Dominant Role of Oncogene Dosage and Absence of Tumor Suppressor Activity in Nras-Driven Hematopoietic Transformation

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Abstract

Biochemical properties of Ras oncoproteins and their transforming ability strongly support a dominant mechanism of action in tumorigenesis. However, genetic studies unexpectedly suggested that wild-type (WT) Ras exerts tumor suppressor activity. Expressing oncogenic NrasG12D in the hematopoietic compartment of mice induces an aggressive myeloproliferative neoplasm (MPN) that is exacerbated in homozygous mutant animals. Here we show that increased NrasG12D gene dosage, but not inactivation of WT Nras, underlies the aggressive in vivo behavior of NrasG12D/G12D hematopoietic cells. Modulating N-RasG12D dosage had discrete effects on myeloid progenitor growth, signal transduction, and sensitivity to MEK inhibition. Furthermore, enforced WT N-Ras expression neither suppressed the growth of Nras mutant cells nor inhibited myeloid transformation by exogenous NrasG12D. Importantly, NRAS expression in increased in human cancer cell lines with NRAS mutations. These data have therapeutic implications and support reconsidering the proposed tumor suppressor activity of WT Ras in other cancers.

Significance—Understanding mechanisms of Ras-induced transformation and adaptive cellular responses are fundamental questions. The observation that oncogenic Nras lacks tumor suppressor
activity while increased dosage strongly modulates cell growth and alters sensitivity to MEK inhibition suggests new therapeutic opportunities in cancer.

Introduction

*Ras* genes encode ubiquitously expressed proteins (N-Ras, H-Ras, K-Ras4A and K-Ras4B) that cycle between active GTP-bound and inactive GDP-bound conformations (Ras-GTP and Ras-GDP)

- Ras-GTP levels are regulated by the competing activities of guanine nucleotide exchange factors and GTPase activating proteins (GAPs), which enhance intrinsic Ras GTPase activity. Proteins encoded by *RAS* oncogenes, which accumulate in the GTP-bound state due to defective intrinsic GTP hydrolysis and resistance to GAPs, are exceedingly difficult targets for anti-cancer drug discovery due to their structural and biochemical properties

Despite compelling evidence that oncogenic Ras proteins have dominant gain-of-function actions in cellular transformation, genetic studies in mice surprisingly suggested that wild-type (WT) Ras exerts tumor suppressor activity in some cancers with oncogenic Ras mutations. However, mechanistic data regarding how normal Ras might antagonize oncogenic signaling are lacking.

Endogenous expression of *Nras*G12D induces a myeloproliferative neoplasm (MPN) in *Mx1Cre, NrasG12D/+* mice that faithfully models human chronic and juvenile myelomonocytic leukemia (CMML and JMML). Hematologic disease is greatly accelerated in homozygous *NrasG12D* mutant mice (*Mx1Cre, NrasG12D/G12D*). We deployed a conditional *Nras* mutant allele to assess the relative contributions of *Nras*G12D oncogene dosage and tumor suppression by WT Ras in myeloid transformation. We find that elevated *Nras*G12D expression drives myeloid transformation *in vivo* and strongly modulates cell growth, Ras signaling, and response to a targeted inhibitor *in vitro*. Consistent with these data, somatic uniparental disomy underlies loss of the WT allele in primary acute myeloid leukemia (AML) cells with *Nras*G12D mutations, resulting in normal-to-increased *Nras* expression. Finally, *NRAS* expression is significantly elevated in human cancer cell lines with *NRAS* mutations while *KRAS* expression is reduced, with a reciprocal pattern seen in cell lines with *KRAS* mutations.

Results

We generated a Cre-dependent conditional *Nras* allele (*Nras*2lox2) by and performed intercrosses to produce hemizygous (*Mx1Cre; LSL-NrasG12D/2lox2*), heterozygous (*Mx1Cre; LSL-NrasG12D/+*), and homozygous (*Mx1Cre; LSL-NrasG12D/G12D*) littermates on a C57Bl/6 strain background (Supplemental Fig. S1A). Use of this conditional *Nras*2lox2 allele avoids potential confounding consequences of eliminating WT *Nras* expression throughout development, and allowed us to simultaneously activate *Nras*G12D expression and inactivate WT *Nras* in the hematopoietic compartment after birth. Efficient recombination of both conditional *Nras* alleles with loss of expression was observed two weeks later (Supplemental Figs. S1B, S1C). Western blot analysis confirmed that N-Ras protein levels are reduced in the bone marrow of hemizygous *Nras* mutant mice (Fig. 1A), which we hereafter refer to as *Nras*G12D*/-*. Consistent with recent reports, ~20% of *Mx1Cre; NrasG12D/G12D* mice died prematurely from T lineage acute lymphoblastic leukemia (T-ALL) (Supplemental Fig. S2A). Surviving animals of all three *Nras* genotypes were euthanized at 6 months of age. All *Nras*G12D/G12D mice had overt MPN, which was characterized by leukocytosis with elevated blood neutrophil counts, splenomegaly, and anemia (Fig. 1B and Supplemental Fig. S2B). By contrast, hematologic parameters were normal in age-matched *Mx1Cre; NrasG12D/+* and
Mx1-Cre; Nras<sup>G12D</sup>/- mice (Fig. 1B and Supplemental Fig. S2B). Flow cytometric analysis revealed increased numbers of immature monocytic (Mac-1<sup>+</sup>, Gr-1<sup>lo</sup>) cells in the hematopoietic tissues of Nras<sup>G12D/G12D</sup> mice, which is also observed in Nf1 and Kras mutant mice with MPN<sup>7</sup> (Fig. 1C). This population was not expanded in hemizygous or heterozygous Nras mutant mice.

We grew colony forming unit granulocyte macrophage (CFU-GM) progenitors to assess the cell intrinsic effects of Nras<sup>G12D</sup> gene dosage and the consequences of inactivating WT Nras. Somatic NRAS mutations are highly prevalent in JMML and CMML, and CFU-GM progenitors from patients with these aggressive cancers are hypersensitive to granulocyte macrophage colony stimulating factor (GM-CSF)<sup>7</sup>. Similarly, Nras<sup>G12D/G12D</sup> bone marrow cells demonstrate cytokine-independent CFU-GM growth and pronounced GM-CSF hypersensitivity (Fig. 1D)<sup>8,9</sup>. In striking contrast, CFU-GM from Mx1-Cre; Nras<sup>G12D</sup> mouse displayed normal cytokine responses (Fig 1D). Phospho-flow cytometric analysis of Lin<sup>-</sup>/cKit<sup>+</sup>/CD105<sup>-</sup>/CD34<sup>+</sup> bone marrow cells, which are highly enriched for myeloid progenitors (Supplemental Fig. S2C), revealed elevated basal levels of phosphorylated ERK (pERK) and enhanced responsiveness to GM-CSF in Nras<sup>G12D/G12D</sup> cells compared to heterozygous and hemizygous Nras<sup>G12D</sup> mutant cells (Fig. 1E). In summary, phenotypic, functional, and biochemical analyses of age and strain-matched mice indicate that WT Nras lacks tumor suppressor activity in the hematopoietic lineage.

To ask if exogenous WT Nras expression might antagonize the abnormal growth of Nras<sup>G12D/G12D</sup> cells, we infected Mx1-Cre; Nras<sup>G12D/G12D</sup> bone marrow with murine stem cell virus (MSCV) vectors encoding N-terminal green fluorescent protein (GFP) fused to WT N-Ras (N-Ras<sup>WT</sup>), WT K-Ras (K-Ras<sup>WT</sup>), or dominant negative N-Ras (N-Ras<sup>N17</sup>)<sup>9</sup>. After sorting to isolate GFP-positive (GFP<sup>+</sup>) cells, CFU-GM colony growth was assayed in the presence of GM-CSF. Mx1-Cre; Nras<sup>G12D/G12D</sup> bone marrow formed significantly more CFU-GM colonies than heterozygous or hemizygous Nras cells (Fig. 2A). Expressing N-Ras<sup>WT</sup> or K-Ras<sup>WT</sup> had no effect on CFU-GM colony growth from Nras<sup>G12D/G12D</sup> bone marrow, while dominant negative N-Ras<sup>N17</sup> reduced growth by greater than two-fold (Fig. 2A).

To further investigate if WT N-Ras interferes with oncogenic N-Ras<sup>G12D</sup>-induced myeloid transformation, we infected WT fetal liver cells with MSCV vectors expressing N-terminal mCherry-tagged N-Ras<sup>G12D</sup> in combination with GFP, GFP-tagged N-Ras<sup>WT</sup>, or GFP-tagged K-Ras<sup>WT</sup>. Flow cytometry and Western blotting revealed an equivalent increase in Ras protein levels in co-transduced cells (Figs. 2B,C). Importantly, neither WT Ras isoform suppressed the aberrant pattern of cytokine-independent CFU-GM progenitor growth induced by exogenous N-Ras<sup>G12D</sup> expression (Fig. 2D).

We next assessed the functional and biochemical consequences of varying Nras oncogene dosage by infecting WT fetal hematopoietic cells with viruses encoding GFP-tagged N-Ras<sup>G12D</sup> and sorting for different levels of GFP expression (Fig. 3A). As expected, increasing GFP intensity correlated with higher N-Ras protein levels (Fig. 3B). Progenitors expressing the highest levels of N-Ras<sup>G12D</sup> demonstrated cytokine-independent CFU-GM colony growth with a threshold level of expression required for myeloid transformation (Figs. 3B,C).

We also interrogated Ras signaling in transduced GFP<sup>+</sup> bone marrow-derived macrophages that were first deprived of cytokines and serum, then stimulated with GM-CSF<sup>10</sup>. Under these conditions, Akt, Erk, and S6 were not phosphorylated in starved macrophages infected with the empty pMIG vector, and these cells responded robustly to GM-CSF stimulation (Fig. 3D). Macrophages expressing low levels of N-Ras<sup>G12D</sup> showed a modest increase in
basal pAkt, pErk, and pS6, which were augmented by cytokine stimulation. By contrast, starved cells expressing the highest levels of N-Ras\textsuperscript{G12D} exhibited a further increase in basal levels of all three phospho-proteins, but were unresponsive to GM-CSF (Fig. 3D). Thus, Akt and ERK activation are uncoupled from cytokine stimulation in primary myeloid cells expressing high levels of oncogenic N-Ras.

Treatment with the potent and selective MEK inhibitor PD0325901 restores a normal pattern of hematopoiesis in \textit{Kras} and \textit{Nf1} mutant mice with MPN\textsuperscript{11,12}. We therefore asked if endogenous \textit{Nras}\textsuperscript{G12D} gene dosage influences the sensitivity of bone marrow-derived macrophages to MEK inhibition. Macrophages grown directly from the bone marrows of \textit{Mx1-Cre; Nras}\textsuperscript{G12D/+} and \textit{Mx1-Cre; Nras}\textsuperscript{G12D/G12D} mice expanded to a similar extent in the presence of a saturating concentration macrophage colony stimulating factor (Fig. 3E). Remarkably, low concentrations of PD0325901 selectively reduced the growth of homozygous \textit{Nras} mutant macrophages (Fig. 3E) despite similar basal levels of ERK activation and sensitivity to inhibition by MEK inhibitor treatment (Supplemental Fig. S3A, B).

\textit{Nras}\textsuperscript{G12D} expression cooperated with the MOL4070LTR retrovirus to efficiently induce AML in \textit{Mx1-Cre; Nras}\textsuperscript{G12D/+} mice that recapitulates morphologic and genetic features of human \textit{Nras} mutant AMLs\textsuperscript{4}. Somatic loss of the WT \textit{Nras} allele occurs in many of these leukemias (Supplemental Fig. S4A)\textsuperscript{4}. Importantly, however, real-time quantitative PCR analysis showed that \textit{Nras} expression is normal or increased in AML blasts (Fig. 4A). Interestingly, \textit{Kras} expression was reduced to levels below that in WT bone marrow (Supplemental Fig. S4B). Consistent with these data, Western blot analysis revealed elevated N-Ras protein levels in AML blasts, including \textit{Nras}\textsuperscript{G12D} leukemias with somatic loss of the normal \textit{Nras} allele (Fig. 4B). We performed fluorescence in situ hybridization (FISH) to investigate the mechanism underlying somatic \textit{Nras} inactivation in leukemias with loss of constitutional heterozygosity (LOH), and also used Taqman PCR to assess \textit{Nras} copy number. Both analyses supported somatic uniparental disomy (UPD) with duplication of the oncogenic \textit{Nras}\textsuperscript{G12D} allele as the genetic basis underlying loss of the WT \textit{Nras} allele in leukemias with LOH (Figs. 4C, D). Together, these studies indicate that genetic and transcriptional mechanisms converge to augment oncogenic N-Ras\textsuperscript{G12D} expression in AML.

To address the broad relevance of increased \textit{RAS} oncogene expression in human cancer, we queried \textit{NRAS/KRAS} mutational status and corresponding expression data across 957 cancer cell lines from different tissues\textsuperscript{13}. Cancer cell lines with \textit{NRAS} mutations showed a highly significant increase in \textit{NRAS} expression (Fig. 4E). Similarly, lines with \textit{KRAS} mutations expressed more \textit{KRAS} transcript on average. Furthermore, cancer cell lines with \textit{NRAS} mutations showed a significant reduction in \textit{KRAS} expression, while those with \textit{KRAS} mutations down-regulated \textit{NRAS} (Fig. 4E). Similar trends were observed when the hematopoietic cell lines in this large collection were analyzed separately (Supplemental Fig. S5A). Gene expression data from a well-annotated collection of human AMLs revealed elevated \textit{NRAS} expression compared to normal CD34\textsuperscript{+} progenitors in leukemias with and without \textit{Nras} mutations (Supplemental Fig. S5B).

**Discussion**

LOH is common in human cancer and classically represents a genetic “second hit” that results in homozygous inactivation of tumor suppressor genes (TSGs) that negatively regulate cell growth through diverse mechanisms. Given this, the finding of frequent somatic oncogenic \textit{Ras} mutations and loss of the corresponding WT allele in mouse cancers induced by chemical carcinogenesis raised the possibility that normal Ras proteins might also restrain malignant growth\textsuperscript{2}. Indeed, subsequent experiments demonstrating that germ
line inactivation of one Ras allele greatly increased the incidence and biologic aggressiveness of genotoxin-induced skin and lung carcinoma supported this idea. While provocative, it has proven difficult to reconcile these genetic data with the dominant transforming ability of oncogenic Ras proteins and their biochemical properties. Although normal Ras genes may function to suppress tumorigenesis in some cell lineages, loss of the normal Ras allele may reflect selective pressure for cancers to increase oncogene dosage as they evolve. This idea is compatible with data showing that spontaneous transformation in primary Hras mutant fibroblasts is associated with amplification of the mutant allele, and with frequent copy number gains, mutant allele specific imbalance, and over-expression of oncogenic KRAS in human cancer cell lines. Interestingly, oncogenic Hras amplification is an early event in murine skin carcinogenesis models and may be associated with somatic UPD.

Somatic NRAS mutations are common in hematologic malignancies, and Mx1-Cre; NrasG12D mice provide a tractable and genetically accurate system for interrogating the putative TSG activity of WT Nras in early (MPN) and late (AML) stage cancers that does not rely on chemical carcinogenesis. Our extensive genetic and functional analysis indicates that WT Nras lacks tumor suppressor activity in vitro and in vivo, and identifies increased oncogene expression as the major “driver” of aberrant growth. Consistent with these data, oncogenic NRAS mutations with UPD have been reported in human AML. A JMML with an Nras mutation that acquired UPD after evolution to AML suggests a role of increased oncogenic NRAS dosage in disease progression. The pattern of Ras gene expression in murine Nras mutant leukemias and in human cancer cell lines with oncogenic RAS mutations also supports the existence of selective pressure to amplify oncogenic signaling by increasing oncoprotein levels while simultaneously down-regulating normal Ras.

The dominant role of mutant Ras amplification is consistent with data showing that expressing oncogenic K-RasG12D and N-RasG12D from their endogenous genetic loci results in remarkably little activation of canonical effectors in primary cells, which likely reflects the inhibitory effects of potent cellular feedback responses. The dramatic effects of modulating N-RasG12D protein levels on basal activation of Ras effectors and on cytokine responses further suggest that cancer cells must titrate an “optimal” level of pathway activation to overcome negative feedback inhibition without evoking cell cycle arrest or senescence.

The idea that normal Ras proteins might antagonize the transforming properties of their oncogenic counterparts suggested potential therapeutic strategies. While this possibility remains viable in tissues such as skin and lung where normal RAS genes are likely to function as tumor suppressors, our data strongly argue that restoring or enhancing normal Ras expression will be ineffective in leukemias with oncogenic NRAS mutations. They also support revisiting this general question in other cancers, particularly given the broad pattern of elevated NRAS or KRAS expression in human cancer cell lines with mutations in each gene. Furthermore, as cancer cells amplify oncogenic signaling to optimize growth, they may become more dependent upon (“addicted to”) these activated pathways, which might be exploited therapeutically. The enhanced dependence of NrasG12D/G12D macrophages on MEK supports this idea. Similarly, somatic UPD is common in AML with oncogenic FLT3 mutations and is associated with resistance to conventional anti-cancer agents. However, these leukemias are more sensitive to FLT3 inhibitors than AMLs without UPD. Thus, selective pressure favoring the outgrowth of clones with increased oncogenic RAS gene expression may also render them more susceptible to inhibitors of critical effector pathways.
Methods

Mouse strains and pathologic analysis

Nras\textsuperscript{2lox2} mice were generated by inserting loxP sites onto each side of exon 2 of the endogenous Nras locus in V26.2 C57BL/6 embryonic stem cells \textsuperscript{24}. A Frt-flanked Neo resistance cassette was also inserted into intron 1 (Supplemental Fig. S1A). After germline transmission of the targeted allele, the Neo resistance cassette was removed by crossing to an Flp deleter strain (Jackson Laboratory, Bar Harbor, ME). Mx1-Cre, Nras\textsuperscript{G12D} mice were intercrossed with Nras\textsuperscript{2lox2} mice to generate Mx1-Cre, Nras\textsuperscript{G12D/G12D}, Mx1-Cre Nras\textsuperscript{G12D/+}, and Mx1-Cre Nras\textsuperscript{G12D/2lox2} mice. All mice received a single intraperitoneal injection of poly-I/poly-C (250 μg) at 3 weeks of age to activate Mx1-Cre expression \textsuperscript{4}. Mice were euthanized at 6 months of age to assess disease. Pathologic examinations were performed as previously described \textsuperscript{4}.

Hematopoietic progenitor assays and flow cytometry

Hematopoietic progenitor assays and flow cytometric analyses were performed as previously described \textsuperscript{4,10}.

Retroviral transduction

Nras\textsuperscript{WT}, Kras\textsuperscript{WT}, Nras\textsuperscript{N17}, and Nras\textsuperscript{G12D} alleles containing either N-terminal green fluorescent protein (GFP) or N terminal mCherry markers were cloned into the murine stem cell virus (MSCV) vector with expression driven by the internal ribosomal entry site (IRES). Retrovirally transduced E14.5 fetal liver cells from C57Bl/6 mice were sorted to isolate GFP-positive cells, which were plated in methylcellulose medium to assess colony-forming unit-granulocyte/macrophage (CFU-GM) growth as described previously \textsuperscript{4}.

Biochemistry

Biochemical analyses were performed on cultured macrophages that were differentiated from transduced GFP-positive (GFP\textsuperscript{+}) fetal liver cells in 50 ng/mL macrophage colony stimulating factor (M-CSF) as described previously \textsuperscript{10}. Quantitative effects of PD0325901 on macrophage ERK signaling were determined by imaging and quantifying blots on an Odyssey imager.

FISH Analysis

A labeled bacterial artificial chromosome (BAC) probe containing the mouse Nras gene (RP23- 280E21; 150 kb) was labeled with 5-(3-aminoallyl)-dUTP by nick-translation, followed by chemical labeling with amine-reactive Alexa fluor 488 using the Ares DNA labeling kit. An 8 kb genomic probe containing the Nras gene was labeled with biotin-dUTP by nick translation and detected with streptavidin conjugated with Alexa fluor 568. FISH was performed as described previously \textsuperscript{25}. Cells were counterstained with 4,6 diamido-2-phenylindole-dihydrochloride. A minimum of 200 interphase nuclei and 10 metaphase cells were scored for each sample.

RNA purification and quantitative PCR analysis

RNA purification and quantitative PCR analyses of primary AML cells that were generated by infecting Nras\textsuperscript{G12D/4} mice with the MOL4070LTR retrovirus were performed as previously described \textsuperscript{4}.
Taqman PCR

Genomic DNA from Nras mutant AMLs was purified using a Qiagen kit. A total of 10 ng of DNA was used as a template for quantitative PCR experiments. The sequence for murine transferrin receptor (mTfrc) was used to normalize total amounts of DNA. The premixed probe and primer assay mixture used to quantify total amounts of genomic Nras were purchased from Applied Biosystems.

Macrophage proliferation assay

8×10^5 bone marrow derived-macrophages from Mx1-Cre, Nras^{G12D/G12D} or Mx1-Cre, Nras^{G12D/+} mice were plated in triplicate in 12 well plates in the presence of 10 ng/mL M-CSF and varying doses of PD0325901. Total numbers of viable cells were counted using the Beckman Coulter Vi-Cell XR at day 5.

Gene expression profiling of cell line and AML

NRAS and KRAS expression in cancer cell lines was extracted from gene-centric RMA-normalized mRNA expression data downloaded from Cancer Cell Line Encyclopedia. KRAS and NRAS mutational status was determined by hybrid capture and Oncomap assays. P values were calculated by two-tailed student’s t test assuming unequal variance. To further examine NRAS and KRAS expression of human leukemia, we performed microarray-based gene expression profiling data of pediatric AML and normal CD34^+ samples generated using Affymetrix U133A microarrays (Affymetrix, Santa Clara, CA) according to the manufacturer’s instructions, with data processed using MAS5 normalization and log2 transformation. This cohort comprised 108 samples including CD34^+ bone marrow cells (N=4), and included 74 without an NRAS mutation and 30 with a mutation. Primary data are available through http://www.ncbi.nlm.nih.gov/geo/ accessions GSE43176 (AML samples) and GSE33315 (CD34^+ bone marrow).

No experiments were performed on any cell lines to generate this data.

All mouse experiments were approved by IACUC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to J Leopold (Pfizer, Inc) for PD0325901 and to A Balmain and F McCormick for suggestions. We would also like to thank T Huang and E Hwang for assistance in critical experiments. This work was supported by NIH grants R37CA72614, P01CA40046, K08CA154649, by a Specialized Center of Research award from the Leukemia and Lymphoma Society (LLS 7019-04), by the ALSAC of St. Jude Children’s Research Hospital, by an American Cancer Society (ACS) Fellowship to J.X., and by a Damon Runyon Cancer Research Foundation Fellowship (DRG-2149-13) to A.J.F. T.J. is an Investigator of the Howard Hughes Medical Institute and K.S. is an ACS Research Professor.

References


Figure 1. Dominant Effects of *Nras*<sup>G12D</sup> Dosage in Hematologic Disease

(A) Western blot analysis of bone marrow lysates from 6 week-old mice shows reduced total N-Ras protein levels in hemizygous *Nras*<sup>G12D</sup> mice. (B) Spleen weights and white blood cell (WBC) counts of 6 month-old heterozygous (n=38), hemizygous (n=32), and homozygous (n=20) *Nras*<sup>G12D</sup> mice. (C) Representative flow cytometric analysis of bone marrow and spleen specimens from all 3 genotypes with the myeloid markers Gr-1 and Mac-1. The percentage of immature monocytic (Gr-1<sup>lo</sup>, Mac-1<sup>hi</sup>) cells is shown on each panel. (D) CFU-GM colony growth from *Nras*<sup>G12D</sup>/+, *Nras*<sup>G12D</sup>−, and *Nras*<sup>G12D</sup>/− bone marrow cells in over a range of GM-CSF concentrations (n= 5-7 per genotype). Note that only *Nras*<sup>G12D</sup>/− cells demonstrate cytokine-independent progenitor growth. (E) Flow cytometric analysis of basal ERK phosphorylation in Lin<sup>−</sup>c-Kit<sup>+</sup> CD105<sup>−</sup>
CD34+ bone marrow cells from 3 month-old mice and response to GM-CSF stimulation (10 ng/mL for 15 min). The vertical black line indicates basal pERK levels in WT cells.
Figure 2. Wild-type Ras Does Not Antagonize Myeloid Transformation by \( \text{Nras}^{\text{G12D}} \)

(A) Left panel. CFU-GM progenitor growth from \( \text{Nras}^{\text{G12D+/+}} \), \( \text{Nras}^{\text{G12D/-}} \), and \( \text{Nras}^{\text{G12D/G12D}} \) (n=6 from each genotype) bone marrow cells in the presence of 1 ng/mL GM-CSF (*** p<0.0001). Right panel. Bone marrow from \( \text{Nras}^{\text{G12D/G12D}} \) mice was infected with MSCV vectors encoding GFP-tagged WT N-Ras (N-Ras\(^{\text{WT}}\)), WT K-Ras (K-Ras\(^{\text{WT}}\)), or a dominant negative N-Ras protein (N-Ras\(^{\text{N17}}\)). After sorting to isolative GFP\(^+\) cells, CFU-GM colonies were grown in the presence of 1 ng/mL GM-CSF. The data are from three independent experiments (* p=0.0135).  

(B) WT E14.5 fetal liver cells were co-transduced with MSCV vectors expressing mCherry-N-Ras\(^{\text{G12D}}\) and either GFP only (pMIG), GFP-N-Ras\(^{\text{WT}}\), or GFP-K-Ras\(^{\text{WT}}\). Flow cytometric analyses indicate equivalent numbers of PE-Texas Red (mCherry) and FITC (GFP) double positive cells. The GFP

\( \text{Nras}^{\text{G12D}} \)
fluorescence is higher in cells expressing GFP only. (C) Immunoblot analysis demonstrates that cells co-transduced with oncogenic N-Ras$^{G12D}$ and WT N-Ras or WT K-Ras express more total Ras than control cells transduced with N-Ras$^{G12D}$ and an “empty” MIG vector. (D) CFU-GM colony growth from sorted cells expressing both GFP and mCherry over a range of GM-CSF concentrations. N-Ras$^{G12D}$ transforms ~40% of myeloid progenitors to cytokine independent growth, and co-expressing WT N-Ras or K-Ras does not alter this phenotype.
Figure 3. Oncogenic Nras$^{G12D}$ Signaling is Dosage Dependent

(A) Subpopulations of WT E14.5 fetal livers cells were infected with a virus expressing GFP-N-Ras$^{G12D}$ and isolated by sorting based on the level GFP fluorescence (L-low, M1-low medium, M2-high medium, H-high). (B) Immunoblot analysis of macrophages grown from fetal liver cells in panel A indicates that N-Ras levels correlate with GFP positivity. (C) CFU-GM colony growth from fetal liver cells expressing L, M1, M2, and H levels of GFP-N-Ras$^{G12D}$. (D) The macrophages shown in panel B were starved overnight, then stimulated with 10 ng/mL of GM-CSF for 15 or 60 min. Levels of phosphorylated Erk, Akt and S6 were assessed by Western blotting. (E) Growth of cultured bone marrow derived-macrophages from 26 week-old Mx1-Cre, Nras$^{G12D/G12D}$ (n = 4) and Mx1-Cre, Nras$^{G12D/+}$ (n = 5) mice. Left panel. Total cell numbers after 5 days in the absence of PD0325901. Right panel. Percentage reduction in cell numbers in cells exposed to 0.01, 0.1, and 1 nM of PD0325901 for 5 days. Asterisks denote significant differences between Nras$^{G12D/G12D}$ and Mx1-Cre, Nras$^{G12D/+}$ (p = 0.0425, ** p=0.0076).
Figure 4. N-Ras Expression in AMLs from Nras\textsuperscript{G12D/+} Mice

(A) Quantitative real-time PCR of Nras mRNA expression in WT bone marrow and primary Nras\textsuperscript{G12D} AMLs with and without LOH (Nras\textsuperscript{G12D} leukemias with loss of or a marked reduction in the WT allele are shown in red and AMLs that retain the WT allele are in black). (B) Western blot of Nras\textsuperscript{G12D} AMLs shows that N-Ras proteins levels are equivalent to or higher than in WT mouse bone marrow. (C) FISH analysis of metaphase and interphase cells from representative AMLs with (#6730; left panel) and without (#6768; right panel) LOH. Arrowheads identify a fused signal from both BAC probe RP23-280E21 and the smaller Nras probe. Hybridizing with the larger BAC clone uniformly revealed signals on both chromosomal homologs in all 5 Nras\textsuperscript{G12D} AMLs. Equal percentages of cells demonstrated one or two hybridization signals (shown here) with the much smaller Nras-specific probe from AMLs with and without LOH. (D) Nras DNA copy number is normal or increased in Nras\textsuperscript{G12D} AMLs with and without LOH as assessed by Taqman quantitative PCR. (E) NRAS and KRAS expression in 957 human cancer cell lines with NRAS mutations (n = 56), KRAS mutations (n = 173), or no RAS mutation (n = 728) (**p=0.0018) (***p=7.7E-16).