Published in final edited form as:
Oncogene. 2010 October 14; 29(41): 5545–5555. doi:10.1038/onc.2010.323.

Analysis of the genome to personalize therapy for melanoma

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Abstract

The treatment of cancer is being revolutionized by an improved understanding of the genetic events that occur in tumors. Advances in the understanding of the prevalence and patterns of mutations in melanoma have recently led to impressive results in trials of personalized, targeted therapies for this disease. In this review, we will discuss the molecular targets that have been validated clinically, additional genetic events that are candidates for future trials, and the challenges that remain to improve outcomes further in this aggressive disease.

Keywords

melanoma; BRAF; NRAS; C-KIT; ERBB4; GNαQ

Introduction

It is estimated that 68 720 patients were diagnosed with melanoma, and 8650 patients died of this disease, in 2009 in the USA (Jemal *et al*., 2009). Melanoma is likely to become an increasingly important public health issue, as a review of the SEER database from 1950 to 2000 found an increase in 619% in the annual incidence and 165% in annual mortality from this disease, more than any other cancer (Tsao *et al*., 2004a). Interferon-α-2b is the only adjuvant therapy approved by the Food and Drug Administration (FDA) for patients with high-risk locally advanced melanoma. This treatment is given for 12 months and ~90% of patients experience significant toxicities (Hauschild *et al*., 2008). Adjuvant Interferon-α-2b initially demonstrated a significant survival benefit versus placebo in the ECOG trial E1684 (Kirkwood *et al*., 1996). However, a subsequent analysis after additional follow-up, and a pooled analysis including three additional large adjuvant Interferon-α-2b trials, failed to show a significant improvement in survival versus controls (Kirkwood *et al*., 2004). For patients with metastatic melanoma, the only FDA-approved therapies are dacarbazine and high-dose interleukin-2 (HD-IL2). Dacarbazine, which is a cytotoxic chemotherapy agent, has a clinical response rate of ~10%, and almost all responses are brief. HD-IL2, which is a biological agent designed to stimulate the immune system, has a similar clinical response

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Conflict of interest

The authors declare no conflict of interest.

rate of 16% (Atkins *et al*., 1999; Phan *et al*., 2001). Long-term disease-free survival has been observed in patients who achieve a complete response with HD-IL2, but this constitutes only B6% of the treated patients (Atkins *et al*., 2000). The use of HD-IL2 is limited by the severe toxicities of this regimen, which requires patients to be hospitalized throughout administration of the treatment, and early trials had a 2% treatment-related mortality rate (Schwartz *et al*., 2002). Numerous trials of combinatorial approaches, including multiagent chemotherapy and biochemotherapy regimens, have failed to improve outcomes in patients (Tsao *et al*., 2004a).

The management of many cancers is entering into an era in which treatments are being used to inhibit pathways activated by mutations in the tumors. This approach, which has been termed ‘targeted therapy’, has demonstrated remarkable clinical benefit in several cancers. The use of kinase inhibitors is now the standard of care in patients with chronic myelogenous leukemia, HER2/neu-amplified breast cancer, clear-cell renal cell carcinoma and gastrointestinal stromal tumors (GIST; Davies *et al*., 2006). Although these results have been impressive, the successful development of these approaches depended upon the identification of genetic abnormalities in these diseases. There is growing evidence that the majority of melanomas harbor genetic changes in key protein kinase signaling pathways (Figure 1). After initial failures, recent clinical trials of targeted therapy agents have demonstrated promising activity in melanoma. We will review the targets that have been validated clinically in melanoma, and discuss new targets that are candidates for testing in the future.

Clinically validated targets

BRAF

In 2002, investigators from the Sanger Institute published the identification of point mutations in *BRAF* in tumors and cell lines (Davies *et al*., 2002). Although mutations were identified in 1–10% of a variety of tumor types, including colon, lung and ovarian cancers, strikingly over half of the tested melanomas had a mutation in the *BRAF* gene. The high prevalence of *BRAF* mutations in melanoma has been validated in multiple studies. A recent meta-analysis of sequencing results for over 2700 samples reported a mutation rate of 65% in melanoma cell lines and 42% in uncultured cutaneous melanomas (Hocker and Tsao, 2007). Mutations in *BRAF* are the most common somatic mutations in this disease.

The BRAF protein is a serine/threonine kinase. BRAF is a component of the RAS-RAF-MEK-MAPK pathway, which is activated in many cancers (Gray-Schopfer *et al*., 2007). Activation of this signaling pathway had previously been implicated in melanoma by the identification of activating mutations in *NRAS*, which occur in 15–25% of melanomas (Hocker and Tsao, 2007). Over 40 different point mutations in *BRAF* have been identified in melanoma. Approximately 90% of the *BRAF* mutations in melanoma affect a single site, the valine at position 600 (V600). The most common mutation, V600E (89% of *BRAF* mutations), markedly increases the *in vitro* catalytic activity of BRAF, and results in constitutive activation of both MEK and MAPK (Davies *et al*., 2002; Wan *et al*., 2004). The *BRAF V600E* mutations are mutually exclusive with activating *NRAS* mutations (Tsao *et al*., 2004b; Goel *et al*., 2006; Haluska *et al*., 2006). However, overlap of *NRAS* mutations has been observed with *BRAF* mutations that do not increase BRAF catalytic activity (Wan *et al*., 2004; Heidorn *et al*., 2010).

The identification of frequent activating *BRAF* mutations in melanoma was rapidly followed by functional testing. Knock down of the V600E mutant BRAF in melanoma cell lines by small interfering RNA inhibited MEK activation, decreased cell proliferation and invasion, and induced cell death (Hingorani *et al*., 2003; Sumimoto *et al*., 2004). Sorafenib is a small

molecule inhibitor of wild-type BRAF, V600E BRAF, CRAF and a number of tyrosine kinase receptors (Strumberg, 2005). Similar to the knockdown studies, sorafenib inhibited the growth and survival of BRAF-mutant human melanoma cells, and slowed the growth of melanoma xenografts (Karasarides *et al*., 2004). Despite these promising preclinical results and the high prevalence of *BRAF V600E* mutations, a Phase II single-agent study of sorafenib in metastatic melanoma reported only one partial clinical response among 34 patients (Eisen *et al*., 2006). A subsequent nonrandomized trial of sorafenib in combination with paclitaxel and carboplatin showed much more promising activity, with a 26% clinical response rate, and the achievement of disease control in the majority of patients (Flaherty *et al*., 2008). However, a randomized Phase III trial demonstrated that sorafenib did not increase the clinical response or disease control rate achieved by the two chemotherapy agents alone (Hauschild *et al*., 2009).

The disappointing clinical results with sorafenib raised the question of whether mutant BRAF was a good therapeutic target in melanoma. The *BRAF V600E* mutation is present in up to 80% of benign nevi, which have virtually no malignant potential (Pollock *et al*., 2003; Yazdi *et al*., 2003). Studies in human cell lines, zebrafish and transgenic mice also demonstrated that expression of the *BRAF V600E* mutation alone failed to fully transform melanocytes (Michaloglou *et al*., 2005; Patton *et al*., 2005; Dankort *et al*., 2009). Thus, it was possible that although *BRAF* mutations are prevalent in melanoma, they were not essential.

The validity of mutant BRAF as a therapeutic target has now been demonstrated by clinical trials with a new, mutant-specific BRAF inhibitor. PLX4032 (also known as RO5185426) is a small molecule inhibitor with selective activity against V600-mutant BRAF. PLX4032 inhibits V600E BRAF catalytic activity with an IC50 of 13nm, as compared with 160nm for wild-type BRAF, and >1000nm for several other related kinases (CSK, SRC, focal adhesion kinase, KDR; Tsai *et al*., 2008). Similarly, the IC50 for the antiproliferative effect of PLX4032 is significantly lower in cells with V600-mutant BRAF (0.3–1.7 μm) versus cells with wild-type BRAF (>10 μm). In the dose escalation portion of the Phase I clinical trial metastatic melanoma, 56% of patients with the *BRAF V600E* mutation had a partial response and an additional 31% had a minor response. No responses were seen in patients without a *BRAF V600E* mutation (Flaherty *et al*., 2008). Preliminary results from the dose expansion cohort of an additional 31 patients with *BRAF V600E* mutations reported that 70% achieved a clinical response (Fisher *et al*., 2010).

The dramatic clinical activity of PLX4032 is very exciting. However, there remain challenges to optimize this new therapeutic approach. Many of the patients who initially responded to PLX4032 have subsequently progressed, with early data demonstrating a median duration of response to PLX4032 of approximately 8 months in the Phase I trial (Fisher *et al*., 2010). The mechanisms that cause resistance are currently unknown. Recently, a number of studies have demonstrated that BRAF inhibitors, including PLX4032, activate MEK and MAPK in melanoma cell lines with wild-type BRAF, including cell lines with mutant NRAS (Halaban *et al*., 2010; Hatzivassiliou *et al*., 2010; Heidorn *et al*., 2010). These studies showed that inhibition of the catalytic activity of wild-type BRAF promotes the formation of a complex between BRAF and CRAF, which increases CRAF catalytic activity. The formation of this complex was RAS-dependent in two of the studies, but not in the third. Activated CRAF activated MEK and ERK, and in some experiments produced increased proliferation and migration of melanoma cells. Although these studies did not specifically demonstrate mechanisms of resistance in *BRAF V600E* mutant cell lines, another study reported that several BRAF-mutant cell lines selected for *in vitro* resistance to the BRAF inhibitor AZ628 had increased CRAF protein expression (Montagut *et al*., 2008). Interestingly, the recent studies in the cell lines with wild-type BRAF reported different

complexes and pathways that are activated by different BRAF inhibitors. This suggests that different approaches may be needed to overcome resistance to the different BRAF inhibitors being tested in patients. If all resistance mechanisms share continued activation of the RAS-RAF-MEK-ERK signaling pathway as a common downstream event, combinatorial approaches that target other components of this pathway, such as MEK, may be effective (Emery *et al*., 2009).

Although most research to this point has focused upon melanomas with *BRAF V600E* mutations, there is evidence that mutations affecting other residues have markedly different signaling effects and may be sensitive to other therapeutic approaches. A number of the BRAF mutations that have been identified in melanoma, including D594V, G596R and G466V, do not activate the catalytic activity of BRAF, but do result in activation of MEK and ERK (Wan *et al*., 2004). Studies have demonstrated that melanoma cell lines with these mutations are critically dependent upon CRAF, and are sensitive *in vitro* and *in vivo* to sorafenib (Smalley *et al*., 2009). Thus, despite the initial negative clinical results, it is possible that pan-RAF inhibitors, like sorafenib may benefit certain subsets of melanoma patients. Testing this hypothesis will require analyzing patients for these relatively rare mutations, and collaborative efforts to accumulate enough patients to be able to definitively characterize clinical responses in this cohort.

The observed effects of the mutant-specific BRAF inhibitors in cell lines without BRAF mutations strongly support that these agents should not be used in patients with wild-type *BRAF*. It remains to be seen if the optimal approach for these patients, which represent over 50% of melanomas, can derive similar clinical benefit from inhibition of the RAS-RAF-MEK-ERK pathway. Alternatively, these tumors may depend upon different pathways, and thus their treatments should be directed against other targets.

c-KIT

Traditionally, melanomas have been categorized by the anatomical site the primary tumor arises from. Although most patients have tumors arising from sun-exposed skin, melanomas also arise from acral surfaces (palms, soles), mucosal surfaces (intestines, reproductive tract) and the uvea of the eye. In 2005, a seminal paper by Dr Boris Bastian demonstrated that melanomas arising from cutaneous, acral and mucosal sites have markedly different patterns of DNA copy gain and loss (Curtin *et al*., 2005). Among the cutaneous tumors, there were also significant differences between tumors with and without evidence of chronic sun damage. In parallel, the investigators demonstrated that the prevalence of *BRAF* and *NRAS* mutations varied markedly between the anatomically defined groups (Table 1). Whereas activating *BRAF* mutations were highly prevalent in cutaneous tumors without chronic sun damage (59%), the mutation rate was much lower in cutaneous tumors with chronic sun damage (11%), acral melanomas (23%) and mucosal melanomas (11%). Other studies have shown that *BRAF* mutations are essentially undetectable in uveal melanomas by traditional technologies (Cohen *et al*., 2003; Rimoldi *et al*., 2003). Analysis with more sensitive technologies suggests that a subpopulation of cells in uveal melanomas may harbor *BRAF* mutations (Maat *et al*., 2008).

The lower prevalence of *BRAF* mutations in the less common melanomas led to investigations to identify other driver mutations. The analysis of DNA copy number changes identified 4q12 as a region of chromosomal gain in the acral and mucosal melanomas, but not in the cutaneous tumors (Curtin *et al*., 2005). Characterization of candidate genes in this region led to the finding of focal copy number gain and mutations in the *c-KIT* gene in melanoma (Curtin *et al*., 2006). c-KIT is a tyrosine growth factor receptor and ~80% of GIST harbor an activating mutation (Hirota *et al*., 1998). In melanoma, c-KIT mutations were identified in 17% chronic sun-damaged cutaneous, 11% acral and 21% mucosal

melanomas. In contrast, no KIT point mutations were detected in areas that were not exposed to chronic sun damage, such as the trunk and the back (Curtin *et al*., 2006). In addition, *KIT* gene amplification was present in 6% of chronic sun-damaged, 7% of acral lentiginous and 8% of mucosal melanomas. Subsequent studies in other panels of melanomas have identified similar rates of *c-KIT* alterations in acral and mucosal tumors, but lower rates (~2%) in chronic sun-damaged cutaneous tumors (Antonescu *et al*., 2007; Beadling *et al*., 2008; Rivera *et al*., 2008; Torres-Cabala *et al*., 2009; Handolias *et al*., 2010). *In vitro* studies demonstrated that point mutations in *c-KIT* result in constitutive activation of the c-KIT protein in melanoma cells, and the activation of downstream proliferative and prosurvival signaling pathways (Jiang *et al*., 2008; Ashida *et al*., 2009).

The identification of activating *c-KIT* mutations was surprising, as a number of lines of research previously rejected a role for c-KIT function in melanoma. Loss of c-KIT protein expression has been shown to correlate with melanoma progression, and enforced expression of c-KIT-inhibited melanoma growth *in vitro* and *in vivo* (Lassam and Bickford, 1992; Huang *et al*., 1996). Three Phase II clinical trials of the c-KIT inhibitor imatinib, which is FDA-approved for GIST, reported only one clinical response among 63 total patients (Ugurel *et al*., 2005; Wyman *et al*., 2006; Kim *et al*., 2008). However, these trials were conducted before the identification of *c-KIT* aberrations in rare melanoma subtypes and, thus, they likely were overwhelmingly composed of patients with cutaneous melanomas. The one patient who did respond had a primary acral melanoma.

There are now several case reports of individual melanoma patients with *c-KIT* mutations who have achieved dramatic clinical responses to c-KIT small molecule inhibitors, including imatinib, sorafenib and dasatinib (Antonescu *et al*., 2007; Hodi *et al*., 2008; Quintas-Cardama *et al*., 2008; Woodman *et al*., 2009). These results support a potential benefit for these inhibitors in melanoma patients with *c-KIT* genetic aberrations, which are currently being tested in several clinical trials. In GIST, mutations in *c-KIT*, but not protein expression levels, are predictive of response to c-KIT inhibitors. In melanoma, imatinib resulted in a 50% clinical response rate among 10 patients with *c-KIT* mutations, but in none of the 10 patients with c-KIT amplification of the wild-type gene (Fisher *et al*., 2010). Although some responses to c-KIT inhibitors have been durable, many responses progress within a relatively short time (Woodman *et al*., 2009). In addition, the c-KIT mutations associated with imatinibresistance in GIST (exons 13, 17 and 18) are more prevalent in melanoma (~15%) as compared with GIST (~1%). Thus, it remains to be seen how effective c-KIT inhibitors will be in melanoma. However, the initial demonstration of activity in melanoma patients with *c-KIT* mutations supports further research to build upon this activity.

Potential targets

NRAS

The RAS family of genes is monomeric guanosine triphosphatases that are normally activated by extracellular signals, which convert them to a guanosine triphosphate-bound active state. Activating mutations of different RAS isoforms (that is, *KRAS, HRAS* and *NRAS*) are common in many types of cancer, including pancreatic (Almoguera *et al*., 1988), colon (Bos *et al*., 1987; Forrester *et al*., 1987), non-small cell lung (Rodenhuis *et al*., 1987), acute myelogenous leukemia and thyroid cancers (Bos *et al*., 1985; Padua *et al*., 1985; Suarez *et al*., 1988; Bos, 1989). These mutations lock RAS in the guanosine triphosphate-bound active state. Mutant RAS activates the RAS-RAF-MEK-MAPK pathway, similar to mutant BRAF. However, research in a variety of cell types has demonstrated that RAS activates a number of other effector pathways, including the phosphatidylinositol 3-kinases (PI3K)-AKT, RALGDS and PLCε cascades (Downward, 2003). Mutations in NRAS have since been identified in ~15–20% of melanomas with 90% of mutations localizing to codon

61 (Brose *et al*., 2002; Davies *et al*., 2002; Hocker and Tsao, 2007). NRAS mutations are mutually exclusive with activating *BRAF* mutations, and their prevalence is relatively consistent across the non-uveal anatomical subtypes (Curtin *et al*., 2005).

Inhibiting mutant RAS proteins has proven to be a daunting challenge (Konstantinopoulos *et al*., 2007). To be activated, RAS must first translocate to the plasma membrane. This translocation is dependent upon the transfer of a farnesyl isoprenoid group to RAS, which is normally catalyzed by farnesyltransferase. A number of farnesyltransferase inhibitors were developed to inhibit this process, and were subsequently evaluated in clinical trials. Unfortunately, these agents failed to demonstrate significant activity. Subsequent studies demonstrated that although HRAS depends exclusively upon farnesylation for membrane recruitment, both KRAS and NRAS alternatively undergo geranylgernylation, which results in recruitment to the plasma membrane (Zhang *et al*., 1997). In addition, farnesyl transferases modify more than 60 proteins, thus making it challenging to interpret the role of RAS in the effects of farnesyltransferase inhibitors.

Because of the difficulty of inhibiting RAS directly, therapeutic strategies have focused upon inhibiting downstream effector pathways. Preclinical studies with MEK inhibitors, such as AZD6244, have demonstrated growth inhibition in most, but not all, NRAS-mutant melanoma cell lines (Dry *et al*., 2010). Studies in several systems, including melanoma, have shown increased efficacy with combined inhibition of targets in multiple RAS effector pathways, such as MEK and PI3K (Engelman *et al*., 2008; Jaiswal *et al*., 2009b). At this time, the efficacy of targeted therapies in melanoma patients with *NRAS* mutations is yet to be reported, but this will be an important area of study in the future.

PI3K-AKT

Activated NRAS is known not only to activate the RAF-MEK-ERK pathway, but also the PI3K-AKT pathway (Stahl *et al*., 2004; Tsao *et al*., 2004a, b). PI3Ks are a family of lipid kinases that phosphorylate the 3′-OH group of the inositol ring (Katso *et al*., 2001; Cantley, 2002; Vivanco and Sawyers, 2002). Class IA PI3Ks are heterodimers comprised of a catalytic subunit encoded by three *p110* genes (*p110*α, *p110*β and *p110*δ) and five different regulatory subunits (p85α, p85β, p85γ, p50α and p55α). The enzymatic product generated by the PI3Ks, PI-(3,4,5)P3, activates AKT, a serine/ threonine kinase that induces a variety of responses including increased cell growth and proliferation (Katso *et al*., 2001; Cantley, 2002; Vivanco and Sawyers, 2002). Phosphatidylinositol (3,4,5) triphosphate levels are tightly controlled by strict regulation of PI3K activity and by phosphatase and tensin homolog (PTEN), a phosphatase which dephosphorylates the 3′-position of the inositol ring (Maehama and Dixon, 1998; Myers *et al*., 1998). *PTEN* is a tumor suppressor gene that is commonly inactivated in human tumors (Li *et al*., 1997; Steck *et al*., 1997), resulting in constitutive activation of the PI3K pathway. Somatic inactivation of PTEN is found in 10–30% of melanomas (Guldberg *et al*., 1997; Robertson *et al*., 1998; Tsao *et al*., 1998; Birck *et al*., 2000; Zhou *et al*., 2000; Chudnovsky *et al*., 2005). PTEN loss usually occurs in melanomas that also harbor a BRAF mutation, but it is mutually exclusive with NRAS mutation. Thus, melanomas frequently have genetic activation of both the PI3K and ERK signaling pathways, either by combined *BRAF* mutation and *PTEN* loss, or by *NRAS* mutation (Tsao *et al*., 2004b). However, quantitative measurement of activation-specific markers has demonstrated that PTEN loss and *NRAS* mutations may not have equivalent effects on AKT activation (Davies *et al*., 2009). Rare activating mutations of the catalytic subunit of PI3K, *PIK3CA*, have also been reported in melanoma, but the mutational status of BRAF and NRAS in those tumors was not reported (Curtin *et al*., 2006; Omholt *et al*., 2006).

Although activation of the PI3K-AKT pathway is frequently implicated in oncogenesis, there is evidence that some mediators of this pathway may be different in melanoma than in other cancers. There are three AKT isoforms, AKT1, 2 and 3. These isoforms have very similar sequences and structures (Brazil *et al*., 2002). Although AKT1 and AKT2 are thought to be the most critical isoforms in most cancers, there are several pieces of evidence supporting a central role for AKT3 in melanoma. First, both immunoprecipitation and small interfering RNA-mediated knockdown of the different AKT isoforms demonstrated that AKT3 is frequently the predominant active/phosphorylated form in melanoma cells (Stahl *et al*., 2004). AKT3 is located on the long arm of chromosome 1, which is an area of frequent chromosomal gain in melanoma (Thompson *et al*., 1995; Bastian *et al*., 1998). Recently, a rare point mutation (E17K) in the regulatory pleckstrin homology domain of AKT3 was identified in melanoma cell lines and clinical specimens (Davies *et al*., 2009). This mutation is in the same residue as the point mutation previously identified in AKT1 in other cancer types, including breast, ovary and colon cancers (Carpten *et al*., 2007). Similar to AKT1 mutations, the AKT3 E17K mutation results in constitutive activation of AKT.

Although activation of the PI3K-AKT pathway appears to be a frequent event in melanoma, to date clinical trials using agents against this pathway have failed to demonstrated significant efficacy. Treatment with CCI-779, an analog of rapamycin that inhibits the AKT effector mammalian target of rapamycin, yielded only a 3% response rate (Margolin *et al*., 2005). Although mammalian target of rapamycin inhibitors were an appealing clinical strategy initially owing to the known tolerance for rapamycin in patients, research has demonstrated that these agents disrupt a feedback loop, resulting in hyperactivation of PI3K and AKT (O'Reilly *et al*., 2006; Tabernero *et al*., 2008). Perifosine is a small molecule inhibitor that interferes with the recruitment of molecules with a pleckstrin homology domain, such as AKT, to the plasma membrane. Treatment of 18 metastatic melanoma patients with perifosine failed to yield any clinical responses (Ernst *et al*., 2005). Unfortunately, no analyses were performed in this trial to see if perifosine treatment inhibited AKT activation in patients. Many new agents that target the PI3K-AKT pathway, including PI3K, dual PI3K/mammalian target of rapamycin and catalytic domain AKT inhibitors, are now in various phases of clinical testing (Courtney *et al*., 2010). It remains to be seen what activity these drugs will have in melanoma as single agents. However, the genetics of melanoma suggests that such agents may be most effective when combined with RAS-RAF-MEK-MAPK pathway inhibitors.

ERBB4

Although the aforementioned targets have generated great interest as therapeutic targets, there remain a significant number (~30%) of melanoma patients for whom no activating mutation is identified. To identify new targets that may contribute to this disease, we recently investigated 86 protein tyrosine kinases for sequence variations in a set of cutaneous melanomas (Prickett *et al*., 2009). After initially screening for mutations in the kinase domains only, we performed whole-exome sequencing for the genes with a somatic mutation in the kinase domain. Using this approach, we identified a total of 99 nonsynonymous somatic mutations in 19 protein tyrosine kinase genes (Table 2). Although a number of these genes are potential therapeutic targets, as an initial candidate we focused on the *ERBB4* gene.

ERBB4 is a member of the ERBB/HER family of receptor tyrosine kinases. Other family members, including ERBB1 (epidermal growth factor receptor) and ERBB2 (HER2), have been implicated by mutations and/or amplifications in a number of cancers, including lung, colon and breast cancers. Genetic abnormalities in these genes are extremely rare in melanoma (Inman *et al*., 2003; Akslen *et al*., 2008). Consistent with these results, we did not detect any somatic mutations in the kinase domain of *ERBB1/2/3* in melanoma samples.

However, the *ERBB4* gene harbored more somatic changes than in any other protein tyrosine kinase. As mutations that arise during tumorigenesis may provide a selective advantage to the tumor cell (driver mutations) or have no functional effect on tumor growth (passenger mutations), it is important to capture both the nonsynonymous and the synonymous alterations for further analysis. The ratio between these two categories of mutations may indicate the likelihood that a gene was selected for during tumorigenesis and therefore is a driver mutation. In the case of *ERBB4*, this ratio was 24:3, which is significantly higher than the NS/S ratio predicted for nonselected passenger mutations ($P > 0.01$; Sjoblom *et al*., 2006). This supports that the frequent *ERBB4* mutations were not random events, but may be functional, driver mutations that contribute to melanoma development or progression. There was overlap with both *BRAF* and *NRAS* in the tumors with *ERBB4* mutations, suggesting that these genes may operate through independent pathways. Indeed, this same scenario is seen for activating mutations in other genes, such as *PIK3CA* (Samuels *et al*., 2004).

The *ERBB4* mutations were localized in several different functional domains of the protein. On the basis of bioinformatics, analyses that localized the identified mutations on the protein crystal structure and evaluated their proximity to the previously identified mutations in *ERBB1* and *ERBB2*, seven of the mutations were cloned into expression vectors for functional experiments. Although these seven mutations affected several different functional domains, all of the mutations induced increased ERBB4 catalytic activity (as measured by autophosphorylation), kinase activity (measured using an *in vitro* kinase assay) and anchorage-independent growth. In addition, all the mutations were shown to be transforming, to a similar extent, to the well-known oncogene *KRAS G12V*. We also examined the effects of inhibiting ERBB4 in melanoma cell lines with the gene mutations. Knockdown of ERBB4 expression with small hairpin RNA had minimal effects on the proliferation of human melanoma cell lines with wild-type ERBB4. In contrast, cell lines with various *ERBB4* mutations were markedly inhibited by knock down of the gene. A similar pattern of selective sensitivity was observed when the cell lines were treated with lapatinib, which is an FDA-approved pan-ERBB family inhibitor. Although lapatinib has been shown to have greatest inhibitory activity against ERBB1 and ERBB2, it showed selective growth inhibition in the melanoma cell lines expressing mutant ERBB4. The mechanism that lies behind this selectivity is as yet unknown, but it could be because of the inhibition of the mutant ERBB4 protein itself, or alternatively to a preferential heterodimerization of mutant ERBB4 with ERBB2. A summary of the genetic and functional investigation of ERBB4 is depicted in Figure 2. Taken together, these findings have identified a novel melanoma ‘driver’ that causes ‘oncogene addiction’ allowing for the investigation of applying targeted therapeutics for melanoma patients harboring *ERBB4* mutations.

Interestingly ERBB4 mutations are dispersed throughout its domains. This is reminiscent to the mutations reported in PIK3CA; although it has two major mutational hotspots, it harbors mutations throughout its domains (Samuels *et al*., 2004; Zhao and Vogt, 2008). A similar observation is seen for p85α, whose identified mutations mainly lie in the iSH2 domain, but are also found in the cSH2, nSH2, iSH2cSH and BCR domains, (Philp *et al*., 2001; TCGA, 2008; Jaiswal *et al*., 2009a), as well as FLT3 (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=gene&ln=FLT3). This suggests that not all mutations in oncogenes must be clustered to be functionally important. As changes that affect protein activity can result from single or multiple mutations within a gene that increase activity or abrogate negative regulatory domains.

GNαQ

As described previously, uveal melanomas are notable for an almost complete absence of *BRAF* and *NRAS* mutations by conventional sequencing approaches (Cruz *et al*., 2003). Previous experiments using a forward genetic screen had identified that hypermorphic mutations in two different G-protein-coupled receptors, GNαQ and GNα11, produced increased proliferation of dermal melanocytes (Van Raamsdonk *et al*., 2009). As conditions that are characterized by increased proliferation of intradermal melanocytes (that is, nevus of Ota) are associated with an increased risk of uveal melanoma, investigators screened uveal melanomas for mutations in these genes. The team lead by Dr. Boris Bastian identified a recurrent point mutation in the *GNαQ* gene in 46% of uveal melanomas and 27% of uveal melanoma cell lines (Van Raamsdonk *et al*., 2009). No *GNαQ* mutations were identified in acral, mucosal or cutaneous melanomas without chronic sun damage; one cutaneous tumor with chronic sun damage harbored a mutation. A study by an independent group of investigators similarly reported a 49% incidence of *GNαQ* mutations in primary uveal melanomas (Onken *et al*., 2008).

All the mutations occurred at the Q209 residue, which is analogous to the Q61 residue in *NRAS*, and all were somatic. GNαQ encodes for the α-subunit of q class of heterotrimeric guanosine triphosphate-binding protein (Gq) that mediates signals between G-protein-coupled receptors (GPCRs) and stimulates phospholipase C (PLC). Activation of PLC catalyzes the hydrolysis of phosphatidylinositol biphosphate to release inositol trisphosphate (IP3) and diacylglycerol (DAG). These second messengers propagate the Gπ-mediated signal through stimulation of protein kinase C (PKC). Expression of the GNαQ Q209L mutation promoted anchorage-independent cell growth and tumorigenicity of melanocytes (Van Raamsdonk *et al*., 2009). In addition, expression of GNαQ Q209L activated protein kinase C signaling and increased expression of P-ERK. A preliminary report has also described point mutations in *GNα11* in uveal melanomas, which were mutually exclusive with the *GNαQ* mutations (Fisher *et al*., 2010).

Future investigation of this promising target should encompass further functional assays to understand the signaling mechanisms regulated by mutant GNαQ and GNα11. In addition, directed therapy against the mutant GNαQ/GNα11 or the signaling pathways activated by these mutations may open up new therapeutic strategies for melanocytic tumors, particularly uveal tumors. This new discovery again points to the importance of the superfamily of monomeric G proteins and GPCRs in cancer.

Looking to the future

The promising results with PLX4032 and c-KIT inhibitors in melanoma patients with *BRAF* and *c-KIT* mutations, respectively, add to the growing list of successful clinical strategies exploiting activating genetic events in cancer. These successes have generated tremendous enthusiasm for the continued development of personalized, targeted therapy approaches for this aggressive disease. However, there remain several critical gaps in our understanding of the role of signaling pathways in melanoma that will be essential for improving outcomes in this disease.

Although the majority of patients with *BRAF* and *c-KIT* mutations have responded to inhibitors against these targets, both primary and secondary resistance have been observed. It will be important to use both preclinical models and clinical specimens to identify the mechanisms that underlie this resistance. It is possible that some patients may only need treatment with a single inhibitor. However, the pattern of mutations in melanoma, such as the frequent co-occurrence of *BRAF* mutations and PTEN loss, suggests that combinatorial approaches against multiple pathways may be more effective. The recent identification of

ERBB4 mutations in tumors with both *BRAF* and *NRAS* mutations suggests other combinatorial approaches. Although candidate approaches may be driven by the identification of genetic changes and/or activated pathways, an alternative approach is the use of broad, unbiased functional screens with libraries of small molecules or small interfering RNAs. Such approaches may identify novel genes or pathways as effective combinatorial targets, and may lead to focused investigations to understand the role or activation of those pathways. In addition, the evidence that immunotherapies, such as HD-IL2, occasionally result in durable cures supports the rationale to see if combinatorial approaches with those agents will improve the duration of the responses that are achieved by targeted therapies.

In a number of diseases, particularly leukemias, the stem cell model has been proposed as a critical mechanism of the failure of anticancer agents (Bonnet and Dick, 1997; Baguley, 2010). The stem cell model hypothesizes a hierarchical organization in which a small subpopulation of cells, the cancer stem cells, is tumorigenic, can proliferate indefinitely, and is ultimately responsible for the long-term outcomes of many anticancer therapies. There is evidence that melanoma does not fit this model. Using a modified xenograft model in which the natural –killer-cell activity of the NOD/SCID mice used to test tumorigenic potential was abrogated, it was demonstrated that on average 25% of cells freshly isolated from melanomas have tumorigenic capacity (Quintana *et al*., 2008). Analysis of 15 different markers, which showed heterogeneous expression in melanoma clinical specimens, including proteins associated with the stem-cell phenotype in other cancers, failed to distinguish tumorigenic from nontumorigenic cells in this model. Thus, at least for melanoma cells, tumorigenic cells appear to be quite prevalent in tumors, not rare (Baker, 2008; Eaves, 2008). Recently, a subpopulation of slow-cycling cells expressing the protein JARID1B has been identified in both melanoma tumors and cell lines (Roesch *et al*., 2010). Experiments demonstrated that expression of this protein was not a prerequisite for tumorigenicity, and independently confirmed the efficient formation of tumors by single melanoma cells in the modified xenotransplantation assay.

In addition to *BRAF* and *c-KIT*, there is ongoing research to identify therapeutic strategies to apply to melanoma patients with *NRAS*, *AKT*, *ERBB4*, *GNαQ* and *GNα11* mutations. However, ~30% of melanoma patients do not have a mutation detected in any of these genes. There is a clear need for focused research efforts on this large cohort of patients. As the cumulative prevalence of mutations in *BRAF* and *NRAS* is so high in this disease, it is possible that these tumors harbor other mutations that activate the RAS-RAF-MEK-MAPK signaling pathway. Alternatively, these tumors may be driven by activation of completely different pathways, and thus require distinct treatment strategies. New technologies, such as whole-exome capture (Ng *et al*., 2009; Turner *et al*., 2009) and whole-genome sequencing (Pleasance *et al*., 2010) may be particularly informative in these patients. These approaches can reveal not only single-base alterations in coding and regulatory regions, but also structural changes such as amplifications, deletions and translocations. Database integration of this information should reveal the major pathways that drive these tumors. Recently, the first publication to describe the results of whole-genome sequencing of a melanoma tumor has been reported (Pleasance *et al*., 2010). This analysis identified ~30 000 somatic alterations, which is at least one order of magnitude higher than other solid tumors. Although many of these changes likely represent passenger events owing to ultraviolet radiation exposure, mutations resulting in over 180 nonsynonymous amino acid substitutions were detected. These numbers suggest that a substantial number of melanomas will need to be sequenced and compared to identify likely driver mutations, with stringent functional testing essential for validation of new targets.

In summary, the treatment of melanoma is rapidly changing owing to the analysis of the melanoma genome. Although such studies to date have focused on the treatment of metastatic tumors, in the future these molecular insights may lead to strategies to improve the early diagnosis of this disease, and perhaps to stratify which patients with local tumors require aggressive interventions to reduce the risk of disease recurrence and spread. Ultimately, improved understanding of the molecular basis of this disease may even lead to preventative strategies. Thus, melanoma is an illness in which future outcomes in patients are likely to be intertwined with a growth in our understanding of the genetic events that occur in this disease.

Acknowledgments

We thank Darryl Leja for graphical expertize and Nature Genetics for permission to use figures from paper doi: 10.1038/ng.438. This work was supported by grants from ASCO (Young Investigator Award) and M D Anderson Cancer Center SPORE in Melanoma (Developmental Research Grant; 5 P50 CA093459 03 PP-DRP3; to MAD) and the Intramural Research Programs of the National Human Genome Research Institute, National Institutes of Health, USA (to YS).

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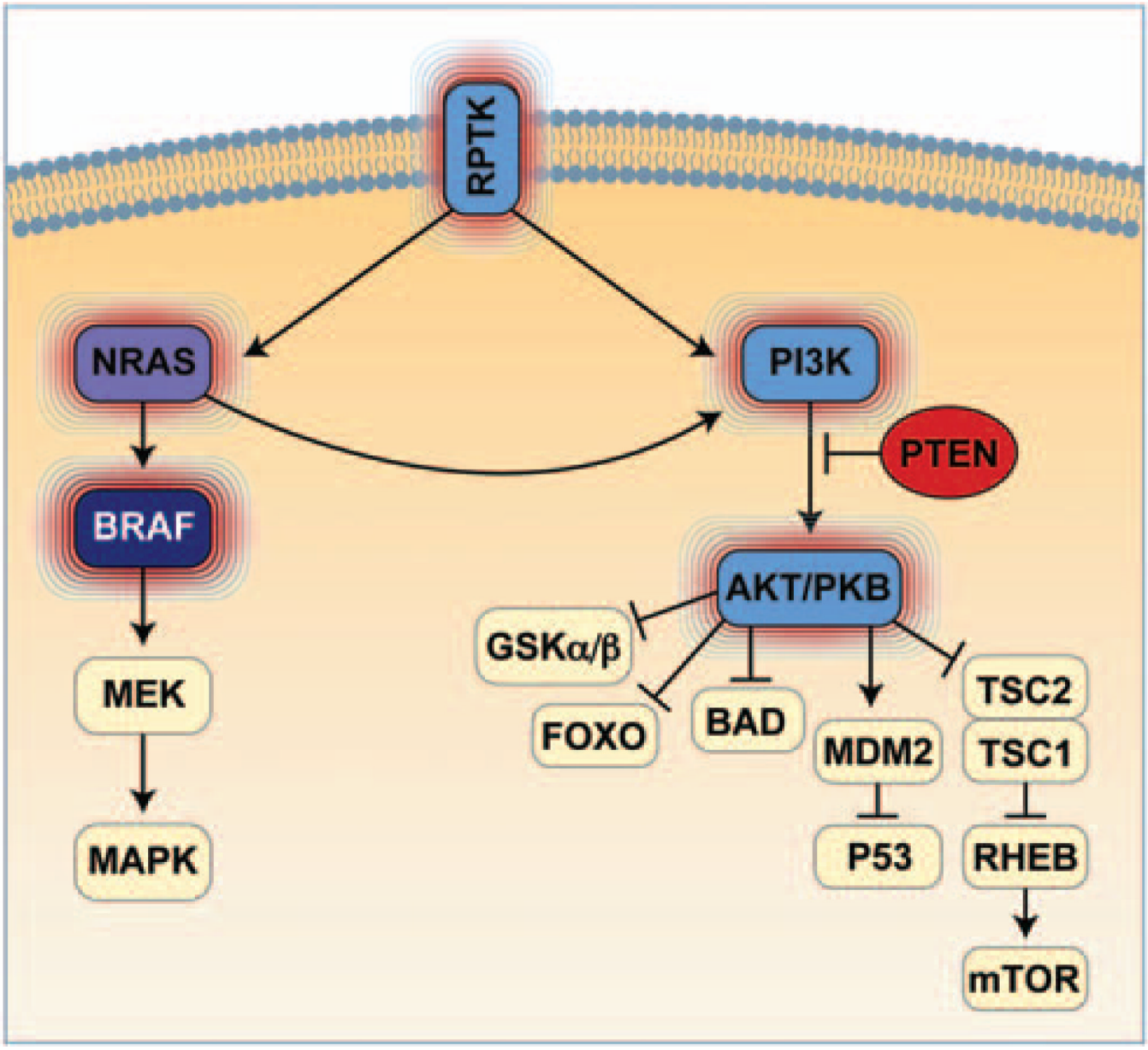

Figure 1.
Kinase signaling pathways in melanoma. The majority of melanomas harbor somatic mutations in the RAS-RAF-MEK-MAPK or PI3K-AKT pathways. Activating mutations are indicated by rippled red and blue circles, with intensity denoting relative prevalence. Loss of function mutations are highlighted in red. RPTK, receptor protein tyrosine kinase (that is, c-KIT, ERBB4).

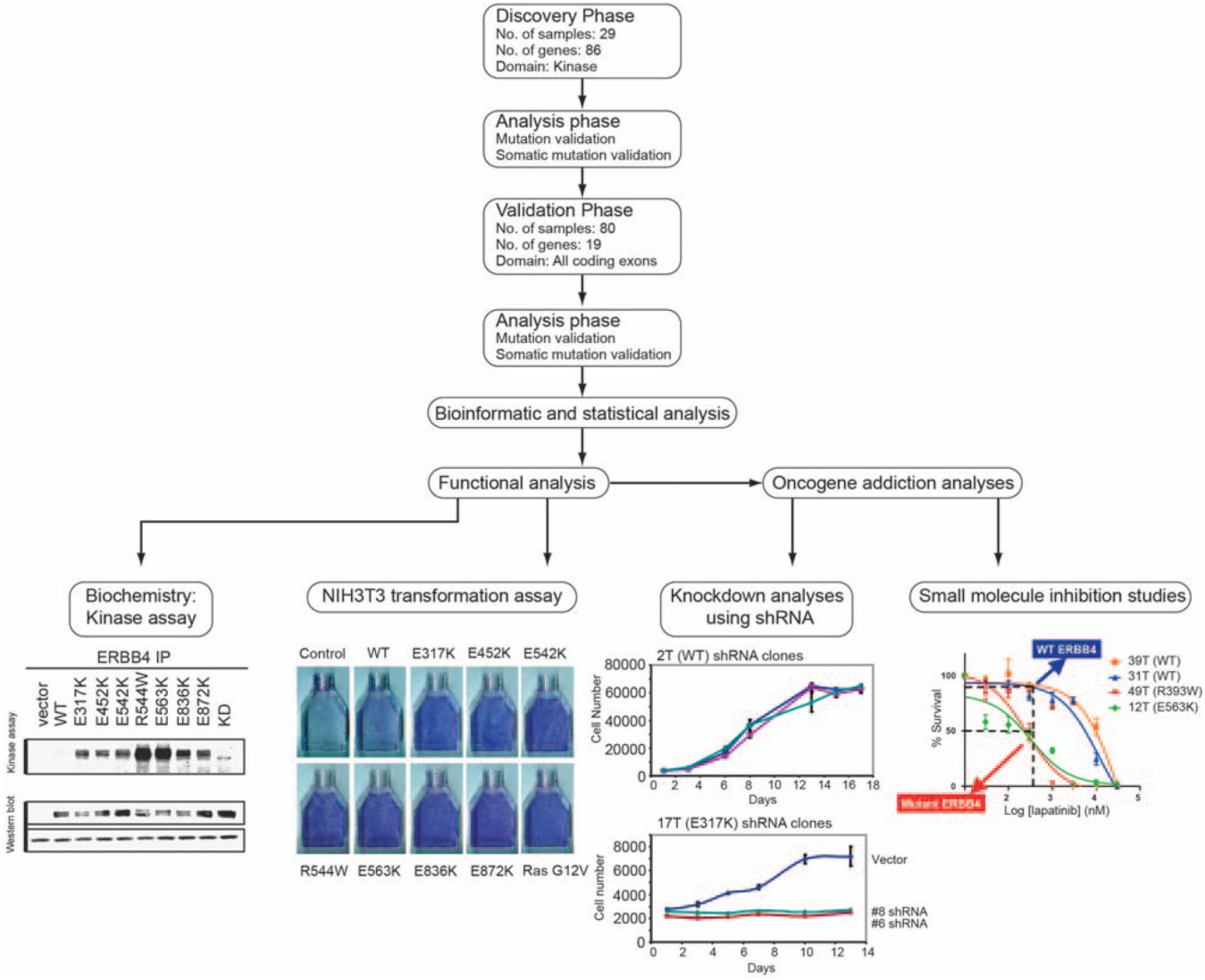

Figure 2.

Genetic and functional analysis of ERBB4 mutations. Flowchart representing the experimental design used to evaluate the presence of somatic mutations in the tyrosine kinome in melanoma and its functional follow-up. Once ERBB4 was found to be the most highly mutated tyrosine kinase, seven of its mutations were shown to (from left to right) increase its kinase activity, induce cell transformation and provide an essential cell survival signal as seen in small hairpin RNA (shRNA) knockdown and small molecule inhibition assays. WT, wild type. Figure adapted from Prickett *et al*., 2009.

Table 1

Frequency of mutations in kinase signaling pathways in melanoma subtypes

	Cutaneous	Acral	Mucosal	Uveal	Not specified
BRAF	40–60	15–20	3–5	<1	
NRAS	15–25	10–15	5–15	<1	
c-KIT (mutation)	<2 (CSD: 2–17)	10–20	15–20	<1	
c-KIT (amplification)	0–7 (CSD: 6)	25–30	25–30	<1	
GNαQ	<1 (CSD: 5)	<1	<1	45–50	
ERBB4					10–20
PTEN					10–30
PIK3CA					1–2
AKT1/3					1–2

Abbreviation: CSD, chronic sun-damaged cutaneous.

Each cell shows the percentage of tumors with the indicated mutation or amplification.

Table 2

Somatic mutations identified in the protein tyrosine kinase screen

Gene	No. of nonsynonymous mutations found	No. of synonymous mutations found	Melanoma cases affected (%)[a]
DDR1	2	0	2.5
FER	2	0	2.5
FLT1	8	0	10.1
EPHA6	5	1	6.3
EPHA10	7	0	6.3
EPHB1	4	1	5.1
EPHB2	7	2	8.9
EPHB6	7	2	8.9
ERBB4	24	3	19
MATK	1	0	1.3
MET	3	0	3.8
NTRK1	2	0	2.5
PDGFRA	5	1	5.1
PTK2	1	1	1.3
PTK2B	8	1	10.1
PTK6	2	0	2.5
PTK7	1	1	1.3
ROR2	4	1	5.1
TIE1	6	3	7.6
Total	99	17	

[a]Number of nonsynonymous and splice-site mutations observed and percentage of tumors affected for each of the 19 genes in the panel of 79 cutaneous melanoma cancers.