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Loss of NF1 in Cutaneous Melanoma Is Associated with RAS Activation and MEK Dependence

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Abstract

Melanoma is a disease characterized by lesions that activate ERK. Although 70% of cutaneous melanomas harbor activating mutations in the BRAF and NRAS genes, the alterations that drive tumor progression in the remaining 30% are largely undefined. Vemurafenib, a selective inhibitor of RAF kinases, has clinical utility restricted to BRAF-mutant tumors. MEK inhibitors, which have shown clinical activity in NRAS-mutant melanoma, may be effective in other ERK pathway-dependent settings. Here, we investigated a panel of melanoma cell lines wild type for BRAF and NRAS to determine the genetic alteration driving their transformation and their dependence on ERK signaling in order to elucidate a candidate set for MEK inhibitor treatment. A cohort of the BRAF/RAS wild type cell lines with high levels of RAS-GTP had loss of NF1, a RAS GTPase activating protein. In these cell lines, the MEK inhibitor PD0325901 inhibited ERK phosphorylation, but also relieved feedback inhibition of RAS, resulting in induction of pMEK and a rapid rebound in ERK signaling. In contrast, the MEK inhibitor trametinib impaired the adaptive response of cells to ERK inhibition, leading to sustained suppression of ERK signaling and significant antitumor effects. Notably, alterations in NF1 frequently co-occurred with RAS and BRAF alterations in melanoma. In the setting of BRAF(V600E), NF1 loss abrogated negative feedback on RAS activation, resulting in elevated activation of RAS-GTP and resistance to RAF, but not MEK, inhibitors. We conclude that loss of NF1 is common in cutaneous melanoma and is associated with RAS activation, MEK-dependence, and resistance to RAF inhibition. Cancer Res; 74(8); 2340-50. ©2014 AACR.
transformed phenotype that may be exploited for therapeutic advantage (9, 10). Here, we performed a functional and genomic analysis of BRAF<sup>WT</sup>/NRAS<sup>WT</sup> melanoma cell lines to determine whether occult MAPK pathway alterations are present in such cells. NF1 alterations that result in RAS activation and MEK dependence were identified in a subset of BRAF<sup>WT</sup>/NRAS<sup>WT</sup> melanoma cell lines. However, NF1 alterations were not mutually exclusive with RAS and BRAF aberrations. Loss of NF1 in cells co-mutated for BRAF was sufficient to overcome the upstream negative feedback that results in suppression of RAS activation in BRAF(V600E) cells and was sufficient to confer resistance to vemurafenib. Furthermore, NF1 loss and corresponding relief of upstream ERK-dependent negative feedback attenuated the antiproliferative effects of the selective MEK inhibitor PD0325901 in NF1-null cells. In contrast, trametinib, an allosteric MEK inhibitor recently approved for use in patients with BRAF-mutant melanoma (11), attenuated the phosphor-ylation of MEK resulting from relief of upstream negative feedback and exhibited greater potency than PD0325901 in NF1-null melanoma cells. In summary, loss of NF1 expression in melanoma results in RAS activation and vemurafenib resistance even in the setting of BRAF mutation. Allosteric MEK inhibitors that impair the adaptive response of cells to ERK inhibition by blocking MEK phosphorylation should be studied in patients with melanoma whose tumors harbor NF1 loss.

Materials and Methods

Cell lines and culture conditions

"SK-Mel" cell lines were provided by Taha Merghoub and Alan Houghton (Memorial Sloan-Kettering Cancer Center, New York, NY), MeWo, Malme3M, A375, and SNF96.2 were purchased from the American Type Culture Collection. M308 was provided by Antoii Ribas (University of California Los Angeles, Los Angeles, CA) and WM3918 by Katherine Nathanson and Meenhard Herlyn (Wistar Institute, Philadelphia, PA). Other than A375 and SNF96.2 (grown in Dulbecco's Modified Eagle Medium), all cell lines were grown in RPMI 1640 as previously described (12).

Genomic studies

Cellular DNA was extracted using the Qiagen DNeasy Tissue Kit. DNA was analyzed using a mass spectrometry–based fingerprinting assay to validate cell line identity as described previously (13). NRAS (G12A, G12D, Q61K, Q61R, Q61L), BRAF (V600E, V600K, V600R, K601E), and c-KIT (D816V) mutations were detected using a mass spectrometry–based assay (Sequenom) and validated by Sanger sequencing (13). Selected cell lines were screened for mutations and copy number alterations in 279 cancer-associated genes using the integrated mutation profiling of actionable cancer targets (IMPACT) assay as has been described (14) and viewed in the integrated genomics viewer (15). Genomic data from the TCGA melanoma project were derived from the cbioportal.org).

RNA-seq

RNA was extracted from cells using the RNeasy Mini Kit (Qiagen Inc.). Quality assessment, poly-A selection, and sequencing with an Illumina HiSeq 2000 were performed by the Genomics Core Laboratory at Memorial Sloan-Kettering Cancer Center. All samples had a minimum RNA integrity number (RIN) of 7.0 (16). Sequencing produced 40 to 120 million 75 bp reads per sample. FASTQ files were generated using CASAVA 1.8.2 software (Illumina). Low-quality bases and adapter sequences were removed with Cutadapt. Trimmed reads were aligned to human genome assembly GRCh37 using TopHat 2.0.8 (17, 18). Gene quantification and differential expression were calculated using Cufflinks 2.1.1 (19). Data visualizations were created with the gplots package for R.

Western blotting

Cells were collected, lysed, and blotted as previously described (12). Secondary antibodies were detected using Super Signal (Thermo) and chemiluminescence imaged using a Fuji LAS-4000 imager (GE Lifesciences). Anti-NF1 (SC-67), cyclin D1 (SC-718), KRAS (SC-30), NRAS (SC-519), HRS (SC-520), and actinin (SC-17829) were from Santa Cruz.
Biotechnology; anti-Ras (#1862335) from Thermo Scientific; and anti-phospho-ERK (pERK) (#9102), pMEK (#9121), and p-CRAF S338 (#9427) from Cell Signaling Technology.

**Proliferation assays/FACS analysis**

Cell viability was measured by trypan blue incorporation using a Vi-CELL XR 2.03 (Beckman Coulter) as previously described (20). Percent growth was calculated using the equation: 

\[
\text{Percent growth} = \frac{[\text{Day 5 drug}] - [\text{Day 0}]}{[\text{Day 5 DMSO}] - [\text{Day 0}]} 
\]

FACS analysis was performed as previously described (21).

**RAS-GTP assay**

GTP-bound RAS was isolated via immunoprecipitation using recombinant RAS binding domain of RAF1 (RAF1-RBD; Active RAS Pull-down and Detection Kit; Thermo Scientific), according to the manufacturer’s instructions. The product was detected using total (pan-RAS) or isoform specific (H-, K-, N-) RAS antibodies.

**RNAi studies**

siRNA studies used ON-TARGET plus siNF1 SMARTpool and ON-TARGET plus nontargeting siRNA#2. siRNA studies were accomplished using GIPZ shNon-targeting RNA or TRIPZ inducible shRNA against NF1. shRNAs (shNF1 #2: CloneID V2THS-260806; shNF1 #4: CloneID V3THS-380114; shRNA#6: CloneID V3THS-380110) were induced with 2 μg/mL doxycycline daily for 1 week before vemurafenib studies.

**Results**

**NF1 expression is lost in a subset of RAS-activated BRAF<sup>WT</sup>/RAS<sup>WT</sup> cell lines**

To identify a cohort of BRAF<sup>WT</sup>/NRAS<sup>WT</sup> melanoma cell lines for genomic and biologic characterization, 191 melanoma cell lines were genotyped for BRAF and NRAS mutations using a mass spectrometry–based (Sequenom) assay (13, 22). This screen identified 66 cell lines that lacked hotspot mutations in BRAF or NRAS (Fig. 1, A and Supplementary Table S1). As this assay was designed to detect only the most common BRAF and NRAS mutations, we further performed Sanger sequencing of BRAF exons 11 and 15 and NRAS exons 2 and 3. This analysis identified BRAF mutations not present in the Sequenom assay in 2 cell lines (D594G in SK-Mel-264 and N581S in SK-Mel-215; Supplementary Table S2). Direct sequencing of KRAS and HRAS further identified activating mutations in KRAS in 2 and HRAS in 1 cell line, respectively (Supplementary Table S2). In summary, 61 cell lines were wild type for RAS and BRAF.

Given the high prevalence of ERK activation in melanoma (10), we hypothesized that a subset of the BRAF<sup>WT</sup>/RAS<sup>WT</sup> cohort likely harbored occult alterations within the MAPK pathway that cause RAS to become refractory to negative feedback and thus confer activation of ERK. We thus measured levels of activated, GTP-bound RAS in a subset of the BRAF<sup>WT</sup>/RAS<sup>WT</sup> cell lines as a surrogate of pathway activation. Similar to human tumors, KRAS- and NRAS-mutant melanoma cell lines exhibit high levels of RAS-GTP, whereas BRAF-mutant cell lines have low to undetectable levels of RAS-GTP (Fig. 1B; refs. 3 and 23). RAS was activated to varying levels in the BRAF<sup>WT</sup>/RAS<sup>WT</sup> melanoma cells, with some expressing levels of activated RAS similar to those present in RAS-mutant cells (Supplementary Fig. S1).

The NF1 gene encodes a protein that functions as the predominant RAS GTPase activating protein (RAS GAP), which suppresses RAS activity and reduces RAS-GTP levels by promoting endogenous RAS GTPase activity. NF1 is inactivated in diverse human cancers (24–27) and would be predicted, if lost, to cause RAS to become refractory to negative feedback. We performed Western blot analysis to determine whether loss of NF1 protein expression occurred in, and was correlated with, elevated RAS-GTP levels in BRAF<sup>WT</sup>/RAS<sup>WT</sup> melanoma cell lines. Complete loss of NF1 expression was noted in 5 of the BRAF<sup>WT</sup>/RAS<sup>WT</sup> cell lines, all of which had high levels of RAS-GTP (Supplementary Fig. S1). Having previously performed high-resolution DNA copy number profiling (array CGH) on 92 melanoma cell lines (22), we identified a sixth NF1-null cell line that harbored homozygous NF1 gene deletion and concurrent NRAS(Q61R) mutation (SK-Mel-103).

NRAS mutations are significantly more prevalent than other RAS mutations in melanoma even though KRAS mutations are predominant in most other cancers (4). To determine which RAS isoforms were activated in NF1-null melanomas, we assayed activated KRAS, HRAS, and NRAS by performing immunoprecipitation with the RAS binding domain of RAF1 (RAF1-RBD; see Materials and Methods) followed by RAS isoform-specific immunoblots. All 4 NF1-null cell lines examined expressed high levels of total active RAS when compared with a BRAF(V600E) control cell line (Fig. 1B and Supplementary Fig. S1). NRAS(Q61K) SK-Mel-30 cells expressed high levels of GTP-bound NRAS, but no detectable levels of activated KRAS, similar to the NRAS(Q61R)/NF1-null SK-Mel-103 line. GTP-bound NRAS was also highly expressed in the other NF1-null cell lines, whereas only a subset had concurrent activation of KRAS, including SK-Mel-217, which harbored KRAS gene amplification. Elevated levels of GTP-bound KRAS and NRAS were also detected in the KRAS(112c)-mutant SK-Mel-285 cell line. Levels of activated HRAS were low or undetectable in all the NF1-null melanoma cell lines (Supplementary Fig. S1).

To define the mechanistic basis for the loss of NF1 expression in the melanoma cell lines, we performed next-generation sequencing of 279 genes commonly mutated in human cancer using an exon capture-based approach (IMpACT assay; refs. 14 and 28). Two cell lines were found to harbor nonsense mutations in NF1 (Fig. 2A): MeWo, a hemizygous Q1336<sup>c</sup> mutation, and Sk-Mel-266, L161<sup>c</sup> and Q282<sup>c</sup> mutations. The remaining 4 cell lines had deletions involving the NF1 gene locus: SK-Mel...
113, focal homozygous loss of the N-terminal domain; SK-Mel-103 and WM3918, focal homozygous loss of the C-terminal domain, and SK-Mel-217, broad monoallelic loss, as well as a focal, intragenic deletion in the second NF1 allele (Fig. 2B). In sum, genomic alterations sufficient to account for complete loss of NF1 protein expression were identified in all 6 NF1-null melanoma cell lines.

Although loss of NF1 was identified in the BRAFWT/RASWT cohort, it was not mutually exclusive with RAS alterations. Notably, concurrent alterations in the NF1 and RAS genes have also been noted in 2 recent whole-exome sequencing studies of melanoma tumors, including Holdis and colleagues and the melanoma study performed by the Cancer Genome Atlas (TCGA) working group (Fig. 2C; ref. 29). CDKN2A and/or TP53 were among the genes most commonly co-altered in the NF1-null melanoma cell lines and tumors, suggesting that these genes may cooperate with NF1 loss in promoting melanomagenesis as has been reported in other cancer types, such as astrocytomas and malignant periphereral nerve sheath tumors (30).

**NF1-null cell lines are sensitive to MEK inhibitors that impair the adaptive response of cells to ERK inhibition**

The growth of NF1-null cell lines derived from human malignant peripheral nerve sheath tumors (MPNST) and MPNSTs that arise in NF1−/− mice have been shown to be dependent on mTORC1 signaling and exquisitely sensitive to the mTORC1 inhibitor rapamycin (31, 32). To determine whether NF1-null melanomas were also mTORC1 dependent, we treated the NF1-null melanoma cell lines with rapamycin and compared their sensitivity with that of SNF96.2, a representative human NF1-null MPNST cell line (Fig. 3A). The NF1-null melanomas as a group were significantly less sensitive to rapamycin (IC50 ranging from 1 to 286 nmol/L) than SNF96.2 (Fig. 3B). However, inhibition of proliferation (Fig. 3B–D). Together, these data suggest that cyclin D1 expression and cell-cycle progression were MEK-dependent in NF1-null melanoma cells, but that rapid rebound in ERK activity may account for the lower sensitivity of NF1-null cells to the MEK inhibitor PD0325901. Induction of cell death was not observed following treatment with the MEK inhibitor in any of the NF1-null cell lines (Fig. 3D). Furthermore, cotreatment with PD0325901 and the pan-AKT inhibitor MK2206 did not augment growth inhibition or induce apoptosis as has been shown in colorectal cells with RAS activation (Supplemental Fig. 5D; ref. 34).

Resistance to allosteric MEK inhibitors can be induced by upstream pathway hyperactivation (35, 36). We have previously shown that treatment of BRAFWT but not BRAF(V600E) cells with PD0325901 leads to increased levels of phosphorylated MEK (pMEK), which results from relief of upstream ERK-dependent negative feedback (3). Consistent with these prior observations, treatment of NF1-null melanoma cells with PD0325901 resulted in increased pMEK (Fig. 3C).

As the induction of pMEK in the NF1-null melanomas paralleled the rebound in pERK activation, we further studied the effects of a second allosteric MEK inhibitor on MEK signaling and cellular proliferation. Trametinib (GSK1120212) has a similar in vitro affinity for MEK1/2 as PD0325901 (IC50s for MEK1 and MEK2 of 0.7 and 0.9 nmol/L, respectively), but in contrast to PD0325901, binding of trametinib to MEK blocks its phosphorylation at serine 217 (11). To compare the relative potencies of PD0325901 and trametinib in vivo, we first exposed BRAF(V600E) SK-Mel-239, and NF1-null SK-Mel-113 cells to increasing concentrations of both drugs and assessed the effect of drug treatment on pERK activation at 1 hour. In BRAF(V600E) SK-Mel-239 cells, both drugs were equipotent in their ability to suppress ERK activation at 1 hour (Fig. 4A). In contrast, in NF1-null SK-Mel-113 cells, trametinib was considerably more potent in its ability to suppress pERK activation than either PD0325901 or 2 additional allosteric MEK inhibitors currently in clinical testing (AZD6244 and MEK162; Fig. 4A and Supplementary Fig. 5D; refs. 37–39). Treatment of NF1-null melanoma cells with either PD0325901 or trametinib resulted in hyperactivation of RAS consistent with relief of upstream negative feedback following inhibition of ERK (Fig. 4B). However, relief of upstream feedback following MEK inhibition was accompanied by a significant increase in pMEK levels in PD0325901-treated NF1-null cells, which was attenuated in cells treated with trametinib (Fig. 4B and Supplementary Fig. 5D). This attenuation of MEK phosphorylation was unique to trametinib and was not observed with PD0325901, AZD6244, or MEK162 (Supplementary Fig. 5D). Furthermore, the resistance of MEK to upstream hyperactivation by RAS in trametinib-treated cells was accompanied by a more durable downregulation of pERK and more potent inhibition of genes whose transcription is dependent upon
ERK, including CCND1 (cyclin D1), ETV1, MYC, and SPRY4, as compared with PD0325901 (Fig. 4B and D). Consistent with these biologic differences among the MEK inhibitors, the antiproliferative effects of trametinib were similar in BRAF (V600E) and NF1-null cells, whereas BRAF (V600E) cells exhibited greater sensitivity than NF1-null cells to PD0325901 (Fig. 4C and Supplementary Fig. S4).

NF1 loss and resulting RAS activation in BRAF(V600E) melanoma cells confers resistance to RAF inhibition

Levels of GTP-bound RAS are low in BRAF (V600E) melanomas as a result of high levels of ERK-dependent negative feedback (23). It was unclear whether loss of NF1 function in this context would be sufficient to overcome the feedback-mediated suppression of RAS activity in BRAF (V600E) cells. We therefore screened a panel of 10 BRAF (V600E) melanoma cell lines for loss of NF1 expression and activation of RAS-GTP. Nine of the 10 BRAF (V600E)–mutant melanoma cell lines expressed low to undetectable levels of RAS-GTP (Fig. 5A). A single BRAF (V600E) cell line (M308) had high levels of RAS-GTP similar to that of an NRAS (Q61K) cell line, and notably, M308 was devoid of NF1 expression by immunoblot (Fig. 5A). Genomic analysis by IMPACT confirmed the presence of a nonsense mutation in the NF1 gene (Q1070X) as the basis for the loss of NF1 protein expression in M308 cells (Supplementary Fig. S5). To determine whether NF1 loss desensitizes BRAF (V600E) cells to RAF inhibition, we assessed the sensitivity of M308 [BRAF (V600E)/NF1-null] cells to vemurafenib. Vemurafenib treatment of SK-Mel-239 failed to significantly reduce cell growth, but in M308 cells, vemurafenib caused a marked decrease in cell growth (Fig. 5B). These findings indicate that NF1 loss in BRAF (V600E) melanoma cells confers resistance to RAF inhibition.

Figure 3. NF1-null melanoma cell lines are MAPK pathway dependent. A, cells were treated with increasing concentrations of rapamycin for 5 days. Results are percent cell growth as a function of drug concentration (nmol/L). B, cells were treated with increasing concentrations of the MEK inhibitor PD0325901 for 5 days. Results as in A. C, cells were treated with 50 or 500 nmol/L PD0325901 for 0, 1, 6, and 24 hours. Phospho- and total levels of MAPK pathway components were determined by immunoblot. D, cells were treated for 24 hours with 50 or 500 nmol/L PD0325901 before undergoing FACS analysis for cell-cycle distribution. Error bars, SEM; n = 3.
239 [BRAF(V600E)/NF1\textsuperscript{WT}] cells resulted in potent down-regulation of phosphorylated MEK and ERK and inhibition of cell growth (Fig. 5B). In contrast, vemurafenib had little effect on levels of phosphorylated MEK and ERK in BRAF (V600E)/NF1-null M308 cells and no effect on cell proliferation (Fig. 5B and C).

RAS activation is sufficient to induce vemurafenib resistance in BRAF(V600E) cells (Supplementary Fig. S6). To determine whether NF1 loss activates RAS sufficiently to overcome ERK-dependent negative feedback and induce vemurafenib resistance, we knocked down NF1 expression in BRAF(V600E)-mutant A375 cells and assessed levels of RAS activation in the presence and absence of vemurafenib. siRNA and shRNA mediated knockdown of NF1 resulted in induction of RAS-GTP and decreased sensitivity to vemurafenib (Supplementary Fig. S6). These data suggest that loss of NF1 function in BRAF (V600E) mutant cells is sufficient to induce RAS-GTP activation and, as a consequence, vemurafenib resistance (Fig. 5A–C and Supplementary Fig. S6).

To assess the MEK dependence of the M308 melanoma cells, we determined the effects of PD0325901 and trametinib treatment on ERK activation and cellular proliferation. Analogous to the results seen with these inhibitors in BRAF(V600E)/NF1-null melanoma cells, exposure of M308 cells to 50 nmol/L PD0325901 was insufficient to durably suppress ERK signaling and cell proliferation (Fig. 5D and E). In contrast, treatment of M308 cells with trametinib resulted in durable suppression of pERK activation, potent downregulation of cyclin D1.
expression, and potent inhibition of cellular proliferation (Fig. 5D and E).

Discussion

Neurofibromatosis Type I is a hereditary disorder caused by germline mutation of the NF1 gene (350 kb genomic DNA; 60 exons; 2,818 amino acids). This syndrome is characterized by the formation of benign neurofibromas as well as pigmented cafe-au-lait spots and Lisch nodules. Individuals with neurofibromatosis are at risk of developing malignant neoplasms at a young age, most commonly optic gliomas, malignant peripheral nerve sheath tumors, and juvenile myelomonocytic leukemia (40). Only recently have somatic alterations in the NF1 gene been implicated in malignancies, including gliomas, breast cancers, and leukemias (24–27). The prior underestimation of the prevalence of NF1 alterations in human tumors is likely attributable to the technical challenges previously inherent in sequencing large genes with tumor suppressive function in which mutations throughout the coding region are potentially oncogenic. With the advent of massively parallel next generation sequencing methods, many of these technical challenges have now been overcome.

Here, we report that a significant subset of melanoma cell lines, including those wild type for BRAF and RAS, exhibit total loss of NF1 protein expression. In all cases, a mutation and/or focal deletion of the NF1 gene, rather than posttranscriptional regulation (41) could be identified as the basis for NF1 loss. In contrast to prior reports (42, 43), all the NF1-null...
melanoma cell lines expressed levels of active GTP-bound RAS comparable with those found in RAS-mutant cells. Notably, NF1 loss was not mutually exclusive with RAS or BRAF mutations. In fact, mutations in NF1 were found to co-associate with additional activating alterations in the RAS/MAP kinase pathway, in particular with exon 11 BRAF mutations (Fig. 2C). Several of these BRAF mutations exhibit impaired kinase activity but induce ERK signaling by dimerizing with and activating CRAF (44). As induction of RAS activity through NF1 loss would be predicted to promote the formation of CRAF homo- and heterodimers, NF1 alterations may cooperate with low and impaired activity BRAF mutants to induce transformation by further enhancing RAF dimer formation.

Studies using genetically engineered mouse models with melanocyte-targeted deletion of NF1 also suggest that NF1 loss cooperates with BRAF(V600E) mutation to promote melanomagenesis and suggest that this cooperativity results, at least in part, through abrogation of oncogene induced senescence (45). We observed that knockdown of NF1 expression was sufficient to overcome the ERK-dependent feedback suppression of RAS observed in BRAF(V600E) cells. However, loss of both copies of NF1 is likely required to maximally elevate RAS-GTP expression, as a heterozygous splice site mutation in NF1 in A375 cells (45) was not accompanied by NF1 loss or increased RAS-GTP levels (Supplementary Fig. S7). These results suggest that loss of NF1 in BRAF(V600E) melanoma cells may provide a selective advantage, even in the absence of RAF inhibitor exposure, by diminishing the oncogene-induced suppression of RAS mediated by ERK-dependent negative feedback. A secondary consequence of this co-mutation pattern is that such tumors exhibit intrinsic resistance to selective RAF inhibitors. Our studies suggest that partial loss of NF1 function may result in a more pronounced and rapid restoration of RAS signaling following RAF inhibitor therapy. This could result in an attenuation of drug response sufficient to promote the emergence of drug-resistant clones.

Efforts to develop clinically useful direct inhibitors of RAF have been unsuccessful to date (46). One alternative pharmacologic strategy for the treatment of tumors with constitutive RAF activation, including those with loss of NF1, is to target the pathways downstream of RAF responsible for maintenance of transformation (Supplementary Fig. S8). We observed that in contrast to NF1-null MPNSTs (31, 32), NF1-null melanomas were dependent on ERK pathway activation and not TORC1 for cell-cycle progression and cell proliferation. This result indicates that the lineage context within which NF1 is inactivated influences the downstream effector pathways that facilitate RAF-mediated transformation and thus likely dictates the potential utility of targeted pathway inhibitors.

Although NF1-null melanomas were dependent upon MEK-ERK activation for cell proliferation, we observed stark differences in the relative potency of allosertic, non–ATP-competitive MEK inhibitors in the NF1-null cohort (11). Specifically, trametinib, which attenuates phosphorylation of MEK by RAF at Serine 217, had greater antitumor effects than PD0325901. Monophosphorylated MEK has only partial activity (11) and the ability of trametinib but not PD0325901 to abrogate the hyperphosphorylation of MEK resulting from relief of upstream negative feedback was associated with more durable inhibition of pERK and cyclin D1 expression and greater antiproliferative effects. A similar lack of potency was also noted in NF1-null melanoma cells with AZD6244 (Supplementary Fig. S4), a second non–ATP-competitive MEK inhibitor incapable of abrogating RAF phosphorylation of MEK. The inability of AZD6244 to block MEK phosphorylation likely accounts for the partial resistance to MEK inhibition observed following NF1 knockdown in a prior study (47). Our data are also consistent with a recent study suggesting that differences in the cellular potency of MEK inhibitors in KRAS-mutant cells can result from differences in the strength of hydrogen bonding with S212 in MEK, which is critical for blocking feedback induced MEK phosphorylation by wild-type RAF (48). In sum, the data imply that MEK inhibitors that block the phosphorylation of MEK by RAF may have greater clinical activity in tumors with activated RAS, including those with loss of NF1 function. Such inhibitors may, however, have a narrow therapeutic index in patients, as they would be predicted to potently inhibit RAS-dependent ERK signaling in normal tissues.

In summary, NF1 loss is common in cutaneous melanomas. Loss of NF1 is associated with RAS activation, MEK dependence and, in the setting of concurrent BRAF mutation, vemurafenib resistance. Upstream hyperactivation of RAS and RAF resulting from loss of negative feedback following ERK pathway inhibition can result in an attenuation of the antitumor activity of allosertic MEK inhibitors. Inhibitors that prevent RAF-mediated phosphorylation of MEK abrogate this adaptive resistance to MEK inhibition and have greater antitumor activity in NF1-null cells. With the recent approval of trametinib by the U.S. Food and Drug Administration for the treatment of BRAF-mutant melanomas, these findings have potential therapeutic implications for patients with melanoma and others tumor types with NF1 alterations.

Disclosure of Potential Conflicts of Interest
P.B. Chapman is a consultant/advisory board member of Glaxo-SmithKline and Roche-Genentech. R. Yaeger is a consultant/advisory board member of GlaxoSmithKline Advisory Board—Combination therapies for BRAF mutant CRC. N. Rosen is a consultant/advisory board member of AstraZeneca, Chugai, and Novartis. D.B. Solit is a consultant/advisory board member of Pfizer.

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