

IN THE SPOTLIGHT

An Unholy Alliance: Cooperation between BRAF and NF1 in Melanoma Development and BRAF Inhibitor Resistance

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Summary: In this issue of *Cancer Discovery*, 2 studies provide new evidence implicating loss of the tumor suppressor neurofibromin (NF1) in the biologic behavior of cutaneous melanoma. The first study from Maertens and colleagues describes a new transgenic mouse model in which mutant *BRAF* cooperates with NF1 loss to drive melanoma development through the abrogation of oncogene-induced senescence. The second, from Whittaker and colleagues, used a high-throughput short hairpin RNA screening approach to identify NF1 loss as a key mediator of acquired and intrinsic BRAF inhibitor resistance. Together these studies provide new insights into the signaling that underlies melanoma initiation and progression and suggests novel therapeutic strategies for patients whose melanomas are *BRAF*-mutant/*NF1*-deficient. *Cancer Discov*; 3(3); 260–3. ©2013 AACR.

See related article by Maertens et al., p. 338 (5).

See related article by Whittaker et al., p. 350 (6).

Ultraviolet radiation (UVR) exposure is the major causative factor of cutaneous melanoma. The central role of UVR in melanoma etiology has been convincingly shown through epidemiologic studies as well as whole genome and exome sequencing, which have shown the overwhelming majority of mutations in melanoma to be “UV-signature” C>T transitions. Despite the wealth of genetic data now available, the exact series of mutational hits required to transform a melanocyte into a melanoma cell is not fully understood. Most research to date has focused upon the role of nevi—common, benign melanocytic proliferations—as the precursor of melanoma development. The link between nevi and melanoma is suggested by the frequent occurrence of activating mutations in *BRAF*, a serine/threonine kinase known to drive the development and progression of 50% of all cutaneous melanomas (1). Despite harboring this deleterious oncogene, most nevi never undergo malignant transformation and remain growth arrested throughout the lifetime of the individual. The explanation for this apparent paradox was suggested by the observation that the introduction of oncogenic *BRAF* into primary melanocytes led to oncogene-induced senescence (OIS) and not cell proliferation (ref. 2; Fig. 1A). Histopathologic examination also showed the majority of human nevi samples to stain positively for senescence-associated β -galactosidase (SA- β -Gal; ref. 2). The mechanism by which some melanocytes harboring oncogenic *BRAF* apparently avoid OIS and undergo malignant transformation remains to be elucidated. Although mutant *BRAF* plays a critical role in melanoma development, inputs from other

signaling cascades are also required. Of these, the best-studied pathway is phosphoinositide 3-kinase (PI3K)/AKT, which is constitutively activated in melanoma through the loss of the tumor suppressor PTEN, increased expression of AKT3, and rarely as the result of mutations in either PI3K or AKT3 (3). Both animal modeling and cell culture studies have shown a cooperating role for the PI3K/AKT pathway in *BRAF*-mediated melanoma development, in which its activity seems to prevent the entry of melanocytes into OIS (ref. 4; Fig. 1A).

In this issue of *Cancer Discovery*, 2 studies provide new data implicating the loss of expression or function of neurofibromin (NF1) in melanoma pathogenesis (5, 6). The first, from Maertens and colleagues from the Cichowski laboratory, describes the development of a transgenic mouse model in which mutant *BRAF* and NF1 loss cooperate to drive melanoma initiation and progression (5). The second, from Whittaker and colleagues from the Garraway laboratory, details a high-throughput short hairpin RNA (shRNA) screen that identified NF1 loss as a mediator of acquired and intrinsic BRAF inhibitor resistance (6). Both of these studies follow earlier work from the 1990s showing loss of neurofibromin expression to be a frequent event in both melanoma cell lines and tissue specimens (7).

NF1 is a known tumor suppressor and a negative regulator of RAS signaling. Under physiologic conditions, it acts to stimulate the GTPase activity of RAS, leading to its accumulation in the GDP-bound (inactive) state (ref. 8; Fig. 1B). Loss of NF1 function releases the negative regulation of RAS, resulting in increased signaling through downstream pathways, including the PI3K/AKT and the mitogen-activated protein kinase (MAPK) signal transduction cascades (Fig. 1B). The *NF1* gene was first described in the context of the familial cancer syndrome neurofibromatosis type I (occurrence: 1 in 3,500), a disorder characterized by inactivating mutations in *NF1* leading to multiple neurofibromas, hyperpigmented macules of the skin (café-au-lait macules), freckling, and iris hamartomas (Lisch nodules; ref. 8). Patients with NF1 typically show an increased cancer incidence and a reduced lifespan, with tumor development occurring following the acquisition of secondary

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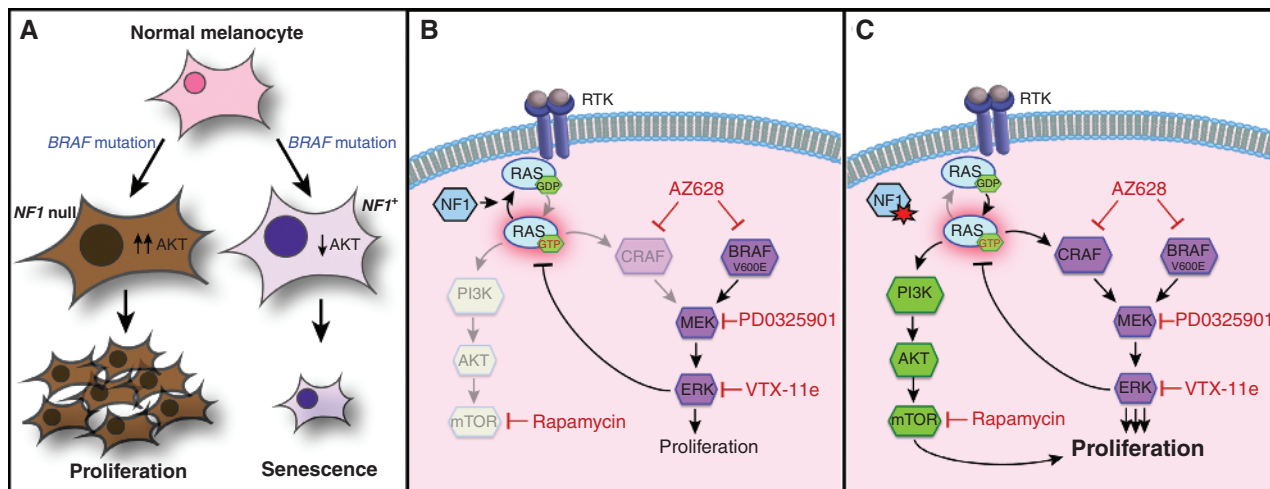


Figure 1. The putative role of NF1 loss in melanoma development and BRAF inhibitor resistance. **A**, loss of NF1 cooperates with mutant BRAF by increasing PI3K/AKT signaling and preventing entry into OIS. **B**, in normal cells NF1 suppresses RAS signaling by increasing its accumulation in the Ras-GDP (inactive state). **C**, when NF1 function is lost, Ras signaling increases and limits the response of BRAF-mutant melanoma cells to the BRAF inhibitor PLX4720 by increasing CRAF and PI3K/AKT signaling. BRAF-mutant/NF1-null cells/tumors show sensitivity to the pan-RAF inhibitor AZ628, the extracellular signal-regulated kinase (ERK) inhibitor VTX11e, and the combination of a MAP-ERK kinase (MEK) + mTOR inhibitor (PD0325901 and rapamycin).

somatic mutations in *NF1* (leading to its homozygous inactivation). Despite pigmentation defects being an important clinical characteristic of *NF1* patients, increases in melanoma incidence have not been reported and the pigmented lesions that arise are not accompanied by increased melanocyte proliferation (9, 10). Café-au-lait macules, which present as large areas of skin pigmentation, result from the increased accumulation of melanosomes in skin melanocytes and keratinocytes. Similarly, freckles are areas of increased skin pigmentation that also occur independently of melanocyte proliferation. In a transgenic mouse model of *Nf1* haploinsufficiency (+/-), neurofibromin regulates the KIT-MITF signaling axis during melanocyte development (9). Melanocytes derived from *Nf1*^{+/-} mice exhibit higher basal MAPK pathway activity than their wild-type counterparts and show increased expression of melanin synthesis genes (9). Likewise, melanocytes from *NF1* patients have higher melanin levels and a higher level of tyrosine hydroxylase activity than do those derived from normal controls.

Although the link between *NF1* loss and melanocyte dysregulation is well established, a role for *NF1* inactivation in melanoma development has never been shown. In their study, Maertens and colleagues (5) began investigating the influence of *NF1* loss upon signaling pathways implicated in the escape from RAF-induced OIS. In cells that retained *NF1* function, RAF activation suppressed RAS and AKT signaling, an effect associated with growth inhibition. In contrast, induction of RAF in *Nf1*-null cells did not inhibit RAS and AKT signaling and proliferation were both maintained (ref. 5; Fig. 1B). The authors next asked whether mutant *BRAF* and *NF1* loss cooperated to drive melanoma development *in vivo*. To explore this hypothesis, a transgenic model was developed in which mice carrying a conditional inactivating mutation in *Nf1* were crossed with those harboring a conditional activating mutation in *Braf*. Significant increases in the incidence of melanoma development were noted in mice harboring mutant *Braf* and *Nf1* loss, compared with those with mutant *Braf* alone (melanoma incidence: 57% vs. 22%,

respectively). A link between melanoma development and escape from BRAF-mediated OIS was suggested by the increased PI3K/AKT signaling and reduced levels of SA-β-Gal staining observed in tumors lacking *Nf1* (5). These data confirm the growing body of evidence implicating PI3K/AKT signaling in BRAF-driven melanomagenesis and suggest the existence of alternate routes to malignant transformation for melanocytes (4).

Recent years have seen major progress in the therapeutic management of disseminated melanoma, with BRAF inhibitors (such as dabrafenib and vemurafenib) showing good levels of tumor regression and increased progression-free survival in patients whose melanomas harbor V600 position mutations in *BRAF* (11, 12). Despite these successes, only half of these patients showed objective responses to therapy, and the median progression-free survival was less than 7 months (11). This heterogeneity in response and the ultimate development of resistance in nearly all patients have been attributed to multiple underlying mechanisms, including BRAF splice forms, PTEN loss, increased receptor tyrosine kinase (RTK) signaling, COT activity, and the acquisition of *NRAS* and *MAP-ERK kinase (MEK)* mutations (13).

Despite reports of a number of potential BRAF inhibitor resistance mechanisms, relatively few unbiased screens have been conducted to identify novel mechanisms of therapeutic escape. To address this, Whittaker and colleagues (6) conducted a high-throughput assay in which a BRAF inhibitor-sensitive melanoma cell line was targeted with a library of 90,000 shRNAs specific for 16,600 genes. Among 31 candidate genes, *NF1* was identified as the top-ranking hit whose knockdown abrogated the growth inhibitory effects of the BRAF inhibitor PLX4720. As expected, the effects of *NF1* loss upon PLX4720 resistance were mediated through the derepression of RAS signaling leading to CRAF-mediated activation of the MAPK pathway (5, 6). These observations agree with previous reports implicating RAS and CRAF in acquired BRAF inhibitor resistance and confirm the central role of the MAPK signaling pathway in the escape from BRAF inhibitor therapy (14). Although

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clinical trials have already shown the use of combining BRAF and MEK inhibitors in patients with melanoma, these may not be effective for every *BRAF*-mutant melanoma genotype (15). In melanoma cell lines harboring mutant *BRAF/NF1*, the BRAF + MEK (PLX4720 + AZD6244) inhibitor combination had little effect upon either MAPK signaling or cell growth, with more impressive results seen following treatment with either a pan-RAF inhibitor (AZ628) or an extracellular signal-regulated kinase (ERK) inhibitor (VTX-11e; ref. 6; Fig. 1C). The lack of MEK inhibitor efficacy in this context is not easily explained, but could be a consequence of either limited drug potency (other MEK inhibitors could be more effective) or differences in feedback inhibition seen following MAPK pathway targeting at different nodes. In the transgenic *Braf/Nf1* mouse model, limited antitumor responses were observed following single-agent MEK inhibition (PD0325901), with enhanced effects seen following combination with the mTOR inhibitor rapamycin (ref. 5; Fig. 1C). Although more work is needed to establish the best combination therapeutic strategy for *BRAF/NF1*-mutant melanomas, the data so far suggest that this genetic subgroup may require similar approaches to those under investigation for *RAS*-mutant melanoma.

The potential clinical significance of these findings was confirmed in a panel of melanoma cell lines and tumors (tissue microarray), in which NF1 expression was found to be reduced or absent in 36% and 18% of samples, respectively (5). However, protein expression assays may underestimate the prevalence of NF1 deficiency, as loss of NF1 function may also result from acquired mutations and altered posttranslational processing without lowering total protein levels. With this in mind, the percentage of patients whose melanomas show impaired NF1 function could be higher than what the above numbers suggest. Although defects in NF1 sometimes overlapped with *BRAF* mutation status, this was not universally exclusive and some co-occurrences with *NRAS* mutations were noted (6). Interestingly, loss of PTEN expression and impaired NF1 function were not mutually exclusive either, suggesting some nonoverlapping functions between the 2 tumor suppressors. An additional analysis of a cohort of 121 melanoma specimens and cell lines identified potentially pathogenic *NF1* mutations in 25% (5/21) of samples that were wild-type for both *BRAF* and *NRAS* (6).

The study of samples from patients with melanoma who received BRAF inhibitor therapy suggested a role for NF1 loss in both intrinsic and acquired drug resistance. Whole-exome sequencing of pre- and posttreatment specimens conducted by Whittaker and colleagues (6) identified 4 patients with *NF1* mutations, with one individual harboring a deleterious nonsense mutation (R2450*) in *NF1* in both pre- and post-drug treatment specimens. A second series of 5 matched pre- and posttreatment melanoma samples analyzed for neurofibromin expression by immunohistochemistry showed that 2 of 5 expressed little or no protein before BRAF inhibitor treatment (5). In the remaining 2 of 3 with initial neurofibromin expression, vemurafenib treatment was associated with loss of protein expression.

As further discoveries in melanoma genomics and proteomics are made, a complex and heterogeneous field of personalized medicine is evolving. It is important to keep in mind that not all mutations and activated pathways identified in the laboratory or in whole-genome studies will actually be causative in patients'

disease. The identification of NF1 as a modifier of BRAF inhibitor response seems supported in both laboratory and patient specimen data but still requires prospective clinical evaluation. Whether patients with *BRAF*-mutant/*NF1*-deficient melanoma will derive more benefit with a pan-RAF, ERK, or MEK + mTOR inhibitor combination remains to be answered. The concept of *BRAF*^{V600} mutational status in melanoma as the sole biomarker for BRAF-targeted therapy is quickly becoming obsolete. Although the phase III studies underway to determine whether BRAF/MEK inhibitor combination therapy is better than BRAF inhibitor monotherapy (NCT01584648; NCT01689519) are important for drug approval, they do not directly address this issue of the complex heterogeneity of patients with *BRAF*-mutant melanoma. An adaptive strategy is needed to integrate real-time genomic and proteomic data for an individualized approach to optimize targeted therapy. It is hoped that greater strides will be achieved by prospectively selecting combination therapies based on broader molecular profiles to optimize a personalized approach in patients with melanoma.

Disclosure of Potential Conflicts of Interest

G.T. Gibney is a consultant/advisory board member of Genentech/Roche. No potential conflicts of interest were disclosed by the other author.

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REFERENCES

- Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. *Nature* 2002;417:949–54.
- Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, et al. BRAFV600E-associated senescence-like cell cycle arrest of human naevi. *Nature* 2005;436:720–4.
- Madhunapantula SV, Robertson GP. The PTEN-AKT3 signaling cascade as a therapeutic target in melanoma. *Pigment Cell Melanoma Res* 2009;22:400–19.
- Vredeveld LC, Possik PA, Smit MA, Meissl K, Michaloglou C, Horlings HM, et al. Abrogation of BRAFV600E-induced senescence by PI3K pathway activation contributes to melanomagenesis. *Genes Dev* 2012;26:1055–69.
- Maertens O, Johnson B, Hollstein P, Frederick DT, Cooper ZA, Mesaien L, et al. Elucidating distinct roles for *NF1* in melanomagenesis. *Cancer Discov* 2013;3:338–49.
- Whittaker SR, Theurillat J-P, Van Allen E, Wagle N, Hsiao J, Cowley GS, et al. A genome-scale RNA interference screen implicates NF1 loss in resistance to RAF inhibition. *Cancer Discov* 2013;3:350–62.
- Andersen LB, Fountain JW, Gutmann DH, Tarle SA, Glover TW, Dracopoli NC, et al. Mutations in the neurofibromatosis 1 gene in sporadic malignant melanoma cell lines. *Nat Genet* 1993;3:118–21.
- Cichowski K, Jacks T. NF1 tumor suppressor gene function: narrowing the GAP. *Cell* 2001;104:593–604.

9. Diwakar G, Zhang D, Jiang S, Hornyak TJ. Neurofibromin as a regulator of melanocyte development and differentiation. *J Cell Sci* 2008;121:167-77.
10. Wang X, Levin AM, Smolinski SE, Vigneau FD, Levin NK, Tainsky MA. Breast cancer and other neoplasms in women with neurofibromatosis type 1: A retrospective review of cases in the Detroit metropolitan area. *Am J Med Genet A* 2012;158A:3061-4.
11. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 2011;364:2507-18.
12. Hauschild A, Grob JJ, Demidov LV, Jouary T, Gutzmer R, Millward M, et al. Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *Lancet* 2012;380:358-65.
13. Fedorenko IV, Paraiso KH, Smalley KS. Acquired and intrinsic BRAF inhibitor resistance in BRAF V600E mutant melanoma. *Biochem Pharmacol* 2011;82:201-9.
14. Paraiso KH, Fedorenko IV, Cantini LP, Munko AC, Hall M, Sondak VK, et al. Recovery of phospho-ERK activity allows melanoma cells to escape from BRAF inhibitor therapy. *Br J Cancer* 2010;102:1724-30.
15. Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med* 2012;367:1694-703.

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