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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 $Nras^{G12D}$ in the hematopoietic compartment of mice induces an aggressive myeloproliferative neoplasm (MPN) that is exacerbated in homozygous mutant animals. Here we show that increased $Nras^{G12D}$ gene dosage, but not inactivation of WT *Nras*, underlies the aggressive *in vivo* behavior of $Nras^{G12D/G12D}$ hematopoietic cells. Modulating $N\text{-}Ras^{G12D}$ 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 $Nras^{G12D}$. 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

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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)[1]. 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[1].

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[2-5]. However, mechanistic data regarding how normal Ras might antagonize oncogenic signaling are lacking.

Endogenous expression of $Nras^{G12D}$ induces a myeloproliferative neoplasm (MPN) in *Mx1-Cre,* $Nras^{G12D/+}$ mice that faithfully models human chronic and juvenile myelomonocytic leukemia (CMML and JMML)[4,6,7]. Hematologic disease is greatly accelerated in homozygous $Nras^{G12D}$ mutant mice *(Mx1-Cre,* $Nras^{G12D/G12D}$*)*[8,9]. We deployed a conditional *Nras* mutant allele to assess the relative contributions of $Nras^{G12D}$ oncogene dosage and tumor suppression by WT *Nras* 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 *(Mx1-Cre; LSL-*$Nras^{G12D/2lox2}$*)*, heterozygous *(Mx1-Cre; LSL-*$Nras^{G12D/+}$*)*, and homozygous *(Mx1-Cre; LSL-*$Nras^{G12D/G12D}$*)* 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[4]. 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[8,9], ~20% of *Mx1-Cre;* $Nras^{G12D/G12D}$ 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 *Mx1-Cre;* $Nras^{G12D/+}$ and

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Mx1-Cre; Nras$^{G12D/-}$ mice (Fig. 1B and Supplemental Fig. S2B). Flow cytometric analysis revealed increased numbers of immature monocytic (Mac-1^{+}, Gr-1^{lo}) cells in the hematopoietic tissues of *Nras*$^{G12D/G12D}$ mice, which is also observed in *Nf1* and *Kras* mutant mice with MPN[7] (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*^{G12D} 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)[7]. Similarly, *Nras*$^{G12D/G12D}$ bone marrow cells demonstrate cytokine-independent CFU-GM growth and pronounced GM-CSF hypersensitivity (Fig. 1D)[8,9]. In striking contrast, CFU-GM from *Mx1-Cre; Nras*$^{G12D/-}$ and *Mx1-Cre; Nras*$^{G12D/+}$ mice displayed normal cytokine responses (Fig 1D). Phospho-flow cytometric analysis of Lin^{-}/cKit^{+}/CD105^{-}/CD34^{+} 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*$^{G12D/G12D}$ cells compared to heterozygous and hemizygous *Nras*^{G12D} 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*$^{G12D/G12D}$ cells, we infected *Mx1-Cre; Nras*$^{G12D/G12D}$ bone marrow with murine stem cell virus (MSCV) vectors encoding N-terminal green fluorescent protein (GFP) fused to WT N-Ras (N-Ras^{WT}), WT K-Ras (K-Ras^{WT}), or dominant negative N-Ras (N-Ras^{N17})[9]. After sorting to isolate GFP-positive (GFP^{+}) cells, CFU-GM colony growth was assayed in the presence of GM-CSF. *Mx1-Cre; Nras*$^{G12D/G12D}$ bone marrow formed significantly more CFU-GM colonies than heterozygous or hemizygous *Nras* cells (Fig. 2A). Expressing N-Ras^{WT} or K-Ras^{WT} had no effect on CFU-GM colony growth from *Nras*$^{G12D/G12D}$ bone marrow, while dominant negative N-Ras^{N17} reduced growth by greater than two-fold (Fig. 2A).

To further investigate if WT N-Ras interferes with oncogenic N-Ras^{G12D}-induced myeloid transformation, we infected WT fetal liver cells with MSCV vectors expressing N-terminal mCherry-tagged N-Ras^{G12D} in combination with GFP, GFP-tagged N-Ras^{WT}, or GFP-tagged K-Ras^{WT}. 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^{G12D} 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^{G12D} 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^{G12D} 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^{+} bone marrow-derived macrophages that were first deprived of cytokines and serum, then stimulated with GM-CSF[10]. 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^{G12D} showed a modest increase in

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basal pAkt, pErk, and pS6, which were augmented by cytokine stimulation. By contrast, starved cells expressing the highest levels of N-Ras^{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 *Kras* and *Nf1* mutant mice with MPN[11,12]. We therefore asked if endogenous *Nras^{G12D}* gene dosage influences the sensitivity of bone marrow-derived macrophages to MEK inhibition. Macrophages grown directly from the bone marrows of *Mx1-Cre; Nras$^{G12D/+}$* and *Mx1-Cre; Nras$^{G12D/G12D}$* 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 *Nras* mutant macrophages (Fig. 3E) despite similar basal levels of ERK activation and sensitivity to inhibition by MEK inhibitor treatment (Supplemental Fig. S3A, B).

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

To address the broad relevance of increased *RAS* oncogene expression in human cancer, we queried *NRAS/KRAS* mutational status and corresponding expression data across 957 cancer cell lines from different tissues[13]. Cancer cell lines with *NRAS* mutations showed a highly significant increase in *NRAS* expression (Fig. 4E). Similarly, lines with *KRAS* mutations expressed more *KRAS* transcript on average. Furthermore, cancer cell lines with *NRAS* mutations showed a significant reduction in *KRAS* expression, while those with *KRAS* mutations down-regulated *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 *NRAS* expression compared to normal CD34^{+} progenitors in leukemias with and without *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 *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[2]. Indeed, subsequent experiments demonstrating that germ

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line inactivation of one *Ras* allele greatly increased the incidence and biologic aggressiveness of genotoxin-induced skin and lung carcinoma supported this idea[5,14]. 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[15], and with frequent copy number gains, mutant allele specific imbalance, and over-expression of oncogenic *KRAS* in human cancer cell lines[16]. Interestingly, oncogenic *Hras* amplification is an early event in murine skin carcinogenesis models and may be associated with somatic UPD[2,17].

Somatic *NRAS* mutations are common in hematologic malignancies, and *Mx1-Cre; $Nras^{G12D}$* 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[18]. A JMML with an *NRAS* mutation that acquired UPD after evolution to AML suggests a role of increased oncogenic *NRAS* dosage in disease progression[19]. 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-Ras^{G12D} and N-Ras^{G12D} 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[20,21]. The dramatic effects of modulating N-Ras^{G12D} 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 $Nras^{G12D/G12D}$ 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[22]. However, these leukemias are more sensitive to FLT3 inhibitors than AMLs without UPD[23]. 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.

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Methods

Mouse strains and pathologic analysis

Nras^{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 [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*^{G12D} mice were intercrossed with *Nras*^{2lox2} mice to generate *Mx1-Cre, Nras*$^{G12D/G12D}$, *Mx1-Cre Nras*$^{G12D/+}$, and *Mx1-Cre Nras*$^{G12D/2lox2}$ mice. All mice received a single intraperitoneal injection of poly-I/poly-C (250 μg) at 3 weeks of age to active Mx1-Cre expression [4]. Mice were euthanized at 6 months of age to assess disease. Pathologic examinations were performed as previously described[4].

Hematopoietic progenitor assays and flow cytometry

Hematopoietic progenitor assays and flow cytometric analyses were performed as previously described [4,10]

Retroviral transduction

Nras^{WT}, *Kras*^{WT}, *Nras*^{N17}, and *Nras*^{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[4].

Biochemistry

Biochemical analyses were performed on cultured macrophages that were differentiated from transduced GFP-positive (GFP^{+}) fetal liver cells in 50 ng/mL macrophage colony stimulating factor (M-CSF) as described previously[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[25]. Cells were counterstained with 4,6 diamidino-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*$^{G12D/+}$ mice with the MOL4070LTR retrovirus were performed as previously described[4].

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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[13]. *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[26]. 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.

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References

1. Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer. 2007; 7:295–308. [PubMed: 17384584]
2. Bremner R, Balmain A. Genetic changes in skin tumor progression: correlation between presence of a mutant ras gene and loss of heterozygosity on mouse chromosome 7. Cell. 1990; 61:407–17. [PubMed: 2185890]

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3. To MD, Wong CE, Karnezis AN, Del Rosario R, Di Lauro R, Balmain A. Kras regulatory elements and exon 4A determine mutation specificity in lung cancer. Nat Genet. 2008; 40:1240–4. [PubMed: 18758463]
4. Li Q, Haigis KM, McDaniel A, Harding-Theobald E, Kogan SC, Akagi K, et al. Hematopoiesis and leukemogenesis in mice expressing oncogenic NrasG12D from the endogenous locus. Blood. 2011; 117:2022–32. [PubMed: 21163920]
5. Zhang Z, Wang Y, Vikis HG, Johnson L, Liu G, Li J, et al. Wildtype Kras2 can inhibit lung carcinogenesis in mice. Nat Genet. 2001; 29:25–33. [PubMed: 11528387]
6. Wang J, Liu Y, Li Z, Du J, Ryu MJ, Taylor PR, et al. Endogenous oncogenic Nras mutation promotes aberrant GM-CSF signaling in granulocytic/monocytic precursors in a murine model of chronic myelomonocytic leukemia. Blood. 2010; 116:5991–6002. [PubMed: 20921338]
7. Ward AF, Braun BS, Shannon KM. Targeting oncogenic Ras signaling in hematologic malignancies. Blood. 2012; 120:3397–406. [PubMed: 22898602]
8. Wang J, Liu Y, Li Z, Wang Z, Tan LX, Ryu MJ, et al. Endogenous oncogenic Nras mutation initiates hematopoietic malignancies in a dose- and cell type-dependent manner. Blood. 2011; 118:368–79. [PubMed: 21586752]
9. Xu J, Hedberg C, Dekker FJ, Li Q, Haigis KM, Hwang E, et al. Inhibiting the palmitoylation/depalmitoylation cycle selectively reduces the growth of hematopoietic cells expressing oncogenic Nras. Blood. 2012; 119:1032–5. [PubMed: 22144181]
10. Schubbert S, Zenker M, Rowe SL, Böll S, Klein C, Bollag G, et al. Germline KRAS mutations cause Noonan syndrome. Nat Genet. 2006; 38:331–6. [PubMed: 16474405]
11. Chang T, Krisman K, Theobald EH, Xu J, Akutagawa J, Lauchle JO, et al. Sustained MEK inhibition abrogates myeloproliferative disease in Nf1 mutant mice. J Clin Invest. 2013; 123:335–9. [PubMed: 23221337]
12. Lyubynska N, Gorman MF, Lauchle JO, Hong WX, Akutagawa JK, Shannon K, et al. A MEK inhibitor abrogates myeloproliferative disease in Kras mutant mice. Sci Transl Med. 2011; 3:76ra27.
13. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature. 2012; 483:603–7. [PubMed: 22460905]
14. To MD, Rosario RD, Westcott PM, Banta KL, Balmain A, et al. Interactions between wild-type and mutant Ras genes in lung and skin carcinogenesis. Oncogene. 2012
15. Finney RE, Bishop JM. Predisposition to neoplastic transformation caused by gene replacement of H-ras1. Science. 1993; 260:1524–7. [PubMed: 8502998]
16. Soh J, Okumura N, Lockwood WW, Yamamoto H, Shigematsu H, Zhang W, et al. Oncogene mutations, copy number gains and mutant allele specific imbalance (MASI) frequently occur together in tumor cells. PLoS One. 2009; 4:e7464. [PubMed: 19826477]
17. Chen X, Mitsutake N, LaPerle K, Akeno N, Zanzonico P, Longo VA, et al. Endogenous expression of Hras(G12V) induces developmental defects and neoplasms with copy number imbalances of the oncogene. Proc Natl Acad Sci U S A. 2009; 106:7979–84. [PubMed: 19416908]
18. Dunbar AJ, Gondek LP, O'Keefe CL, Makishima H, Rataul MS, Szpurka H, et al. 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. Cancer Res. 2008; 68:10349–57. [PubMed: 19074904]
19. Matsuda K, Nakazawa Y, Sakashita K, Shiohara M, Yamauchi K, Koike K. Acquisition of loss of the wild-type NRAS locus with aggressive disease progression in a patient with juvenile myelomonocytic leukemia and a heterozygous NRAS mutation. Haematologica. 2007; 92:1576–8. [PubMed: 18024411]
20. Tuveson DA, Shaw AT, Willis NA, Silver DP, Jackson EL, Chang S, et al. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. Cancer Cell. 2004; 5:375–87. [PubMed: 15093544]
21. Chandarlapaty S, Sawai A, Scaltriti M, Rodrik-Outmezguine V, Grbovic-Huezo O, Serra V, et al. AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell. 2011; 19:58–71. [PubMed: 21215704]

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22. Pratz KW, Sato T, Murphy KM, Stine A, Rajkhowa T, Levis M. FLT3-mutant allelic burden and clinical status are predictive of response to FLT3 inhibitors in AML. Blood. 2010; 115:1425–32. [PubMed: 20007803]

23. Gale RE, Green C, Allen C, Mead AJ, Burnett AK, Hills RK, et al. The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. Blood. 2008; 111:2776–84. [PubMed: 17957027]

24. Haigis KM, Kendall KR, Wang Y, Cheung A, Haigis MC, Glickman JN, et al. Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. Nat Genet. 2008; 40:600–8. [PubMed: 18372904]

25. Le Beau MM, Espinosa R 3rd, Davis EM, Eisenbart JD, Larson RA, Green ED. Cytogenetic and molecular delineation of a region of chromosome 7 commonly deleted in malignant myeloid diseases. Blood. 1996; 88:1930–5. [PubMed: 8822909]

26. Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature. 2012; 481:157–63. [PubMed: 22237106]

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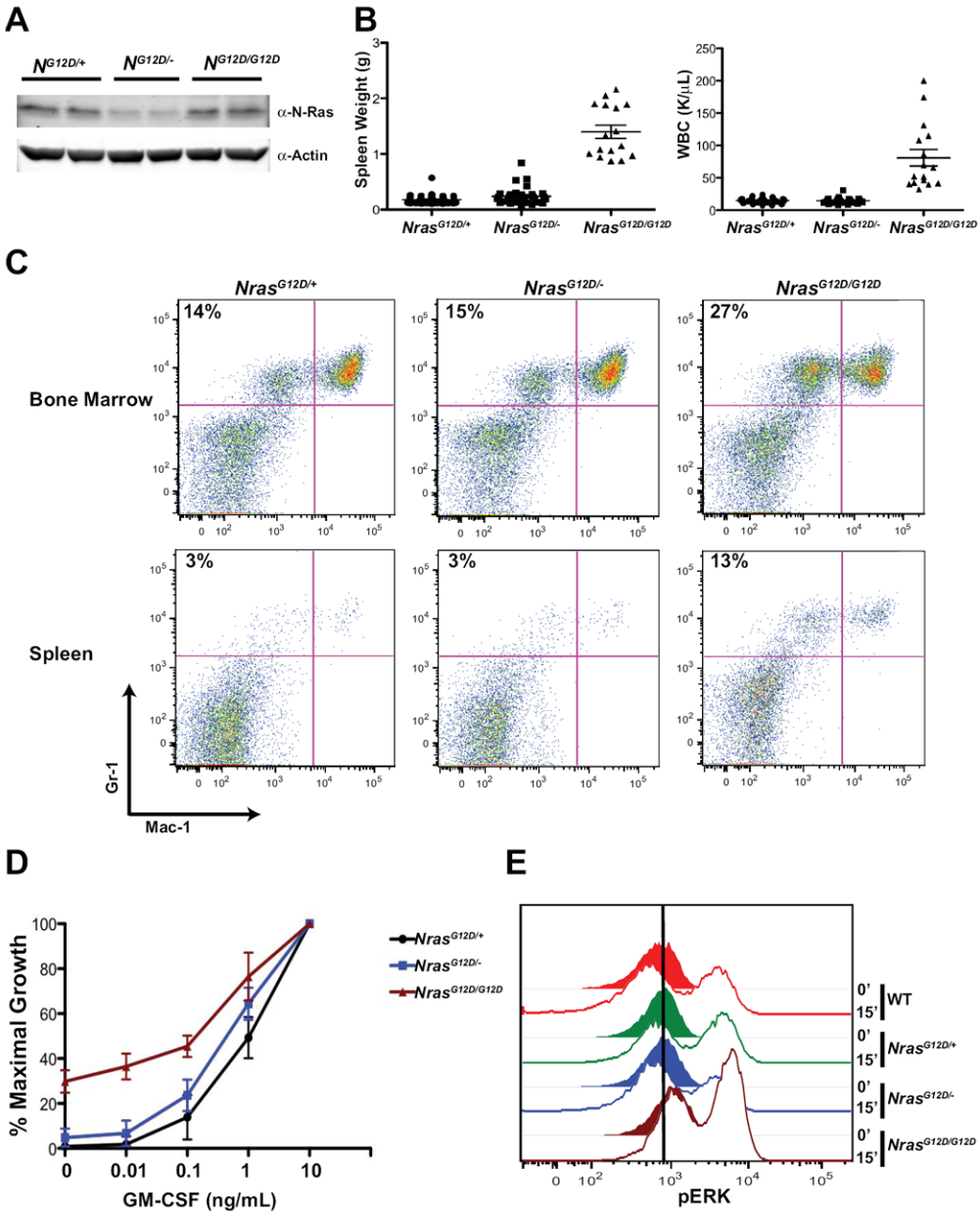

Figure 1. Dominant Effects of *Nras*^{G12D} Dosage in Hematologic Disease

(**A**) Western blot analysis of bone marrow lysates from 6 week-old mice shows reduced total N-Ras protein levels in hemizygous *Nras*^{G12D} 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*^{G12D} 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^{lo}, Mac-1^{hi}) cells is shown on each panel. (**D**) CFU-GM colony growth from *Nras*$^{G12D/+}$ (black line), *Nras*$^{G12D/-}$ (blue line), and *Nras*$^{G12D/G12D}$ (red line) bone marrow cells in over a range of GM-CSF concentrations (n= 5-7 per genotype). Note that only *Nras*$^{G12D/G12D}$ cells demonstrate cytokine-independent progenitor growth. (**E**) Flow cytometric analysis of basal ERK phosphorylation in Lin^{-} c-Kit^{+} CD105^{-}

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.

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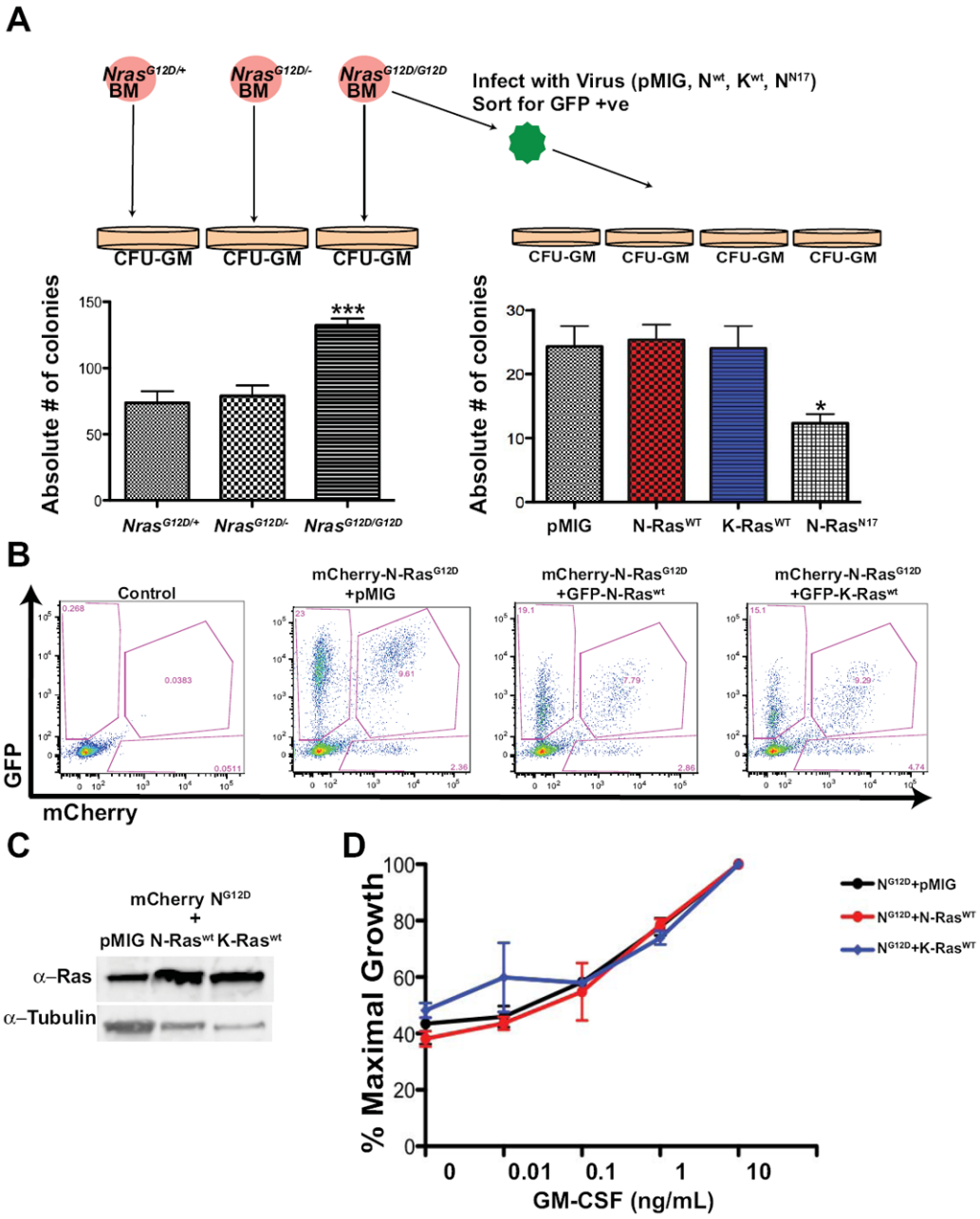

Figure 2. Wild-type Ras Does Not Antagonize Myeloid Transformation by *Nras*^{G12D}

(**A**) Left panel. CFU-GM progenitor growth from *Nras*$^{G12D/+}$, *Nras*$^{G12D/-}$, and *Nras*$^{G12D/G12D}$ (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 *Nras*$^{G12D/G12D}$ mice was infected with MSCV vectors encoding GFP-tagged WT N-Ras (N-Ras^{WT}), WT K-Ras (K-Ras^{WT}), or a dominant negative N-Ras protein (N-Ras^{N17}). After sorting to isolative GFP^{+} cells, CFU-GM colonies were grown in the presence of 1ng/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^{G12D} and either GFP only (pMIG), GFP-N-Ras^{WT}, or GFP-K-Ras^{WT}. Flow cytometic analyses indicate equivalent numbers of PE-Texas Red (mCherry) and FITC (GFP) double positive cells. The GFP

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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.

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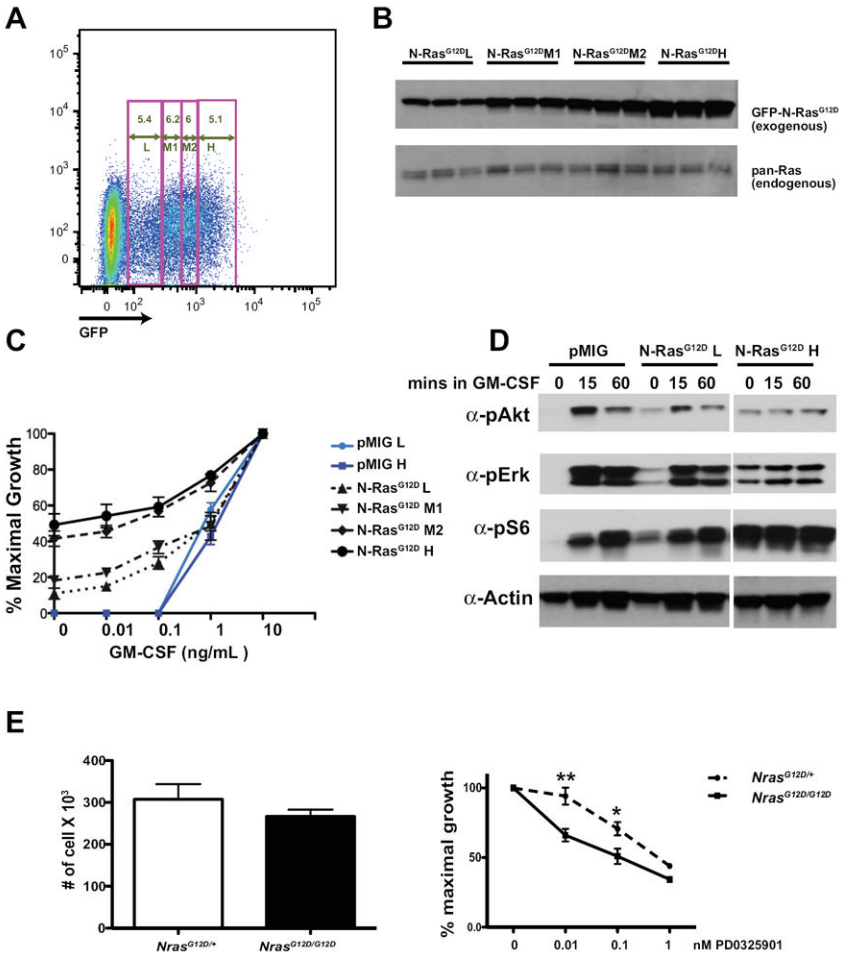

Figure 3. Oncogenic *Nras*^{G12D} Signaling is Dosage Dependent

(**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).

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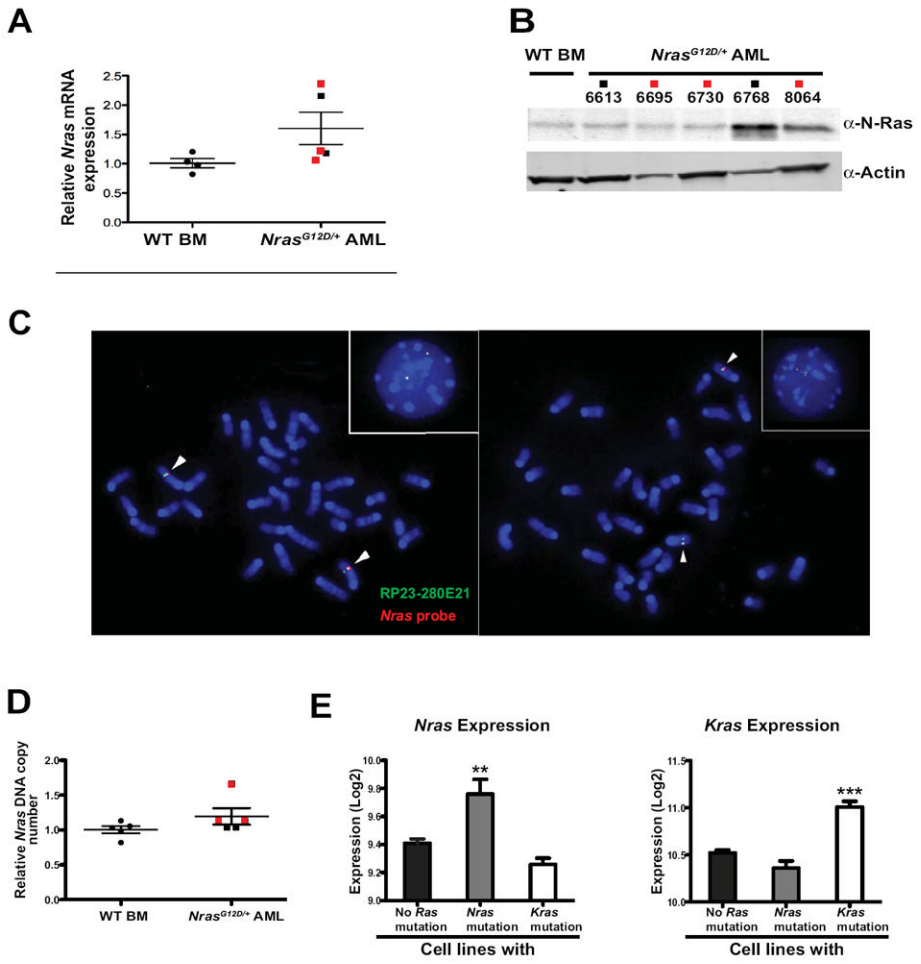

Figure 4. N-Ras Expression in AMLs from *Nras*$^{G12D/+}$ Mice

(A) Quantitative real-time PCR of *Nras* mRNA expression in WT bone marrow and primary *Nras*^{G12D} AMLs with and without LOH (*Nras*^{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*^{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*^{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*^{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).