Chapter 9
Moving Forward: Making BRAF-Targeted Therapy Better

Keith T. Flaherty

Abstract For half of the advanced melanoma population, selective BRAF inhibitor therapy has transformed the natural history of disease and provided a platform for developing molecularly targeted therapy combinations. The clinical utility of vemurafenib, FDA approved BRAF inhibitor, has been validated by another potent and selective agent, dabrafenib. However, two clinical limitations of BRAF inhibitor therapy frame the problem for the melanoma field: *de novo* and acquired resistance. Insights into the mechanisms underlying both of these phenomena have set the stage for clinical investigation of several novel BRAF inhibitor based combination therapies. Foremost among them is the combination of a MEK inhibitor with BRAF inhibitor. Preliminary clinical evidence suggests that this combination may supplant single agent BRAF inhibitor therapy in the near future as the standard approach for metastatic patients. Yet resistance remains a challenge and strategies to target non-MAP kinase pathway dependent mechanisms are needed. This chapter will outline the preclinical evidence that supports the categorization of resistance mechanisms and the framework for clinical investigation of novel combination therapies.

Keywords Melanoma · BRAF · Resistance · Receptor tyrosine kinases · PI3K pathway · Cyclin dependent kinases · Apoptosis

9.1 Introduction

Selective BRAF inhibitors induce tumor regression in approximately 90% in patients with activating *BRAF* mutations that harbor the V600 position, with complete responses in 5% [1, 2]. Disease control is achieved for 6–7 months, on average. However, responding patients relapse as quickly as 2 months after the first evi-

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dence of tumor regression and a small subset of patients remain progression-free for more than 2 years [3]. Early clinical studies that incorporated early assessment of metabolic response (fluorodeoxyglucose positron emission tomography) suggest that all patients have at least metabolic responses to therapy within the first several weeks [4]. Based on this data, it would appear that BRAF inhibitor therapy is able to impact all tumors, but to a highly variable degree with regard to magnitude of initial effect. Similarly, the time to emergence of resistance is highly variable. These clinical observations give rise to two question that chapter will attempt to address:

1. what cell survival mechanisms underlie survival of some *BRAF* mutant tumors?
2. how do melanoma cells restore proliferation in the face of ongoing BRAF inhibition?

Addressing these questions will explain the rationale for the BRAF inhibitor-based combination therapy regimens that are currently being pursued clinically.

9.2 Genetic Complexity

BRAF mutant melanomas vary significantly with regard to the number of somatic genetic alterations that co-occur with *BRAF* mutations [5]. Typically arising on intermittently sun-unexposed skin, many of these tumors lack the very large number of cytosine to thymidine mutations, thought to derive from ultraviolet radiation, that can be found in melanomas that arise on chronically sun-exposed skin. Additionally, several of the oncogenic pathways that are known to contribute to melanoma formation in some instances are genetically normal in a distinct subset of *BRAF* mutant melanomas. Two examples of tumor suppressor genes that are commonly inactivated through mutation or deletion are *CDKN2A* and *PTEN*. Amongst *BRAF* mutant melanoma cell lines, those that harbor *PTEN* loss are more resistant to BRAF inhibitors than cell lines that lack of these abnormalities [6, 7]. Preliminary analysis of patient tumor samples from a subset of participants in phase II trials of vemurafenib and dabrafenib appears to confirm this association, and suggests that CDKN2A loss is also associated with worse outcome [8]. Conversely, those patients in whom *CDKN2A* and *PTEN* are wild-type, are amongst those who achieve the most long-lasting responses. These observations raise a very simple hypothesis: lesser degree of genetic complexity is associated with greater response and duration of response. With relatively long-term follow-up of patients treated on phase II and phase III trials with vemurafenib and dabrafenib and pretreatment tumor samples being available for the vast majority of patients, this can be readily confirmed using deep sequencing methods to characterize genetic alterations across all expressed regions of the genome. A corollary to this hypothesis that patients receive BRAF inhibitor therapy in the metastatic setting would be more likely to achieve initial and long-lasting responses if were treated when there is less, rather than more burden of disease. Subset analyses from vemurafenib and dabrafenib trials support this hypothesis [1, 2].

9.3 MAP Kinase Pathway-Dependent Resistance

Following 2 weeks of treatment with vemurafenib, analysis of tumor biopsy specimens reveals profound but incomplete inhibition of ERK activation/phosphorylation [9]. In vitro, exposure of *BRAF* mutant melanoma cell lines to a selective BRAF inhibitor at concentrations that are comparable to those achieved in human plasma similarly results in incomplete inhibition of ERK [10]. The addition of a MEK inhibitor to these same concentrations of BRAF inhibitor results in greater ERK suppression and significantly more cell death, thus validating that melanomas depend on the residual amount of MAP kinase pathway activity to survive.

At the time of disease progression on single agent BRAF inhibitor therapy, analysis of tumor biopsies patients treated with vemurafenib revealed that most tumors demonstrate reactivation of MAP kinase pathway, measured by immunohistochemistry for phosphorylated ERK [9]. As reviewed elsewhere in this volume, significant insight has been gained into the molecular mechanisms that account for this mechanism (Fig. 9.1). Taken together with the evidence of low level, persistent ERK activation early in the course of therapy, these finding suggests that *BRAF* mutant melanomas can survive with markedly reduced ERK signaling, but need to restore ERK activation to near-normal levels in order to proliferate. Both lines of evidence supported the clinical evaluation of BRAF/MEK combination therapy.

In a phase I/II clinical trial, dabrafenib and trametinib (a potent and selective MEK 1/2 inhibitor) were combined at a range of doses including the full single-agent

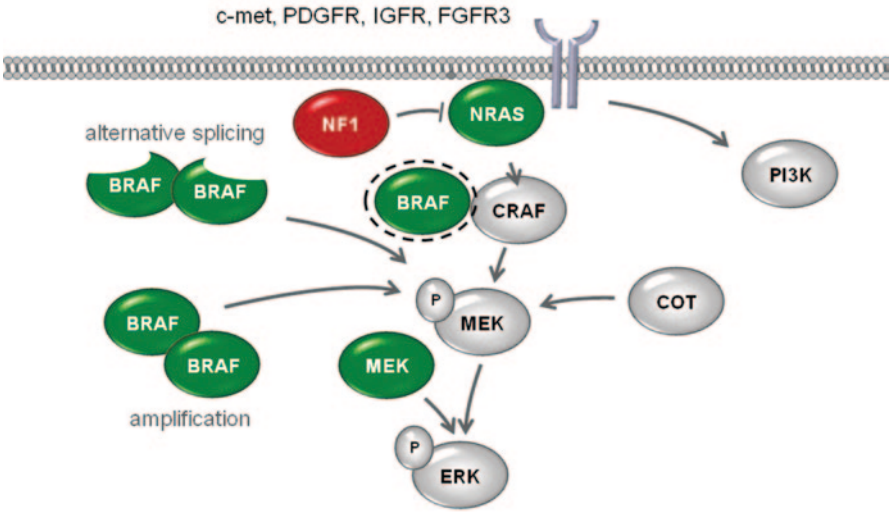

Fig. 9.1 BRAF inhibitor acquired resistance mechanisms c-met, PDGFR, IGFR, FGFR3

doses of both drugs [11]. Remarkably, the combination of both drugs at full doses produced a lower rate of dose limiting toxicity than either agent alone previously conducted phase I trials. This is thought to be a consequence of the MEK inhibitor counteracting inhibitor associated paradoxical activation, and BRAF inhibitor associated paradoxical activation attenuating MEK inhibitor related toxicities. With regard to efficacy, the dabrafenib/trametinib combination was associated with a significantly higher response rate, including a complete response rate of 10%, compared to single agent dabrafenib which was evaluated concurrently in a randomized phase II component of this trial. These results support the preclinical observation that suppression of residual ERK activation by co-administering a MEK inhibitor results in cell death. A similar outcome was observed when vemurafenib was combined with another experimental MEK inhibitor [12].

Duration of response was also significantly improved with the dabrafenib/trametinib combination compared to single agent dabrafenib, with a near doubling of median response duration from 5.6 to 10.5 months [11]. This confirmed that reactivation of ERK following BRAF inhibitor monotherapy was clinically relevant and that some mechanisms of restored MAP kinase pathway signaling can be successfully suppressed, if not prevented, with a MEK inhibitor. It is not currently known which *BRAF* mutant tumors are most susceptible to BRAF/MEK combination therapy compared to BRAF inhibitor monotherapy. A small subset of patients with disease progression on single-agent BRAF inhibitor therapy have persistently suppressed ERK phosphorylation and are presumed to depend on mechanisms outside of the MAP kinase pathway to drive tumor proliferation at that time [9]. For this group, BRAF/MEK combination therapy may add little to efficacy of single-agent BRAF inhibitor therapy. Additionally, there are no methods available to predict which tumors will emerge with *NRAS* mutations, splice variants of BRAF, *BRAF* amplification, or activating *MEK* mutations. And therefore, there is no basis by which one can tailor the use of BRAF/MEK combination therapy at the present time.

9.4 BRAF/MEK Resistance and ERK Inhibition

With most *BRAF* mutant tumors demonstrating evidence of restored ERK activation at the time of resistance to single agent BRAF inhibitor therapy, there is increased interest in exploring agents that block MAP kinase pathway signaling further downstream. As discussed above, MEK inhibition has been explored extensively preclinically and clinically. While the preclinical evidence suggests that MEK inhibitors can inhibit cell growth and induce cell death comparably to selective BRAF inhibitors in vitro and in vivo, the clinical evidence suggests that the antitumor effects achieved at tolerable doses are slightly less robust compared to BRAF inhibitors [13, 14]. This raises the issue of therapeutic index for each point of intervention in the pathway with regard to normal tissue dependencies and effects in tumor tissue relative to normal tissue.

BRAF and MEK inhibitors have a starkly different profile with regard to their impact on the MAP kinase pathway in *BRAF* mutant tumor tissue versus normal tissue as discussed previously [15]. Specifically, the BRAF inhibitors for which the

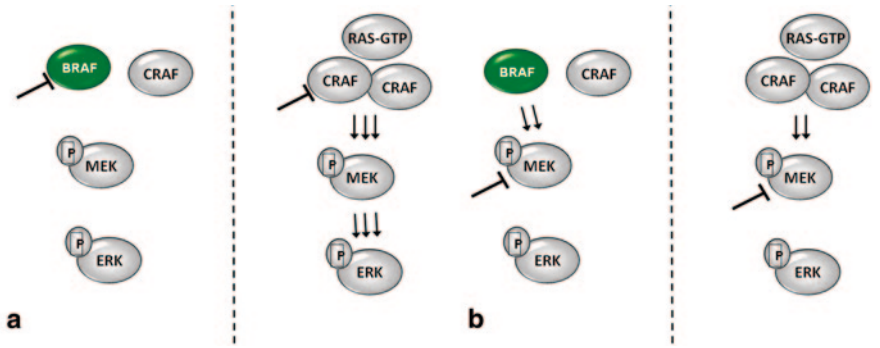

Fig. 9.2 Inhibition of MEK/ERK in the setting of BRAF mutation. **a** Activation of MEK/ERK in the setting of activated RAS. **b** Inhibition of MEK/ERK in the setting of activated RAS

most clinical experience exists do not appear capable of overcoming paradoxical activation and having a net inhibitory effect on the MAP kinase pathway at doses/exposures that can be safely administered to patients (Fig. 9.2a). Therefore, BRAF inhibitor related toxicities appear to occur as a consequence of paradoxical activation or via inhibition of kinases other than BRAF and CRAF [16]. MEK inhibitors, on the other hand, are associated with ERK inhibition in nearly all cell types analyzed to date, including cancer cell lines with a variety of oncogenic drivers as well as normal cell lines (Fig. 9.2b) [13, 17]. Therefore, MEK inhibitors are thought to mediate toxicity via inhibition of the MAP kinase pathway inhibition in normal tissues [10, 16]. Co-administration of BRAF and MEK inhibitors results in greater degrees of MAP kinase pathway suppression in tumor suppression, but less activation or inhibition of the pathway in normal tissues. The reduction in the rate of cutaneous squamous cell carcinomas and keratoacanthoma when BRAF and MEK inhibitors are co-administered is taken as clinical validation of these biochemical observations [18].

ERK inhibition represents a novel strategy that has not been fully explored (Fig. 9.3). Perhaps the most compelling current evidence in support of development of ERK inhibitors is the presence of activating *MEK* mutations at baseline and, in a larger subset of patients, following exposure to BRAF inhibitor therapy in some patients [9]. Preclinically, these mutations appear to confer resistance to the currently available allosteric MEK 1/2 inhibitors [19]. But, the known differences in feedback regulation of BRAF and MEK and the absence of such feedback loops that effect ERK, provides another rationale for considering this point of intervention in hopes that compensatory feedback mechanisms would not erode the pharmacodynamic effects of an ERK inhibitor as they would BRAF or MEK inhibitors [20].

Two ATP competitive, selective ERK 1/2 inhibitors have recently entered clinical development and extensive preclinical data is now available for one of these agents (SCH772984) [NCT01781429 & NCT01358331]. Like MEK inhibitors, SCH772984 is able to inhibit MAP kinase pathway signaling in both *BRAF* mutant and *RAS* mutant models [21]. But, more relevant to the issue of BRAF inhibitor resistance, this agent inhibits the MAP kinase pathway and cell proliferation in BRAF mutant melanoma cell lines with acquired or engineered resistance to BRAF inhibitors. Specifically, cell lines into which activating *RAS* mutation, the truncating

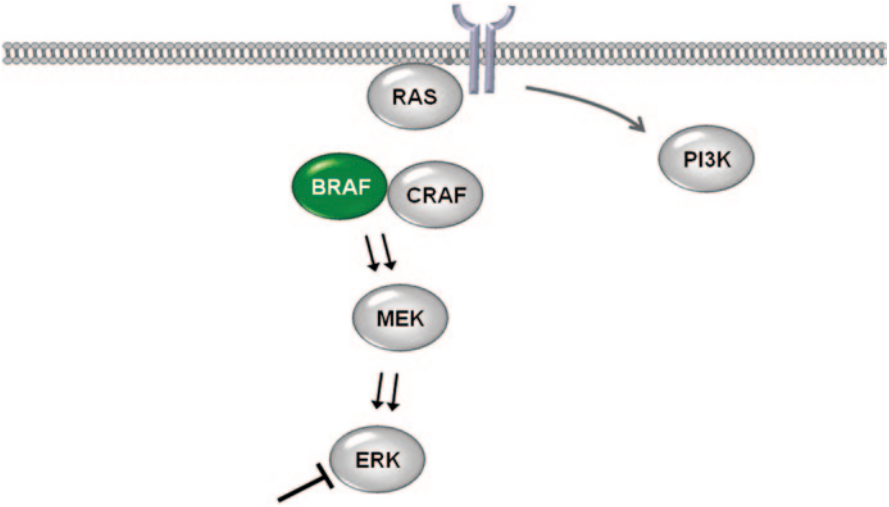

Fig. 9.3 Blocking downstream in the MAPK pathway: ERK inhibitors

BRAF splice variants, forced overexpression of BRAF, or activating *MEK* mutation are sensitive to single agent SCH772984, but not a BRAF or MEK inhibitor. And, in a xenograft established with a melanoma cell line with acquired resistance to concomitant BRAF and MEK inhibitor exposure, SCH772984 produces growth control as a single agent and in combination with continued BRAF/MEK combination therapy. These data point to a very clear potential application for selective ERK inhibitors in *BRAF* mutant/BRAF inhibitor refractory patients. But, it remains possible that ERK inhibition could have greater single-agent efficacy than either BRAF or MEK inhibitors in the BRAF inhibitor naïve setting. Or, an ERK inhibitor could be a more optimal component of a BRAF inhibitor-based combination approach, supplanting MEK inhibition. This possibility is particularly intriguing in light of the observation that concomitant administration of a BRAF inhibitor with a MEK inhibitor attenuates the frequency and severity of the typical MEK inhibitor associated toxicities: acneiform rash and diarrhea [11, 14]. Presumed to be a consequence of paradoxical activation associated with selected BRAF inhibitors, ERK inhibitors could benefit from this compensatory signaling effect in normal tissues as well.

9.5 Alternative Schedules

Alternative strategies to continuous suppression of the MAP kinase pathway with either BRAF inhibitor monotherapy or BRAF/MEK inhibitor combination therapy are scheduled interruption of therapy and pulsatile dosing.

The concept of introducing interruptions in the dosing of BRAF inhibitor therapy stems from the observed mechanisms of resistance that have been described in patient tumor specimens procured and characterized at the time of disease progression following initial response to BRAF inhibitor therapy. Knowing that over activation of oncogenic pathways has been previously demonstrated to induce senescence

(oncogene-induced senescence) or cell death in other contexts, investigators explored the consequences of withdrawal of vemurafenib following development of acquired resistance in vitro [21, 22]. They observed the hypothesized effect: in cells that restored MAP kinase pathway signaling in the face of chronic BRAF inhibition, withdrawal of the BRAF inhibitor resulted in hyperactivation of ERK, cell cycle arrest and cell death. They demonstrated that *BRAF* mutant melanoma cells require ERK activation within a certain range to survive and proliferate. Too little MAP kinase pathway output (in the setting of initial BRAF inhibitor therapy) impacts proliferation and survival and, based on these recent findings, too much pathway output is similarly toxic. This suggests the possibility of exposing cells to a BRAF inhibitor long enough for them to reset their ability to survive in the face of decreased MAP kinase pathway output, followed by withdrawal of the BRAF inhibitor, and then reinstitution of the BRAF inhibitor after MAP kinase pathway output reequilibrates. This strategy successfully postponed the outgrowth of resistant clones in vitro. Using a patient-derived xenograft from a *BRAF* mutant melanoma patient whose tumor acquired high-level *BRAF* amplification, these investigators demonstrated potential clinical relevance of this strategy by showing that interrupted schedule of administration resulted in longer duration of tumor control compared to continuous dosing. While this results support the clinical investigation of interrupted schedule of vemurafenib, dabrafenib, or other selected BRAF inhibitors, one wonders whether greater clinical impact could be achieved by investigating an interrupted schedule of administration for BRAF/MEK combination therapy.

Pulsatile dosing refers to the strategy of administering higher doses of therapy than can be safely administer continuously for a short duration of time. This is not a new concept in cancer therapeutics, as nearly all conventional cytotoxic chemotherapy are administered in this way. However, this strategy has not yet been explored in a widespread fashion with molecularly targeted therapies. Promising preclinical evidence has been generated for small molecule EGFR inhibitor therapy given in a pulsatile fashion in combination with conventional cytotoxic chemotherapy to patients with metastatic non-small cell lung cancer [23]. One might hypothesize that an even greater incremental benefit could be observed if one were to this strategy in an oncogene-defined subpopulation receiving the relevant oncogene targeted therapy. Preclinical evidence has been generated in support of this concept for abl kinase inhibitors in chronic myelogenous leukemia harboring BCR-ABL translocations [24]. This strategy has not yet been explored for BRAF inhibitor-based therapy in *BRAF* mutant melanoma, but certainly warrants consideration.

9.6 More Potent and Selective BRAF Inhibitors

Based on the evidence supporting greater initial antitumor effect in vitro, in vivo, and in patients when a MEK inhibitor is combined with a BRAF inhibitor, it remains possible that further optimization in the properties of a selected BRAF inhibitor could result in greater efficacy than is observed with vemurafenib or dabrafenib. With this motivation, LGX818 was selected for further development as a more potent and more selective BRAF inhibitor than the currently available agents [25]. Other than allowing for lower doses of drug, greater potency it is not intuitively

expected to produce improvement in therapeutic effect. However, careful analysis of several BRAF inhibitors and their capacity for inducing paradoxical activation has shown that, for most agents, activation can be overcome with sufficiently high concentrations of drug [15]. Some BRAF inhibitors are associated with a narrow range of concentrations at which initial activation is observed and then overcome. Vemurafenib, for example has a particularly broad range of doses over which these phenomena are observed, likely making it impossible to achieve sufficient drug concentrations in patients to overcome paradoxical activation LGX818, on the other hand, is several-fold more potent for V600E BRAF, and has a relatively narrow range of concentrations over which paradoxical activation can be induced and then overcome. Thus, it is possible that this type of BRAF inhibitor could be dosed in a fashion that produces not only greater MAP kinase pathway inhibition in *BRAF* mutant tumors, but is not associated with paradoxical activation and the toxicities that appear to be a consequence. Increased selectivity raises the possibility of producing a greater impact on BRAF signaling without perturbing signaling mediated by the next most potently inhibited kinases. To date, it is not clear what BRAF inhibitor toxicities are a consequence of effects on non-RAF kinases. But, photosensitivity, for vemurafenib, and fever, with dabrafenib, appear to be compound specific effects and may not relate to RAF inhibition [26, 27].

9.7 Degrading BRAF

The appearance of BRAF splice variants at the time of disease progression on a BRAF inhibitor as well as the smaller number of cases associated with high-level *BRAF* amplification point to the possibility that targeting BRAF in ways other than ATP competitive kinase inhibition may be useful [28]. It has been known for several years that oncogenic BRAF is a client protein for the chaperone heat shock protein 90 (HSP90). Disruption of the HSP90/BRAF interaction would be hypothesized to lead to accelerated BRAF degradation, lower expression, and decreased oncogenic potential (Fig. 9.4).

Various HSP90 inhibitors have been developed over the past decade and represent an opportunity for exploring this mechanism of action. In vitro, it is clear that both geldanamycin-derivative and novel chemical classes of HSP90 inhibitors produce this effect [29, 30]. However, the immediate concern with regard to clinical application is that HSP90 has a large number of client proteins not all of which are uniquely relevant to cancer pathophysiology. Assuaging this concern regarding potential low therapeutic index are data supporting tumor growth control in xenograft experiments at doses that do not produce overt toxicity [30]. However, it is clear from single agent phase I and phase II clinical trials with HSP90 inhibitors that toxicity does occur at doses that produce drug exposures that are not high above the threshold for antitumor effects in preclinical models [31, 32]. Specifically, severe fatigue is a common, class-effect toxicity that is not well appreciated in preclinical toxicology or in vivo efficacy experiments, yet is commonly observed in patients receiving potentially therapeutic doses of HSP90 inhibitor therapy. In one clinical study amongst patients with metastatic melanoma, evidence of decreased BRAF

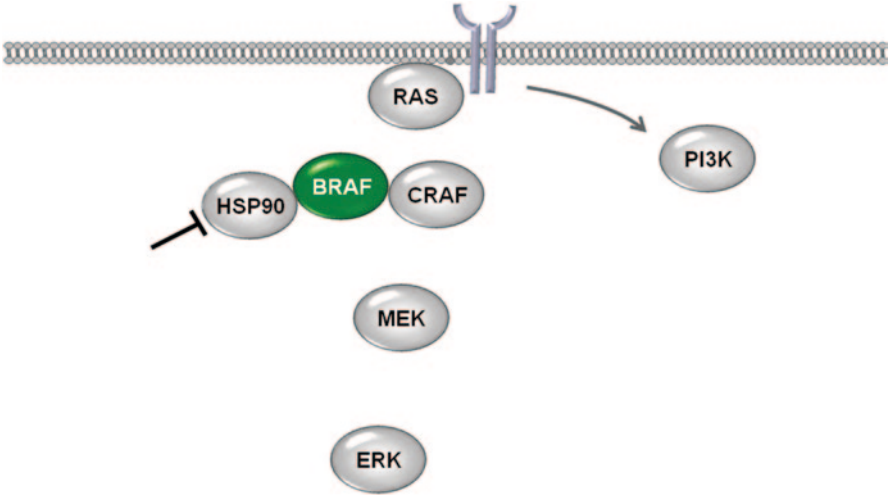

Fig. 9.4 Targeting BRAF protein stability with HSP90 inhibitors

expression was documented in patient tumor biopsies obtained on therapy and compared to biopsies from immediately before initiation of treatment [31]. However, the lack of significant single-agent efficacy in the same trial suggested that the magnitude of effect on BRAF expression is insufficient and higher doses/exposures not possible due to dose limiting toxicities.

While single agent HSP90 inhibition may have limited clinical application at least in the BRAF inhibitor naïve setting, it is possible that these agents would serve as compelling agents to investigate in combination with BRAF or BRAF/MEK dual inhibitor strategies. By decreasing expression of oncogenic BRAF, the pool of V600 mutated BRAF molecules would be diminished and presumably could be occupied with a lower concentration of a selective BRAF inhibitor. This potential interaction is supported by preclinical evidence in vitro, demonstrating not only decreased BRAF expression, but greater inhibition of proliferation and induction of cell death with combined BRAF/HSP90 inhibition [30]. And, in vivo, this combination produces more durable tumor control then BRAF inhibitor monotherapy. This strategy is currently being explored in a phase I/II clinical trial in which XL888 is combined with vemurafenib[NCT01657591]. But, given the apparently greater efficacy and attenuated toxicity of BRAF/MEK combination therapy, this may be the preferred MAP kinase pathway targeting strategy with which to combine an HSP90 inhibitor.

9.8 CRAF Dependent Resistance and RAF Dimer Blockers

The canonical MAP kinase pathway signaling cascade is comprised of RAS, RAF, MEK and ERK. In the setting of oncogenic V600 mutated BRAF, RAS activation is not required and, in fact, low concentrations of RAS-GTP (activated RAS) are

observed [15]. Notably, this creates a state in which oncogenic BRAF is responsible for nearly all RAF related signaling as low RAS-GTP results in decreased CRAF and ARAF activation. However, in the setting of acute and chronic BRAF inhibition, RAS-GTP levels are increased in vitro. This causes CRAF activation and is thought to be responsible for the rapid rebound in MEK and ERK activation observed after just 48–72 hours of exposure to a selected BRAF inhibitor [10]. At the time of acquired resistance to BRAF inhibitor therapy in patients, a notable minority are found to have activating *NRAS* mutations along with persistence of BRAF V600 mutations [33]. In the absence of a *BRAF* mutation, activating *NRAS* mutations have been shown to drive MAP kinase pathway signaling primarily through CRAF, not BRAF [34]. These clinical and preclinical observations lend support to the hypothesis that restoration of CRAF signaling is a potentially important component of BRAF inhibitor resistance.

Independent investigations outside of the context of *BRAF* mutant cancers have shown that CRAF, but not BRAF, has other activities beyond direct phosphorylation of MEK. In the setting of elevated RAS-GTP, CRAF is recruited to the plasma membrane and complexes with several scaffolding proteins in association with MEK, which CRAF directly phosphorylates. However, activated CRAF can localize to two additional intracellular compartments: the outer membrane of mitochondria and the mitotic spindle (Fig. 9.5) [35, 36]. When localized to the mitochondria, activated CRAF complexes directly with proapoptotic BAD tipping the balance of apoptosis-related proteins toward cell survival. At the mitotic spindle, CRAF colocalizes with polo-like kinase 1 and contributes to cell cycle progression through mitosis [37]. Therefore, in the setting of BRAF inhibitor therapy mechanisms that restore RAS activation could, indirectly, lead to CRAF-mediated cell survival and cell cycle progression in the face of ongoing BRAF inhibition. Additionally, acti-

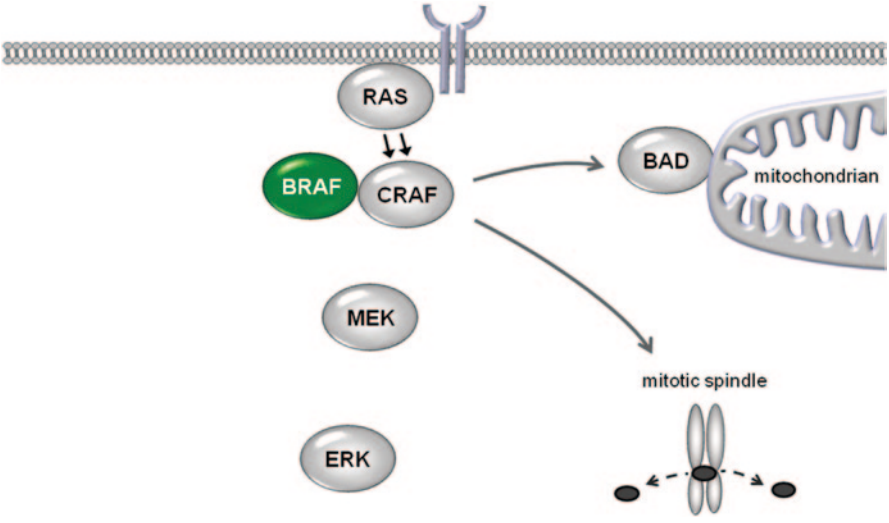

Fig. 9.5 The MEK-independent functions of activated CRAF

vated CRAF would be expected to phosphorylate MEK. The addition of a MEK inhibitor to the BRAF inhibitor would be anticipated to overcome only this last component of CRAF-dependent signaling. To overcome CRAF-mediated effects on cell survival and cell cycle that are MEK/ERK independent, CRAF itself could targeted or the downstream effectors of the pro-survival or mitotic progression effects. There is considerable interest in the possibility of developing potent and selective CRAF inhibitors, with relative selectivity for CRAF as opposed to BRAF. This is a critical design feature as both vemurafenib and dabrafenib are equipotent for BRAF and CRAF in isolated kinase assays, but ultimately have the net effect of contributing to paradoxical activation of the MAP kinase pathway in *BRAF* wild-type cells [15, 38, 39]. So, to function as an inhibitor of the MAP kinase pathway via CRAF inhibition, more potent and selective activity is needed against CRAF. Such an agent has not yet been described. With regard to downstream effectors, BH3 or SMAC mimetics may overcome the CRAF/BAD mediated pro-survival signal. And, aurora kinase inhibitors may intercept the effect of activated CRAF at the mitotic spindle.

An alternative strategy for disrupting both CRAF mediated resistance to BRAF inhibitors and paradoxical activation as a consequence of RAF dimer formation is to develop agents that inhibit the dimer interface. This region has been well-characterized from the crystal structure of BRAF and CRAF [40]. However, designing drugs that would selectively disrupt this protein-protein interaction without having widespread interactions with other intracellular proteins may be a far greater challenge than developing ATP competitive RAF inhibitors. Early attempts to identify such compounds suggest that it is feasible to identify small molecules with drug-like properties that could serve as the basis for further developing clinical candidates [41].

9.9 MITF Dependent Resistance

MITF, the transcription factor considered the master regulator of the melanocyte lineage, is suppressed by oncogenic BRAF and constitutively active MAP kinase pathway signaling [42, 43]. It is not surprising, then, that BRAF inhibitor therapy is associated with significant increases in MITF expression in vitro and in human tumors [44]. While this has the potentially positive consequence of upregulating the expression of melanocyte lineage antigens that could allow for more effective immune surveillance [45], MITF itself is a known oncogene [46]. The potential adverse consequences of increased MITF expression have only recently been elucidated. MITF directly regulates the expression of the BCL-2 family member, BCL2A1 [21]. Following exposure to a BRAF inhibitor, BCL2A1 expression is significantly increased. The pro-survival effects of BCL2A1 expression are supported by evidence that greater cell death can be induced by genetically silencing BCL2A1 expression in conjunction with BRAF inhibitor therapy. In patient tumor specimens, induction of BCL2A1 expression early in the course of BRAF inhibitor therapy is associated with lesser response to therapy compared to those patients in whom BCL2A1 expression is not induced. Overcoming this pro-survival impact of BRAF

inhibitor therapy would require either an agent that can suppress MITF expression or antagonize BCL2A1. Only agents that nonspecifically impact MITF or BCL2A1 currently exist. HDAC inhibitors appear to cause degradation of MITF [47], but have far-reaching effects on gene transcription and protein stability beyond MITF, and the BH3 mimetic, obatoclax, is able to bind BCL2A1 in addition to other BCL-2 family members [48]. More direct or selective pharmacologic inhibitors of MITF or BCL2A1 may be to address this particular mechanism of resistance.

9.10 BCL-2

Independent of the connection between MITF and BCL2A1, elevated expression of BCL-2 has been documented in melanoma [49]. The functional relevance of BCL-2 in terms of contributing to melanoma cell survival is supported by genetic silencing experiments in cell culture. Attempts to modulate BCL-2 clinically with an antisense oligonucleotide (oblimersen) were ultimately unsuccessful. Early clinical investigations with this agent in metastatic melanoma patients suggested only moderate impact on BCL-2 expression in patient tumor specimens assayed after treatment with oblimersen compared to pretreatment tumor specimens [50]. More recently, small molecule, BH3 mimetics have been developed to antagonize BCL-2 in, perhaps, a more effective fashion. In preclinical models, two BH3 mimetics (ABT-737 & ABT-263) results and down regulation of BCL-2 and potentiates BRAF inhibitor induced cell death and produces more durable tumor regression in vivo [51, 52]. This agent has been explored clinically in chronic lymphocytic leukemia and small cell lung cancer, both of which are associated with nearly ubiquitous high-level expression of BCL-2 [53, 54]. In those settings ABT-737 appears to augment the effect of conventional cytotoxic chemotherapy to some extent. It is hoped that in melanoma, where BRAF inhibitor therapy is a more active cytotoxic backbone, ABT-737 may contribute to an even greater therapeutic impact.

9.11 FOXO/ERBB3

Analogous to the relationship between oncogenic BRAF and MITF, inhibition of mutated BRAF appears to have more widespread consequences with regard to transcription factor expression and activity. Specifically, FOXD3 has been identified as another transcription factor for which expression is suppressed by oncogenic BRAF and upregulated as a consequence of BRAF inhibition [55, 56]. Combining gene expression profiling with chromatin immunoprecipitation assays, several FOXD3 regulated genes were identified that might relate to counterproductive downstream consequences of BRAF inhibition. The epidermal growth factor receptor family member, ERBB3, was identified as one of the genes whose expression was restored when FOXD3 was overexpressed in melanoma cells [57]. Genetic silencing of

FOXD3 or ERBB3 potentiated the efficacy of BRAF inhibition. Given that ERBB3 is a far more tractable potential therapeutic target than FOXD3, the therapeutic value of targeting ERBB3 has been further explored preclinically. ERBB3 is unique in comparison to ERBB1, ERBB2, and ERBB4 in that it lacks intrinsic kinase activity. It is thought that ERBB3 activates downstream signal transduction through heterodimer formation with these other ERBB family members. Therapeutic strategies that are currently being explored clinically in other tumors for which ERBB3 is thought to be a potential target include monoclonal antibodies that block ligand-dependent activation, both ligand-dependent and ligand-independent activation, or the kinase activity of ERBB1, ERBB2, and ERBB4. To date, evidence has been generated with lapatinib, a small molecule inhibitor of of ERBB1, ERBB2, and ERBB4 in combination with BRAF inhibition in both *BRAF* mutant melanoma and thyroid cancer [58, 59]. The availability of lapatinib for further clinical investigation in this setting as well as an increasing number of ERBB3 monoclonal antibodies in clinical development provides the opportunity to rapidly conduct clinical trials in combination with BRAF inhibition. A challenge that remains is that there are not currently predictive biomarkers that can be used to restrict the investigation of these combinations to those patients whose tumors will ultimately manifest FOXD3/ERBB3 upregulation.

9.12 PI3K/pS6

The PI3 kinase pathway has been associated with melanoma pathophysiology for many years. Specifically, *BRAF* mutations are commonly accompanied by deletion or inactivating mutations in *PTEN* or *AKT3* amplification in melanoma, supporting their role of this pathway in contributing to melanocytic transformation [60, 61]. In advanced melanoma, there is compelling evidence that the PI3K pathway can confer resistance to BRAF inhibitor therapy, particularly in melanomas that have loss of PTEN expression [6, 7]. In the same models, synergy is observed when a BRAF inhibitor is combined with a selective PI3K inhibitor and points toward one combination targeted therapy approach which may be particularly well-suited for the *BRAF* mutant/*PTEN* deleted subset of patients.

Emerging evidence suggests that downstream elements of the PI3K pathway can be differentially regulated in *BRAF* mutant melanoma cells. Outside of the cancer context, crosstalk between downstream elements of the MAP kinase pathway and PI3K/AKT pathway has been demonstrated. Specifically, activated RSK, a substrate for ERK, directly phosphorylates S6 kinase, which is otherwise known to be regulated by mTOR(). In some *BRAF* mutant melanoma cell lines, S6 kinase is under the control of the MAP kinase pathway whereas, in others, it is not. This has been documented in vitro and inhibition of S6 kinase is strongly associated with robust induction of cell death in comparison to cells with persistent S6 kinase phosphorylation which do not undergo apoptosis [62]. Combined inhibition of the MAP kinase pathway and mTOR results in inhibition of S6 kinase in these refrac-

tory cells and induces a comparable degree of cell death compared to MAP kinase pathway inhibition alone in S6 kinase responsive tumors. In patient tumor samples obtained immediately before and soon after initiation of BRAF inhibitor therapy, the inhibition of S6 kinase versus persistent phosphorylation correlates with improved progression free survival.

This observation points to the possibility that some *BRAF* mutant melanomas have an adequate signaling response to single agent BRAF inhibitor therapy and that monitoring S6 kinase phosphorylation early in the course of therapy could identify patients who should continue on single agent therapy and those who should pursue combination therapy. The challenge of testing this hypothesis in the clinic is that real-time molecular monitoring of an activated phosphoprotein has not been previously attempted. The research methods described above would need to be developed into a robust and reproducible pathology assay in the proper, quality-controlled environment. The absence of a predictive biomarker that identifies which patients will have an adequate versus inadequate signal transduction inhibitory effect forces consideration of this cutting-edge approach in an attempt to personalize BRAF inhibitor-based single agent in combination therapy. The same can be said for monitoring the upregulation of ERBB3 as discussed above.

9.13 Microenvironment-Mediated Resistance

As discussed extensively thus far, much of the focus of the melanoma field has been to elucidate mechanisms of acquired resistance to single agent BRAF inhibitor therapy and to understand the role of concomitant somatic genetic alterations in de novo resistance. Undoubtedly, these tumor cell autonomous factors are promising therapeutic co-targets with BRAF inhibition, or BRAF/MEK combination therapy. However, an unanswered question in the field is how growth factor receptor tyrosine kinases become activated and contribute to BRAF inhibitor resistance in the absence of activating mutations or amplification (Fig. 9.6).

Two seminal preclinical investigations have shed light into the potential interaction of the tumor microenvironment with *BRAF* mutant cells under the selective pressure a BRAF inhibitor therapy [63, 64]. In one set of experiments, cell types known to exist in the tumor microenvironment were individually co-cultured with *BRAF* mutant melanoma cell lines (in parallel with other oncogene defined tumor models), including fibroblasts, endothelial cells, pericytes, and others [63]. Fibroblasts were uniquely capable of conferring resistance in *BRAF* mutant melanoma cell lines exposed to a selective BRAF inhibitor. It was subsequently shown that conditioned media from fibroblasts was similarly able to induce resistance. And a large-scale screen of all known secreted growth factors and cytokines identified hepatocyte growth factor (HGF) as the molecule that was able to mimic this effect. In an independent laboratory-based investigation, this same approach of exposing *BRAF* mutant melanoma cell lines in the context of BRAF inhibition to a large panel of growth factors, again identified HGF as

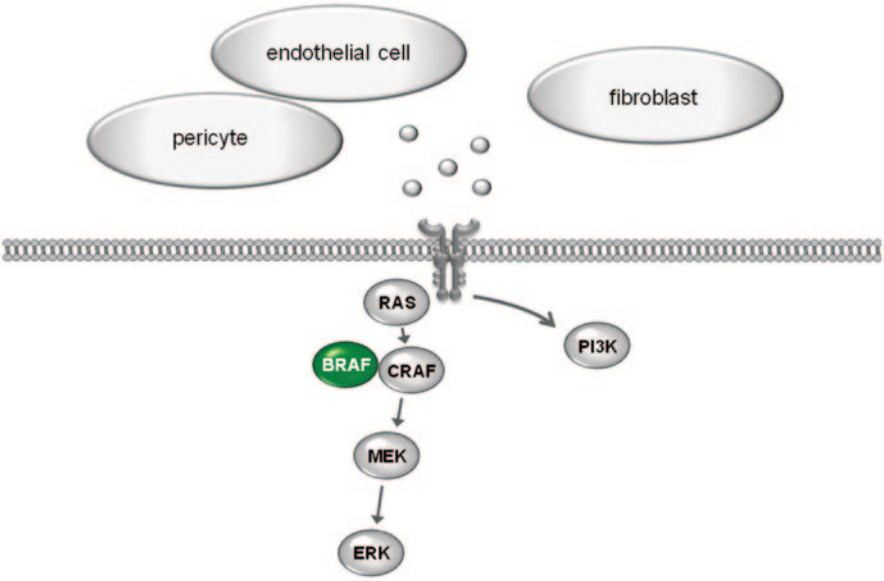

Fig. 9.6 Tumor microenvironment mediated BRAF inhibitor resistance

the most capable of protecting *BRAF* mutant melanoma cells from apoptosis following exposure to a BRAF inhibitor [64].

Two lines of clinical evidence support the potential relevance of stroma-derived HGF to de novo resistance to BRAF inhibitor therapy. First, HGF can be readily detected at the periphery of metastatic melanomas, where fibroblasts typically reside [63]. And, the presence of stromal HGF in and around the tumors of patients who receive BRAF or BRAF/MEK inhibitor therapy predicts lesser degrees of tumor regression compared to patients with no detectable stromal HGF. Second, elevated levels of serum HGF prior to treatment with a BRAF inhibitor predict shorter progression-free survival to BRAF inhibitor therapy compared to lower than average HGF levels [64]. A large number of HGF targeted monoclonal antibodies and small molecule c-met inhibitors are currently in clinical trials and potentially available to investigate in combination with BRAF or BRAF/MEK combination therapy. Experimentally, c-met inhibitors appear capable of overcoming this mechanism of resistance.

9.14 Summary and Conclusions

BRAF inhibitor therapy has changed the landscape of treatment options and the ability to rapidly ascertain common mechanisms of acquired resistance has led to a further clinical advance in BRAF/MEK combination therapy. As the field now focuses on mechanisms of *de novo* and acquired resistance to BRAF/MEK com-

bination therapy a number of additional questions rise to the top of the research agenda. Optimal schedule of administration has not yet been explored clinically and represents an opportunity to maximize the impact of already available agents. Opportunities to further exploit the profound dependence that BRAF mutant tumors have on the MAP kinase pathway are evident with emerging preclinical data with HSP90 and ERK inhibitors. Intercepting pathways that are activated as a consequence of BRAF inhibitor therapy, such as up regulation of BCL2A1 and ERBB3, represent tractable strategies for improving on the early impact of therapy. And, blocking compensatory pathways not impacted by BRAF inhibitor therapy such as the PI3K pathway (in some cases) and growth factor receptor activation derived from the tumor microenvironment provide further opportunities for improving on a backbone of optimal MAP kinase pathway inhibition. As this broad array of novel therapeutic strategies are investigated clinically, an immediate need arises for the development of predictive biomarkers that allow for the novel combinations to be deployed in as personalized a fashion as BRAF inhibitor therapy was itself.

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