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Resistance mechanisms to genetic suppression of mutant *NRAS* in melanoma

James P. Robinson^{1,*}, Vito W. Rebecca^{2,*}, David A. Kircher³, Mark R. Silvis⁴, Inna Smalley^{5,6}, Geoffrey T. Gibney⁷, Kristin J. Lastwika⁸, Guo Chen¹⁰, Michael A. Davies¹⁰, Douglas Grossman^{3,9,11}, Keiran S.M. Smalley^{5,6}, Sheri L. Holmen^{3,4,9,#}, and Matthew W. VanBrocklin^{3,4,9,#}

¹Hormel Institute, University of Minnesota, Austin, Minnesota, USA

²Department of Medicine and Abramson Cancer Center; University of Pennsylvania School of Medicine, Philadelphia, PA USA

³Department of Oncological Sciences, University of Utah Health Sciences Center, Salt Lake City, Utah, USA

⁴Department of Surgery, University of Utah Health Sciences Center, Salt Lake City, Utah, USA

⁵Tumor Biology, Moffitt Cancer Center, Tampa, Florida, USA

⁶Cutaneous Oncology, Moffitt Cancer Center, Tampa, Florida, USA

⁷Lombardi Comprehensive Cancer Center, MedStar Georgetown University Hospital, Washington DC, USA

⁸Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA

⁹Huntsman Cancer Institute, University of Utah Health Sciences Center, Salt Lake City, Utah, USA

¹⁰Department of Melanoma Medical Oncology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

¹¹Department of Dermatology, University of Utah Health Sciences Center, Salt Lake City, Utah, USA

Abstract

Targeted therapies have revolutionized cancer care but the development of resistance remains a challenge in the clinic. To identify rational targets for combination strategies, we used an established melanoma mouse model and selected for resistant tumors following genetic suppression of *NRAS* expression. Complete tumor regression was observed in all mice but 40% of tumors recurred. Analysis of resistant tumors revealed that the most common mechanism of

[#]Correspondence should be addressed to: Matthew W VanBrocklin, Huntsman Cancer Institute, 2000 Circle of Hope Dr., Salt Lake City, UT 84112; phone: 801.213.4236; fax: 801.585.0900; matthew.vanbrocklin@hci.utah.edu or Sheri L. Holmen, Huntsman Cancer Institute, 2000 Circle of Hope Dr., Salt Lake City, UT 84112; phone: 801.213.4235; fax: 801.585.0900; sheri.holmen@hci.utah.edu.

^{*}These authors contributed equally.

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resistance was overexpression and activation of receptor tyrosine kinases (RTKs). Interestingly, the most commonly overexpressed RTK was Met and inhibition of Met overcame NRAS resistance in this context. Analysis of NRAS mutant human melanoma cells revealed enhanced efficacy of cytotoxicity with combined RTK and MEK inhibition. In this study, we establish the importance of adaptive RTK signaling in the escape of NRAS mutant melanoma from inhibition of RAS and provide the rationale for combined blockade of RAS and RTK signaling in this context.

Keywords

Melanoma; mouse model; NRAS; resistance

Introduction

Metastatic melanoma is well known for its aggressive clinical behavior and resistance to both conventional and targeted therapies. Key mutations in the mitogen-activated protein kinase (MAPK) pathway have been identified in this disease including gain-of-function alterations in *BRAF*, which are present in ~50% of all cutaneous melanomas [1]. Single agent BRAF kinase inhibitors such as vemurafenib and dabrafenib are associated with impressive but mostly short-lived regression of disease in *BRAF*-mutant melanomas [2,3]. More recently, co-targeting BRAF and mitogen activated protein kinase kinase (MEK) enhanced clinical response rates and progression-free survival (PFS) compared to treatment with a BRAF inhibitor alone [4–7], prompting FDA approval of this combination in 2015. The future of targeted therapy for *BRAF*-mutant melanoma lies in the development of novel combination strategies that negate mechanisms of acquired resistance resulting from target-based therapies.

NRAS is mutated in 15–20% of melanomas but efforts to pharmacologically inhibit mutant RAS directly have been unsuccessful to date [8]. RAS signals to several downstream effectors including phosphatidylinositol-3-kinase (PI3K)/AKT, RAL, and the RAF serine/threonine protein kinases, which activate MEK and extracellular signal-regulated kinase (ERK). Highly specific inhibitors of MEK have been developed and investigated clinically for their therapeutic efficacy in treating advanced melanomas, including those harboring *NRAS* mutations [9,10]. In a phase II clinical trial, binimetinib (MEK162) had a response rate of 20% in NRAS mutant melanoma making it the first targeted therapy to demonstrate activity in this molecular subgroup. Despite this response, PFS was brief (median: 3.6 months) [9], highlighting the need for identification of resistance mechanisms in order to direct future therapeutic strategies.

A powerful way to identify rational targets for combination strategies is to use an *in vivo* genetic approach. We recently described the development of a novel mouse model of melanoma based on the RCAS/TVA retroviral vector system, which allows for tissue- and cell-specific targeted infection of mammalian cells through ectopic expression of the viral receptor [11,12]. This system utilizes a viral vector, RCASBP(A) hereafter referred to as RCAS, derived from the avian leukosis virus (ALV). The receptor for RCAS is encoded by the TVA gene, which is normally expressed only in avian cells. Transgenic mice expressing

TVA under the control of the dopachrome tautomerase (DCT) promoter were generated to allow specific targeting of melanocytes [13].

The highest heritable risk for melanoma results from mutations in the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus [14,15], which encodes p16/INK4a and p14/ARF (p19/Arf in mice). This locus is also lost or mutated in ~40% of sporadic melanomas [16,17]. p16 inhibits CDK4/6-mediated phosphorylation of the Retinoblastoma protein allowing for unregulated cell division [18], while loss of p14 enhances ubiquitination and destruction of p53 by HDM2, leading to pervasive genetic instability and a higher risk of melanoma in individuals with germline *CDKN2A* mutations [19,20].

To model human melanoma, we generated *Dct::TVA;Cdkn2a^{lox/lox}* mice and virally delivered *NRAS^{Q61R}* and *Cre*-recombinase postnatally to somatic melanocytes expressing TVA. Tumors developed in two-thirds of the mice following a mean latency of only seven weeks. In this model, tumors evolve from gene mutations in developmentally normal somatic cells in the context of an unaltered microenvironment and therefore closely mimic the human disease [12]. We have further expanded the utility of this model by engineering the viral vectors to permit regulation of gene expression post-delivery using the tetracycline (tet)-regulated system [21–23].

Mouse models that permit regulated expression of oncogenes are especially useful for modeling the effects of targeted therapies because abrogation of oncogene expression mimics pharmacological inhibition of the target [11]. These models often develop resistance and elucidation of the mechanisms of resistance can inform therapeutic strategies. The goal of this study was to identify rational targets for combination therapy in mutant *NRAS*-driven melanoma. To accomplish this goal, we expressed *NRAS^{Q61R}* under the control of a tet-responsive element (TRE) and examined mechanisms of resistance to suppression of *NRAS* expression *in vivo*. Interestingly, the most common mechanism of resistance responsible for the continued growth of these tumors in the absence of mutant *NRAS* was overexpression and activation of receptor tyrosine kinases (RTKs). Analysis of *NRAS* mutant human melanoma cell lines demonstrated that escape from MAPK inhibition can be overcome by suppression of RTK signaling. These data suggest that combined inhibition of RTK and MAPK signaling is a rational combination therapeutic strategy that may prevent the development of resistance or treat recurrent melanoma driven by mutant *NRAS*.

Methods

Mice and genotyping

Dct::TVA;Cdkn2a^{lox/lox} mice and genotyping have been described [12].

Drugs

AZD6244 and XL-184 were purchased from Selleck Chemical. Trametinib was purchased from ChemieTek.

Establishment of melanoma cell lines and culture conditions

Mouse melanoma cell lines were established following dissection of primary tumors by physical disruption in to single cells using scalpels and trypsin. Melanocyte/melanoma cultures were maintained in 254 medium containing human melanocyte growth supplement (Invitrogen), 10% FBS, and 50 µg/ml gentamicin at 37 °C with 5% CO₂. Human melanoma cell lines WM1361A, WM1361AR, WM1346, WM1366, M245, and M318 were maintained in RPMI-1640 with 5% FBS, at 37 °C with 5% CO₂. WM1361AR cells are resistant to the MEK inhibitor AZD6244 and were maintained in the presence of the drug. DF-1 cells were grown in DMEM-high glucose supplemented with 10% FBS, 1 × penicillin/streptomycin and maintained at 39 °C as described [12].

Viral constructs

The retroviral vectors used in this study are replication-competent ALV LTR, splice acceptor, and Bryan polymerase-containing vectors of envelope subgroup A [RCASBP(A)] or no splice acceptor [RCANBP(A)]. RCASBP(A)Tet-off and RCANBP(A) have been described. To generate RCANBP(A)TRE-*NRAS*^{Q61R}-*IRES-Cre*, TRE was isolated from pTRE2Hyg (Clontech) via Nco I and Xho I restriction digestion and cloned into pENTR-3C *NRAS*^{Q61R}-*IRES-Cre* digested with Nco I and Xho I [12]. The subsequent LR Clonase II (Invitrogen) reaction with the Gateway Destination vector RCANBP(A) [24] and pENTR-3C-TRE-*NRAS*^{Q61R}-*IRES-Cre* generated RCANBP(A)TRE-*NRAS*^{Q61R}-*IRES-Cre*. The final vector was sequence verified.

Virus production

Virus infection was initiated by calcium phosphate transfection of plasmid DNA that contained the retroviral vector in proviral form in DF-1 cells as previously described [12].

Met copy number analysis

DNA was extracted from formalin fixed paraffin embedded (FFPE) tissue and cell lines using the QIAamp DNA Mini Kit (QIAGEN) and quantified using a NanoDrop Spectrophotometer (ThermoFisher). Met copy number analysis was performed in replicates of six on the ViiA™ 7 Real-Time PCR System using 20ng of DNA, TaqMan Universal Master Mix (ThermoFisher), Met TaqMan copy number probes (intron 2, mm0734318CN, ThermoFisher) and TfrC TaqMan Copy Number Reference probes (4458368, ThermoFisher). The results were analyzed according to manufacturer's instructions, normalized against internal TfrC control probes and compared to tail genomic DNA and FFPE tissue from wild-type mice.

RNA interference

Production of the pLenti6/Block-iT M1, M2 (targeting Met) and Luc (targeting luciferase) lentiviruses has been described [25]. Mouse melanoma cells were seeded in 6-well plates at a density of 5×10^4 cells/well. After the cells attached, 1 ml of filtered virus-containing medium was added in the presence of 8 µg/ml polybrene (Sigma) for 2h at 37 °C.

In vivo infection

Infected DF-1 cells from a confluent culture in a 10-cm dish were trypsinized, pelleted, and resuspended in 50 μ l PBS, and placed on ice. Newborn mice were injected subcutaneously with 10 μ l of cells as described [12]. All mice were monitored for tumor development daily. Tumor size was evaluated by caliper measurements, and tumor volume was calculated by length $\times$ width $\times$ depth.

Immunohistochemistry (IHC)

Tumor samples were fixed in 10% neutral buffered formalin and paraffin-embedded. Four μ m sections were cut, deparaffinized in Xylene and rehydrated in a graded alcohol series. NRAS^{Q61R}-HA expression was detected using an antibody to the HA epitope (HA.11, Covance) (1:1000). Detection of MAPK activation was performed using a 1:100 dilution of an antibody to phospho-Erk (4370, CST). Met expression was detected using a rabbit polyclonal primary antibody to a peptide mapping within a C-terminal cytoplasmic domain of Met (Sc-162; Santa Cruz) diluted 1:200 in Renaissance diluent for 1 hour at room temperature (RT). Detection of Met phosphorylation was performed overnight at 4°C using a 1:100 dilution of a rabbit polyclonal antibody to phosphorylated (Y1003) Met (ab61024; Abcam) in Renaissance diluent. The sections were washed with TBS-T and then probed with Mach 4 polymer reagent (Biocare) for 30 min at RT. The signals were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Biocare). Detection of Akt activation was performed using a 1:50 dilution in SignalStain® 4060, CST). Visualization was performed using the Rabbit SignalStain® Boost IHC Detection system and the SignalStain® DAB Substrate Kit (CST). Cell proliferation was detected using an antibody diluted 1:50 to Ki67 (M7246, Dako). Apoptosis was detected by TUNEL staining using the *in situ* cell death detection kit (Roche) per the manufacturer's specifications. All sections were counterstained with hematoxylin.

Immunoblotting

Blots were immunostained using the following primary antibodies from CST: MET (8198), P-MET (Y1234/5; 3077) as well as HA (HA.11, Covance), RAS10 (05-516, EMD Millipore, Billerica, MA), and Tubulin (ab21058, Abcam). Detection was performed using anti-mouse or anti-rabbit IgG-HRP secondary antibodies as appropriate (CST). The blots were incubated in ECL solutions per the manufacturer's specifications (Amersham) and exposed to film.

Colony formation assay

Cells were plated in 6-well plates at 2×10^4 per well. Media with vehicle (DMSO) or 3 μ M XL-184 were added the following day and replaced twice a week. After 4 weeks, the plates were stained with crystal violet. Relative colony density was determined by solubilizing the crystal violet dye in 10% acetic acid followed by measurement of absorbance at 450 nm.

Flow Cytometry

Cells were treated with vehicle (DMSO), 1 μ M AZD6244, 3 μ M XL-184, 30 nM trametinib or AZD6244 and XL-184 or trametinib and XL-184 in combination and harvested after 120

hours. Cells were subsequently stained with Annexin-V/TMRM and apoptosis was assessed via an LSR-II flow cytometer. Results were analyzed with Flowjo and results representing the mean $\pm$ S.E.M. from 3 independent experiments are shown.

Three-dimensional spheroid assays

Melanoma spheroids were prepared using the liquid overlay method. Spheroids were either treated for 144 hours with vehicle control DMSO, 1 μ M AZD6244, 3 μ M XL-184 or AZD6244 and XL-184 in combination before being washed and analyzed as previously described.

Reverse phase protein array analysis

Frozen tumor tissue was embedded in optimum cutting temperature (OCT) compound. H&E stained slides were reviewed by an experienced dermatopathologist (AJL) to identify areas that contained >70% tumor cells. Regions with extensive necrosis, fibrosis, or hemorrhage within the tumor specimens were excluded. The H&E slides were used as a guide to macrodissect the OCT blocks and isolate tumor-enriched regions for further analysis. 10–20 μ m tumor shears were generated by cryostat and were used for protein and total RNA extraction. Proteins were isolated from the tumor shears and RPPA was performed as previously described [26].

Illumina gene expression analysis

Microarray expression profiling was performed by the Microarray Core Facility of The University of Texas Health Science Center at Houston, Houston, TX. The RNA was amplified in to cRNA and biotinylated by *in vitro* transcription using the Illumina® TotalPrep RNA Amplification Kit (Ambion) per the manufacturer's instructions. Biotinylated cRNAs were purified, fragmented, and subsequently hybridized to an Illumina Mouse-8 V2 BeadChip (Illumina). All analyses were performed using GenomeStudio software (Illumina). The gene expression data described in this manuscript have been deposited in the NCBI Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) database and are accessible through GEO Series accession number GSE51906.

Statistical Analysis

Censored survival data was analyzed using a log-rank test of the Kaplan-Meier estimate of survival. For colony formation assays, each cell line was assayed according to the *n* indicated and the data is represented as the mean $\pm$ S.E.M. Quantitative analysis of TUNEL and Ki67 positive cells was performed using ImageJ and the data is represented as mean $\pm$ S.E.M. Student's t-test was used to determine significance. Student's t-test was also used to compare protein expression levels between groups in the RPPA analysis. Protein-protein coefficients were determined by the Pearson correlation method, and the significance of the interactions was determined by the t statistic using R software. Unsupervised hierarchical clustering of mean-centered protein expression values was done using Cluster 2.1 and Treeview software.

Study approval

All animal experimentation was performed in AAALAC approved facilities at the University of Utah. All animal use protocols were reviewed and approved prior to experimentation by the Institutional Animal Care and Use Committee at the University of Utah.

Results

Delivery of viruses containing *Tet-off* and TRE-*NRAS*^{Q61R}-*IRES-Cre* results in highly penetrant tumor formation in *Dct::TVA;Cdkn2a*^{lox/lox} mice

To allow for regulation of NRAS expression post-delivery using the tet-regulated system, we utilized the RCANBP(A) vector, hereafter referred to as RCAN, that permits the use of an internal promoter [24]. A TRE promoter was inserted upstream of the *NRAS*^{Q61R}-*IRES-Cre* cassette in the RCAN vector such that both NRAS and Cre expression was driven from the TRE and not the viral long-terminal repeat (LTR). Delivering mutant NRAS and Cre in the same virus and linking their expression with an IRES ensured that all tumors developed in the context of mutant NRAS. We have previously demonstrated that tumors do not form by delivery of either mutant NRAS or Cre viruses alone in this model [12]. Expression from the TRE requires the presence of a tetracycline transcriptional activator (tTA) such as Tet-off. In the context of Tet-off, the Tet-responsive genes are repressed in the presence of doxycycline (Dox). Expression and activity of both NRAS and Cre was confirmed *in vitro* in the context of Tet-off prior to *in vivo* experiments (data not shown).

We have previously shown that expression of *NRAS*^{Q61R}-*IRES-Cre* from these retroviruses efficiently induces melanoma in *Dct::TVA;Cdkn2a*^{lox/lox} mice [12]. Tumors developed at the site of injection in 72% of the *Dct::TVA;Cdkn2a*^{lox/lox} mice infected with both Tet-off and TRE-*NRAS*^{Q61R}-*IRES-Cre* viruses ($n=46$) while no tumors developed in *Dct::TVA*-negative;*Cdkn2a*^{lox/lox} mice. Importantly, *Dct::TVA;Cdkn2a*^{lox/lox} mice infected with viruses containing TRE-*NRAS*^{Q61R}-*IRES-Cre* or Tet-off alone remained tumor free for the duration of the study ($n=20$). The median overall survival of *Dct::TVA;Cdkn2a*^{lox/lox} mice injected subcutaneously with viruses encoding Tet-off and TRE-*NRAS*^{Q61R}-*IRES-Cre* was 42.5 ± 4.3 days (Figure 1). Animals were sacrificed due to primary tumor burden. Cell lines established from the primary melanomas expressed virally delivered mutant NRAS. As expected, administration of Dox to the culture medium resulted in loss of mutant NRAS expression. Importantly, expression of endogenous Ras was not affected by Dox treatment (Figure 1 inset).

Tumors induced by expression of Tet-off and TRE-*NRAS*^{Q61R}-*IRES-Cre* were indistinguishable from tumors induced by delivery of *NRAS*^{Q61R} and Cre where expression was driven from the viral LTR [12]. Macroscopically, the tumors were highly vascular and amelanotic as a result of the albino FVB/n background. Microscopically, high-grade nuclear features were observed along with prominent nucleoli and numerous mitotic figures. The tumors consisted primarily of short spindle cells, though some possessed giant cells with epithelioid features (Figure 2A). A hemagglutinin (HA) epitope tag was engineered on the N-terminus of *NRAS*^{Q61R} to allow for the distinction between virally delivered *NRAS*^{Q61R} and endogenous NRas. Expression and activity of *NRAS*^{Q61R} was verified by

immunohistochemistry (IHC) for HA (Figure 2B) and phosphorylated Erk (P-Erk, Figure 2C).

Suppression of NRAS expression promotes tumor regression and significantly increases survival

To determine whether down-regulation of NRAS expression in Tet-off and TRE-*NRAS*^{Q61R}-*IRES-Cre*-initiated melanomas results in tumor regression, tumors were induced in *Dct::TVA;Cdkn2a^{lox/lox}* mice by co-delivery of viruses containing Tet-off and TRE-*NRAS*^{Q61R}-*IRES-Cre* at birth. When tumors reached 1,000 mm³, mice were randomly assigned to receive standard feed (untreated) or Dox-containing food to suppress *NRAS*^{Q61R} expression. In the Dox-treated cohort, tumors progressed for 2–3 days after initiating treatment followed by rapid tumor regression in all animals (Supplemental Figure S1). To evaluate the kinetics of virally delivered NRAS^{Q61R} expression and activity following Dox treatment, we treated tumor bearing mice with Dox for 24 or 72 hours. Reduced expression and activity of NRAS^{Q61R} was detected by IHC for HA and P-Erk following 24 hours of Dox treatment (Figure 2D–F), and by 72 hours of Dox treatment, NRAS^{Q61R} and P-Erk expression were undetectable by IHC (Figure 2G–I). This correlates with the continued tumor growth observed in the Dox-treated mice 24–48 hours following Dox administration and rapid tumor regression beginning at 72 hours after the start of Dox treatment (Supplemental Figure S1). Since the tumors from all Dox-treated mice regressed, TdT-mediated dUTP nick end labeling (TUNEL) assays were performed to quantitate apoptotic cells. A significant difference in the mean number of TUNEL positive cells was observed between the tumors from mice treated with Dox for 72 hours [123±25.3 per high-power field (HPF)] and untreated animals [45.6±9.98 per high powered field (HPF); $P=0.047$] (Figure 2J–K). A significant difference was also observed between the two groups in the mean number of cells staining positively for the proliferation marker Ki67. While 303±16.3 Ki67 positive cells were detected per HPF in the untreated tumor samples, 150±10.2 Ki67 positive cells were detected per HPF in the samples treated with Dox for 72 hours ($P=0.004$) (Figure 2L–M). These data suggest that tumor regression resulted from increased apoptosis as well as decreased proliferation.

Survival rates were compared between control mice ($n=38$) and mice given Dox ($n=25$) using a log-rank test of the Kaplan-Meier estimate of survival. Administration of Dox, and subsequent loss of NRAS^{Q61R} expression, significantly increased survival. The median survival for control mice was 35.5±2.1 days vs. 137.5±15.1 days ($P=2.5\times10^{-14}$) for Dox-treated mice that developed resistance. Complete responses were observed in 60% (15/25) of the treated mice (Figure 3).

Resistant tumors develop after a long latency in the absence of NRAS^{Q61R} expression

Forty-percent (10/25) of the Dox-treated mice became resistant while on Dox treatment after a median latency of 61.5±11.3 days from the start of treatment (Supplemental Figure S1). To delineate the mechanism(s) of resistance responsible for mediating tumor recurrence, we first evaluated all tumor tissue for expression of virally delivered NRAS^{Q61R}. IHC for NRAS^{Q61R} (HA) revealed that 4/10 tumors re-expressed NRAS^{Q61R} (Supplemental Figure S2). Cell lines were successfully established from two of these tumors and *in vitro*

examination confirmed expression of virally delivered NRAS even in the presence of higher concentrations of Dox (Supplemental Figure S3). Virally delivered NRAS^{Q61R} expression was not observed in the other six resistant tumors. Therefore, these tumors were further evaluated for activation of the canonical MAPK pathway by IHC for P-Erk. In contrast to the four tumors that retained NRAS^{Q61R}-HA expression, MAPK pathway activation was not evident in the remaining six resistant tumors (Supplemental Figure S2).

PI3K/Akt pathway activation is a characteristic of the resistant tumors

To further define the signaling molecules active in the resistant tumors we employed unbiased reverse-phase protein array (RPPA), which allows quantitative analysis of protein expression and activation [27]. Tumor-enriched protein isolates from seven resistant tumor samples and three controls not subjected to NRAS extinction were analyzed by RPPA. The tumor samples were separated into “NRAS on” and “NRAS off” groups based on levels of virally delivered mutant NRAS expression in each tumor. The heat map in Figure 4 shows the results of unsupervised hierarchical clustering of the proteins that showed a significant difference in expression ($n=29$; $P<0.05$ by unpaired t-test) between the NRAS on and NRAS off samples. Importantly, activation of several RTKs was observed using this approach including ErbB1 (EGFR, Y1068), and ErbB2 (HER2, Y1248) as well as increased levels of VEGFR2. Interestingly, higher expression of several phospho-proteins (AKT, PRAS40, GSK3 α/β) in the PI3K/Akt pathway was observed in the NRAS off tumors, suggesting that increased activation of this pathway is a characteristic of the resistant tumors (Figure 4). Phospho-Akt was also confirmed in several resistant tumors by IHC (Supplemental Figure S4).

Genetic suppression of NRAS *in vivo* increases expression of receptor tyrosine kinases

To further characterize the mechanisms of resistance to genetic extinction of NRAS, we performed gene expression microarray analysis on eight tumor samples, including six resistant tumors and two untreated tumors. Analysis of the most highly up-regulated genes in the resistant samples relative to untreated controls revealed significant increases in expression of several RTKs including c-Met, Fgfr1, Egfr, and Igfr1. Increased expression of Met appeared to be a common mechanism in three resistant tumor samples. Furthermore, the levels of Met expression were significantly different in these samples compared to the other tumors analyzed (Supplemental Figure S5). Therefore, we further assessed Met expression in all of the tumor samples by IHC. Increased Met protein was observed in four of the ten resistant tumors (Supplemental Figure 6A–J). Met activity was confirmed in all of these samples by IHC for phosphorylated Met (P-Met; Y1003) and a representative image for sample 3194 is shown in Supplemental Figure 6K. Akt activity was also observed in this sample by IHC for phosphorylated Akt (P-Akt; S473) (Supplemental Figure 6L). To assess if copy number alterations were responsible for the increase in Met expression in these tumors, we used TaqMan® Copy Number Assays. Using this approach, we observed that the tumors with increased Met protein expression, and the cell lines created from them, contained highly significant increases in Met DNA copy number compared to untreated tumors and normal genomic controls (Figure 5A–B). Two of the resistant tumors (2908, 3194) contained over 30 copies of Met DNA. This increase in Met copy number was similar to what we have previously reported in DNA from melanoma xenografts (305, 303) [25].

Inhibition of Met overcomes NRAS resistance generated *in vivo*

To evaluate a role for Met in mediating resistance, Met-directed M1 and M2 lentiviral shRNAs were delivered to the 2908 resistant tumor cell lines *in vitro*. Significantly reduced Met expression and activity was confirmed in these cells by Western blot (Figure 5C). The requirement for Met expression in the escape from NRAS inhibition was demonstrated by the ability of Met knockdown to prevent the outgrowth of clones in a 4-week colony formation assay (Figure 5D). Similarly, it was noted that XL-184, which has anti-Met activity, also significantly prevented the growth of the resistant cell lines, 2908 and 3267, which have increased Met expression and activity. XL-184 less efficiently inhibited cell line 3019, which retained mutant NRAS expression and does not have increased Met activity (Figure 5E).

Escape from MEK inhibition can be overcome through suppression of RTK signaling

Most patients with *NRAS* mutant melanoma that initially respond to MEK inhibition generally relapse [9]. Therefore, we next sought to determine whether co-abrogation of RTK signaling prevented escape from MEK inhibition in human cells. The importance of adaptive RTK signaling in the escape from MEK inhibition was demonstrated by the ability of the combination of either AZD6244 or trametinib with XL-184 to enhance human melanoma cell cytotoxicity in a 3D spheroid assay (Figure 6A), increase levels of apoptosis in an Annexin-V binding flow cytometry assay (Figure 6B), and prevent the outgrowth of clones in a 4-week colony formation assay in human melanoma cell lines (Figure 6C and Supplemental Figure S7). We further validated our findings in the MEK inhibitor resistant, *NRAS* mutated patient-derived cell line WM1361AR. Combination treatment with AZD6244 and XL-184 significantly reduced colony formation in these cells (Figure 6C).

Discussion

Significant progress has recently been made due to the development of targeted therapies for melanoma, particularly for those tumors that harbor activating BRAF^{V600E} mutations [2,4,5,28]. Attention has now turned to other genetic subgroups of melanoma, with clinical trials showing the potential utility of targeting the MEK/ERK signaling pathway in *NRAS* mutant melanoma [8,9,29]. Despite initially encouraging results, the duration of response to MEK inhibitors in this setting is limited, with treatment failure being rapid and resistance being near universal. Thus, it is becoming increasingly clear that intracellular signaling networks are highly dynamic and subject to adaptation, and that this inherent plasticity is critical in the escape from small molecule kinase inhibitors. In melanoma, this phenomenon has been best studied in the context of mutant *BRAF*, where signaling from RTKs provides important parallel signals that allow BRAF inhibition to be circumvented [30,31]. Although this adaptive signaling has been little explored in *NRAS* mutant melanoma, an extensive understanding of these cellular processes will likely prove critical for the development of effective combination therapy strategies [8,29,32].

In the current study we used an *in vivo* mouse model of oncogene escape in which *NRAS* mutant melanomas were allowed to establish, regressed following genetic suppression of *NRAS* expression, and later developed resistance. The generation of oncogene resistance

using a genetic approach in a spontaneous model of *NRAS* mutant melanoma offers a number of major advantages. Most importantly, this model provides a means to precisely determine whether inactivation of *NRAS* is an effective treatment for melanoma, with specific inhibition of oncogenic *NRAS* signaling accomplished *in vivo* and in the context of a normal microenvironment and intact immune system. In cases where responses are not durable, this model enables further study of the mechanisms of resistance that develop *in vivo*. We observed that targeting mutant *NRAS* caused significant tumor regression, demonstrating that continuous *NRAS* signaling is required for tumor maintenance and progression while implicating the inhibition of mutant *NRAS* as a therapeutic target. Despite decades of research, specific pharmacological inhibition of *NRAS* has been unsuccessful to date, and efforts to extinguish oncogenic *NRAS* signaling continue [8]. Genetic suppression of *NRAS*^{Q61R} in our model was not sufficient to prevent tumor recurrence as resistant tumors developed after a long latency in the absence of mutant *NRAS*. Analysis of these *NRAS*-resistant murine melanoma specimens revealed re-activation of mutant *NRAS* in 40% of the samples and increased RTK expression in the remaining 60% of resistant tumors. The most commonly altered RTK was c-Met. We previously reported increased c-Met gene copy number in the context of *Ink4a/Arf* loss is sufficient to drive melanomas *in vivo* [25]. Accordingly, we conducted an analysis of c-Met gene copy number in our resistant tumors and found that the increased c-Met expression corresponded with copy number alterations.

Met is an RTK whose expression is frequently increased in many tumor types, including melanoma [33–35]. Constitutive Met signaling, which often occurs following the establishment of an autocrine signaling loop through its ligand HGF, promotes the growth and metastatic behavior of cancer cells through the activation of multiple signaling pathways including PI3K/AKT, MAPK, and Wnt [36]. For instance, Met functions as a melanoma oncogene in transgenic mice that are wild-type for both *BRaf* and *NRas* by initiating a Met/HGF autocrine loop [37]. In melanoma patient specimens, increased MET expression has been implicated in tumor progression, with receptor expression correlating with melanoma growth and metastasis [38,39]. Recent studies have revealed the concomitant overexpression of transcription factors ETS1, PAX3, and SOX10 that drive up-regulation of Met in melanoma cell lines and primary tumors [35,40]. We report herein that increased Met expression mediates the generation of *NRAS* resistant melanoma *in vivo*, while inhibition of Met overcomes this resistance, indicating that combination strategies that suppress RTK signaling with MAPK pathway inhibition may be therapeutically beneficial.

There is already evidence from epithelial cancers that upregulated RTK signaling overcomes MAPK pathway inhibition. In colorectal carcinoma, escape from knockdown of *KRAS* is associated with increased AKT signaling mediated through IGF1R [41]. In this context, suppression of *KRAS* expression decreases the throughput of the MAPK signaling pathway leading to the relief of feedback inhibition mediated through the adaptor protein IRS-1 [41]. Similar signaling adaptations have also been observed in triple-negative breast cancer, where inhibition of MEK leads to therapeutic escape associated with the increased expression and activity of a number of RTKs including HER2, RON, AXL, VEGF, and PDGFR- β [42]. In a similar manner, rewiring of the kinome has also been observed in melanoma cells treated with BRAF inhibitors, and it has been suggested that increased RTK signaling arising from both tumor intrinsic growth factor autocrine loops and host-derived factors is a key survival

adaptation [30,43,44]. Of these, increased ERBB3 signaling, secondary to an enhanced expression of the transcription factor, FOXD3, has been implicated in short-term tumor-intrinsic adaptive resistance [43]. HGF released from host fibroblasts has also been shown to confer resistance to the BRAF inhibitor PLX4720, with levels of stromal HGF being found to correlate with innate resistance to BRAF inhibitor therapy [44]. More recently, HGF was shown to rescue both mutant NRAS and BRAF melanoma cell lines from the growth inhibitory effects of MAPK pathway inhibition [45].

Additional studies have also implicated growth factors such as EGF, IGF1R and PDGF in acquired BRAF inhibitor resistance [46–48]. Met activation in melanoma cell lines containing mutant BRAF has been demonstrated to confer primary resistance to PLX4032 as well [46]. Most recently, comprehensive transcriptome analysis of serial tumor biopsies from patients before and after mutant BRAF inhibitor therapies, including BRAF alone or combination with MEK inhibition, revealed an increase in *c-MET* transcripts in resistant tumors [49]. This increased expression mediated MAPK inhibitor resistance. Furthermore, *c-MET* mRNA up-regulation denoted poor survival prognosis [49].

Melanoma cell lines and tumor specimens express a large number of cell surface growth factor receptors, all with the potential to mediate escape signaling. The exact complement of RTKs responsible for driving innate or acquired resistance to small molecule kinase inhibitors in melanoma remains to be determined, and targeting these modalities remains a significant clinical challenge. Amuvatinib (MP-470), which inhibits MET, KIT, PDGFRA, and AXL, was shown to attenuate NRAS mutant melanoma cell growth and induce apoptosis *in vitro* [50]; however, MP-470 has not been assessed clinically. The small molecule MET, RET, KIT, FLT-1, FLT-3, FLT-4, TIE2, AXL, PDGFR- β kinase inhibitor XL-184 (cabozantinib) was assessed in a phase II randomized trial and demonstrated tumor regression in 55% of melanoma patients (5% objective response rate), with no correlation noted between response and *BRAF* status [51]. We demonstrate that the addition of XL-184 to NRAS mutant human melanoma cells increased the cytotoxicity of the MEK inhibitors, AZD6244 and trametinib, as measured by 3D spheroid assays, apoptosis assays, and long-term colony formation assays. The observation that RTK inhibitors prevent escape from MEK inhibition in *NRAS* mutant melanoma is supported by preclinical studies of breast cancer, *BRAF*-mutant colorectal carcinoma, and *KRAS* mutant colon cancer showing that RTK inhibitors (including pan-RTK, EGFR/HER kinase and IGF1R) enhance the anti-tumor activity of RAS/MEK/ERK signaling ablation [41–43].

In this study we show for the first time the importance of adaptive RTK signaling in the escape of NRAS mutant melanoma from the inhibition of signaling through RAS *in vivo*. We propose that adaptive RTK signaling and copy number variations provide a selective survival advantage; resistant cells are then initially reliant on localized stromal signaling before undergoing clonal evolution for increased RTK copy number or further mutation leading to independence from stromal signaling and recurrence. Our results provide additional rationale for the co-targeting of both RAS/MEK and RTK signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Delivery of viruses containing Tet-off and TRE-NRAS^{Q61R}IRES-Cre results in highly penetrant tumor formation in DCT-TVA;*Ink4a/Arf*^{lox/lox} mice

Newborn DCT-TVA;*Ink4a/Arf*^{lox/lox} mice were injected with viruses containing Tet-off and/or TRE-NRAS^{Q61R}-IRES-Cre. No tumors formed in DCT-TVA positive *Ink4a/Arf*^{lox/lox} mice injected with Tet-off or TRE-NRAS^{Q61R}-IRES-Cre viruses alone ($n = 20$, dashed line). However, tumors developed in 72% of mice injected with both Tet-off and TRE-NRAS^{Q61R}-IRES-Cre viruses ($n = 46$). Censored overall survival data was analyzed using a log-rank test of the Kaplan-Meier estimate of survival, $P = 8.3 \times 10^{-7}$. *Inset*: Tumor cells were derived from a mouse injected with viruses containing Tet-off and TRE-NRAS^{Q61R}-IRES-Cre. The tumor cells were grown in the absence (–) or presence (+) of 4 ug/ml Dox for 1 week. Forty-eight hours prior to lysis the cells were maintained in media containing 10% serum (10%) or incubated in serum-free medium (SFM). Lysates were separated on a 16% Tris-glycine polyacrylamide gel, transferred to nitrocellulose, and probed with an antibody directed against RAS. The membrane was re-probed with an antibody against α -tubulin to ensure equal loading. The RAS blot shows endogenous Ras (lower band) and the virally delivered NRAS^{Q61R} (upper band). The virally delivered NRAS^{Q61R} is a higher molecular weight as a result of the HA epitope tag.

Figure 2. Time course of doxycycline administration *in vivo* demonstrates significant loss of NRAS^{Q61R} expression by 72 hours

Mice were injected with viruses containing Tet-off and TRE-NRAS^{Q61R}-IRES-Cre at birth.

When tumors reached 1,000 mm³, tumor-bearing mice were given standard feed or administered food containing Dox to suppress NRAS^{Q61R} and Cre expression for 24 or 72 hours. (A–C) Tumor sections from a control untreated mouse. (D–F) Tumor sections from a mouse treated with Dox for 24 hours. (G–I) Tumor sections from a mouse treated with Dox for 72 hours. (A, D, and G) H&E stained tumor sections; (B, E, and H) IHC for the HA epitope tag on virally delivered NRAS; (C, F, and I) IHC for phosphorylated Erk (P-Erk). Tumor sections from control untreated and 72 hour Dox-treated mice were subjected to TUNEL staining as a measure of apoptosis (J–K) and Ki67 staining as a measure of

proliferation (L–M). All IHC sections were counterstained with hematoxylin. Scale bars represent 100 μm .

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Figure 3. Inhibition of NRAS^{Q61R} expression significantly increases survival
DCT-TVA;*Ink4a/Arf*^{lox/lox} mice were injected at birth with viruses containing Tet-off and TRE-NRAS^{Q61R}-IRES-Cre. When tumors reached 1,000 mm³, one cohort of tumor-bearing mice was treated with Dox continuously via the food to suppress NRAS^{Q61R} and Cre expression ($n = 25$, dashed line) while a separate cohort of tumor-bearing mice received standard feed ($n = 38$, solid line). Censored survival data was analyzed using a log-rank test of the Kaplan-Meier estimate of survival; $P = 2.5 \times 10^{-14}$.

Figure 4. RPPA analysis of protein from control and resistant melanomas

Protein was isolated from both untreated control tumors (noted by *) and NRAS resistant tumors; expression was assessed by RPPA. Log2 expression data were subjected to unsupervised clustering and the results are presented as a heat map. Tumors were labeled NRAS on (purple squares) or NRAS off (gray squares) based on expression of virally delivered mutant NRAS in the tumor samples. The heat map shown represents the proteins with $P < 0.05$ in t-tests between the NRAS on ($n = 7$) and NRAS off ($n = 6$) tumors. Green is decreased levels and red is increased levels.

Figure 5. Increased Met expression resulting from Met copy number changes drives resistance to NRAS inhibition

(A) Relative Met DNA copy number in tumor tissue. Gene copy number analysis was performed on fourteen FFPE NRAS melanoma samples and two from mouse melanomas (303, 305) known to be driven by extensive Met copy number amplifications. A highly significant increase in Met copy was observed in 2908, 3267, 3270, 3194, 303, and 305. (B) Met amplification was also observed in cell lines established from 2908, 3267, 303, and 305. (C) Cells from resistant tumor 2908 were established in culture and Met expression was reduced by shRNA targeting Met (M1 and M2). Luc represents cells infected with the lentiviral vector containing a shRNA sequence targeting luciferase (control). The blots were incubated with antibodies to Met and pMet (Y1234/5) as indicated to the right of each panel.

The membrane was re-probed with an antibody against α -tubulin to ensure equal loading. A significant reduction in Met expression and phosphorylation was observed following delivery of M1 and M2 shRNAs. (D) The importance of Met signaling in the escape from NRAS inhibition was demonstrated by the ability of the combination of Met shRNA and Dox to prevent the outgrowth of clones in a 4 week colony formation assay. Colony formation of untreated control 2908 cells or 2908 cells with expression of either an shRNA targeting luciferase or Met was assessed. The data from 2908 cells expressing M1 and M2 shRNAs were combined as biological replicates. Colonies were visualized following staining with crystal violet and relative colony density was determined by solubilizing the crystal violet dye in 10% acetic acid followed by measurement of absorbance at 450 nm. Relative clonogenic survival (%) as compared to the control is shown. (E) The pan-RTK inhibitor XL-184 also prevented the colony growth of resistant cell lines 2908 and 3267, but less efficiently inhibited 3019 cells, which do not overexpress Met. Colony formation was assessed as in D.

Figure 6. Escape from MEK inhibition is overcome through pan-RTK inhibition

(A) Co-treatment with AZD6244 and XL-184 reduces the survival of collagen-implanted spheroids compared to either inhibitor alone. Pre-formed WM1361A, WM1366 and M318 spheroids were embedded in collagen and treated with vehicle (control), AZD6244 (AZD), XL-184 (XL), or AZD6244 + XL-184 (AZD +XL) for 120 hours before being stained for cell viability (Green = live cells, Red = dead). (B) XL-184 enhances the level of AZD6244 and trametinib-induced apoptosis in *NRAS* mutant melanoma cell lines. Cells were treated with DMSO (Control), 1 μ M AZD6244 (AZD), 3 μ M XL-184 (XL), 30nM trametinib (TRA) or AZD6244 and XL-184 or trametinib and XL-184 in combination and harvested after 120 hours. Cells were subsequently stained with Annexin-V/TMRM and apoptosis was assessed via an LSR-II flow cytometer. Results were analyzed with flowjo and results represent the mean $\pm$ S.E.M. from 3 independent experiments. (C) Co-treatment with AZD6244 (AZD) and XL-184 (Combo) reduces colony formation compared to either inhibitor alone. Cultures were treated with either vehicle, AZD2644 (1 μ M), XL-184 (3 μ M)

or the two drugs in combination for 4 weeks. Colonies were visualized following staining with crystal violet. Relative colony density was determined by solubilizing the crystal violet dye in 10% acetic acid followed by measurement of absorbance at 450 nm. Relative clonogenic survival (%) as compared to the control is shown. ** $P = 0.01$, *** $P = 0.001$, **** $P = 0.0001$.