NRAS mutation at codons 12, 13, or 61 is associated with transformation; yet, in melanoma, such alterations are nearly exclusive to codon 61. Here, we compared the melanoma susceptibility of an NrasQ61R knock-in allele to similarly designed KrasG12D and NrasG12D alleles. With concomitant p16INK4a inactivation, KrasG12D or NrasQ61R expression efficiently promoted melanoma in vivo, whereas NrasG12D did not. In addition, NrasQ61R mutation potently cooperated with Lkb1/Stk11 loss to induce highly metastatic disease. Functional comparisons of NrasQ61R and NrasG12D revealed little difference in the ability of these proteins to engage PI3K or RAF. Instead, NrasQ61R showed enhanced nucleotide binding, decreased intrinsic GTPase activity, and increased stability when compared with NrasG12D. This work identifies a faithful model of human NRAS-mutant melanoma, and suggests that the increased melanomagenicity of NrasQ61R over NrasG12D is due to heightened abundance of the active, GTP-bound form rather than differences in the engagement of downstream effector pathways.

SIGNIFICANCE: This work explains the curious predominance in human melanoma of mutations of codon 61 of NRAS over other oncogenic NRAS mutations. Using conditional "knock-in" mouse models, we show that physiologic expression of NRASQ61R, but not NRASG12D, drives melanoma formation.

Cancer Discov; 4(12); 1–10. © 2014 AACR.
INTRODUCTION

One third of all human cancers harbor activating KRAS, HRAS, or NRAS mutations, which localize predominantly to codons 12, 13, or 61 (1, 2). RAS proteins function as canonical GTPase switches, binding to effectors in the presence of GTP and activating downstream signaling pathways to influence cellular proliferation, differentiation, and survival. The return of RAS to an inactive, GDP-bound state is catalyzed by GTPase-activating proteins (GAP), which stimulate the weak, intrinsic GTPase activity of these proteins. Mutations at codons 12 or 13 render RAS proteins insensitive to GAP activity, resulting in constitutive, oncogenic signaling (3). Similarly, mutation of Q61, a catalytic residue required for efficient GTP hydrolysis, impedes the return of RAS to an inactive GDP-bound state (4).

Historically, RAS proteins with codon 12, 13, or 61 alterations have been considered oncogenic equivalents; however, recent clinical observations suggest functional differences for each RAS mutation. For example, in colorectal cancer, KRAS mutational status is used as a prognostic indicator of resistance to therapy with EGFR antibodies (e.g., cetuximab; refs. 5–8). Retrospective analyses, however, of “all-comer” trials suggest mutational specificity in this regard: patients harboring KRAS codon 13 mutations appear to benefit from cetuximab therapy, whereas those with codon 12 mutations were unresponsive (9–13). Moreover, progression-free survival on targeted therapies may also be codon-specific in non–small cell lung cancer (NSCLC; ref. 14). Here, molecular modeling and reverse-phase protein analysis pinpointed differential effector engagement and downstream signaling as potential mediators of mutation-specific therapeutic response (14). Together, these results suggest that distinct, codon-specific properties of RAS mutations have important clinical and biologic implications.

Cancers display tissue-specific preferences for mutation of the RAS homologs (Supplementary Table S1). In melanoma, NRAS is by far the most frequently mutated RAS isoform, and notably, 84% of these mutations localize to codon 61 versus only 7% to glutamine 12 (Supplementary Table S1). A similar preference for codon 61 mutations is noted in thyroid cancer, but is not observed in other cancer types. Codon 12 and 13 mutations constitute more than 90% of KRAS mutations observed in human colon, pancreatic, lung, and ovarian cancers (Supplementary Table S1 and ref. 1). Likewise, glycine 12 is the most common site of NRAS mutation in acute myeloid leukemia (Supplementary Table S1). The mechanistic basis for codon 61 selection in melanoma and thyroid cancer is unclear. Some have suggested that cytosine to thymidine transversions caused by ultraviolet (UV) light may explain the preference for certain mutations in melanoma, but the majority of codon 61 mutations do not exhibit a characteristic UV-damage signature (15). Alternatively, it is possible that codon mutation preferences reflect differences in oncogenic signaling.

Comparing the oncogenic potential of various RAS mutants is challenging for several reasons. RAS gene dosage clearly influences downstream signaling, and artifacts of RAS overexpression are well described. Likewise, endomembrane localization is critical for physiologic RAS signal transduction (16), and may not be adequately recapitulated using
exogenous protein expression. In addition, genetic alterations private to a given cell line or tumor sample could obscure distinct functions of individual RAS mutants. To circumvent these issues, we generated a knock-in allele (LSL-Nras\textsuperscript{G12D}) allowing for the conditional, tissue-specific, and somatic expression of NRAS\textsuperscript{Q61R} under control of the endogenous promoter. We used this allele in combination with similarly designed knock-in Nras\textsuperscript{G12D} (17) and Kras\textsuperscript{G12D} (18) models to compare the transforming potential of each mutant when expressed at physiologic levels in melanocytes.

**RESULTS**

**Generation and Characterization of LSL-Nras Alleles**

To compare the ability of NRAS mutants to promote melanoma formation, we used three conditional knock-in alleles: LSL-Nras\textsuperscript{G12D} (17), LSL-Kras\textsuperscript{G12D} (18), and LSL-Nras\textsuperscript{Q61R}. Each allele contains a floxed transcriptional stop sequence followed by a single missense mutation in the endogenous Ras gene (G12D or Q61R, respectively; Fig. 1A). The codon 12 LSL-Kras and Nras alleles have been previously described (17, 18). We generated and confirmed a related LSL-Nras\textsuperscript{Q61R} allele using standard homologous recombination followed by Southern blot, PCR, and genomic sequencing (Supplementary Fig. S1A–S1D). To minimize strain-specific effects, all alleles were backcrossed more than seven generations to C57BL/6J in the presence of a conditional p16\textsuperscript{INK4a} knockout allele (p16\textsuperscript{L}; ref. 19) and a melanocyte-specific, 4-hydroxytamoxifen (4-OHT)–inducible CRE recombinase (Tyr-CRE-ERT\textsubscript{2}; ref. 20). Cohorts of all three alleles were born at normal Mendelian ratios (data not shown) and showed no defects in development or fertility.

Using primary melanocytes derived from syngeneic Tyr-CRE-ERT\textsubscript{2} p16\textsuperscript{L} LSL-Nras\textsuperscript{G12D/G12D} (Tp\textsuperscript{G12D}/12D) or LSL-Nras\textsuperscript{Q61R/Q61R} (Tp\textsuperscript{N61R}/61R) neonates, we verified the functionality of the LSL alleles. Melanocyte purities of >99% were confirmed using tyrosinase-related protein 1 (TRP1) as a marker for flow cytometry
Codon Specificity in RAS-Driven Melanoma

Research Article

( Supplementary Fig. S2A; ref. 21). In culture, melanocytes were treated with ethanol vehicle or 4-OHT to induce CRE activity. CRE-dependent excision of the transcriptional stop element was verified by PCR (Fig. 1B), and resulted in the production of mutant Nras mRNA (Supplementary Fig. S2B). Activation of either allele did not induce changes in melanocyte morphology or pigmentation (Supplementary Fig. S2C), but induced a decrease in melanocyte proliferation as measured by EdU incorporation (Fig. 1C). Despite codeletion of p16INK4a, physiologic expression of either NRAS mutant caused comparable antiproliferative effects (Fig. 1C). In addition, both TpN12D/12D and TpA61R/61R melanocytes failed to bypass senescence in the presence of 4-OHT, and the cells invariably ceased proliferating after two to three passages in culture (data not shown). Therefore, when expressed under the control of an endogenous promoter, neither NRASG12D nor NRASG61R was capable of immortalizing p16INK4a-deficient melanocytes.

NRAS-Driven Melanogenesis Is Codon-Specific

We next sought to examine the phenotypic effects of melanocyte-specific Ras mutations in vivo. Toward that end, we generated contemporaneous colonies of p16INK4a-deficient Kras- or Nras-mutant mice in a common genetic background. We elected to study Nras mutations in the homozygous state for two reasons: (i) we noted no melanoma formation despite 80 weeks of monitoring in a large cohort of Tyr-CRE-ERT2 p16+/−/LSL-NrasG12D/WT (TpN12D/WT) animals (Supplementary Fig. S3A), and (ii) deep sequencing of nine NRAS-mutant human melanoma cell lines failed to detect the presence of a wild-type allele, demonstrating consistent NRAS loss of heterozygosity in vivo (22). Prior work from our group has demonstrated that melanocytic expression of KRASG12D efficiently promotes melanoma in vivo in the setting of p16INK4a inactivation (19, 23). Herein, syngeneic Tyr-CRE-ERT2 p16+/−/LSL-KrasG12D/WT (TpK12D/WT) mice were maintained in a heterozygous state due to the homozygous lethality of Kras deletion (24). All mice in these cohorts were treated neonatally with 4-OHT (19) to induce melanocyte-specific expression of the desired Ras mutant and delete p16INK4a.

Initial examination of the skin, paws, and tails of these mice revealed the presence of nevi and hyperpigmented regions on the paws and tails (Supplementary Fig. S4A). The penetrance and severity of these phenotypes was allele-specific with TpK12D/WT animals having the most pronounced effect and the NRAS codon 12 mutation producing the least (TpK12D/WT > TpN12R/61R > TpN12D/12D). To quantify nevi frequency, nevi presence was scored weekly for 10 weeks. As expected, nevi were rarely observed on Tyr-CRE-ER T2 p16+/−/ (Tp) mice, but was consistently found on TpK12D/WT animals (Supplementary Fig. S4B). Members of both the TpA12D/12D and TpN12R/61R cohorts had more nevi than control Tp mice (P < 0.001). However, the frequency with which NRASQ61R triggered nevus formation was significantly higher than that observed in TpK12D/WT animals (P = 0.03). These data suggest that in p16INK4a-deficient melanocytes, physiologic NRASQ61R expression more efficiently promotes nevus formation than NRASG12D.

These established colonies of syngeneic TpN12D/12D, TpN12R/61R, and TpK12D/WT mice were aged and serially assessed for melanoma formation. In accordance with previous data, TpK12D/WT animals developed tumors with high penetrance (Fig. 2A; Table 1; ref. 19). Tumors were very rare in TpA12D/12D mice (one tumor found in 29 mice observed for 80 weeks), whereas TpN12R/61R mice readily developed melanoma with high penetrance and a median latency of 26.3 weeks (Fig. 2A and B and Table 1). To explain this result, we considered the possibility that the LSL-NrasQ61R and LSL-NrasG12D alleles had different recombination efficiencies. Toward that end, we examined allelic recombination in primary melanocyte cultures and noted that, if anything, the codon 61 allele recombined with lower efficiency than the codon 12 allele (Supplementary Fig. S5A). We also considered the possibility that the recombined LSL-NrasG12D allele was poorly expressed. Sequencing of cDNA from treated melanocytes confirmed 4-OHT-dependent expression of NrasG12D mRNA.

![Figure 2](http://example.com/figure2.jpg)
(Supplementary Fig. S2B). Finally, functional validation of the LSL-NrasG12D allele was accomplished by inducing allelic recombination in the hematopoietic lineage using an interferon-inducible MsI-CRE driver. Expression of NrasG12D in the hematopoietic compartment efficiently induced a myeloproliferative syndrome in accord with prior findings (ref. 25, and data not shown). These data establish that when expressed at physiologic levels in melanocytes, NRASQ61R and KRASG12D are inherently more transforming than NRASG12D.

We performed additional analyses to determine whether there were phenotypic differences in the tumors of TpN12D/12D, TpN61R/61R, and TpK12D/WT mice. Similar growth rates were observed in tumors from TpN12D/12D, TpN61R/61R, and TpK12D/WT mice, albeit only one tumor was found in the TpN12D/12D cohort (Supplementary Fig. S5B). Moreover, melanomas from all three groups were histologically similar, containing both spindle-cell and desmoplastic cell types with no overt signs of macrometastatic spread (Fig. 2C and Table 1). In the TpN61R/61R tumors, we confirmed the presence of the recombined LSL-NrasQ61R allele (Supplementary Fig. SSC) and used quantitative real-time PCR to look for potential gene amplifications. Compared with TpK12D/WT tumors, in which endogenous Nras expression is unaltered, TpN61R/61R melanomas displayed no significant change in Nras mRNA levels, suggesting that the allele was not amplified during tumorogenesis (Supplementary Fig. SSD). These data show that NRASQ61R, NRASG12D, and KRASS12D tumors, once established, are phenotypically similar, and suggest that oncogenic differences between the alleles are most pronounced during tumor initiation.

### Table 1. Comparative summary of melanomagenesis and macrometastasis in LSL-Ras-driven models

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tumors/treated mice, %</th>
<th>Median tumor latency, wks</th>
<th>Macrometastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tp61R</td>
<td>1/24 (4.1%)</td>
<td>&gt;80</td>
<td>None</td>
</tr>
<tr>
<td>TpN12D/12D</td>
<td>1/29 (3.4%)</td>
<td>&gt;80</td>
<td>None</td>
</tr>
<tr>
<td>TpN61R/61R</td>
<td>14/20 (70%)</td>
<td>26.3</td>
<td>None</td>
</tr>
<tr>
<td>TpK12D/WT</td>
<td>16/21 (76%)</td>
<td>36.3</td>
<td>None</td>
</tr>
<tr>
<td>TpLN61R/61R</td>
<td>17/20 (85%)</td>
<td>22.1</td>
<td>5/14 (36%)</td>
</tr>
</tbody>
</table>

Lkb1 Loss Promotes Melanoma Metastasis in TpN61R/61R Mice

Melanoma mortality is predominantly attributed to metastatic disease; however, very few RAS-driven genetically engineered mouse models, including the TpN61R/61R model, show evidence of macrometastases (Table 1; ref. 26). We recently demonstrated that the loss of liver kinase B1 (Lkb1/Stk11) promotes widespread macrometastases in a KRASG12D-driven mouse melanoma model (23). LKB1 is mutated in approximately 10% of malignant human melanomas (15, 27, 28) and functionally inactivated by oncogenic BRAF (22, 29, 30). To test whether Lkb1 loss promotes metastasis in TpN61R/61R tumors, we crossed these animals to a previously described conditional Lkb1 knockout allele (Lkb1f/f; ref. 31). TpLN61R/61R mice were treated neonatally or at adulthood with 4-OHT to stimulate melanocyte-specific deletion of both Lkb1 and pE6K54a and induce NRASQ61R expression. The onset of tumor formation in TpN61R/61R neonates was not affected by Lkb1 loss (Fig. 3A and Table 1); however, the propensity for distant metastasis was markedly affected. TpLN61R/61R animals, treated as adults or neonates, exhibited enhanced nevus formation relative to Lkb1-proficient counterparts (compare Supplementary Fig. S4A with Fig. 3B). Upon sacrifice due to increasing tumor burden, many of TpLN61R/61R showed signs of metastasis. We performed thorough autopsies on 14 TpLN61R/61R mice, noting lymph node enlargement and other signs of macroscopic disease (Fig. 3C) in the majority of animals. The presence of visceral metastatic disease to the lung, spleen, and/or liver was confirmed in 5 of these animals by histologic examination (36%; Fig. 3D and Table 1). Metastases appeared similar in morphology and incidence to those observed in a prior KRAS-driven, Lkb1f/f melanoma model (23). Flow cytometric analyses of TpLN61R/61R mice with primary melanomas showed the presence of infiltrating tumor cells expressing the melanocyte marker TRP1 (Fig. 3E; note that CD45+ cells were excluded from splenic analyses). Unlike cells from TpN61R/61R tumors, we were able to culture TpLN61R/61R melanoma cells in vitro. These cell lines exhibited varied morphologies, but were invariably TRP1 positive (Supplementary Fig. S6). Together, these findings establish TpLN61R/61R as a faithful murine model of metastatic, cutaneous melanoma driven by an endogenous, oncogenic NRAS allele.

**NRASQ61R and NRASG12D Similarly Bind Melanogenic Effector Pathways**

The distinct oncogenicity of HRAS, KRAS, and NRAS is often attributed to the isoform-specific, preferential engagement of downstream effector pathways (17, 32–35). We postulated that a similar mechanism might also drive codon-specific melanomagenesis. To this end, the interaction of purified KRAS, NRAS, NRASG12D, and NRASQ61R with PI3K and RAF [i.e., the BRAF RAS binding domain (RBD)] was examined in vitro. Each GTPase was first loaded with a fluorescent GTP analogue [2’-/3’-O-(N’-methylanthraniloyl)-guanosine-5’- [(β,γ-imido) triphosphate (mMGMPPNP)]. Next, purified PI3K or RAF-RBD was titrated into reactions containing a constant amount of GMPNP-RAS protein at both 5 mmol/L and 100 μmol/L Mg2+ concentrations. Fluorescence quenching, caused by effector binding, was monitored to determine the Ki for each protein–protein interaction. In these assays, NRASQ61R bound both PI3K and the RAF-RBD with a lower affinity than either wild-type NRAS or NRASG12D (Table 2). However, the noted differences were small (<1-fold for PI3K; ~4-fold for the RAF-RBD) and unlikely to translate into an in vivo phenotype. Likewise, isothermal titration calorimetry experiments did not reveal significant differences in the RAF-RBD-binding affinities of NRASQ61R, NRASG12D, and wild-type NRAS (Supplementary Fig. S8). These data indicate that distinct engagement of the oncogenic RAS effector pathways frequently targeted in melanoma (i.e., RAF and PI3K) is not the cause of codon-specific melanomagenesis.

**Activation of MAPK and ERK Is Codon-Independent in NRAS-Mutant Melanomas**

The distinct subcellular localizations of HRAS, KRAS, and NRAS are suggested to influence effector availability and contribute to isoform-specific RAS oncogenicity (17, 32–35). To determine whether the availability of specific effector pools...
in vivo contributes to codon-specific signaling, we examined MAPK and PI3K activation in a variety of human melanoma cell lines harboring a mutation in NRAS codon 12, 13, or 61 (n = 11). These cell lines exhibited variable levels of activated ERK and AKT that did not correlate with genotype (Fig. 4A and B). NRAS<sup>G12D</sup>/<sup>G13D</sup>-mutant cell lines exhibited variable and codon-independent (Supplementary Fig. S10A and S10B, top). Therefore, we analyzed these cells at an early time point following CRE induction (2 days). In both cases, we found that phospho-ERK and AKT levels were particularly resistant to sustained allelic recombination (Supplementary Fig. S10B, top). Therefore, we analyzed these cells at an early time point following CRE induction (2 days). In both cases, we found that phospho-ERK and AKT levels were variable and codon-independent (Supplementary Fig. S10A and S10B, bottom). These data, along with observations in human melanoma cell lines, demonstrate that melanoma-geneic NRAS mutants similarly activate the MAPK and PI3K pathways.

Because of the confounding potential of secondary mutations acquired in human melanoma cell lines, we also examined ERK and AKT activation in primary and immortalized melanocytes derived from the Tp<sup>N61R/N61R</sup> and Tp<sup>N61R/N61R</sup> models. Expression of each Nras allele was induced in vitro using CRE recombinase and verified by genomic PCR (Supplementary Fig. S10A and S10B, top). Immortalized Tp<sup>N61R/N61R</sup> melanocytes were particularly resistant to sustained allelic recombination (Supplementary Fig. S10B, top). Therefore, we analyzed these cells at an early time point following CRE induction (2 days). In both cases, we found that phospho-ERK and AKT levels were variable and codon-independent (Supplementary Fig. S10A and S10B, bottom). These data, along with observations in human melanoma cell lines, demonstrate that melanoma-geneic and nonmelanomageneic NRAS mutants similarly activate the MAPK and PI3K pathways.

**NRAS<sup>G61R</sup> Exhibits Distinct Biochemical Properties**

Surprised by the finding that NRAS codon 12, 13, and 61 mutants appear equally to engage the oncogenic PI3K and MAPK effector pathways, we further investigated the biochemical properties that differentiate these two mutants. We observed during RAS·<sup>mantGMPPNP</sup>-based binding assays that the nucleotide exchange rate of NRAS<sup>G61R</sup> was significantly retarded compared with WT or NRAS<sup>G12D</sup> (data not shown). To follow up on this observation, we measured the intrinsic and son of sevenless (SOS)-mediated nucleotide disassociation

**Table 2. Binding affinities of RAS·<sup>mantGMPPNP</sup> to PI3K and RAF-RBD**

<table>
<thead>
<tr>
<th>RAS mutant</th>
<th>&lt;sup&gt;mantGMPPNP&lt;/sup&gt; binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;sup&gt;K&lt;sub&gt;a&lt;/sub&gt;&lt;/sup&gt; (µM)</td>
</tr>
<tr>
<td></td>
<td>BRAF-RBD</td>
</tr>
<tr>
<td>WT</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>G12D</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>G61R</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>G12D</td>
<td>0.06 ± 0.03</td>
</tr>
</tbody>
</table>
rates of purified NRAS, NRAS<sup>G12D</sup>, and NRAS<sup>Q61R</sup>. To monitor exchange, each NRAS variant was preloaded with either <sup>32</sup>P-methylguanosine diphosphate (m<sub>32</sub>P-GDP) or 1,000-fold excess unlabeled nucleotide. The resulting change in fluorescence over time was used to monitor exchange rates. NRAS, NRAS<sup>G12D</sup>, and NRAS<sup>Q61R</sup> showed a similar ability to exchange GDP (Table 3). However, the GMPPNP exchange rate of NRAS<sup>G12D</sup> was significantly lower than NRAS and NRAS<sup>Q61R</sup> (Table 3). This distinction became more pronounced when SOS was added to each reaction (Table 3). Specifically, SOS was unable to stimulate either GDP or GMPPNP exchange in the codon 61 variant (Table 3). These results indicate that NRAS<sup>Q61R</sup> possesses a clear affinity for GTP that cannot be overcome by guanine nucleotide exchange factor (GEF) interaction at the concentrations used in our assays.

These data, along with previous observations in other RAS isoforms (36), suggest a decreased intrinsic GTPase activity of NRAS<sup>Q61R</sup>. Indeed, when directly assessed, the intrinsic GTP hydrolysis rate of NRAS<sup>Q61R</sup> was much slower than NRAS<sup>G12D</sup> or NRAS (1,150 and 2,300 times slower, respectively; see Table 3). These results, taken together, suggest that NRAS<sup>Q61R</sup> has higher affinity for GTP relative to NRAS and NRAS<sup>G12D</sup>. Therefore, we examined the relative stability of NRAS, NRAS<sup>G12D</sup>, and NRAS<sup>Q61R</sup> using thermal unfolding measurements for both GDP- and GMPPCP-bound proteins. NRAS<sup>Q61R</sup> remained stable until reaching 80°C ± 5°C, whereas the wild-type and NRAS<sup>G12D</sup> proteins were destabilized at much lower temperatures (67°C ± 3°C and 70°C ± 3°C, respectively; see Supplementary Table S2). The thermostability of NRAS<sup>Q61R</sup> was lower in the GDP-bound versus GMPPCP-bound state (74°C ± 4°C vs. 80°C ± 5°C; see Supplementary Table S2), suggesting that NRAS<sup>Q61R</sup> adopts a conformation that stabilizes nucleotide binding, especially in the GTP-bound state. Together, our work reveals that NRAS<sup>Q61R</sup> exhibits distinct nucleotide-binding capacity, stability, and GTPase resistance likely responsible for its exceptional melanomagenic properties.
DISCUSSION

Our data explain a long-standing mystery in the field, demonstrating through biochemical and genetic analyses that the predominance of codon 61 mutants in human melanoma can be attributed to their distinct oncogenic properties. In our novel suite of mouse models, endogenous levels of NRASQ61R, but not NRASG12D, were able to efficiently drive in vivo melanomagenesis (Fig. 2). Although prior work has linked isoform-specific RAS oncogenicity to different effector binding (17, 32–35), we found that NRASQ61R and NRASG12D similarly engaged the PI3K and MAPK pathways (Table 2; Fig. 4; Supplementary Figs. S8 and S10). Our data suggest an alternative model for codon-specific oncogenicity, demonstrating that the melanogenic NRASQ61R mutant possesses distinct biochemical properties not found in NRASG12D (Table 3).

Before this study, it was unclear whether the prevalence of NRAS codon 61 mutations in melanoma reflected a preferential pattern of mutagenesis or codon-specific differences in the biologic function of each allele. We now show that NRASG12D does not efficiently drive cutaneous melanomagenesis (Fig. 2), and therefore codon-specific RAS biology influences tumorogenic potential. Tissue and temporal specificity are also likely to affect oncogenic RAS activity. For instance, activation of the LSL-NRASG12D allele in hematopoietic stem cells and early melanocyte progenitors drives leukemogenesis and melanoma of the central nervous system (refs. 25, 37, 38; data not shown). Moreover, it is possible that our experimental system, which relies upon p16INK4a loss to facilitate melanomagenesis, is biased to favor NRAS codon 61 mutations. Patient studies have suggested exquisite cooperation between NRAS codon 61 mutations and p16INK4a inactivation (39, 40), and NRASQ61R transgenic mice appear to be “addicted” to the activity of CDK4/6, which is enhanced by p16INK4a loss (41). Therefore, perhaps other cooperating oncogenic events commonly found in melanoma would more efficiently synergize with NRASG12D in tumor formation. Nonetheless, p16INK4a loss is found in the majority of human melanomas, occurring through a variety of mechanisms (i.e., epigenetic silencing, mutation, and deletion). Given the significance of this event in the clinical setting, we believe that the choice of the p16INK4a-deficient genetic context is highly relevant to the human disease.

Wildly efficacious and durable treatment options are not available for the 17% to 30% of patients with NRAS-mutant, metastatic melanomas (1, 15, 42). With mounting data to suggest that genetically engineered mouse models of cancer more faithfully report therapeutic efficacy (43–45), a number of RAS-mutant melanoma models have been developed (see Supplementary Table S3). Most of these models use transgenic technologies that can alter the expression, subcellular compartmentalization, intracellular signaling, and transforming potential of RAS (16). To this end, the activation of endogenous RAS oncogenes triggers minimal ERK and AKT activation in our system and others (Supplementary Fig. S10; refs. 17, 18, 25); yet, transgenic RAS alleles robustly stimulate these pathways (46, 47). Other murine melanoma models use oncogenic RAS isoforms rarely observed in human melanomas (i.e., HRAS and KRAS mutants). As each RAS isoform can initiate a unique set of downstream signals, it is unclear to what degree these models faithfully recapitulate the oncogenic mechanisms found in human melanomas. For these reasons, we believe that the TpNO61R and TpLA561R models will be extremely valuable for preclinical drug testing, especially given the metastatic nature of the Lkb1-deficient tumors.

NRASQ61R Exhibits Unique Biochemical Properties

In contrast to prior studies showing that preferential effector usage drives isoform-specific RAS oncogenesis (17, 32–35), we found that distinct biochemical properties inherent to each RAS mutant likely drive codon-specific melanomagenesis (Table 2 and Supplementary Table S2). Nearly three decades ago, Der and colleagues (48) reported that HRAS codon 61 mutants, regardless of their transforming potential, exhibited an approximately 10-fold decrease in intrinsic GTP hydrolysis. Later, Donovan and colleagues (49) used predicted RAS-GTP levels to form the hypothesis that defects in intrinsic GTPase activity and nucleotide exchange dictate the transforming potential of individual RAS mutants. Our experimental results are consistent with this model, demonstrating that the melanomogenic NRASQ61R variant exhibits high-affinity GTP binding, increased stability, and reduced intrinsic GTPase activity when compared with NRASG12D (Table 2 and Supplementary Table S2).

These results suggest that NRASQ61R may more efficiently activate downstream effectors in vivo. However, the activation of ERK and AKT was similar in both primary melanocytes and human melanoma cultures harboring an NRAS codon 12 or 61 mutation (Fig. 4 and Supplementary Fig. S10). Moreover, even through unbiased probes of the kinome using PhosphoScan technology (Cell Signaling Technology), we were unable to detect consistent differences between NRASG12D and NRASQ61R signaling in primary melanocytes (data not shown). Because of feedback mechanisms within many pathways downstream of RAS, changes in signaling flux may not be readily apparent. Alternatively, enhanced cooperation with secondary oncogenic events during the initial stages of tumor development may be responsible for the selection of NRASQ61R mutants in melanoma. In line with this observation, RAF activation is a common event in both thyroid carcinomas and melanomas, the two major tumor types wherein NRAS codon 61 mutations predominate (1, 2). Mounting evidence supports the idea that the intensity of oncogenic RAS signaling can influence transformation potential (38). In addition, RAF binding in conjunction with unidentified allosteric regulators is speculated to further reduce the intrinsic GTPase activity of HRAS codon 61 mutants (36). These findings, along with our work, suggest that NRAS codon 61 mutants exhibit unique biochemical properties that promote the transformation of RAF-responsive tissues. Parsing the mechanism of this specific dependency will require the structural analyses of multiple NRAS mutants as well as comprehensive functional screens using endogenous RAS expression systems and relevant cell types. However, our data suggest that this work would be extremely valuable, identifying codon-specific tumor vulnerabilities for therapeutic targeting.

METHODS

Murine Alleles and Husbandry

Animal work was conducted in accordance with protocols approved by the Institutional Care and Use Committee for animal research at the University of North Carolina (Chapel Hill, NC) and the Ohio State University (Columbus, OH) as well as with local animal care and use regulations. Murine genetic background and genetic deletions were confirmed by standard genotyping methods.

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State University (Columbus, OH). The Tyt-CRE-ER173, p16i, Lkbhi, LSL-Kras12D, and LSL-Nra12D alleles have been previously described (17–20, 31). All animals in this study were backcrossed more than seven generations to C57BL/6. The Lkbhi allele was initially established on an albino C57BL/6 background and then crossed into the TpLN61R colony. Therefore, some of the mice in this cohort are albino.

Analysis of tumor-free survival was conducted using GraphPad Prism software. To determine statistical significance, the log-rank (Mantel–Cox) test was performed for each experimental pairing.

**Generation of the LSL-Nras61Q Allele**

Standard homologous recombination procedures were used to insert a conditionally excisable transcriptional stop codon and point mutation into the endogenous Nras locus (see Supplementary Fig. S1A). Founding mice were first crossed to C57BL/6 P16i mice (The Jackson Laboratory; #0005703) to remove the neomycin resistance cassette. Southern blotting was performed on embryonic stem (ES) cells and founder DNA using the standard procedures. PCR primers and cycling conditions used for genotyping LSL-Nras61Q were as follows: Q61R GENO2–5′- GCAAGAGGCCGCCGACTTACA-3′ (0.15 µmol/L); Primer 1–5′-AG ACGCGGAGCTTCGACCAG-3′ (0.15 µmol/L); Primer 2–5′-GCTGG ATCGTCAAGGCGCTTTTCC-3′ (0.15 µmol/L); cycling—95°C 15 minutes, 35 × [94°C 30 seconds, 62°C 30 seconds, 72°C 45 seconds], 72°C 5 minutes. The resulting PCR products were 487 (wild-type), 371 (LSL- Nras61Q), and 562 (LSL-Nras61Q + CRE) base pairs in size.

**Primary Melanocyte Culture**

Skin was isolated from newborn pups and placed dermis side down in 0.25% trypsin, 0.1% EDTA for 3 hours at 37°C, and 5% CO2. Using forceps, the epidermal layer was separated from the dermis. Using surgical scissors, epidermal cells were minced in phosphate-buffered saline supplemented with 0.02% EDTA. To further dissociate the cell suspension, each sample was subjected to two rounds of program A on the GentleMACs dissociator (Miltenyi Biotec). The cell suspension, each sample was subjected to two rounds of program A on the GentleMACs dissociator (Miltenyi Biotec). The resulting suspensions were spun down and plated on rat tail collagen-coated dishes in melanocyte growth medium (Ham F10, 10 µg/mL insulin, 0.5 ng/mL bovine serum albumin, 5% fetal bovine serum, 1 µmol/L ethanolamine, 1 µmol/L phosphoethanolamine, 10 mmol/L sodium selenite, 20 nmol/mL TPA, 50 pmol/L cholera toxin, 1x penicillin/streptomycin, 100 nmol/L melanocyte stimulating hormone, and 0.05 mmol/L di-butylcylic AMP). Media was changed every other day.

**Induction of CRE Recombinase**

For neonatal induction, pups were painted dorsally (postnatal days 2–4) with 25 mg/mL 4-OHT dissolved in DMSO. Adult induction of CRE was performed as previously described (30). For cell culture studies in primary melanocytes, 4-OHT was dissolved in ethanol and added to the growth media. Cells were treated for 6 consecutive days replacing the media and 4-OHT every other day. In the immortalized TpLN61R cultures, which were resistant to recombination, an adenosine-recombinase was used (Ad5-MCV-Cre-GFP; Baylor College of Medicine Vector Development Laboratory, Houston, TX). Before proliferation or morphologic assessment, melanocyte cultures were returned to untreated media for 3 days. Allocin recombination in tumors and primary cultures was confirmed by genomic PCR using the genotyping primer sets and conditions described for each allele.

**Scoring of Murine Nevi**

Nevi on a 4-cm2 dorsal area were counted by staff, blinded of the animal’s genotype, every week for 10 weeks. So as not to interfere with melanogenesis, we chose not to depilate or shave these animals during the study. The presence of a single nevus at any time point was scored as a “1.” If no nevus was observed a “0” was entered for that time point. The percentage of time nevus positive was calculated as: (sum of all 10 measurements/10) × 100. Each dot represents 1 animal with the mean indicated by a line. Comparisons between sample pairs were conducted using the Student t test.

**Culture of Murine and Human Melanoma Cell Lines**

The NZM24 and NZM63 cell lines were developed in the laboratory of Dr. Bruce C. Baguley (51) and obtained from the Cell Line Collection maintained by the Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, The University of Auckland (Auckland, New Zealand). The MaMe17 cell line was generated by Drs. G. Finlay, C. Posch, and S. Ortiz (Mount Zion Cancer Research Center, San Francisco, CA; ref. 52). WM3670, WM3629, and WM1366 cells were created in the laboratory of Meenhard Herylin and obtained from the Wistar Institute (Philadelphia, PA; ref. 53). VMM39 cells were the kind gift of C. Slingluff (University of Virginia, Charlottesville, VA; ref. 54). The SK-Me1-119, SK-Mel-103, and SK-Mel-147 cell lines were generously provided by Dr. A. Houghton (Memorial Sloan Kettering, New York, NY; ref. 55). The Mel224 cell line was produced and supplied by J. Hansson (Karolinska Institutet, Solna, Sweden). Tumor-derived murine cell lines from the TpLN61R and TpK61R/61R models were generated, genotyped, and maintained as previously described (23).

**Authentication of Cell Lines**

All cell lines were obtained from their original sources or authorized distributors and maintained as previously described (51–56). Cell lines from the Wistar Institute (WM3670, WM3629, and WM1366) are routinely validated by short-tandem repeat (STR) profiling using the AmpFISTR Identifier PCR Amplification Kit (Life Technologies) and were used for the experiments shown within 3 months of receipt. For primary cell lines where original STR data is not currently available, NRAS mutations were validated by PCR followed by Sanger sequencing.

**Flow Cytometry**

**Cell-cycle analysis of primary melanocytes** After 10 days in culture, 4.4 × 105 primary melanocytes were seeded onto collagen-coated 60-mm dishes. A day later, the medium was changed and cells were allowed to recover overnight. EdU was added to each culture at a concentration of 2.5 µg/mL for a period of 16 hours. Cells were harvested and processed as described in the Click-iT EdU Flow Cytometry Assay Kit (Invitrogen) using saponin for permeabilization.

**Cell-cycle analysis of human melanoma cell lines** Human melanoma cell lines grown to 50% to 70% confluence and were treated with 10 µmol/L EdU for a period of 6 hours. Cells were harvested, processed, stained for EdU incorporation, and analyzed on a FlowSight cytometer (Amnis).

**TRP1 staining** To validate that our cultures contained a pure population of melanocytes and verify the identity of TpLN61R metastases, PEP1 antibody (αTrp1; ref. 21) was added at a 1:100 dilution to saponin-permeabilized cells followed by incubation with an Alexa Fluor 488-conjugated anti-rabbit secondary (Molecular Probes, 1:1,000 dilution). In the analysis of splenic tissues (Fig. 3E), co-staining with CD45 was first used to gate out any CD45+ cells.

**In Vitro Protein Purification**

A vector encoding human NRAS (1-172) was acquired from Addgene (vector #25256, NRAS-A). The G12D and Q61R mutations were each introduced into this vector using standard quick change mutagenesis. All proteins were expressed and purified from Rosetta BL21(DE3) cells (Novagen). Briefly, cells were grown at 37°C in a shaking culture of Terrific Broth media supplemented with kanamycin. Once the cells reached an OD600 between 0.6 and 1.0, the culture...
was chilled in an ice bath (to −20°C) and 0.1 mmol/L IPTG added. The cultures were then shaken for an additional 12 to 15 hours at 18°C. Cells were harvested by centrifugation and pellets stored at −80°C. NRAS proteins were purified on Ni Sepharose 6 columns following the manufacturer’s protocol (GE Life Sciences). To further purify the proteins from contaminants, size-exclusion chromatography was performed (Superdex 75 10/300 GL; GE Life Sciences). Purity exceeding 95% was confirmed by SDS-PAGE analysis.

The minimum catalytic domain of the human protein SOS<sup>mut</sup> was expressed in the pQinkH vector (Addgene) and purified as previously described (57). The E. coli codon-optimized RAS-binding domain of BRAF (amino acids 149–232) with an N-terminal purification tag (MGHHHHHSSGVNLGTFQDS) was synthesized by Genewiz, subcloned into pET28a and expressed in BL21 (DE3) cells. The resulting BRAF-RBD was purified on Ni Sepharose, cleaved overnight with TEV protease, and then subjected to subtractive Ni column purification. The tag-less BRAF-RBD was further purified using size exclusion chromatography and verified to be >95% pure by SDS-PAGE analysis. Purified PI3K<sub>y</sub> (amino acids 144–1102) was kindly provided by Genentech.

**Loading of NRAS with Nucleotide Derivatives**

To observe nucleotide dissociation activity in the GTP-bound state, NRAS was loaded with <sup>mut</sup>GTP<sub>NNP</sub>. NRAS was exchanged out of excess MgCl<sub>2</sub> into a buffer containing 20 mmol/L HEPES, 50 mmol/L NaCl, 125 mmol/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 1 mmol/L MgCl<sub>2</sub> at pH 7.4. The concentration of NRAS was adjusted to 100 µmol/L and a 5-mol/L excess of <sup>mut</sup>GTP<sub>NNP</sub> was added. Alkaline phosphatase conjugated to sepharose beads was added to 1:10 volume and EDTA was added to 1 mmol/L to increase the nucleotide exchange rate. The protein mixture was incubated at 4°C until all unlabeled nucleotide was converted to guanosine. The alkaline phosphatase beads were removed by centrifugation and 10 mmol/L MgCl<sub>2</sub> was added to induce nucleotide binding. The concentration of <sup>mut</sup>GTP<sub>NNP</sub>-bound NRAS was determined by measuring the absorbance of the mant fluorophore [<i>e<sub>530</sub> = 5,700/(mol/L*cm)] for assams requiring unlabeled NRAS in the GTP-bound state, NRAS-GMP<sub>NNPP</sub>PCP was prepared in an identical manner.

For assays observing NRAS nucleotide dissociation activity in the GDP-bound state, the protein was loaded with <sup>mut</sup>GDP<sub>0</sub> as previously described (58). NRAS was exchanged into a buffer containing 20 mmol/L HEPES, 125 mmol/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 100 µmol/L EDTA, and 1 mmol/L MgCl<sub>2</sub> at pH 8.0. A 5-mol/L excess of <sup>mut</sup>GDP<sub>0</sub> was added. Alkaline phosphatase conjugated to sepharose beads was added to 1:10 volume and EDTA was added to 1 mmol/L to induce complete nucleotide exchange. The protein mixture was incubated at 4°C until all unlabeled nucleotide was removed from buffers before use to minimize background fluorescence. NRAS was incubated with GTP and passed through a PD-10 column to remove trace Mg<sup>2+</sup> before analysis. A thermal melt scan from 20°C to 90°C was performed to determine the temperature (T<sub>m</sub>) at which half of the protein is unfolded.

**Determination of Nucleotide Exchange in the Absence and Presence of SOS<sup>cat</sup>**

Nucleotide dissociation rates were measured using a PerkinElmer LS50B fluorimeter as previously reported (58). NRAS was loaded with mant-labeled nucleotide and added to a final concentration of 1 µmol/L in 1 ml of exchange buffer (20 mmol/L HEPES, 50 mmol/L NaCl, 5 mmol/L MgCl<sub>2</sub>, and 100 µmol/L DTPA, pH 7.4). A 1,000-fold excess of unlabeled nucleotide was added to initiate the dissociation reaction, and the rate of nucleotide dissociation was measured by monitoring the change in fluorescence (λ<sub>ex</sub> = 365 nm; λ<sub>em</sub> = 435 nm). For reactions that showed dissociation rates too slow to reach completion within the time frame of the assay, 25 mmol/L EDTA was added to induce complete nucleotide dissociation. For GEF-facilitated nucleotide exchange, the minimum catalytic domain of SOS<sup>mut</sup> was added to a 1:2 RAS-to-SOS<sup>cat</sup> ratio (chosen to limit RAS binding to the allosteric site in SOS<sup>mut</sup>). The fluorescence data were normalized and fit to one-phase exponentials to determine rates.

**Determination of Intrinsic GTP Hydrolysis Activity of NRAS**

To measure GTP hydrolysis rates, the phosphate sensor FLIPPi (Addgene) was used for the reaction, which binds to free phosphate in solution (61). Because of the nature of the assay, all trace phosphate was removed from buffers before use to minimize background fluorescence. NRAS was exchanged into a buffer containing 20 mmol/L HEPES, 20 mmol/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 mmol/L EDTA, and 1 mmol/L inosine, pH 8.0. NRAS was incubated with GTP and passed through a PD-10 column to remove trace Mg<sup>2+</sup> (62). NRAS was diluted to 10 µmol/L and combined with 10 µmol/L FLIPPi, and 2 mmol/L MgCl<sub>2</sub> was added to initiate the hydrolysis reaction. A phosphat standard curve was used to convert the raw fluorescence output to a measurement of [GTP] hydrolyzed.

**Circular Dichroism Spectroscopy**

A Jasco J-815 CD Spectrometer was used for circular dichroism (CD) measurements. NRAS was exchanged into a buffer containing 10 mmol/L KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> at pH 7.45 and diluted to 15 µmol/L MgCl<sub>2</sub> and guanine nucleotide (GDP or GMPPCP) were added to a final concentration of 500 and 80 µmol/L, respectively, immediately before analysis. A thermal scan from 20°C to 90°C was performed to determine the temperature (T<sub>m</sub>) at which half of the protein is unfolded.

**RAS Mutational Analysis in Human Cancers**

Data were downloaded from COSMIC (release v64, March 26, 2013; ref. 1). Cell lines and cultured cells were removed from the analysis to prevent inclusion of events secondary to culture. Remaining
events were limited to observations of somatic missense mutations in codons 12, 13, or 61 of HRAS, NRAS, or KRAS. Multiple subtypes and histologies were then summed to give information for the displayed tumor types.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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