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# *NRAS* mutant melanoma: biological behavior and future strategies for therapeutic management

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## Abstract

The recent years have seen a significant shift in the expectations for the therapeutic management of disseminated melanoma. The clinical success of BRAF targeted therapy suggest that long-term disease control may one day be a reality for genetically defined sub-groups of melanoma patients. Despite this progress, few advances have been made in developing targeted therapeutic strategies for the 50% of patients whose melanomas are *BRAF* wild-type. The most significant sub-group of *BRAF* wild-type tumors is the 15–20% of all melanomas that harbor activating *NRAS* mutations. Emerging preclinical and clinical evidence suggests that *NRAS* mutant melanomas have patterns of signal transduction and biological behavior that is distinct from *BRAF* mutant melanomas. This overview will discuss the unique clinical and prognostic behavior of *NRAS* mutant melanoma and will summarize the emerging data on how *NRAS*-driven signaling networks can be translated into novel therapeutic strategies.

## Introduction

For many years, the prognosis for patients with disseminated melanoma has been poor. Responses to standard chemotherapy have been historically low and associated with median survival times of 6–10 months (1). While immunotherapy with interleukin-2 and ipilimumab provides more durable responses in approximately 10% of metastatic melanoma patients, there are no established biomarkers to select patients and serious toxicities are common (2). The recent years have seen significant shifts in our understanding of the drivers of malignant transformation. Cancer is now known to be a genetic disease, with defined tumor histologies often being uniquely dependent upon (or "addicted" to) the signaling activity of a restricted number of oncogenes. This knowledge, and the ability to target "druggable" oncogenes using small molecule inhibitors, has led to significant therapeutic breakthroughs in a number of cancers including the targeting of the Bcr-Abl fusion protein in chronic myeloid leukemia (CML), the ALK-EML4 fusion and mutant EGFR in non-small cell lung cancer (NSCLC) and c-KIT in gastrointestinal stromal tumors (GIST) (3–5). A major turning point in our understanding of melanoma biology was the 2002 discovery that the majority (>50%) of all cutaneous melanomas harbored activating mutations in the serine/threonine kinase BRAF (6). The identification of mutant *BRAF* as a *bona fide* oncogene in melanoma led to the rapid development of small molecule BRAF inhibitors (such as vemurafenib and dabrafenib),

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which were subsequently shown to increase the overall survival of patients with metastatic *BRAF* mutant melanoma (6–8). Following the FDA-approval of vemurafenib in August of 2011, attention has now turned to the 50% of melanoma patients whose tumors are *BRAF* wild-type, with the hope of making similar therapeutic breakthroughs. Among the cutaneous melanomas that lack *BRAF* mutations, 15–20% harbor mutations in the small GTPase *NRAS* (9). At present, little progress has been made in developing targeted therapy strategies for *NRAS* mutant melanoma. In this review we will discuss the biological and signaling characteristics that are unique to *NRAS* mutant melanomas and will outline possible future strategies for their therapeutic management.

## Ras proteins

Ras genes were first identified as encoding for the proteins that mediated the transforming activity of Rat sarcoma viruses. It is now known that three Ras family members, *NRAS*, *HRAS* (Harvey Rat Sarcoma Virus) and *KRAS* (Kirsten Rat sarcoma virus) are often mutated in human cancers, and that >20% of all tumors harbor activating mutations in one of their *RAS* genes (10). Ras proteins constitute a major family of low molecular weight plasma membrane-associated GTP-binding proteins. Within this super-family (which also includes Rab, Rho, and Arf), the Ras proteins primarily regulate growth, and function as molecular switches that link signals from receptor tyrosine kinases (RTKs) at the cell surface to transcription factors and cell cycle proteins in the nucleus (11, 12). Under physiological conditions, Ras proteins exist in either an active (GTP-bound) state or an inactive (GDP-bound) state. The exchange of GDP for GTP, and the switching of Ras to its activated state is catalyzed by a family of guanine nucleotide exchange factors (GEFs), such SOS1, SOS2 and RASGRF (10) (Figure 1), and is opposed by the GTPase stimulatory activity of Ras GTPase activating proteins (Ras-GAPs) (13)(Figure 1). In normal cells, Ras activation typically proceeds following the binding of ligand to its cognate RTK. This then leads to RTK autophosphorylation and dimerization, and the recruitment of adaptor molecules, such as GRB2. Signals from the activated RTK are relayed to Ras by the binding of Grb2 to the RTK at its SH2 domain. This then recruits SOS to the plasma membrane through binding of two SH3 domains, and SOS in turn catalyzes the GDP to GTP exchange on Ras (14) (Figure 1). Once activated, Ras recruits and stimulates a number of intracellular signaling pathways including the Raf/MEK/ERK mitogen activated protein kinase (MAPK) pathway, the phosphoinositide 3-kinase (PI3K)/AKT pathway, the Ral guanine nucleotide exchange factors (RAL-GEFs) such as Ral-GDS and phospholipase C epsilon (Figure 2) (15).

## Mutant *Ras* as an initiating melanoma oncogene

Despite much attention being focused upon *BRAF* mutant melanoma, *NRAS* was actually the first melanoma oncogene to be identified. In1984, a screen of melanoma cell lines for genes that possessed transforming properties identified activating mutations in *NRAS* in 4/30 samples (16). In a further observation, now highly pertinent to the use of BRAF inhibitors in melanoma, it was noted that mutant *Ras* expression was heterogeneous within metastatic lesions derived from the same patient (found only in 1/5 samples) (16). Currently, mutations in *NRAS*, *KRAS* and *HRAS* are known to be present in 20%, 2% and 1% of all melanomas, respectively (17). With regards to *NRAS*, the most common oncogenic change (>80% of all *NRAS* mutations) reported is a point mutation leading to the substitution of leucine to glutamine at position 61, with mutations at positions 12 and 13 occurring less frequently (9). Position 61 mutations also account for the majority of *HRAS* mutations in melanoma, whereas most *KRAS* mutations are at position 12 (http://www.sanger.ac.uk/genetics/CGP/cosmic/). From a mechanistic standpoint, *Ras* mutations at position 61 are associated with impaired GTPase activity and the locking of the Ras protein into its activated (GTP-associated) conformation. Mutations at positions 12 and 13 render Ras insensitive to the

physiological mechanisms of inactivation by the Ras-GAPs (9). Once mutated, Ras drives cellular transformation through a network of signal transduction pathways involved in growth, motility and survival (Figure 2). Although it is not clear why *NRAS* mutations are more frequent in melanoma compared to *HRAS* or *KRAS* mutations, there is some suggestion that *NRAS* may be overexpressed in melanocytes relative to other Ras isoforms. It is also possible that *NRAS* may activate different signaling pathways to *KRAS* and *HRAS*; an idea supported by the observation that *NRAS* has greater transforming activity than *KRAS* in experimental models of melanoma (18).

The available evidence suggests that *Ras* mutations are acquired at an early stage in the oncogenic process. Benign common nevi most commonly (>80%) harbor mutations in *BRAF*, with *NRAS* mutation being less frequent (~14%)(19). In contrast, congenital nevi (e.g. those that develop *in utero*, independently of UV exposure) do not possess *BRAF* mutations and instead frequently (81%) harbor *NRAS* mutations (20). Spitz nevi, large benign melanocytic lesions that are histologically indistinguishable from melanoma often (~11% of cases) exhibit concurrent amplification and mutation in *HRAS*, in the absence of significant proliferative capacity (21). Taken together, these data suggest that although *Ras* mutations are found in melanoma precursor lesions, these do not appear to initiate oncogenic transformation in isolation and require other co-operating genetic events. These ideas are supported by the observation that most nevi rarely progress to melanoma and typically exhibit signs of replicative senescence (22, 23).

Genetically engineered mouse models of melanoma driven through mutant *HRAS* or *NRAS* have suggested a role for concurrent loss or inactivation of the tumor suppressor $p16^{INK4A}$, deletion of p53 or exposure to ultraviolet light (24, 25). These studies are supported by the finding that *NRAS* mutant, but not *BRAF* mutant, melanoma is frequently associated with methylation of the $p16^{INK4A}$ promoter (26). Cell culture studies have confirmed the role of *NRAS* mutations in the growth of melanoma cell lines, with the siRNA mediated knockdown of *NRAS* found to inhibit growth associated with decreased expression of cyclins D1 and Cyclin E2 (27). At the same time it was noted that siRNA knockdown of *NRAS* reversed invasive capacity, an effect associated with the decreased expression of the cytoskeletal proteins paxillin, leupaxin and vinculin (27).

One other sub-group of melanomas with Ras-dependence is those with “low-activity” *BRAF* mutations, such as those found at positions 465, 463 and 596 (28). Cell lines with low activity *BRAF* mutations, show an impaired activation of MAPK signaling in isolated kinase assays and often harbor concurrent *NRAS* mutations at positions 12 and 13 (29). Unlike melanoma cell lines with *BRAF* V600E/D mutations, low activity mutant *BRAF* cell lines are sensitive to the growth inhibitory effects of Ras-neutralizing antibodies as well as cell death induced by the CRAF/multi-kinase inhibitor sorafenib (28, 30). It is therefore likely that strategies designed to target *Ras* mutant melanoma could also be effective in low activity *BRAF* mutant melanoma.

## Clinical and prognostic significance of *NRAS* mutations in melanoma

It is now accepted that melanoma is a diverse collection of melanocytic neoplasms whose biological behavior can be defined by their pattern of oncogenic mutations (31, 32). A recent histopathological analysis of a large series of melanoma specimens, grouped according to their *BRAF/NRAS* mutational status showed *BRAF*-mutated melanomas to possess morphological features that were distinct from tumors that were *BRAF* wild-type (31). It was found that *BRAF*-mutated melanomas had an increased tendency to upward migration and nest formation and gave rise to larger, rounded and more pigmented tumor cells. In contrast,

*NRAS* mutated melanomas were not found to exhibit these morphological and phenotypic characteristics (31).

The presence of an *NRAS* mutation in melanoma is also of prognostic significance. The typical patient with *NRAS* mutant melanoma tends to be older (>55 years of age) with a more chronic pattern of UV exposure than a patient with a *BRAF* mutant melanoma (33, 34). Overall, patients with *NRAS* mutant melanomas have been found to have thicker tumors at presentation. These tumors are typically located at the extremities and have greater rates of mitosis than *BRAF* mutant melanoma (33, 35). Despite *NRAS* mutant melanomas having lower rates of ulceration than *BRAF* mutant melanoma, overall survival for *NRAS* mutant melanoma patients is worse than their wild-type counterparts (33–35).

## Intracellular signaling in *NRAS* mutant melanoma

Although *NRAS* and *BRAF* mutant melanomas share a set of common signaling pathways, significant differences exist in how these are regulated. In normal human melanocytes, MAPK activation occurs following stimulation with growth factors such as stem cell factor (SCF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) (36) (Figure 3). Under these normal physiological conditions stimulation of the pathway occurs following the Ras-mediated activation of Raf (37). MAPK signaling in melanocytes proceeds through BRAF rather than CRAF (also called Raf-1), with Raf-isoform selectivity being determined through the concurrent growth factor mediated activation of adenylate cyclase (AC) (37, 38) (Figure 3). Increased AC activity leads to the accumulation of cyclic AMP (cAMP) and the activation of protein kinase A (PKA). This then inactivates CRAF via its direct phosphorylation at two inhibitory sites (S43 and S233) (39, 40) (Figure 3). The high frequency of *BRAF* mutations found in melanoma likely stems from the lineage-specific reliance of melanocytes upon BRAF signaling for their growth factor mediated MAPK activation.

Interestingly, BRAF is not required for MAPK activation in every melanoma, with *NRAS* mutant melanomas being instead reliant upon CRAF signaling (37) (Figure 3). CRAF-mediated MAPK signaling in *NRAS* mutant melanoma cells is dependent upon two parallel mechanisms; the inactivation of BRAF (permitting Raf isoform switching) and deregulation of PKA signaling (that prevents CRAF inactivation) (37). The inactivation of BRAF is achieved through a negative feedback mechanism in which ERK limits RAF signaling by phosphorylating it at S151, T401, S750 and T753 (41) (Figure 3). The S151 site in BRAF is close to the Ras-binding domain, and its phosphorylation appears to prevent the Ras/BRAF interaction (38). Phosphorylation of BRAF at T401, S750 and T753 disrupts the dimerization of BRAF with CRAF (41). The deregulation of PKA signaling occurs as a result of increased expression of the cAMP degrading enzyme phosphodiesterase IV (PDE4) that limits PKA activity and in turn prevents CRAF from being phosphorylated at its inhibitory sites (38) (Figure 3). The potential therapeutic implications of these findings were demonstrated by the ability of selective PDE4 inhibitors to prevent the growth and increase apoptosis in *NRAS* mutant melanoma cell lines (38).

Constitutive MAPK activity is known to be essential for melanoma initiation and progression (42). Signals through this pathway are responsible for the uncontrolled growth that characterizes melanoma, and occur via regulation of the G1 checkpoint through the increased expression of cyclin D1 and downregulation of the cell cycle inhibitor $p27^{KIP1}$ (43, 44). MAPK signaling also plays a critical role in melanoma survival through the phosphorylation (at S69) and proteasomal degradation of the pro-apoptotic protein BIM and via p90RSK-mediated effects upon BAD (45, 46).

In addition to the well-known role of MAPK signaling in melanomagenesis, *in vitro* and animal modeling studies have suggested that the PI3K/AKT cascade also plays a critical role in melanoma development (47, 48). Once activated at the plasma membrane, PI3K phosphorylates phosphotidylinositol-4,5, biphosphate (PIP2) to PIP3 which in turn activates the downstream kinases PDK1 and AKT. Once active, AKT promotes melanoma cell survival through direct phosphorylation of BAD and through the negative regulation of BIM expression via the phosphorylation and inactivation of FOXO3a (45, 49–51). Signals through the PI3K/AKT pathway are also critical for cell cycle entry, and regulate the G1 cell cycle checkpoint by phosphorylating and inactivating glycogen-3 synthase kinase (GSK3)-β, leading to modulation of cyclin D1 (52). PI3K/AKT signaling also has important downstream effects upon protein turnover and cell glucose metabolism via the regulation of the mTOR/S6K and GSK3β signaling pathways (53). Like the MAPK pathway, PI3K/AKT signaling is differentially regulated between *NRAS* and *BRAF* mutant melanoma, with *NRAS* mutant melanomas rarely harboring either mutations in, or silencing of the negative regulator of the PI3K pathway, phosphatase and tensin homolog (PTEN) (54, 55). Additionally, activating mutations in AKT3, which are sometimes found in *BRAF* mutant melanoma, have not been observed in *NRAS* mutant melanomas (56). Despite data implying that mutant *NRAS* should activate MAPK and PI3K/AKT signaling concurrently, a recent analysis of clinical melanoma specimens and cell lines, showed *NRAS* mutant melanomas to exhibit lower levels of constitutive AKT signaling than those with *BRAF* mutations (55). The significance of this observation is still under investigation.

Another family of less well-characterized mediators of mutant *NRAS* are the Ral-GEFs such as Ral-GDS, RGL1 and RGL2 (15). Ral signaling is known to play a role in cell growth through transcriptional activation of cyclin D1, and (in concert with AKT) in cell survival, through the regulation of BIM and FasL expression (15, 57). Increased Ral activity has been also implicated in cytoskeletal reorganization, associated with increased filopodia formation (57). Although there is little evidence of melanomas harboring Ral-GDS mutations, Ral activity appears to be required for the development of *NRAS* mutant melanoma (58–60). In animal xenograft models, shRNA knockdown of RalA was noted to delay the growth of human *NRAS* mutant melanoma cell lines (59). A recent analysis of the role of Raf, PI3K and Ral signaling in immortalized melanocytes revealed distinct requirements for each pathway in oncogenic transformation (60). Specifically, it was shown that whereas Raf and PI3K were important for overcoming oncogene-induced senescence and invasion, signaling through Ral was a prerequisite for anchorage independent growth (60). The requirement for multiple signaling pathways in the development of *NRAS* mutant melanoma development was demonstrated by the fact that no one individual signaling pathway fully recapitulated the transforming activity of oncogenic Ras (60).

## Therapeutic strategies for *NRAS* mutant melanoma

### Farnesyl transferase inhibitors

*NRAS* has so far proven to be intractable to conventional drug discovery. The main “Ras-directed” therapies to be investigated thus far have been the farnesyl transferase inhibitors (FTIs), a class of drugs designed to prevent the post-translational modification of Ras and its insertion into the plasma membrane (61). Although this strategy showed some preclinical promise, the clinical experience has been disappointing with serious off-target effects reported and few responses observed, even in tumors with high rates of Ras mutations (such as colorectal carcinoma) (61–63). The lack of success with FTIs is attributable to the fact that many critical cellular proteins are farnesylated in addition to Ras and the fact that alternative post-translational modifications exist that allow for membrane targeting of Ras in the absence of FT activity. In melanoma, a small phase II trial of FTIs in genetically unselected patients showed very little clinical activity. Other attempts to directly target Ras

have involved the downregulation of Ras protein expression using either antisense oligonucleotides or small interfering RNAs (siRNAs). Despite preclinical evidence that Ras knockdown can be achieved using siRNA, the approach has proven technically challenging in the clinical setting (27). Current approaches are now centered upon improved methods of siRNA delivery *in vivo*, using nanoparticle-based delivery systems (64).

## MEK inhibitors

Given the lack of success thus far in directly targeting *NRAS*, focus has instead shifted to targeting the critical signal transduction pathways that drive Ras-mediated transformation. Of these, targeting the MAPK pathway, using allosteric MEK inhibitors has received the most attention. In preclinical studies, melanoma cell lines with oncogenic *NRAS* showed variable response to MEK inhibitors, with some cell lines exhibiting high sensitivity and some demonstrating near-total resistance (43, 65). A recent high throughput genomic analysis, correlating cell line mutational status with drug response (the cancer cell line "encyclopedia"), demonstrated a link between the sensitivity of *NRAS* mutant melanoma cell lines to MEK inhibition and elevated expression of the aryl hydrocarbon receptor (AHR) (66). The dependence of *NRAS* mutant melanoma cell lines upon AHR receptor signaling was confirmed through shRNA knockdown and it was noted that MEK inhibition in these cell lines was associated with inhibition of AHR signaling function (66).

Although MEK inhibitors, such as CI-1040 and AZD6244 have been investigated in melanoma patients, most of the studies were performed prior to the era of routine genetic testing. In most of the published trials to date, patients were unselected and significant intratumoral phospho-ERK inhibition was not observed at the maximum tolerated doses (67). There were some notable exceptions with one *NRAS* mutant melanoma patient remaining on AZD6244 therapy for 3 cycles, associated with >70% tumor shrinkage and near-total inhibition of intratumoral phospho-ERK (67). Overall the results of clinical trials with early generation MEK inhibitors were disappointing (~10% objective response rate) and retrospective genotyping for the *NRAS/BRAF* mutations did predict for clinical benefit.

Enthusiasm for targeting MEK in *NRAS* mutant melanoma patients has received new attention with the clinical development of the third generation MEK inhibitors trametinib (GSK1120212) and MEK162 (ARRY-438162). Both trametinib and MEK162 are potent inhibitors of MEK1/2 with sustained MAPK pathway inhibition at clinically achievable doses (68, 69). Trametinib has now demonstrated strong activity in *BRAF* mutant melanoma patients treated in the phase III METRIC study (70). The overall response rate (ORR) with trametinib was 24% with a median progression free survival (mPFS) of 4.8 months, both significantly better than standard chemotherapy. Similarly, MEK162 has shown promising clinical activity (ORR 23%) in *BRAF* mutant melanoma patients in a recent phase II study (71). While these response rates are lower than the reported data for first line treatment with the *BRAF* inhibitors vemurafenib and dabrafenib, it is important to note that patients in both of the MEKi studies had received multiple prior therapies and perhaps had more aggressive disease biology.

The first prospective clinical data on *NRAS* mutant melanoma patients treated with targeted therapy was presented at ASCO 2012 (71). As part of a combined study of *BRAF* and *NRAS* mutant melanoma patients, 30 metastatic melanoma patients whose tumors harbored an *NRAS* mutation were enrolled and treated with MEK162. The ORR was 21% and the mPFS was 3.65 months. Further study in this patient population will be necessary to confirm its clinical activity in comparison to other standard therapies. While prospective data with trametinib in *NRAS* mutant melanoma patients is not available, early retrospective data from ongoing clinical studies suggests that trametinib may have activity in a subset of *NRAS* mutant melanoma patients (72).

## Combination therapy with MEK and PI3K pathway inhibitors

The evolving lesson of using small molecule BRAF inhibitors in patients with *BRAF* V600E mutant melanoma suggest that resistance will always limit responses to single agent therapy and that multi-drug combinations are necessary (8, 73–76). There is already good evidence from animal models of Ras-driven cancers that the simultaneous inhibition of MEK + PI3K leads to greater extent of tumor regression than either inhibitor alone (77). From a mechanistic standpoint, the MEK and PI3K/AKT pathways seem to converge at the level of cell survival, through the coregulation of the pro-apoptotic protein BAD, as well as the level of cap-dependent protein translation, through the translational repressor 4E-BP1 (78, 79). Similar findings have also been reported in xenograft studies of *NRAS* mutant human melanoma cell lines, with shRNA knockdown of BRAF + CRAF or BRAF + PI3K expression being found to delay and inhibit tumor formation (80). In melanoma cell lines where BRAF inhibitor resistance is mediated through an acquired *NRAS* mutation, the combination of a MEK inhibitor (trametinib) with a PI3K/mTOR inhibitor (GSK2126458) was noted to overcome drug resistance and inhibit cell survival (75).

It is not yet clear what the optimal combination of signal transduction inhibitors will be for *NRAS* mutant melanoma and even whether any one combination will be effective for all melanomas with oncogenic *NRAS*. Presently, there are 14 active or recently completed phase I clinical trials investigating the combination of PI3K pathway and MEK inhibitors (Table 1). Data presented on the dose escalation of the phase I study of BKM120 and trametinib was promising for tolerability and potential efficacy (81). A total 66 patients were treated at increasing doses of both drugs, reaching the maximum tolerated doses of 70mg daily for BKM120 and 1.5mg daily for trametinib. Of note, the dose of trametinib in phase III study of BRAF mutant patients (METRIC study) was 2mg daily (70). Enrollment focused on *RAF* and *RAS* mutant tumors, with 10 melanoma patients treated in total. While the strongest clinical activity was seen in *KRAS* mutant ovarian carcinoma patients (3 partial responses by RECIST criteria), there was tumor regression that did not meet RECIST criteria seen in 3 melanoma patients. Completed data on this study and others are eagerly awaited.

## Feedback inhibition and RTK signaling

The experience of targeted therapy in many cancer types has demonstrated that resistance can often occur through the derepression of feedback inhibition and compensatory upregulation of signaling through parallel pathways (82). Understanding and developing strategies that limit these complex adaptive signaling responses is likely to prove critical in the use of targeted therapy regimens for the long-term management of disease. The scale of this problem was recently indicated by an integrated genomic/proteomic study of triple negative breast cancer in which MEK inhibition led to the rapid remodeling the RTK kinome associated with increased expression of multiple RTKs (83). Mechanistically, it was observed that the short-term inhibition of MAPK signaling decreased the expression c-Myc that in turn initiated autocrine signaling through HER2, RON, Axl, VEGFR and PDGFRβ (83). The drug-induced increase in RTK signaling allowed the cells to escape from MEK inhibitor therapy and could be reversed through the combination of a MEK inhibitor with the pan-RTK inhibitor sorafenib (83).

Compensatory signaling of a similar nature was also observed following the siRNA knockdown of RAS, with recent studies showing that inhibition of *KRAS* in colorectal carcinoma led to a rebound increase in PI3K signaling (84). Mechanistically this appeared to result from a suppression of MEK-mediated TORC1 activity leading to derepression of IRS-1 and increased AKT signaling mediated through insulin like growth factor receptor (IGFR1) (84). Although not yet studied in the context of *NRAS* mutant melanoma, functional IGF-1R autocrine signaling loops are known to be present in both melanoma cell lines and

tumor specimens, raising the possibility these may contribute to the escape from MEK inhibitor therapy (85). A possible role for rebound IGF-1R signaling in the *de novo* resistance of a subset of *BRAF* mutant melanoma cells to MEK inhibition has already been demonstrated (86). In this instance, MEK inhibitor sensitivity was restored through combination with either a TORC1/2 inhibitor or an IGF-1R inhibitor (86). In addition to IGF-1R, comprehensive phospho-proteomic screening of melanoma cell lines revealed a diverse array of RTKs to be expressed including TYRO3, Axl, MERTK, EPHB2, MET, IGFR1, EGFR, KIT, HER3 and HER4 (85, 87). The potential role of these RTKs in *NRAS* mutant melanoma, and their involvement in adaptive signaling responses following kinase inhibition remains to be determined. Of the RTKs expressed in melanoma, the TAM family (Tyro3, Axl, MER) receptor kinase Axl was noted to be overrepresented in *NRAS* mutant melanoma (87, 88). In the majority of *NRAS* mutant cell lines examined, Axl was activated through a Gas6-driven autocrine loop and was linked to activation of AKT signaling (88). Functional studies indicated that Axl signaling was required for the invasive behavior of *NRAS* mutant melanoma cell lines (88).

While inhibition of the PI3K pathway downstream of RTK signaling may be one approach to disrupt cellular adaptation to RAF and MEK inhibition, preclinical and early clinical data is promising for direct inhibition of RTKs and other tyrosine kinases Recent preclinical work with RAF-265, an inhibitor of BRAF, CRAF, VEGFR2, PDGFR, and c-Kit, has shown induction of tumor regression in *BRAF* wild-type melanoma xenografts, including one specimen with a Q61 mutation in *NRAS* (89). RAF-265 is now being studied in combination with MEK162 in patients with *BRAF* or *RAS* mutant tumors, including *NRAS* mutant melanoma (NCT01352273). A similar concept is being explored with the combination of pazopanib (VEGFR, PDGFR, c-Kit and FGFR inhibitor) with trametinib in a phase I study (NCT01438554).

Recent data on broad tyrosine kinase inhibition has also been presented in metastatic melanoma patients treated with the combination of ARQ 197, a specific c-Met inhibitor, and sorafenib, an inhibitor of VEGFR, PDGFR, c-Kit, and RAF (90). In this dose-escalation study, a total of 16 melanoma patients were treated at the recommended phase II dose level. The ORR was 25% with a mPFS of 5.3 months. Interestingly, half of the patients in this cohort had *NRAS* mutant melanoma. Efficacy data showed one complete response and one partial response in this subgroup, along with an overall mPFS of 9.2 months. These findings suggest that tyrosine kinase inhibition may prove to be valuable therapeutic strategy in *NRAS* mutant melanoma and further study is warranted.

## *NRAS* mutations as mechanism of therapeutic escape from BRAF inhibitor therapy: mutational heterogeneity in melanoma?

Despite the increases in both progression-free and overall survival seen in *BRAF* mutant melanoma patients receiving vemurafenib therapy, resistance occurred in nearly every case (8, 73). In a number of instances therapeutic escape was associated with the acquisition of position 61 mutations in *NRAS* that seemed to be lacking in the original tumor (91). This phenomenon was also reported in cell culture studies, with the emergence of *RAS* mutations being noted in *BRAF* mutant melanoma cell lines following chronic vemurafenib/dabrafenib treatment (75, 92, 93). Insights into the potential mechanism underlying this apparent mutational switch came from a recent study into the role of acquired *KRAS* mutations in the resistance of colorectal carcinoma to EGFR inhibition (94). Using clinical specimens and mathematical modeling, it was demonstrated that low-levels of mutant *KRAS* pre-existed within ostensibly *KRAS* wild-type tumors, and that EGFR blockade led to the expansion of these sub-clones (94).

Although *BRAF* and *NRAS* mutations are mutually exclusive at the single melanoma cell level there is evidence that a minor fraction of patients have melanomas containing co-existent clones with different oncogenic mutations. At least two independent studies have reported the presence of *BRAF* mutant and *NRAS* mutant cells within the same melanoma specimen (95, 96). If confirmed in a larger patient cohort, the issue of mutational polyclonality could have important clinical implications, particularly in light of the overwhelming pre-clinical evidence that BRAF inhibitors confers a growth and survival advantage to *NRAS*-mutant melanoma cells (97–99).

The paradoxical activation of MAPK signaling observed in *Ras* mutant cells following BRAF inhibitor treatment occurs following the increased formation of Raf dimers consisting of either CRAF homodimer pairs or dimers between drug-bound BRAF and CRAF (99, 100) (Figure 4). Once formed, the dimers activate downstream MEK signaling. The requirement for dimer formation in the paradoxical activation of MAPK signaling was confirmed by the lack of MAPK activation seen in BRAF inhibitor treated *Ras* mutant cell lines transfected with dimerization-deficient isoforms of Raf (99, 101). Other studies have provided evidence that the BRAF-inhibitor-mediated paradoxical MAPK activation also proceeds via upstream Ras signaling through a mechanism involving the disruption KRAS and NRAS nanoclustering at the cell membrane (102). Intriguingly, it was noted that although Ras nanoclustering promoted Raf signaling, this unexpectedly led to an inhibition of AKT signaling (102). From a clinical standpoint, the ability of BRAF inhibitors to paradoxically stimulate the growth of *Ras* mutant cells underlies the emergence of *HRAS*-mutant squamous cell carcinomas, commonly seen in patients on vemurafenib/trametinib therapy (103, 104). There is evidence that BRAF inhibitor therapy may also lead to the development of new, *BRAF* wild-type primary melanomas (of which 1/12 was noted to be *NRAS* mutant) (105). Together these findings underscore the need for the future development of highly sensitive *BRAF/NRAS* mutational testing protocols that gives a systemic readout of mutational status prior to the initiation of BRAF inhibitor therapy.

### NRAS mutations as a predictor of immune therapy response

Despite the current lack of effective targeted therapies for *NRAS* mutant melanoma, there is some evidence that *NRAS* mutational status may predict for response to other therapies. A recent retrospective analysis of patients treated with high dose interleukin (IL)-2 demonstrated that the majority of the responders were *NRAS* mutant and that patients with either *BRAF* mutant or *BRAF/NRAS* wild-type melanoma were less likely to respond (106). It is not yet clear whether *BRAF* or *NRAS* mutational status predicts for better responses in melanoma patients receiving the anti-CTLA-4 antibody ipilimumab or anti-PD-1 antibodies. In the only published analysis to date, the objective response rate of melanoma patients on ipilimumab therapy was noted to be 30% and 33% for those with and without *BRAF* mutations, respectively (107). A further breakdown of the *BRAF* wild-type patients into those with *NRAS* mutations was not available.

## Future strategies

While the current immunotherapeutic agents may offer some hope to *NRAS* mutant melanoma patients, these therapies are not mutation specific, are associated with modest response rates, and carry the risk of significant toxicities. Many targeted strategies are now being evaluated for *NRAS* mutant melanoma, although these tumors appear to be more heterogeneous than those with *BRAF* mutations. It is therefore likely that multiple strategies may have to be developed for *NRAS* mutant melanoma in concert with more sophisticated mutational screening. So far, the most promising data from clinical investigations are with MEK inhibition. However, the relatively short progression free survival indicates that either combination strategies or other targeted approaches will be necessary to achieve more

clinically important disease responses. Further prospective biomarker-driven investigations will be crucial for PI3K pathway, RTK, and other targeted therapeutic approaches.

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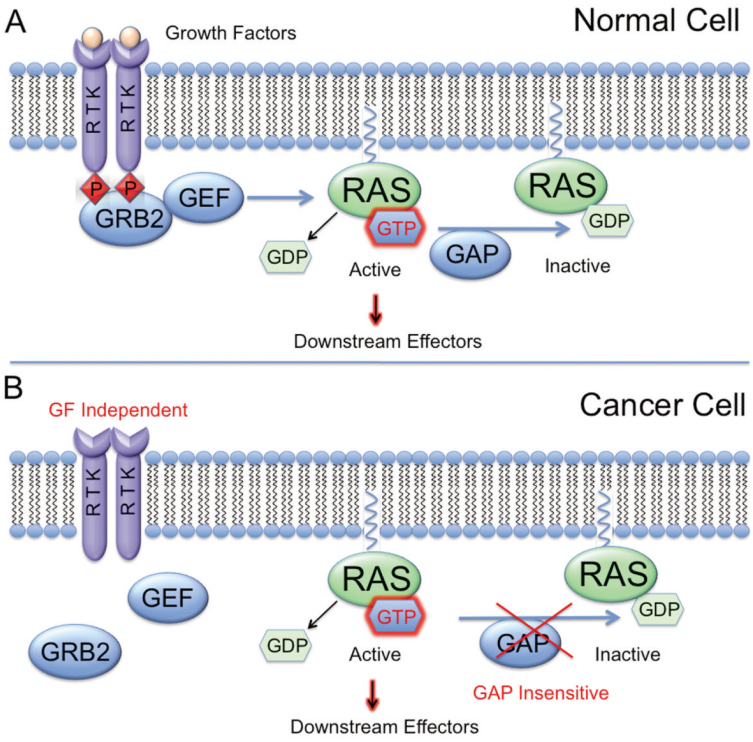


**Figure 1. Mechanisms of Ras activation in normal and cancer cells**

**A.** Upon RTK stimulation, GRB2 adaptor molecules facilitate signal transduction to Ras via guanine nucleotide exchange factors (GEFs). GEFs accelerate GDP release by Ras, allowing GTP binding and activation. The activating effects of GEFs are opposed by GAPs which promote a rapid rate of GTP hydrolysis, returning Ras to its inactive state. **B.** While Ras activation is dependent on growth factor signaling in normal cells, mutations in oncogenic Ras "lock" the GTPase in an active, GTP-bound conformation and render it insensitive to inactivation by GAPs.

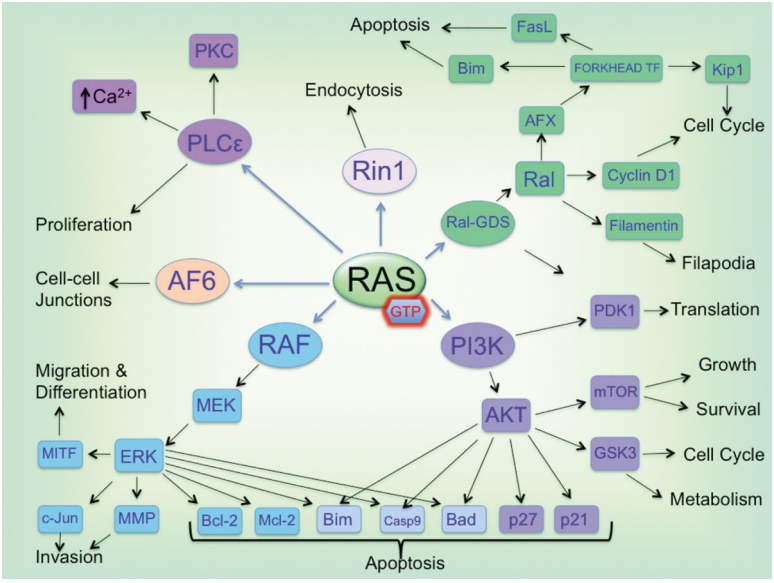


**Figure 2. Downstream mediators of Ras signaling**

Activated Ras can stimulate a wide array of downstream effectors, mediating many cellular processes such as proliferation, survival, invasion, endocytosis, cell-cell signaling and differentiation.

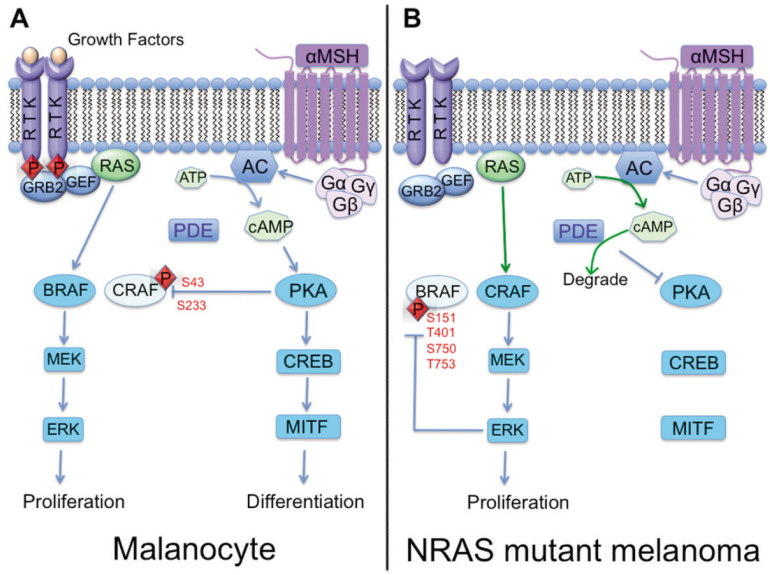


**Figure 3. Raf isoform switching in *NRAS* mutant melanoma**

**A.** In normal melanocytes, cyclic AMP pathway activation promotes PKA-mediated inhibition of CRAF. Therefore MAPK signaling proceeds through BRAF rather than CRAF in these cells (left panel). **B.** In *NRAS* mutant melanoma, inhibition of PKA signaling due to enzymatic activity of phosphodiesterase (PDE) and negative feedback inhibition of BRAF due to activation of the MAPK pathway promotes CRAF-mediated MAPK signaling instead (right panel).

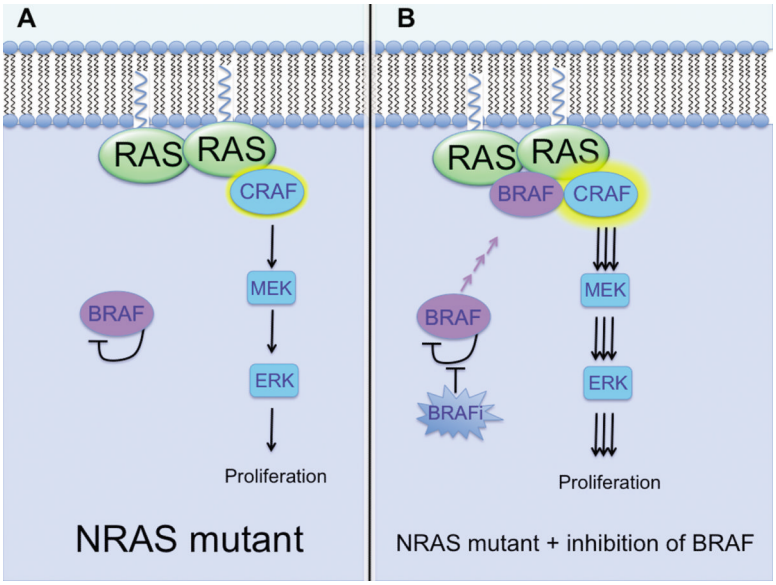


**Figure 4. Transactivation of Raf signaling by BRAF inhibitors in *Ras* mutant melanoma**

**A.** Typically, *NRAS* mutant melanomas rely on CRAF-mediated MAPK activation, with BRAF locked into an inactive conformation. **B.** BRAF inhibitors relieve the auto-inhibition of BRAF leading to its recruitment to the plasma membrane. This then leads to the dimerization of BRAF and CRAF leading to enhanced MAPK pathway signaling and proliferation.

## Table 1

Active or recently completed phase I studies with combination MEK and PI3K pathway inhibitors.

| **PI3K inhibitor** | **MEK inhibitor** | **Clinical Trial Number** | **Status** |
|---|---|---|---|
| BKM120 | MEK162 | NCT01363232 | Recruiting |
| BKM120 | Trametinib | NCT01155453 | Recruiting |
| BAY86-9766 | BAY90-6946 | NCT01392521 | Recruiting |
| BYL719 | MEK162 | NCT01449058 | Recruiting |
| GDC-0941 | GDC-0973 | NCT00996892 | Recruiting |
| GSK2126458 | Trametinib | NCT01248858 | Recruiting |
| **Dual PI3K/mTOR inhibitor** | **MEK inhibitor** | **Clinical Trial Number** | **Status** |
| BEZ235 | MEK162 | NCT01337765 | Recruiting |
| SAR245409 | MSC1936369B | NCT01390818 | Recruiting |
| PF-04691502 | PD-0325901 | NCT01347866 | Recruiting |
| **AKT inhibitor** | **MEK inhibitor** | **Clinical Trial Number** | **Status** |
| GSK2110183 | Trametinib | NCT01476137 | Recruiting |
| GSK2141795 | Trametinib | NCT01138085 | Completed |
| MK-2206 | AZD6244 | NCT01510444 | Recruiting |
| GDC-0068 | GDC-0973 | NCT01562275 | Recruiting |
| **mTOR inhibitor** | **MEK inhibitor** | **Clinical Trial Number** | **Status** |
| Temsirolimus | AZD6244 | NCT01166126 | Active, not recruiting |

* www.clinicaltrials.gov accessed July 7, 2012.