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 \textit{Nras}^{G12D} in the hematopoietic compartment of mice induces an aggressive myeloproliferative neoplasm that is exacerbated in homozygous mutant animals. Here, we show that increased \textit{Nras}^{G12D} gene dosage, but not inactivation of WT \textit{Nras}, underlies the aggressive in vivo behavior of \textit{Nras}^{G12D/G12D} hematopoietic cells. Modulating \textit{Nras}^{G12D} dosage had discrete effects on myeloid progenitor growth, signal transduction, and sensitivity to MAP-ERK kinase (MEK) inhibition. Furthermore, enforced WT N-Ras expression neither suppressed the growth of \textit{Nras}-mutant cells nor inhibited myeloid transformation by exogenous \textit{Nras}^{G12D}. Importantly, \textit{NRAS} expression increased in human cancer cell lines with \textit{NRAS} mutations. These data have therapeutic implications and support reconsidering the proposed tumor suppressor activity of WT Ras in other cancers.

\textbf{SIGNIFICANCE:} Understanding the mechanisms of Ras-induced transformation and adaptive cellular responses is fundamental. The observation that oncogenic Nras lacks tumor suppressor activity, whereas increased dosage strongly modulates cell growth and alters sensitivity to MEK inhibition, suggests new therapeutic opportunities in cancer.

Abstract:

\textbf{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 \textit{Nras}^{G12D} in the hematopoietic compartment of mice induces an aggressive myeloproliferative neoplasm that is exacerbated in homozygous mutant animals. Here, we show that increased \textit{Nras}^{G12D} gene dosage, but not inactivation of WT \textit{Nras}, underlies the aggressive in vivo behavior of \textit{Nras}^{G12D/G12D} hematopoietic cells. Modulating \textit{Nras}^{G12D} dosage had discrete effects on myeloid progenitor growth, signal transduction, and sensitivity to MAP-ERK kinase (MEK) inhibition. Furthermore, enforced WT N-Ras expression neither suppressed the growth of \textit{Nras}-mutant cells nor inhibited myeloid transformation by exogenous \textit{Nras}^{G12D}. Importantly, \textit{NRAS} expression increased in human cancer cell lines with \textit{NRAS} mutations. These data have therapeutic implications and support reconsidering the proposed tumor suppressor activity of WT Ras in other cancers.

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\textbf{INTRODUCTION} 

\textit{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; ref. 1). Ras-GTP levels are regulated by the competing activities of guanine nucleotide exchange factors and GTPase-activating proteins (GAP), which enhance intrinsic Ras GTPase activity. Proteins encoded by RAS oncogenes, which accumulate in the GTP-bound state due to defective

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intrinsic GTP hydrolysis and resistance to GAPs, are exceedingly difficult targets for anticancer 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 have 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 NrasG12D induces a myeloproliferative neoplasm in Mx1-Cre\textbackslash NrasG12D/+ mice that faithfully models human chronic and juvenile myelomonocytic leukemia (CMML and JMML; refs. 4, 6, 7). Hematologic disease is greatly accelerated in homozygous NrasG12D/mutant mice (Mx1-Cre; NrasG12D/G12D, refs. 8, 9). We deployed a conditional Nras mutant allele to assess the relative contributions of NrasG12D oncogene dosage and tumor suppression by WT Nras in myeloid transformation. We find that elevated NrasG12D 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 NrasG12D mutations, resulting in normal-to-increased Nras expression. Finally, NRAS expression is significantly elevated in human cancer cell lines with NRAS mutations, whereas KRAS expression is reduced, with a reciprocal pattern seen in cell lines with KRAS mutations.

RESULTS

We generated a Cre-dependent conditional Nras allele (Nrascre(lox)) and conducted intercrosses to produce hemizygous (Mx1-Cre\textbackslash NrasG12D/lox), heterozygous (Mx1-Cre\textbackslash NrasG12D/+), and homozygous (Mx1-Cre\textbackslash NrasG12D/G12D) littermates on a C57Bl/6 strain background (Supplementary Fig. S1A). Use of this conditional Nraslox allele avoids potential confounding consequences of eliminating WT Nras expression throughout development and allowed us to simultaneously activate NrasG12D 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 2 weeks later (Supplementary Fig. S1B and 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 NrasG12D−/−.

Consistent with recent reports (8, 9), approximately 20% of Mx1-Cre\textbackslash NrasG12D/G12D mice died prematurely from T lineage acute lymphoblastic leukemia (Supplementary Fig. S2A). Surviving animals of all three Nras genotypes were euthanized at 6 months of age. All NrasG12D/+ mice had overt myeloproliferative neoplasm, which was characterized by leukocytosis with elevated blood neutrophil counts, splenomegaly, and anemia (Fig. 1B and Supplementary Fig. S2B). In contrast, hematologic parameters were normal in age-matched Mx1-Cre\textbackslash NrasG12D/+ and Mx1-Cre\textbackslash NrasG12D−/− mice (Fig. 1B and Supplementary Fig. S2B). Flow cytometric analysis revealed increased numbers of immature monocyte (Mac-1+), Gr-1+ cells in the hematopoietic tissues of NrasG12D/G12D mice, which is also observed in Nf1- and Kras-mutant mice with myeloproliferative neoplasm (ref. 7; Fig. 1C). This population was not expanded in hemizygous or heterozygous NrasG12D mice.

We grew colony-forming unit granulocyte macrophage (CFU-GM) progenitors to assess the cell-intrinsic effects of NrasG12D 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; ref. 7). Similarly, NrasG12D/G12D bone marrow cells show cytokine-independent CFU-GM growth and pronounced GM-CSF hypersensitivity (Fig. 1D; refs. 8, 9). In striking contrast, CFU-GM from Mx1-Cre\textbackslash NrasG12D−/− and Mx1-Cre\textbackslash NrasG12D−/− mice displayed normal cytokine responses (Fig. 1D). Phospho-flow cytometric analysis of Lin−/Kit+/CD105−/CD34− bone marrow cells, which are highly enriched for myeloid progenitors (Supplementary Fig. S2C), revealed elevated basal levels of phosphorylated extracellular signal-regulated kinase (pERK) and enhanced responsiveness to GM-CSF in NrasG12D/G12D cells compared with heterozygous and hemizygous NrasG12D−/− 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 determine whether exogenous WT Nras expression might antagonize the abnormal growth of NrasG12D/G12D cells, we infected Mx1-Cre\textbackslash NrasG12D/G12D bone marrow with murine stem cell virus (MSCV) vectors encoding N-terminal GFP fused to WT N-Ras (N-RasWT), WT K-Ras (K-RasWT), or dominant negative N-Ras (N-RasN17; ref. 9). After sorting to isolate GFP-positive (GFP+) cells, CFU-GM colony growth was assayed in the presence of GM-CSF. Mx1-Cre\textbackslash NrasG12D/G12D bone marrow formed significantly more CFU-GM colonies than heterozygous or hemizygous Nras cells (Fig. 2A). Expressing N-RasWT or K-RasWT had no effect on CFU-GM colony growth from NrasG12D/G12D bone marrow, whereas dominant negative N-RasN17 reduced growth by more than twofold (Fig. 2A).

To further investigate whether WT N-Ras interferes with oncogenic N-RasG12D-induced myeloid transformation, we infected WT fetal liver cells with MSCV vectors expressing N-terminal mCherry-tagged N-RasG12D in combination with GFP, GFP-tagged N-RasWT, or GFP-tagged K-RasWT. Flow cytometry and Western blotting revealed an equivalent increase in Ras protein levels in cotransduced cells (Fig. 2B and C). Importantly, neither WT Ras isoform suppressed the aberrant pattern of cytokine-independent CFU-GM progenitor growth induced by exogenous N-RasG12D 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-RasG12D 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-RasG12D showed cytokine-independent CFU-GM colony growth with a threshold level of expression required for myeloid transformation (Fig. 3B and C).

We also interrogated Ras signaling in transduced GFP+ bone marrow–derived macrophages that were first deprived of cytokines and serum and then stimulated with GM-CSF (10).
Oncogene Dosage Is Crucial; Loss of WT Nras Is Not

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 three 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 over a range of GM-CSF concentrations (\(n = 5–7\) per genotype). Note that only Nras\(^{G12D}/G12D\) cells show 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 minutes). The vertical black line indicates basal pERK levels in WT cells.
Figure 2. Wild-type Ras does not antagonize myeloid transformation by Nras\(^{G12D}\).

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

**B**, WT E14.5 fetal liver cells were cotransduced with MSCVs expressing mCherry-N-Ras\(^{G12D}\) and either GFP-only (pMIG), GFP-N-Ras\(^{WT}\), or GFP-K-Ras\(^{WT}\). Flow cytometric analyses indicate equivalent numbers of PE-Texas Red (mCherry) and FITC (GFP) double-positive cells. The GFP fluorescence is higher in cells expressing GFP only.

**C**, Immunoblot analysis shows that cells cotransduced with oncogenic N-Ras\(^{G12D}\) and either GFP-N-Ras\(^{WT}\) or GFP-K-Ras\(^{WT}\) express more total Ras than control cells cotransduced 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 approximately 40% of myeloid progenitors to cytokine-independent growth, and coexpressing WT N-Ras or K-Ras does not alter this phenotype.
Oncogene Dosage Is Crucial; Loss of WT Nras Is Not

Under these conditions, AKT, ERK, and S6 were not phosphorylated in starred macrophages infected with the empty pMIG vector, and these cells responded robustly to GM-CSF stimulation (Fig. 3D). Macrophages expressing low levels of N-RasG12D showed a modest increase in basal pAKT, pERK, and pS6, which were augmented by cytokine stimulation. In contrast, starred cells expressing the highest levels of N-RasG12D 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 MAP-ERK kinase (MEK) inhibitor PD0325901 restores a normal pattern of hematopoiesis in Kras- and Nf1-mutant mice with myeloproliferative neoplasm (11, 12). We therefore asked whether endogenous NrasG12D gene dosage influences the sensitivity of bone marrow–derived macrophages to MEK inhibition. Macrophages grown directly from the bone marrows of Mx1-Cre;NrasG12D/+ and Mx1-Cre;NrasG12D/G12D mice expanded to a similar extent in the presence of a saturating concentration macrophage colony-stimulating factor (M-CSF; 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 (Supplementary Fig. S3A and S3B).

NrasG12D expression cooperated with the MOL4070LTR retrovirus to efficiently induce AML in Mx1-Cre;NrasG12D/+ 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 (Supplementary...
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 (Supplementary Fig. S4B). Consistent with these data, Western blot analysis revealed elevated N-Ras protein levels in AML blasts, including \textit{Nras} \textit{G12D} leukemias with somatic loss of the normal \textit{Nras} allele (Fig. 4B). We conducted 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} \textit{G12D} allele as the genetic basis underlying loss of the WT \textit{Nras} allele in leukemias with LOH (Fig. 4C and D). Together, these studies indicate that genetic and transcriptional mechanisms converge to augment oncogenic N-Ras \textit{G12D} expression in AML.

To address the broad relevance of increased RAS oncogene expression in human cancer, we queried \textit{NRAS}/\textit{KRAS} mutational status and corresponding expression data across 957 human cancer cell lines with \textit{NRAS} mutations (\textit{n} = 56), \textit{KRAS} mutations (\textit{n} = 173), or no RAS mutation (\textit{n} = 728). **, \textit{P} = 0.0018; ***, \textit{P} = 7.7E-16.

**Figure 4.** N-Ras expression in AMLs from \textit{Nras} \textit{G12D/+} mice. \textbf{A,} quantitative real-time PCR of \textit{Nras} mRNA expression in WT bone marrow (BM) and primary \textit{Nras} \textit{G12D} AMLs with and without loss of constitutional heterozygosity (LOH) (\textit{Nras} \textit{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). \textbf{B,} Western blot analysis of \textit{Nras} \textit{G12D} AMLs shows that N-Ras proteins levels are equivalent to or higher than in WT mouse bone marrow. \textbf{C,} FISH analysis of metaphase and interphase cells from representative AMLs with (6613; left) and without (6695; right) LOH. Arrowheads identify a fused signal from both BAC probe RP23-280E21 and the smaller \textit{Nras} probe. Hybridizing with the larger BAC clone uniformly revealed signals on both chromosomal homologs in all 5 \textit{Nras} \textit{G12D} AMLs. Equal percentages of cells showed one or two hybridization signals (shown here) with the much smaller \textit{Nras}-specific probe from AMLs with and without LOH. \textbf{D,} \textit{Nras} DNA copy number is normal or increased in \textit{Nras} \textit{G12D} AMLs with and without LOH as assessed by TaqMan quantitative PCR. \textbf{E,} \textit{NRAS} and \textit{KRAS} expression in 957 human cancer cell lines with \textit{NRAS} mutations (\textit{n} = 56), \textit{KRAS} mutations (\textit{n} = 173), or no RAS mutation (\textit{n} = 728). **, \textit{P} = 0.0018; ***, \textit{P} = 7.7E-16. BAC, bacterial artificial chromosome.
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, whereas those with KRAS mutations downregulated NRAS (Fig. 4E). Similar trends were observed when the hematopoietic cell lines in this large collection were analyzed separately (Supplementary Fig. SSA). Gene expression data from a well-annotated collection of human AMLs revealed elevated NRAS expression compared with normal CD34+ progenitors in leukemias with and without Nras mutations (Supplementary Fig. SSB).

**DISCUSSION**

LOH is common in human cancer and classically represents a genetic “second hit” that results in homozygous inactivation of tumor suppressor genes (TSG) 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 showing that germline inactivation of one Ras allele greatly increased the incidence and biologic aggressiveness of genotoxin-induced skin and lung carcinoma supported this idea (5, 14).

Though 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, significant allele-specific imbalance, and overexpression 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 Mxl-Cre;NrasG12D mice provide a tractable and genetically accurate system for interrogating the putative TSG activity of WT Nras in early (myeloproliferative neoplasm) and late (AML) stage cancers that do not rely on chemical carcinogenesis. Our extensive genetic and functional analysis indicates that WT Ras activity may also render them more susceptible to inhibitors of critical effector pathways.

**METHODS**

**Mouse Strains and Pathologic Analysis**

NrasG12D mice were generated by inserting lnsP 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 (Supplementary Fig. S1A). After germline transmission of the targeted allele, the Neo resistance cassette was removed by crossing to an Flp deleter strain (Jackson Laboratory). Mxl-Cre;NrasG12D mice were intercrossed with NrasG12D mice to generate Mxl-Cre; NrasG12D;G12D, Mxl-Cre;NrasG12D/+ , and Mxl-Cre;NrasG12D/neo mice. All mice received a single intraperitoneal injection of poly-l-lysine-C (250 μg) at 3 weeks of age to activate Mxl-Cre expression (4). Mice were euthanized at 6 months of age to assess disease. Pathologic examinations were conducted as previously described (4).

**Hematopoietic Progenitor Assays and Flow Cytometry**

Hematopoietic progenitor assays and flow cytometric analyses were conducted as previously described (4, 10).

**Retroviral Transduction**

NrasWT, KrasWT, NrasG12D, and NrasG12D alleles containing either N-terminal GFP or N-terminal mCherry markers were cloned into the MSCV with expression driven by the internal ribosomal entry
Biochemistry

Biochemical analyses were conducted on cultured macrophages that were differentiated from transduced GFP+ fetal liver cells in 50 ng/mL 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 BAC probe containing the mouse Nras gene (RP23-28082; 150 kb) was labeled with 5-(3-aminooallyl)-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 conducted 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 NrasG12D+ mice with the MOL407LTR retrovirus were conducted as previously described (4).

Gene Expression Profiling of Cell Line and AML

NRRs and KRAS expression in cancer cell lines was extracted from gene-centric RNA-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 t test assuming unequal variance. To further examine NRAS and KRAS expression in human leukemia, we conducted microarray-based gene expression profiling data of pediatric AML and normal CD34+ samples generated using Affymetrix U133A microarrays (Affymetrix) according to the manufacturer’s instructions, with data processed using MAS 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/ accession numbers GSE43176 (AML samples) and GSE53315 (CD34+ bone marrow).

No experiments were carried out on any cell lines to generate these data. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J. Xu, K. Shannon
Development of methodology: J. Xu, T. Jacks
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Xu, K.M. Haigis, A.J. Firestone, Q. Li, J. Downing, M.M. Le Beau
Writing, review, and/or revision of the manuscript: J. Xu, J. Nakitandwe, J. Downing, M.M. Le Beau, K. Shannon
Study supervision: M.M. Le Beau, K. Shannon
Performed the FISH analysis of the samples: E. Davis

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REFERENCES

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

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