild-type growth rates over time with the expected switch to cells that were Mac1^{−} and c-kit[+] (Fig. 4*D*). Cytospin preparations during this proliferative stage confirmed a predominance of mature myeloid cells with some immature forms.

Diseased animals were injected with BrdUrd to measure proliferation *in vivo*. *Mx1-Cre Kras*[G12D] bone marrows contained a similar percentage of BrdUrd-positive cells as wild-type marrow, but with a marked increase within the Mac1^{+} fraction (Table 1). The percentage of *Mx1-Cre Kras*[G12D] splenocytes that incorporated BrdUrd was also elevated. Most proliferating cells in *Mx1-Cre Kras*[G12D] spleens were in the Mac1^{+}/Gr1^{lo} fraction or expressed the erythroid marker Ter119 (Table 1 and data not shown). In contrast, the few cycling cells identified in normal spleens were mostly lymphocytes (Table 1 and data not shown). These data show that the spleen contains a large number of proliferating cells in *Mx1-Cre Kras*[G12D] spleens and suggest that these populations contribute to the MPD.

Discussion

We find that somatic activation of a latent *Kras*[G12D] allele rapidly induces a fatal MPD with complete penetrance. By contrast, mice in which oncogenic *Kras* was activated by spontaneous recombination (6) or by a CMV–Cre transgene (8) did not develop myeloid malignancies after months of observation. It is possible that the latent *Kras* allele was not activated in a susceptible leukemia-initiating cell in either strain. The *Mx1-Cre* transgene directs efficient recombination in bone marrow (15) and in hematopoietic cells with high repopulating potential (16). Furthermore, the *Kras*[V12] allele designed by Guerra *et al.* (8) was modified with a 3′ internal ribosome entry site (IRES)–βgeo cassette, which may have altered the expression of *Kras* transcripts. Consistent with this idea, we find that activating *Kras*[G12D] expression in pancreatic and colonic epithelia induces proliferation and initiates tumor formation *in vivo* (D.A.T. and T.E.J., unpublished data). Thus, the proposal that oncogenic Ras can only initiate tumorigenesis in a narrow range of cell types (8) must be reevaluated.

Ras-GTP levels are consistently elevated in *Mx1-Cre Kras*[G12D] bone marrow cells. However, our analysis of MEK and Akt phosphorylation surprisingly uncovered no differences between mutant and control cells, despite profound effects of *Kras*[G12D] expression on myeloid proliferation. Similarly, mouse embryonic fibroblasts expressing *Kras*[V12] from the endogenous locus paradoxically demonstrated reduced levels of phosphorylated ERK at steady state, and showed only modest prolongation of ERK and Akt activation after starvation and stimulation (8). Our data show that the biochemical consequences of expressing oncogenic Ras in primary cells are remarkably different from what has been observed in transformed cell lines. Furthermore, although mutant Ras proteins are frequently described as "constitutively active," neither Ras-GTP nor downstream effector cascades are saturated in *Kras*[G12D] marrow, which respond to growth factors with enhanced myeloid progenitor colony formation and by activating MEK, ERK, and Akt. Because hematopoietic cells at various stages of differentiation are present in *Kras*[G12D] mice

Table 1. Percentages of nucleated spleen and bone marrow cells staining with Mac-1, TER119, or BrdUrd

| Source | Genotype | % positive cells | | | | |
		BrdUrd	Mac-1	TER119	BrdUrd and Mac-1	BrdUrd and TER119
Bone marrow	*Kras*[wt]	18	45	10	5	5
Bone marrow	*Kras*[G12D]	17	65	5	10	2
Spleen	*Kras*[wt]	6	16	4	2	1
Spleen	*Kras*[G12D]	26	40	12	7	8

with MPD, aberrant activation of effector cascades within a minor subpopulation would not be detected in lysates of whole bone marrow. For example, the highly proliferative colony-forming unit granulocyte-macrophage fraction comprises ≈0.1% of the nucleated cells present in *Mx1-Cre Kras^{G12D}* marrow. Assessing the activation status of signaling cascades in rare subsets of primary cells is a challenging problem that will likely require new methodologies such as the flow cytometry-based approach recently described by Perez and Nolan (20).

Primary cells might also respond to chronic Ras activation by modifying signaling networks. Recent studies have uncovered extensive biochemical crosstalk between linear pathways, feedback from downstream components, and effects of previous exposure to a ligand on subsequent responses (21). Bhalla and colleagues (22) found that ERK activation of NIH 3T3 cells by platelet-derived growth factor (PDGF) induced MAP kinase phosphatase (MKP) expression, which extinguished signaling by dephosphorylating ERK. MKP levels remained elevated for some time after initial exposure to PDGF, and this dampened the response to subsequent stimulation. It is possible that hematopoietic cells adapt to chronically elevated Ras-GTP levels by attenuating effector cascades. This idea is consistent with recent data showing that acute and chronic loss of *Rb1* function had differential effects on quiescence and senescence phenotypes in mouse embryonic fibroblasts, which were explained, in part, by modulating levels of p19^{ARF} and p107 (23).

Somatic activation of *Kras^{G12D}* expression results in pronounced proliferation of myeloid lineage cells that are hyperresponsive to hematopoietic growth factors. *Nf1* encodes neurofibromin, a GTPase-activating protein for Ras (4, 24). Interestingly, although murine *Nf1*-deficient myeloid progenitors are hypersensitive to GM-CSF *in vitro*, this defect is less severe than in *Mx1-Cre Kras^{G12D}* marrow (25, 26). Furthermore, *Nf1* inactivation initiates an MPD that is less aggressive than the corresponding disorder in *Mx1-Cre Kras^{G12D}* mice (ref. 25 and our unpublished data). Whereas oncogenic *RAS* mutations both markedly impair the intrinsic Ras GTPase activity and confer resistance to GTPase-activating proteins, inactivation of *Nf1* is a less severe biochemical lesion that does not alter intrinsic GTP hydrolysis. The correlation between the predicted biochemical consequences of expressing *Kras^{G12D}* or ablating *Nf1* and phenotypic severity argues in favor of a quantitative relationship between the activation status of Ras and responsiveness to growth factors in myeloid cells. *Mx1-Cre Kras^{G12D}* progenitors demonstrate normal self-renewal in colony replating assays, which is in contrast to cells that are immortalized by *AML1-ETO* or *MLL* fusion proteins (27, 28). However, the effects of *Kras^{G12D}* on the self-renewal of hematopoietic stem cells can only be definitively addressed in competitive repopulation and serial transplantation experiments.

Ineffective erythropoiesis is prominent in MDS and is observed in some patients with MPD. Similarly, *Mx1-Cre Kras^{G12D}* mice show large numbers of proliferating splenic Ter119^{+} cells in the face of a progressive anemia. Although we have not observed morphologic features of dysplasia, the uniform finding of marked splenic erythropoiesis with a increased proportion of immature elements in anemic *Mx1-Cre Kras^{G12D}* mice suggests that erythropoiesis is ineffective. Interestingly, transduction of human or murine erythroid progenitors with oncogenic *RAS* results in hyperproliferation (29–31) but with defective differentiation resulting in reduced production of mature erythrocytes. Our *in vivo* data are consistent with these findings and support the notion that mutant *RAS* directly impairs erythropoiesis in patients with MPD and MDS. Harnessing the culture system recently described by Zhang *et al.* (29) to analyze erythroid cells from *Mx1-Cre Kras^{G12D}* mice will help to elucidate how expressing K-Ras^{G12D} from its normal locus perturbs erythroid development.
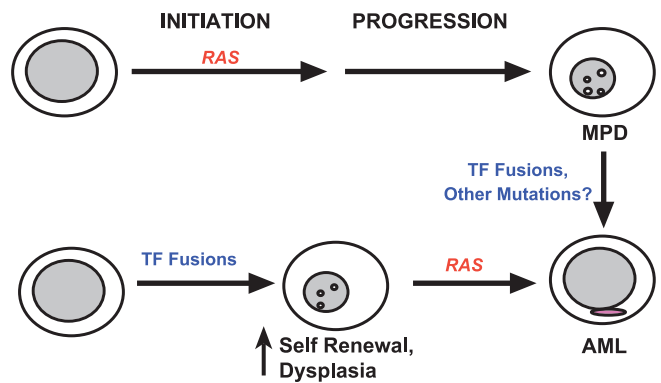

Fig. 5. Proposed role of *RAS* mutations in MPD and AML. Our results and other human and murine data support the idea that initiating events that result in hyperactive Ras induce MPD (upper pathway). Transcription factor (TF) fusions are detected in some cases of CML at the time of evolution to blast crisis. Other acquired mutations are likely to exist as well. By contrast, leukemia-associated TF fusions may initiate AML by perturbing cell fates but appear to require a cooperating mutation that drives proliferation to cause overt disease. Here, oncogenic *RAS* (or activated *FLT3*) represent secondary mutations that contribute to leukemogenesis (lower pathway). The ability of oncogenic *RAS* to either initiate disease or cooperate with preexisting genetic lesions is likely to account for the finding of *RAS* mutations in myeloid malignancies with distinct phenotypes.

The MPD that arises in *Mx1-Cre Kras^{G12D}* mice closely resembles chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML). *RAS* mutations are common in CMML (32) and genetic analysis of JMML supports the idea that hyperactive Ras can initiate myeloid leukemogenesis. Children with neurofibromatosis type 1 and Noonan syndrome (NS) are predisposed to JMML (33, 34). The *NF1* tumor suppressor undergoes homozygous inactivation in JMML bone marrow, which results in hyperactive Ras (26, 35). Germ-line mutations in *PTPN11* cause ≈50% of NS. *PTPN11* encodes SHP-2, a tyrosine phosphatase that relays signals from activated growth factor receptors to Ras (36, 37). Germ-line and somatic *PTPN11* mutations that activate SHP-2 phosphatase activity have recently been detected in JMML (38). Importantly, *RAS* mutations are also found in ≈25% of JMML samples, and are largely restricted to cases that do not show *NF1* or *PTPN11* mutations (38, 39). Together, these data support the idea that JMML is initiated by hyperactive Ras, which results from a mutation in *RAS, NF1,* or *PTPN11. Mx1-Cre Kras^{G12D}* mice provide a tractable model system for investigating the pathogenesis of CMML and JMML.

Leukemia-associated mutations that result in hyperactive Ras such as *BCR-ABL*, mutant *FLT3*, and loss of *NF1* all induce MPD in mice (25, 40, 41). Together with these observations, our data argue that deregulated Ras signaling in a susceptible hematopoietic precursor initiates MPD *in vivo* (Fig. 5). If this is true, why are *RAS* mutations also found in patients with AML and MDS? Genetic data from studies of AML specimens support a model in which *RAS* point mutations and other genetic lesions that deregulate signal transduction pathways cooperate with transcription factor fusion proteins in leukemogenesis (42). In most children who develop AML associated with *AML1-ETO*, the fusion gene can be detected in neonatal blood specimens, which implies the need for secondary mutations (43). Furthermore, transgenic or knock-in mice engineered to express *AML1-ETO*, *PML-RARA*, or *MLL-AF9* either do not spontaneously develop AML or show incomplete penetrance and prolonged latency (16, 44, 45). Based on these data, it is likely that AML is initiated by a transcription factor fusion and that oncogenic *RAS* serves as a cooperating event (Fig. 5). Although less is

known about MDS, we envision a similar role of oncogenic *RAS* as in AML (i.e., a mutation that is acquired during disease evolution). Thus, we propose that oncogenic *RAS* can either initiate leukemogenesis or occur as a secondary event, and that the disease phenotype will be dictated by both the constellation of mutations and by the order in which they are acquired. This idea is consistent with observations in chronic myeloid leukemia (CML), which is caused by the *BCR-ABL* fusion. Patients with CML present with MPD but subsequently develop a phenotype of acute leukemia during the "blast crisis" phase of the disease, which may be accompanied by translocations involving transcription factors such as *AML1* or *HOXA9* (46, 47). We speculate that intercrossing mice that express AML-associated fusion proteins with the *Mx1-Cre LSL-Kras* strain will generate accurate and highly penetrant models that recapitulate the clinical and biologic features of human AML.

The proteins encoded by mutant alleles of *FLT3* and *CKIT* and the *BCR-ABL* fusion not only result in hyperactive Ras, but interact biochemically with other signaling molecules cascades (48, 49). Despite this, a broad implication of this study is that hyperactive Ras is a pivotal biochemical alteration that promotes the growth of malignant myeloid lineage cells in the context of

each of these genetic lesions. This idea is consistent with studies showing that *RAS* mutations are rare in CML (50), in JMML samples with mutations in either *PTPN11* or *NF1* (38, 39), and in AML specimens with *FLT3* mutations (51). Hyperactive Ras therefore represents a common molecular target in myeloid malignancies. The acquisition of resistance to imatinib (52) suggests that combining this drug with a second agent directed against Ras or one of its effectors is a rational therapeutic strategy for patients with *de novo* or resistant CML (53). Hematopoietic cells from *Mx1-Cre Kras*^{G12D} mice provide a tractable system for exploring therapeutic approaches directed against hyperactive Ras in myeloid malignancies that may be applicable to many human cancers.

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