A Genome-Scale RNA Interference Screen Implicates NF1 Loss in Resistance to RAF Inhibition

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ABSTRACT

RAF inhibitors such as vemurafenib and dabrafenib block BRAF-mediated cell proliferation and achieve meaningful clinical benefit in the vast majority of patients with BRAF<sup>V600E</sup>-mutant melanoma. However, some patients do not respond to this regimen, and nearly all progress to therapeutic resistance. We used a pooled RNA interference screen targeting more than 16,500 genes to discover loss-of-function events that could drive resistance to RAF inhibition. The highest ranking gene was NF1, which encodes neurofibromin, a tumor suppressor that inhibits RAS activity. NF1 loss mediates resistance to RAF and mitogen-activated protein kinase (MAPK) kinase kinase (MEK) inhibitors through sustained MAPK pathway activation. However, cells lacking NF1 retained sensitivity to the irreversible RAF inhibitor AZ628 and an ERK inhibitor. NF1 mutations were observed in BRAF–mutant tumor cells that are intrinsically resistant to RAF inhibition and in melanoma tumors obtained from patients exhibiting resistance to vemurafenib, thus showing the clinical potential for NF1-driven resistance to RAF/MEK-targeted therapies.

SIGNIFICANCE: This work identifies functional loss of NF1 as a mediator of resistance to RAF inhibitors in BRAF<sup>V600E</sup>-mutant cancers. Furthermore, we nominate new therapeutic modalities to treat this mechanism of resistance. Cancer Discov; 3(3); 350–62. ©2012 AACR.

See related commentary by Gibney and Smalley, p. 260.

INTRODUCTION

Cancer therapy has arguably entered a transformation fueled by the success of targeted agents such as kinase inhibitors deployed against tumors harboring “druggable” oncogene mutations. Unfortunately, resistance to these therapies remains a formidable challenge (1). In some cases of therapeutic resistance, cancer patients fail to respond to treatment at the outset (termed de novo or innate resistance). Alternatively, resistance may emerge after several weeks or months of clinical response (termed acquired resistance).

The identification of BRAF mutations as key driver events in malignant melanoma spurred the development of small-molecule inhibitors of the mitogen-activated protein kinase (MAPK) pathway in an effort to block dysregulated signal transduction engendered by the mutant BRAF oncoprotein. As a result, RAF inhibitors such as vemurafenib or dabrafenib, or mitogen-activated protein kinase (MAPK) kinase kinase (MEK) inhibitors such as trametinib, elicited striking clinical response rates when administered as single agents in patients with BRAF<sup>V600E</sup>-mutant melanomas (2–4). The use of RAF and MEK inhibitors in combination further extends the magnitude and duration of clinical benefit (5). However, intrinsic or acquired resistance to these regimens remains a major clinical problem. Systematic characterization of resistance to these agents is therefore needed to further the development of combined therapeutic strategies that either complement existing therapies or provide alternative treatment avenues.

Several mechanisms of resistance to vemurafenib have been described, most of which involve reactivation of downstream MEK/extracellular signal-regulated kinase (ERK) signaling. Interestingly, secondary mutations involving the BRAF gatekeeper residue (a threonine at codon 529), common in drug-resistant chronic myelogenous leukemia (CML) and epidermal growth factor receptor (EGFR)-mutant lung cancers, have not been observed, although preclinical data may support such a mechanism (6–9). A number of laboratories have generated resistant cell line subclones by chronic exposure to RAF inhibitors in vitro, which may facilitate identification of resistance mechanisms by functional or genomic characterization of these cells. Such efforts have implicated amplification or mutation of RAS isoforms (10), enhanced CRAF expression (11), activation of receptor tyrosine kinases (12), and a splice variant of BRAF that constitutively dimerizes in the presence of inhibitor, producing sustained MEK/ERK signaling (13). In addition, systematic gain-of-function screens identified COT (MAP3K8) as a resistance effector (14). Moreover, secretion of hepatocyte growth factor (HGF) by stromal cells may activate MET to promote resistance to BRAF inhibition (15, 16). In aggregate, these studies provided a rationale for the combined use of RAF and MEK inhibitors and, potentially, additional therapeutic avenues that may overcome specific resistance mechanisms.

Genome-scale RNA interference (RNAi) screens may offer a systematic and unbiased genetic approach to study tumor biology and therapeutic resistance. To elucidate potential loss-of-function mechanisms of resistance to BRAF or MEK inhibition, we conducted a pooled, lentiviral short hairpin RNA (shRNA) screen in drug-sensitive BRAF–mutant melanoma cells exposed to high RAF inhibitor concentrations. Characterization of surviving cells identified inactivation of NF1 as a novel mechanism of resistance to both BRAF and MEK inhibition.
RESULTS

To identify genes capable of suppressing resistance to BRAF inhibition, we used a library of 90,000 shRNAs targeting approximately 16,600 genes expressed in A375 cells, which harbor the BRAFV600E mutation and are sensitive to small-molecule RAF and MEK inhibitors (17). Following infection with the lentiviral shRNA library, these cells were cultured in the presence of either dimethyl sulfoxide (DMSO) or 3 μmol/L PLX4720 for up to 14 population doublings. shRNA sequences were amplified by PCR, and the relative abundance of each shRNA was determined by illumina sequencing. PD, population doubling.

B, growth of A375 cells infected with approximately 90,000 shRNAs and cultured in the presence of either DMSO or 3 μmol/L PLX4720 for up to 14 population doublings. C, the number of reads per shRNA was normalized and log 2 transformed, and shRNA data for 2 DMSO controls and 6 PLX4720-treated replicas were analyzed using a "second-best shRNA" 2-class comparison of log-fold change (LFC) in RIGER to generate a ranked list of genes that were enriched in the PLX4720-treated cells. The screening hits are visualized by plotting the function y = 1/normalized enrichment score (NES). The top 5-ranking candidate genes are indicated. D, heat map of shRNA representation across early time point (ETP), DMSO-, and PLX4720-treated replicas for the screen. shRNAs 39714 and 39717 were enriched across all drug-treated replicates. E, validation of shRNA-mediated knockdown of NF1 in A375 cells. A375 cells were infected with shRNAs against luciferase or NF1; after selection in puromycin, cell lysates were made and proteins were determined by Western blotting. F, A375 cells infected with either shLuc or shNF1 were cultured in the presence or absence of 3 μmol/L PLX4720 for 2 weeks. Cells were fixed, then stained with crystal violet and photographed.

The presence of either dimethyl sulfoxide (DMSO) or 3 μmol/L PLX4720 for up to 14 population doublings. The relative abundance of each shRNA was determined by Western blotting. Knockdown of PLX4720-treated replicas for the screen. shRNAs 39714 and 39717 were enriched across all drug-treated replicates. A ranked gene list was generated on the basis of the degree of enrichment of shRNAs targeting a given gene in the PLX4720-treated arm compared with the DMSO-treated arm. Using RNAi gene enrichment (RIGER) analysis (18), a ranked gene list was generated on the basis of the degree of enrichment of shRNAs targeting a given gene in the PLX4720-treated arm (Fig. 1C). Only 31 genes had 2 or more shRNAs ranked in the top 1,000 enriched subgroup (a criterion for candidacy as a “hit” from this screen; Supplementary Table S1). The top-ranking gene was NF1, which encodes the RAS-GTPase-activating protein (RAS-GAP) neurofibromin (19). In particular, enrichment of 2 shRNAs targeting NF1 was clearly observed across 6 PLX4720-treated replicates (Fig. 1D). These data raised the possibility that suppression of
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NF1 activity might permit proliferation of BRAF-mutant melanoma cells in the presence of the RAF inhibitor.

To validate this observation, A375 cells were reinfected with 2 distinct shRNAs targeting NF1. Reduced expression of NF1 protein was then confirmed by Western blotting (Fig. 1E), and cell proliferation was assessed in the presence or absence of 3 μmol/L PLX4720 for 2 weeks. Both NF1 shRNAs permitted robust cell proliferation (as measured by a colony formation assay in vitro) in the presence of PLX4720 (Fig. 1F). These results suggested that loss of NF1 in A375 cells might reduce cellular dependency on oncogenic BRAF for proliferation.

To determine whether suppression of NF1 could provide a generalizable loss-of-function mechanism of resistance to RAF inhibition, the BRAF-mutant melanoma cell lines SKMEL28 and UACC62 were treated with PLX4720 alongside A375 cells following infection with the shNF1 constructs. In each case, sustained cell proliferation in the presence of the RAF inhibitor was observed following NF1 silencing, compared with cells expressing a control shRNA (Fig. 2A).

Indeed, suppression of NF1 conferred a 5- to 31-fold shift in the concentration required to inhibit cell proliferation by 50% (GI50) for PLX4720 (e.g., up to 2.2–3.5 μmol/L compared with 0.112 μmol/L in control A375 cells). By comparison, ectopic expression of oncogenic KRASG12V, a positive control for resistance to PLX4720 (and which is functionally equivalent to oncogenic forms of NRAS; ref. 14), produced a GI50 of 26.3 μmol/L.
In parallel, cells were treated with 1 μmol/L PLX4720 for 16 hours, and cell lysates were analyzed by Western blotting for ERK phosphorylation (Fig. 2B). Loss of NF1 enabled sustained ERK phosphorylation in the presence of PLX4720 compared with mock-treated or shLuc-treated cells, although to a lesser extent than the KRAS G12V control (Fig. 2B). Thus, NF1 silencing was sufficient to confer resistance to RAF inhibition in multiple BRAFV600E melanoma cell contexts.

Recent whole-exome sequencing studies of melanoma tumors suggest that mutant NF1 might provide a “driver” genetic event that dysregulates MAPK signaling in some melanoma cells that lack BRAF and NRAS mutations. BRAF/NRAS wild-type melanoma cells are typically unresponsive to RAF inhibition (20–22). We therefore sought to test the ability of NF1 silencing to compensate for mutated BRAF and to modulate sensitivity to RAF inhibitors in an immortalized melanocyte model system (23). Knockdown of NF1 in primary human melanocytes expressing oncogenic BRAF caused a 10-fold shift in the PLX4720 GI50, permitted robust proliferation in the presence of 3 μmol/L PLX4720, and allowed sustained ERK phosphorylation in the presence of 0.2 and 1 μmol/L PLX4720 (Supplementary Fig. S1A–S1C). These experiments provide independent evidence that silencing of NF1 could confer resistance to RAF inhibition in a MAPK pathway-dependent manner.

Given that NF1 is a known negative regulator of RAS activity (24), we queried the activation state of RAS in A375 cells following NF1 knockdown using a RAS-GTP pull-down assay. As expected, NF1 suppression caused a substantial increase in the level of active GTP-bound RAS (Fig. 3A). Associated with the increased RAS-GTP, we observed a concomitant increase in CRAF activation, as measured by phosphorylation.

In Fig. 3B, combinatorial knockdown of NF1 and CRAF abrogates NF1-mediated resistance to BRAF inhibition at the level of ERK phosphorylation (pERK). A375 cells were infected with NF1 shRNA and treated with either DMSO or PLX4720 for 16 hours. Cell lysates were analyzed for the indicated proteins. C, combinatorial knockdown of NF1 and CRAF abrogates NF1-mediated resistance to RAF inhibition. Quantitative analysis of the Western blots from Fig. 3B for pERK normalized to ERK2 (red) and for cyclin D1 normalized to vinculin (green). Data from 3 independent experiments is presented. D, combinatorial knockdown of NF1 and CRAF abrogated NF1-mediated resistance to RAF inhibition. shRNA-infected cells were treated with a 10-point concentration response of the inhibitors for 4 days, and cell proliferation was determined using CellTiter-glo.

Figure 3. Activation of RAS and CRAF drives resistance to PLX4720. A, A375 cells were depleted of NF1 using shRNA, and RAS-GTP levels in A375 cells were determined by a RAS-GTP affinity pull-down, followed by Western blotting for the indicated proteins. B, combinatorial knockdown of NF1 and CRAF abrogates NF1-mediated resistance to BRAF inhibition at the level of ERK phosphorylation (pERK). A375 cells were infected with NF1 shRNA and treated with either DMSO or PLX4720 for 16 hours. Cell lysates were analyzed for the indicated proteins. C, combinatorial knockdown of NF1 and CRAF abrogates NF1-mediated resistance to RAF inhibition. Quantitative analysis of the Western blots from Fig. 3B for pERK normalized to ERK2 (red) and for cyclin D1 normalized to vinculin (green). Data from 3 independent experiments is presented. D, combinatorial knockdown of NF1 and CRAF abrogated NF1-mediated resistance to RAF inhibition. shRNA-infected cells were treated with a 10-point concentration response of the inhibitors for 4 days, and cell proliferation was determined using CellTiter-glo.
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**Figure 4.** Both MEK inhibition and combined RAF/MEK inhibition do not completely reverse NF1-mediated resistance. A, A375 cells infected with shRNAs targeting NF1 were treated with a 10-point concentration response of AZD6244 for 4 days and cell proliferation determined using CellTiter-glo. Cell proliferation was determined by CellTiter-glo. B, combined BRAF/MEK inhibition did not fully block NF1-mediated resistance at the level of ERK phosphorylation. A375 cells were infected with shRNAs/ORFs as above and then treated with DMSO (D), 1 μmol/L PLX4720 (P), 0.2 μmol/L AZD6244 (A), or a combination of PLX4720 and AZD6244 (C) for 16 hours. Cell lysates were analyzed by Western blotting for the indicated proteins.

Having established that the loss of NF1 could promote resistance to RAF inhibition, we sought to determine whether NF1 silencing could also affect sensitivity to pharmacologic MEK inhibition. In particular, we hypothesized that inhibition of MEK, a substrate of both BRAF and CRAF, might have equal potency regardless of NF1 expression (given that MEK inhibition of MEK is a limiting MAPK effector downstream of CRAF). Interestingly, cells exhibiting NF1 knockdown were partially resistant to AZD6244 (Fig. 4A). Here, NF1 silencing increased the GI50 to AZD6244 by approximately 7-fold. ERK phosphorylation was also sustained in the presence of AZD6244 (Fig. 4B). Combined inhibition of BRAF and MEK by simultaneous treatment with PLX4720 and AZD6244 achieved greater efficacy than either agent alone; however, NF1 knockdown was still associated with residual resistance to combined RAF/MEK inhibition in vitro (Fig. 4C). Moreover, a clear correlation was evident between residual ERK phosphorylation and inhibition of cell proliferation (Fig. 4D). Thus, a resistance phenotype remained apparent in the setting of NF1 knockdown even under the conditions of combined RAF/MEK inhibitor exposure.

In the presence of activated RAS, PLX4720 can activate CRAF through loss of feedback inhibition and induction of RAF dimerization (25–27). Conceivably, then, the RAS activation engendered by NF1 loss might promote a biochemical state wherein the activated RAF/MEK complex is less vulnerable to

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MEK inhibition, particularly under conditions of concomitant RAF inhibitor exposure. To test this hypothesis, we used the irreversible RAF inhibitor AZ628. This compound prevents CRAF activation due to persistent occupation of the ATP-binding site (25). In contrast with the aforementioned drug conditions, only minimal resistance (2-fold) was observed to AZ628 (Fig. 5A). Moreover, ERK phosphorylation was effectively suppressed (between 80%–90%) by this irreversible inhibitor, even in the setting of NF1 knockdown, but only by 65% in the presence of oncogenic KRAS (Fig. 5B). Furthermore, combined NF1/CRAF knockdown resensitized cells to treatment with the MEK inhibitor (Supplementary Fig. S3A and S3B). These results provide additional support for the notion that CRAF signaling is required for the resistance phenotype induced by NF1 loss.

We also tested the effect of pharmacologic ERK inhibition in this setting using the compound VTX-11e (29). Interestingly, BRAFV600E melanoma cells harboring NF1 knockdown remained sensitive to ERK inhibition (Fig. 5C). Consistent with this observation, phosphorylation of the ERK substrate FRA1 (FOS-like antigen 1) was inhibited equally in the presence or absence of NF1 knockdown (Fig. 5D). This pattern of activity was confirmed in colony formation assays, whereby robust resistance was observed to the selective RAF inhibitors PLX4720, PLX4032, GDC0879, and the MEK inhibitor AZD6244. However, NF1 knockdown produced only modest resistance to the irreversible RAF inhibitor AZ628 and caused no resistance to the ERK inhibitor VTX-11e (Supplementary Fig. S3A and S3B).

To understand the potential for endogenous NF1 inactivation to mediate resistance to RAF inhibition, we leveraged the Cancer Cell Line Encyclopedia (CCLE; 30) to identify cell line models that contained putative inactivating NF1 mutations in the context of oncogenic BRAFV600E mutation. Toward this end, over 500 CCLE lines have previously been profiled for pharmacologic sensitivity to RAF and MEK inhibition (30). We identified 5 BRAFV600E cell lines (3 melanomas and 2 colorectal tumors) with co-occurring mutations in NF1 (Supplementary Fig. S4). Of these, 3 contained obvious “damaging” NF1 mutations (HS695T: BRAFV600E, LOXIMVI: NF1 R2805Q and RKO: NF1 L626F, Q2340fs, V2205A), whereas 2 contained point mutations (WM88: BRAFV600E, LS411N: NF1 T2805Q), deemed unlikely to affect NF1 function according to the Mutation Assessor algorithm (data not shown; ref. 31). HS695T and RKO cells also exhibited reduced NF1 mRNA expression. First, we assessed NF1 protein expression by Western blotting, and those cell lines with damaging mutations in NF1 expressed significantly less protein than the wild-type cells (Fig. 6A). The sensitivity of HS695T, LOXIMVI, and RKO cells to MAPK pathway inhibition by PLX4720 was assessed alongside A375, SKMEL28, and UACC62 BRAFV600E/NF1WT melanoma cells. BRAFV600E cell lines that expressed no or undetectable levels of NF1 protein (LOXIMVI and RKO) were
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**Figure 6.** Loss of NF1 is observed in some BRAF-mutant human melanoma and colorectal cell lines. A, melanoma and colorectal cancer cell lines from the Broad Cancer Cell Line Encyclopedia were assessed for expression of BRAFV600E and damaging mutations in NF1. Three such lines were identified (HS695T, LOXIMVI, and RKO, in red) and analyzed alongside a panel of BRAFV600E/NF1WT cells (blue) for expression of NF1 protein by quantitative Western blotting. A 2-tailed t test was conducted to determine whether the expression of NF1 protein was significantly different between cells expressing either wild-type (WT) or mutant (MT) protein. B–D, Cells with wild-type NF1 are shown as blue, and those with mutant NF1 are shown as red. B, reduced expression of NF1 protein is associated with resistance to PLX4720. NF1 wild-type and mutant cell lines were treated with a 10-point titration of PLX4720 for 16 hours, and ERK phosphorylation was assessed using an In-cell Western assay. C, reduced expression of NF1 protein is associated with resistance to PLX4720. NF1 wild-type and mutant cell lines were treated with a 10-point titration of PLX4720 for 4 days, and cell proliferation was assessed using CellTiter-glo. D, human BRAF- and NF1-mutant melanoma and colorectal cancer cell lines display only modest resistance to inhibition of ERK. Cells were exposed to a 10-point titration of the ERK inhibitor VTX-11e for 4 days and cell proliferation was assessed using CellTiter-glo.

highly resistant to the inhibition of ERK phosphorylation by PLX4720, whereas HS695T cells that expressed low levels of NF1 protein were still relatively sensitive (Fig. 6B). In accord with this finding, NF1 mutation was correlated with resistance to PLX4720 in these cells (Fig. 6C). The mean GI\(_50\) was 0.247 μmol/L in NF1 wild-type cells, which increased more than 50-fold in the NF1-mutant cell lines to 15.8 μmol/L.

Given that NF1 knockdown did not confer resistance to pharmacologic ERK inhibition in A375 cells (as described above), we tested the hypothesis that BRAF\(^{V600E}\)/NF1-mutant cancer cell lines might remain sensitive to VTX-11e. Notably, the ERK inhibitor showed activity against NF1-mutant cell lines with a mean GI\(_{50}\) of 0.781 μmol/L compared with 0.219 μmol/L in BRAF-mutant cells with wild-type NF1 (Fig. 6D). These results suggest that pharmacologic ERK inhibition might in principle provide a viable therapeutic strategy in BRAF\(^{V600E}\) cancer cells harboring NF1 loss.

To confirm that NF1 alterations may confer resistance to BRAF inhibition in human melanoma, we assessed the status of NF1 using whole-exome sequencing of tumors from patients...
Figure 7. Whole-exome sequencing identifies NF1 mutations in tumors of melanoma patients exhibiting resistance to vemurafenib. A, patients with melanoma who progressed on treatment with vemurafenib were biopsied pre- and posttreatment. Whole-exome sequencing was conducted and NF1 alterations assessed. The NF1 mutations are listed alongside the functional impact on the expressed protein. Silent mutations were assessed for their potential effects on splicing sites using the human splicing finder. The splicing motif affected by the mutation (in red) is indicated and “site broken” indicates potentially damaging effects on splice site function.

B, PFS data were extrapolated from Chapman and colleagues (2), and the number of patients who progressed per month is shown in blue, the cumulative number of progressing patients is in gray. NF1 mutations observed in our cohort of patients are overlaid at their respective PFS times. Patients with a PFS of less than 3 months were nominated for de novo resistance, whereas those with a PFS of 3 months or more showed acquired resistance. Three NF1 alterations were observed in pretreatment BRAF–mutant melanoma patient samples, and all 3 patients showed de novo resistance. A fourth patient with a PFS of 5 months exhibited acquired resistance.

C, Integrative Genomics Viewer (48) plot showing an NF1 silent mutation observed in the post-relapse biopsy sample only (c.4023G>A) in a patient with a PFS of 5 months.

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DISCUSSION

In malignant melanoma, activating BRAF mutations confer exquisite dependency on RAF/MEK/ERK signaling, which
has been successfully targeted using small-molecule RAF and MEK inhibitors. However, therapeutic resistance inevitably develops following a period of disease stabilization or regression, and some BRAFV600E-mutant melanomas exhibit intrinsic resistance to these agents (2). Understanding the mechanistic basis of drug resistance is essential to the development of new therapeutic strategies that maximize clinical benefit. Here, we queried resistance to RAF inhibition using an unbiased, genome-scale RNAi screen for modifiers of sensitivity to small-molecule RAF inhibition. Our approach builds upon complementary studies using gain-of-function approaches, such as systematic open reading frame (ORF) overexpression screens (14) or the generation of resistant cell lines by chronic exposure to inhibitors (10, 11, 13, 34).

The identification of NF1 loss as a resistance effector describes one of the first loss-of-function events capable of mediating resistance to RAF inhibitors identified by systematic functional approaches. The protein encoded by the NF1 gene, neurofibromin, is a known tumor suppressor gene and negative regulator of RAS proteins. This function is achieved through stimulation of the GTPase activity of RAS by neurofibromin, thus converting it from an active, GTP-bound form to its inactive GDP-bound form. Therefore, loss of NF1 activates RAS and provides an upstream stimulus to activate CRAF, driving resistance to a RAF inhibitor by reactivation of the MAPK pathway. This finding is consistent with other reported mechanisms whereby RAS is mutated or amplified in vemurafenib-resistant cell lines, signaling through CRAF to maintain MAPK signaling (10, 35). These findings are therefore consistent with several published reports indicating that the majority of resistance mechanisms reported for RAF inhibitors involve reactivation of the MAPK pathway. This underscores a fundamental dependency on RAS signaling potentiated by NF1 loss. This event also confers a CRAF dependency in the setting of pharmacologic RAF inhibition, as evidenced by a near-complete reversal of the resistance phenotype by CRAF knockdown. This observation raises the possibility that, in the future, saturating pharmacologic inhibition of both BRAF and CRAF (and possibly ARAF) could reduce the capacity for resistance to develop. Such inhibition might be achieved by an irreversible RAF inhibitor that promotes an inactive conformation of RAF. Consistent with this notion is the observation that NF1 was unable to confer resistance to AZ628, a tool compound with these characteristics (25). Consequently, we hypothesized that either a single-agent MEK inhibitor or combined RAF and MEK inhibition might circumvent NF1-mediated resistance. Indeed, combined exposure displayed more potent activity in vitro than did either agent alone in the setting of NF1 knockdown. However, the drug combinations tested herein did not fully restore sensitivity, at least in vitro. Conceivably, this limitation may be overcome by more potent MEK inhibitors or clinical RAF/MEK regimens that enable more complete suppression of MEK/ERK signaling. Alternatively, CRAF may have MEK-independent roles in tumor cell survival and proliferation (36–39). Therefore, although combined RAF/MEK inhibition certainly improves clinical responses in BRAF-mutant melanoma (5), the potential for NF1 loss (and perhaps other mechanisms of upstream RAS activation) to drive resistance may remain. On the other hand, melanoma cells harboring NF1 knockdown remained sensitive to a selective ERK inhibitor, suggesting that ERK may represent a pivotal integration point for upstream MAPK signaling and may offer therapeutic potential for circumventing multiple resistance mechanisms.

To ascertain whether NF1 loss might provide an endogenous resistance mechanism, we analyzed cancer cell lines in which NF1 mutation co-occurred with BRAFV600E mutation. Using the CCLE, we identified 5 BRAFV600E cell lines that had NF1 mutations, 3 of which were nonsense mutations. Each of these cell lines was insensitive to PLX4720, consistent with the NF1 shRNA screening and validation results in RAF inhibitor–sensitive cell lines. Furthermore, by integrating our findings with a published cohort of 121 primary melanoma tumors, metastatic tumors, and short-term cultures (20) previously analyzed by whole-exome sequencing, we gained additional insights into the potential clinical relevance of NF1 mutations. In this cohort, putatively damaging NF1 mutations were observed in 25% (5/21) of samples that lacked highly recurrent mutations in either BRAF or NRAS. Genomic studies of an independent melanoma cohort have also shown this association (21). However, NF1 mutations were also observed in BRAF- or NRAS-mutant tumors, including a splice site mutation (NF1P1166S), a nonsense mutation (NF1P1324L) in a BRAFV600E tumor. Given that both melanoma and colorectal tumors harbor coexisting BRAF and NF1 mutations, it therefore seemed likely that clinical scenarios could arise in which NF1 loss serves as a gating resistance effector in the setting of BRAF/MEK inhibition. Toward this end, we assessed NF1 alterations by whole-exome sequencing in the tumors of patients with melanoma treated with vemurafenib who progressed while on treatment. We identified 4 patients whose tumors had NF1 alterations that could have an impact on protein function or expression. One event resulted in a nonsense mutation in NF1 (p.R2450*), likely to result in nonfunctional protein. The other 3 mutations were silent events, but importantly, they occurred in putative splice regulatory or exon splicing enhancer sites predicted to regulate proper mRNA processing. These data affirm the potential for NF1 alterations to drive both de novo and acquired resistance to vemurafenib in melanoma. As deep genomic/molecular characterization accrues for tumor specimens obtained before treatment, during treatment, and following relapse, the relevance of NF1 loss to clinical resistance should become clearer.

Our data may also endorse the development of irreversible RAF inhibitors. Such agents may, in principle, show benefit in NRAS-mutant tumors because of their enhanced dependency upon CRAF signaling for tumor cell growth. Alternatively, the use of ERK inhibitors may overcome numerous melanoma resistance mechanisms if such agents can be administered safely at doses that achieve robust target inhibition. Toward this end, both irreversible RAF and ERK inhibitors are entering preclinical and clinical trials, thereby offering forthcoming opportunities to evaluate their efficacy in BRAF–mutant cancers. In the aggregate, these results show the promise of systematic preclinical studies combined with knowledge of tumor genetic/molecular alterations to elucidate cancer drug
resistance mechanisms that may inform novel therapeutic avenues for many types of cancer.

METHODS

Cell Culture and Reagents

Cell lines were obtained from the American Type Culture Collection (ATCC) or the National Cancer Institute (NCI) and cultured in either RPMI-1640 or Dulbecco modified Eagle’s medium (Cellgro) and 10% FBS (Gemini Bio-Products) as recommended by the suppliers. Cells were passaged for less than 6 months after receipt and authenticated by short tandem repeat profiling. PLX4720, PLX4032, AZ628, GDC0879, and AZD6344 were purchased from Selleck Chemicals, and the ERK inhibitor VTX-11e was synthesized as previously reported (29). Expression constructs for KRAS 

Pooled Lentiviral shRNA Screen

A375 cells were seeded into 12-well plates at a density of 3 × 10^5 cells per well, and a total of 7.2 × 10^5 cells per replicate were infected with a 90,000 shRNA pooled library at a multiplicity of infection of 0.3 to 0.5 (18). Cells were centrifuged for 2 hours at 2,000 rpm, followed by an immediate medium change. The next day, cells were pooled and transduced with plasmids using the expression protocol in the presence of 4 μg/mL polybrene for 3 days. Cells were then seeded into T225 flasks for each replicate and treated with either DMSO or 3 μmol/L PLX4720. DMSO-treated cells were passaged for up to 14 population doublings, and PLX4720-treated cells were passaged for approximately 7 population doublings. Cells were harvested by trypsinization and stored at −80°C in PBS. Genomic DNA was extracted as described previously (18), and the shRNA sequences were amplified by PCR (41). Here, 140 μg of genomic DNA was split over 60 reactions per sample, and reactions were then re-pooled per sample before a secondary PCR step. Illumina adapters and independent sample barcodes were incorporated during this secondary PCR step using a scaled-up secondary PCR reaction consisting of six 100 μL reactions, followed by massively parallel sequencing (Illumina). The number of reads for each shRNA was incorporated into the following calculation to normalize between samples: Log2[(shRNA reads/total reads for sample) ×166]. The enrichment of shRNAs in the PLX4720-treated arm relative to the DMSO arm was determined using the RIGER algorithm, “second best shRNA” analysis in GENE-E (http://www.broadinstitute.org/cancer/software/GENE-E; ref. 18) to produce a ranked list of statistically significant genes based on the degree of corresponding shRNA enrichment in the PLX4720-resistant population. By ranking genes according to the ranking of the “second-best shRNA” per gene, results are influenced less by high-ranking, single shRNA hits for a given gene.

shRNA/ORF Constructs and Lentiviral Infections

All shRNA expression constructs were obtained from The RNAi Consortium and the Broad Institute RNAi Platform. shRNAs were expressed from the lentiviral expression plasmid pLKO.1 or pLKO-TRC005, and virus was produced by transfection of approximately 2.4 × 10^6 293T cells with 3 μg pLKO.1, 2.7 μg Δ8.9 (gag-pol), and 0.3 μg VSV-G plasmids using 18 μL Fugene6 (Promega). Viral supernatant was harvested 72 hours after transfection. Mammalian cells were infected at a 1:10 dilution of virus in the presence of 4 μg/mL polybrene (Millipore) and centrifuged for 30 minutes at 2,000 rpm. The culture medium was changed immediately after spin infection, and 24 hours later puromycin at a concentration of 0.5–1 μg/mL was added to select for infected cells. pLX304 plasmids for LacZ and KRAS were used to generate lentivirus as described above. Cells were infected in the same manner except 10 μg/mL blasticidin (Life Technologies) was used to select for infected cells.

shRNA Constructs Used

The shRNA constructs used in this study include the following:

1. SHC002 MISSION pLKO.1-puro Non-Mammalian shRNA Control, sequence CACAGAGATGAAGAGCAACAA (referred to as shCtrl)
2. TRC0000472243, Luciferase
3. TRC000039714, NM_000267-1:84685c1, NF1
4. TRC000039717, NM_000267-1:86275c1, NF1
5. TRC000001066, NM_002880.x:12365c1, RAFI

Cell Proliferation Assays

Cells were seeded at a density of 0.5 to 5 × 10^5 cells per well in 96-well plates. The next day, cells were treated with 4 μg/mL polybrene and transduced with shRNA-expressing lentivirus by centrifugation for 30 minutes at 2,000 rpm followed by an immediate medium change. After 3 days, the medium was again changed and compounds were added to target-qualified concentrations. After a further 4 days, cell proliferation was assessed using the CellTitre-glo reagent. Glow values were determined using GraphPad Prism. For colony formation assays, shRNA-infected cells were seeded at 300 cells per well of a 12-well plate and incubated with 3 μmol/L PLX4720 or DMSO for 2 weeks. Medium containing either DMSO or PLX4720 was changed every 3 to 5 days. Cells were fixed with 4% formaldehyde and stained with 0.5% crystal violet; plates were then washed with distilled water and photographed.

Protein Analysis

Cells were seeded at 5 to 10 × 10^4 cells per well in 6-well plates. Following treatment, cells were washed with PBS and lysed in 1% SDS lysis buffer. Following protein normalization using the BCA reagent (Sigma), equal amounts of protein were resolved by SDS-PAGE (Life Technologies) and transferred to PVDF-FI membranes (Bio-Rad). Western blots were blocked with Li-Cor blocking buffer and then probed with the desired antibodies overnight at 4°C. Bands were detected using infrared fluorescence and an Odyssey scanner (Li-Cor Biosciences). Antibodies were obtained from Cell Signaling, Santa Cruz Biotechnology, Sigma, Millipore, BD Biosciences, Li-Cor Biosciences, and Thermo-Fisher Scientific. The determination of RAS-GTP levels in cell lysates was conducted with the RAS Activation Assay Kit (Millipore).

In-cell Western Assay

Cells were seeded in 96-well clear-bottomed, black microtiter plates at 2 × 10^4 cells per well. The next day, cells were treated with a 10-point titration of PLX4720 for 16 hours. Cells were fixed in 4% formaldehyde, 0.1% TX-100 in PBS for 30 minutes. The wells were blocked with Li-Cor blocking buffer for 30 minutes and incubated with antibodies to phospho-ERK (Sigma) and ERK2 (Santa Cruz Biotechnology) overnight at 4°C. Cells were washed 3 times with 200 μL 0.1% Tween 20 and incubated with Li-Cor secondary antibodies (anti-mouse IR800, anti-rabbit IR680) for 1 hour. The plates were scanned using an Odyssey scanner (Li-Cor) and quantified using the manufacturer's software. ERK phosphorylation was calculated as a percentage of vehicle-treated controls.

Clinical Samples

All melanoma and matched normal samples analyzed were collected and sequenced under an Institutional Review Board–approved protocol (COUHES#0806002814). Standard Broad Institute Sequencing Platform techniques were used for DNA extraction and quality assessment (20).
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Library Preparation, Assembly, and Quality Control
Exome capture and library construction were conducted as previously described (42), and libraries were sequenced on Illumina HiSeq 2000 machines. The resulting sequencing data obtained from the Illumina pipeline were assembled using the Picard pipeline (43). Cross-contamination of samples was estimated using ContEst (44), and samples with more than 5% contamination were excluded from this study. Single-nucleotide polymorphism fingerprints from each lane of a tumor/normal pair were crosschecked to confirm concordance, and nonmatching lanes were removed from analysis.

Identification of Somatic Mutations
Somatic single-nucleotide base-pair substitutions were identified using MuTect (45). Annotation of identified mutations for mutation effect was done using Oncotator (46). These algorithms were executed using the Broad Firehose Infrastructure (47).

Disclosure of Potential Conflicts of Interest
N. Wagle has an ownership interest (including patents) and is a consultant/advisory board member for Foundation Medicine. G.S. Cowley and D. Schadendorf have received honoraria from service on the speakers’ bureaus from MSD, Roche, and Bristol-Myers Squibb and are consultant/advisory board members of Bristol-Myers Squibb, Roche, GlaxoSmithKline, Amgen, and Novartis. L.A. Garraway has a commercial research grant from Novartis, has ownership interest (including patents) in Foundation Medicine, and is a consultant/advisory board member for Millennium/Takeda, Novartis, and Foundation Medicine. No potential conflicts of interest were disclosed by the other authors.

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