REVIEW

Melanoma: from mutations to medicine

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Melanoma is often considered one of the most aggressive and treatment-resistant human cancers. It is a disease that, due to the presence of melanin pigment, was accurately diagnosed earlier than most other malignancies and that has been subjected to countless therapeutic strategies. Aside from early surgical resection, no therapeutic modality has been found to afford a high likelihood of curative outcome. However, discoveries reported in recent years have revealed a near avalanche of breakthroughs in the melanoma field—breakthroughs that span fundamental understanding of the molecular basis of the disease all the way to new therapeutic strategies that produce unquestionable clinical benefit. These discoveries have been born from the successful fruits of numerous researchers working in many—sometimes-related, although also distinct—biomedical disciplines. Discoveries of frequent mutations involving BRAF(V600E), developmental and oncogenic roles for the microphthalmia-associated transcription factor (MITF) pathway, clinical efficacy of BRAF-targeted small molecules, and emerging mechanisms underlying resistance to targeted therapeutics represent just a sample of the findings that have created a striking inflection in the quest for clinically meaningful progress in the melanoma field.

The disease

Melanomas can arise within any anatomic territory occupied by melanocytes. Although cutaneous melanoma, which develops from epidermal melanocytes of the skin, represents the most common site of origination, noncutaneous melanocytes such as those lining the choroidal layer of the eye, respiratory, gastrointestinal, and genitourinary mucosal surfaces, or the meninges do occasionally undergo malignant transformation, albeit at a low frequency.

Clinical morphologists have traditionally divided the cutaneous disease into several subgroups, including superficial spreading melanoma, nodular melanoma, acral lentiginous melanoma, and lentigo maligna melanoma (Fig. 1), and other uncommon variants such as desmoplastic melanoma and nevoid melanoma. Histological patterns have been well described, and microscopic features that correlate with clinical subgroups have been thoroughly codified. Perhaps one of the most unusual and tested aspects of melanoma physiology is the mathematical relationship between tumor thickness (i.e., diameter of tumor sphere) and outcome (Balch et al. 2009). Other features, including mitotic rate and ulceration, also play significant roles in determining prognosis. Despite decades of study, an understanding of the melanoma subsets that are destined to be lethal remains incomplete.

Despite recent therapeutic advances in management of advanced melanoma, several crucial biological questions remain, including: (1) What is the relationship between environmental exposures and melanoma risk? (2) Do biomarkers exist that may predict clinical behavior and thus guide therapies? (3) Which genomic alterations drive invasion, metastasis, and drug resistance? (4) Which molecular lesions underlie tumor maintenance? (5) Which aberrant pathways and targets are amenable to either preventative or therapeutic intervention?

Genetic loci and variants that confer melanoma risk

A family history of melanoma occurs in 10% of melanoma patients and confers an approximately twofold increase in melanoma risk (Gandini et al. 2005). One can argue that melanoma is fundamentally a genetic disease, since the range of heritable risk factors—from physical characteristics such as light complexion, an inability to tan, red hair, and blue eyes to the familial atypical mole/melanoma (FAMM) syndrome—are all determined by distinct genetic elements. Hereditary melanoma itself is often associated with (1) multiple cases of melanoma in several generations on one side of the family, (2) multiple primary melanomas in a given individual, and (3) early onset of disease. In this section, we review recent discoveries in melanoma predisposition and survey known risk loci, especially those uncovered through genome-wide association studies (GWAS).

High-risk melanoma loci

To date, the weight of evidence suggests that the retinoblastoma (RB) pathway, which serves to regulate the

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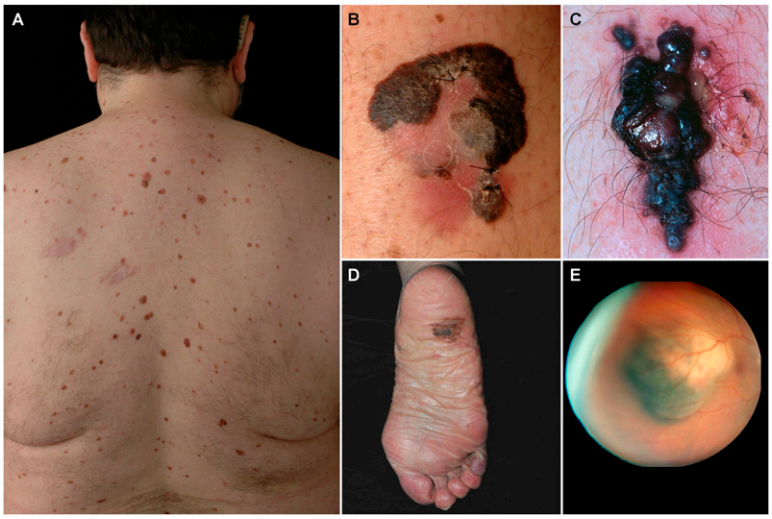

Figure 1. Clinical disease. (*A*) A patient with multiple clinically atypical moles. Since ~80% of all acquired nevi harbor BRAF(V600E) mutations, hundreds of BRAF-activating events occur in such nevi-prone individuals without progression to melanoma. It is thought that melanocyte growth arrest results from OIS. Common forms of cutaneouos melanoma include superficial spreading melanoma (*B*) and nodular melanoma (*C*). Both of these subtypes are associated with *BRAF* or *NRAS* mutations. (*D*) Acral lentiginous melanoma is the most common subtype among darker-skinned individuals and is more often associated with *KIT* aberrations. (*E*) Ocular melanoma is rarer than cutaneous melanoma and is not associated with *BRAF*, *NRAS*, or *KIT* changes, but rather with *GNAQ* or *GNA11* alterations (picture courtesy of Dr. Ivana Kim, Massachusetts Eye and Ear Infirmary).

G1/S checkpoint, is uniquely vulnerable in melanoma susceptibility.

Cyclin-dependent kinase N2A (CDKN2A) It has been recognized for decades that there are families with an increased occurrence of both melanoma and clinically atypical moles (i.e., dysplastic nevi) (Fig. 1A). Through the systematic collection of these kindreds worldwide, linkage analysis on melanoma families led to putative loci on chromosomes 1p36 (Bale et al. 1989) and 9p21 (Cannon-Albright et al. 1992). Within the 9p21 region, the *p16* (now *CDKN2A*) gene quickly became an attractive candidate for the melanoma locus, since p16 was shown to be a potent cell cycle inhibitor through a direct negative interaction with CDK4 (Serrano et al. 1993). Hussussian et al. (1994) then demonstrated deleterious germline mutations in *CDKN2A* among a subset of melanoma-prone families that exhibited linkage to chromosome 9p21 markers, thereby establishing the first high-risk susceptibility locus in melanoma. Around this time, several groups also reported homozygous deletions and deleterious mutations of *CDKN2A* in a variety of cancer cell lines (Kamb et al. 1994; Nobori et al. 1994). Thus, within a span of a few years, *CDKN2A* catapulted into the center of cancer biology as a critical target of inactivation at both the germline and somatic levels.

The *CDKN2A* locus is composed of four exons and encodes for two distinct proteins through alternative splicing: $p16^{INK4a}$ and $p14^{ARF}$ (Fig. 2; for review, see Chin 2003); interestingly, both proteins are potent tumor suppressors with distinct but equally crucial roles in cell cycle and apoptosis regulation. $p16^{INK4a}$ binds to and inhibits CDK4/6, thereby preventing CDK4/6 from phosphorylating the RB protein (Koh et al. 1995). Since hyperphosphorylation of RB triggers the release of E2F1, a transcriptional inducer of S-phase genes, loss of $p16^{INK4a}$ encourages G1–S transition and re-entry into the cell cycle. On the other hand, $p14^{ARF}$ binds to human double

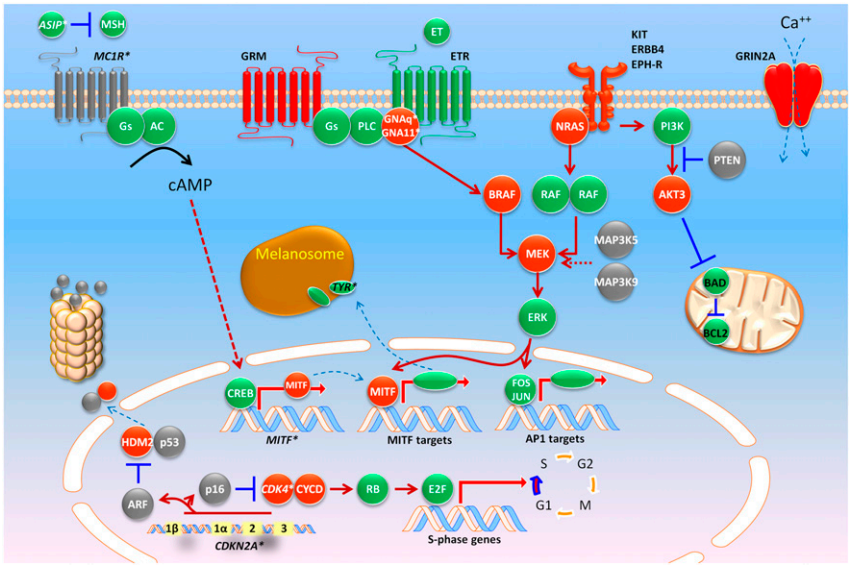

Figure 2. A molecular map of melanoma. Heritable loci with risk alleles or SNPs are shown in italics with asterisks (e.g., *CDKN2A**). Red and gray colors indicate somatic alterations that result in gain of function (i.e., oncogenes such as *BRAF*) or loss of function (i.e., tumor suppressor genes such as *PTEN*), respectively. See the text for abbreviations.

minute-2 (HDM2) protein at its N terminus and promotes the rapid degradation of HDM2. Since HDM2 in turn ubiquitinates and condemns p53 to destruction, the net effect of $p14^{ARF}$ loss is a destabilization of p53 (Kamijo et al. 1998; Stott et al. 1998; Zhang et al. 1998). Thus, in true genetic economy, *CDKN2A* lesions eliminate both the RB and p53 pathways through loss of $p16^{INK4a}$ and $p14^{ARF}$, respectively (Lin et al. 2008).

Most germline mutations that confer melanoma risk occur in exons 1α and 2, which suggests that $p16^{INK4a}$ is the preferentially targeted, and functionally dominant, component of *CDKN2A*. For $p14^{ARF}$, rare deletions and a 16-base-pair (bp) insertion at exon 1β with a mutational hot spot at the exon 1β splice site (Hewitt et al. 2002; Harland et al. 2005b) have also been reported. The presence of exon 1β-specific alterations suggest that p14ARF is a bona fide melanoma susceptibility gene independent from p16. Even rarer deep intronic mutations of *CDKN2A* have also been described, although these account for very few cases worldwide (Harland et al. 2001, 2005a).

Several studies now have estimated the risk of developing cutaneous melanoma among *CDKN2A* mutation carriers. For familial cases, the overall penetrance has been calculated to be 30% by age 50, and 67% by age 80, although this risk is higher in sunnier climes. The risk of melanoma by age 50 reaches 13% in Europe, 50% in the United States, and 32% in Australia; by age 80, it is 58% in Europe, 76% in the United States, and 91% in Australia (Bishop et al. 2002). Among sporadic *CDKN2A* carriers, the risk appears lower: 14%, 24%, and 28% by ages 50, 70, and 80, respectively (Begg et al. 2005). Other coinherited modifiers (such as *MC1R* variants) (Demenais et al. 2010) may enhance risk, although the familial ascertainment itself may introduce bias and produce a higher calculated penetrance. Absolute melanoma risk among *CDKN2A* mutation carriers is clearly modulated by pedigree structure and environmental input.

Several animal models have provided biological proof of *CDKN2A*'s involvement in carcinogenesis, particularly in melanoma. These models demonstrated formation of fibrosarcomas and lymphomas with high frequency in mice containing a targeted disruption of *Cdkn2a* (Serrano et al. 1996). Evidence for involvement of this locus in melanoma has been shown using $Cdkn2a^{-/-}$ mice with alleles containing activating mutations in the oncogenes *HRAS* (Chin et al. 1997) and *NRAS* (VanBrocklin et al. 2010). These mice were readily induced to form melanoma-like cutaneous tumors. Within this family of oncogenes, however, observations have revealed *NRAS* as the most frequently mutated family member in human tumors (Hocker and Tsao 2007). Further proof of *Cdkn2a* involvement is shown in a gene exposure model through ultraviolet (UV) irradiation of $Cdkn2a^{-/-}$ mice. In this model, in the absence of DNA excision repair (e.g., inactivation or loss of *Xpc*), UV irradiation of mice led to rapid formation of cutaneous melanomas. It is interesting to note that the melanomas induced in this background harbored activating mutations in *KRAS* (Yang et al. 2007). This model illustrates the importance of fidelity in DNA repair and that any UV-induced damage to the DNA without this repair machinery in place may result in activation of the *RAS* pathway, thereby leading to melanomagenesis.

CDK4 Recurrent mutations of *CDK4*, which lead to cell cycle progression, have been reported in the germline of melanoma-prone families and in tumors (Wolfel et al. 1995; Zuo et al. 1996; Soufir et al. 1998; Tsao et al. 1998a; Molven et al. 2005). These mutations were observed to occur at a conserved arginine residue (Arg 24) that abolished regulatory interactions with $p16^{INK4a}$. Carcinogen treatment of a knock-in mouse model expressing the R24C mutation in CDK4 had a marked increase in potential to acquire melanomas after exposure. In concordance with these observations, some human melanomas have been known to amplify and/or mutate CDK4 (Muthusamy et al. 2006).

RB1 Hereditary retinoblastoma has been linked to inactivated copies of *RB1* in the germline; however, such carriers have a fourfold to 80-fold elevated risk to develop melanoma (Draper et al. 1986; Sanders et al. 1989; Eng et al. 1993; Fletcher et al. 2004). Development of sarcomas in patients with loss of *RB1* function decreases proportionately with decreased use of radiotherapy. However, the incidence of melanomas does not track with radiotherapy use, which is likely due to loss of heterozygosity (LOH) for the *RB1* allele. This demonstrates the intricate linkages between the CDKN2A/CDK/RB pathways of tumor suppression in humans.

Low-to-moderate-risk melanoma loci

In contrast to high-risk alleles, low-to-moderate disease variants often lack familial clustering and dictate cancer-susceptible traits, such as skin color, rather than cancer itself. Although some of these variants are quite common in the general population, some risk-conferring alleles have minor allele frequencies (MAFs) below 1%. These low-to-moderate-risk loci are outlined in the following sections.

Determinants of pigmentation and melanoma risk

MC1R A host of epidemiological studies long established a direct positive connection between light skin color and melanoma risk (for review, see Gandini et al. 2005). Among the plethora of genes known to regulate constitutive and facultative pigmentation, the melanocortin-1 receptor (*MC1R*) has emerged as one of the leading moderate-risk loci for melanoma susceptibility (for review, see Miller and Tsao 2010).

MC1R is a seven-transmembrane G-protein-coupled receptor that activates adenylate cyclase (Fig. 2) in response to α-MSH binding (Garcia-Borron et al. 2005). Subsequent increases in cAMP up-regulates the microphthalmia-associated transcription factor (MITF), which consequently induces the transcription of pigment synthetic genes and the production of eumelanin—the major source of UV attenuation in darkly pigmented skin. Germline variants of *MC1R* that disrupt this signaling cascade are present in ~80% of individuals with red hair color (RHC), <20% of people with brown or black hair,

and <4% of persons with a robust tanning response (Valverde et al. 1995). Association studies have found that the MC1R variants p.D84E, p.R151C, p.R160W, p.D294H, p.R142H, and p.I155T are strongly associated with the RHC phenotype (i.e., "R" variants), while the p.V60L, p.V92M, and p.R163Q variants seem to have weaker association with the RHC phenotype (i.e., "r" alleles) (Raimondi et al. 2008). A recent meta-analysis found that all but the p.V60L and p.V92M variants were associated with melanoma risk with odds ratios (ORs) ranging from 1.42-fold for p.R163Q to 2.45-fold for p.I155T (Raimondi et al. 2008). Interestingly, some variants are associated with melanoma risk but not pigmentary phenotypes, suggesting that MC1R may harbor subtle cancer effects that go beyond hair and skin color (Kennedy et al. 2001).

MITF As discussed above, engagement of α-MSH and MC1R leads to increased cAMP and the induction of MITF by the cAMP response element-binding protein (CREB) (Fig. 2). Although MITF's role in melanoma risk was thought to be purely responsive to MSH signaling, it now appears that variants in MITF itself are also instructive of risk. Through a whole-genome sequencing effort, a novel germline MITF variant (p.E318K) was discovered in a patient with melanoma. Among families harboring this variant, linkage analysis generated a log-of-odds (LOD) score of 2.7, implicating MITF(E318K) as a potential risk variant. In a large Australian/United Kingdom case control series of 3940 melanoma patients and 4036 controls, the p.E318K variant was found to confer a 2.19-fold risk, which is the range calculated for MC1R variants, although the prevalence of the MITF(E318K) alteration is significantly lower (<1%) in the general white population compared with MC1R RHC variants. Functional analysis (see "MITF—Melanocyte Master Regulator") showed that the missense mutation at codon 318 abrogated a conserved SUMOylation site, thereby altering the transcription of several MITF targets, including hypoxia-inducible factor 1a (HIF1a), a regulatory event that appears to also confer renal cell carcinoma risk among MITF(E318K) carriers (Bertolotto et al. 2011; Yokoyama et al. 2011).

Lessons from GWAS

Multiple GWAS have also yielded several risk-associated single-nucleotide polymorphisms (SNPs) across the genome (for review, see Chatzinasiou et al. 2011). The most substantiated loci include *MC1R* (per allele OR = 1.83; 95% confidence interval [CI] = 1.44–2.32), *ASIP* (per allele OR = 1.35; 95% CI = 1.08–1.68), *TYR* (per allele OR = 1.34; 95% CI = 1.14–1.58), *IRF4* (per allele OR = 0.80; 95% CI = 0.67–0.95), and *SLC45A2* (per allele OR = 0.72; 95% CI = 0.44–1.18). Additional loci that have recently been found include loci at *CASP8* (OR = 1.11; 95% CI = 1.06–1.18), *CCND1* (OR = 1.07; 95% CI = 1.01–1.13), *ATM* (OR = 0.87; 95% CI = 0.81–0.94), *MX2* (OR = 0.91; 95% CI = 0.86–0.96), and chromosome 1q21.3 (OR = 0.89; 95% CI = 0.85–0.95), including a region containing the *ARNT* and *SETDB1* genes (Chatzinasiou et al. 2011; Macgregor et al. 2011). It is clear from careful correlative analysis that some of these SNPs define overlapping phenotypes such as sun sensitivity, propensity for nevus formation, and skin cancer risk, including melanoma and nonmelanoma skin cancers.

In summary, a harvest of novel low–moderate melanoma risk loci has now been discovered through candidate and systematic genome-wide approaches. At this point, it is unlikely that there are many undisclosed recurrent high-risk loci (e.g., *CDKN2A*). Rather, mutations in multiple high-penetrant genes or the presence of several moderate-risk alleles in a single kindred may explain the balance of strong melanoma pedigrees. On a population level, most of the attributable risk for melanoma may in fact result from an untoward conspiracy of moderate-risk SNPs.

Biological drivers and therapeutic targets in melanoma

Receptor tyrosine kinase (RTK) activation

Over the years, a considerable amount of evidence has accrued in support of the notion that RTKs may contribute importantly to melanoma biology. Whereas earlier studies focused primarily on receptor overexpression, recent genomic studies suggest that genetic dysregulation may also play a role in some cases. These advances raise the possibility that small-molecule therapeutics targeting certain RTKs may prove clinically useful against specific subsets of melanoma. An integrated diagram of signaling molecules and biological drivers in melanoma is shown in Figure 2.

KIT The c-KIT gene encodes the RTK for stem cell factor (SCF). Although early reports described a sequential loss of c-KIT expression from benign to primary and metastatic melanomas (Montone et al. 1997; Shen et al. 2003; Zhu and Fitzpatrick 2006), reconstitution of the RTK in metastatic melanoma cells apparently restored sensitivity to SCF-induced apoptosis in vitro (S Huang et al. 1996). The role of c-KIT in melanoma biology had been uncertain until more recent genomic screens.

A survey of copy number imbalances in primary melanomas found the *KIT* locus (chromosome 4q11) to be amplified and/or mutated in 39% of mucosal, 36% of acral, and 28% of melanomas on chronically sun-damaged skin (CSD), respectively (Curtin et al. 2006). However, subsequent large series from Australia (Handolias et al. 2010) and China (Kong et al. 2011) revealed much lower rates of aberrations. It is notable that many of the activating *KIT* mutations described in melanomas overlap with those reported for gastrointestinal stromal tumors (GISTs) (Curtin et al. 2006; Antonescu et al. 2007; Rivera et al. 2008; Smalley et al. 2008; Ashida et al. 2009), suggesting that a similar trophic signaling pathway is shared between the two tumor types.

Given the success of imatinib mesylate in *KIT*-mutated GISTs, a pharmacological framework for targeting *KIT*-mutated melanomas became evident. Several early reports of dramatic responses using imatinib (Hodi et al. 2008; Lutzky et al. 2008) led to the development of two phase II (open label) trials of imatinib for *KIT*-mutated

melanomas. In one trial (Carvajal et al. 2011), 28 patients (N = 25 evaluable) with metastatic *KIT*-altered melanomas were treated, and two complete responses (CRs; 94 and 95 wk), two durable partial responses (PRs; 53 and 89 wk), and two transient PRs (12 and 18 wk) were reported. The median progression-free survival (PFS) and overall survival (OS) were 12 wk and 46.3 wk, respectively. In terms of the genetics, 23.4% of the cases harbored *KIT* mutations and/or amplifications, with the most significant responses occurring in patients with c-KIT(K642E) or c-KIT(L576P) mutations and those with tumors that enriched for the presence of a *KIT* mutation (i.e., mutant:allele ratio >1). In the second trial (Guo et al. 2011), 43 patients with metastatic melanomas harboring *KIT* aberrations (i.e., exons 9, 11, 13, 17, and 18 mutations and/or copy number gains) were treated with imatinib. Partial responses were observed in 10 patients (23.3%), with disease stabilization observed in another 13 patients (30.2%), and progressive disease observed in an additional 20 patients (46.5%). The median PFS and OS were 3.5 mo and 14.0 mo, respectively, in this second trial. Genotype–phenotype correlations did not reveal any evident relationships between response and *KIT* mutations. It is clear that imatinib exhibited only modest effects, although other RTK inhibitors (e.g., sunitinib, nilotinib, and dasatanib) are being tested with hopes of being more efficacious.

Epidermal growth factor receptor (EGFR) The gene encoding the *EGFR* is located on chromosome 7, which has long been known to undergo frequent polysomy in advanced melanoma (Koprowski et al. 1985; Bastian et al. 1998; Udart et al. 2001). Whereas enforced activation of *EGFR* may promote metastatic progression in cell line studies (de Wit et al. 1992; TS Huang et al. 1996), neither activating *EGFR* mutations nor focal *EGFR* amplifications have been observed in melanoma. Thus, most studies linking EGFR activation to melanoma biology have relied on protein expression or activation studies (Topcu-Yilmaz et al. 2010; Tworkoski et al. 2011). An assortment of in vitro studies has suggested that ectopic EGFR expression may enhance melanoma cell growth (Diaz et al. 2007; Ueno et al. 2008) and that pharmacological blockade of EGFR using small-molecule inhibitors or monoclonal antibodies may suppress growth in melanoma cell lines (Boone et al. 2011), either alone or in combination with other targeted agents (Ivanov and Hei 2005; Schicher et al. 2009). Also, preliminary results raise the possibility that EGFR activation may contribute to uveal melanoma pathogenesis in some instances (Wu et al. 2012). However, some studies found that the gefitinib GI_{50} values reached the 2–3 μM range and that melanoma cells continued to grow even at 10 μM gefitinib (Djerf et al. 2009). Together with the paucity of driver EGFR mutations in melanoma, such results have dampened preclinical enthusiasm for EGFR as a target in this malignancy. In fact, one recent study found that EGFR expression may modestly suppress melanoma growth in a B16 model (Diaz et al. 2009).

The limited clinical experience with EGFR inhibitors has been similarly disappointing. A phase II trial of the EGFR inhibitor gefitinib in 46 metastatic melanoma patients resulted in a median PFS of only 1.4 mo and a median OS of only 9.7 mo. During treatment, there were no reproducible changes in tumoral p-ERK1/2, p-AKT, and PAK1 and serum vascular endothelial growth factor (VEGF) and IL-8 levels (Patel et al. 2011). Thus, despite the aforementioned experimental evidence of EGFR's involvement in melanoma progression, there are scant clinical data to support single-agent anti-EGFR therapy.

Despite the relatively poor support for either EGFR dependency or monotherapy in melanoma, recent results raise the possibility that the combination of EGFR and RAF inhibitors might prove beneficial in some cases of de novo resistance to RAF inhibition. Bernards and colleagues (Prahallad et al. 2012) performed a synthetic-lethal RNAi screen in the setting of RAF inhibition in BRAF mutant colon cancer cells, which show limited sensitivity to RAF inhibitor monotherapy. They found that EGFR knockdown and pharmacological blockade were synergistic with vemurafenib in the suppression of BRAF mutant cells that were intrinsically resistant to vemurafenib alone. In this study, the addition of EGFR inhibition appeared to interdict a feedback up-regulation engendered by vemurafenib treatment. Although these results were seen in BRAF mutant colorectal cancer, they raise the intriguing possibility that at least some BRAF mutant melanomas that exhibit de novo resistance to RAF inhibition may be candidates for concomitant EGFR inhibition in future clinical trials.

MET The RTK c-MET is normally activated by binding of its ligand, hepatocyte growth factor/scatter factor (HGF). Autocrine activation of HGF-MET has been described in melanoma progression (for review, see Vande Woude et al. 1997; Li et al. 2001). Although increased c-MET expression has been observed in metastatic melanoma (Natali et al. 1993), the *MET* locus also resides on chromosome 7, which commonly undergoes polysomy in this setting (as described above). As with *EGFR*, *MET* amplifications and activating point mutations thus far have not been described in melanoma. Thus, the evidence that MET activation constitutes a bona fide melanoma dependency remains scant. However, several lines of experimental evidence suggest that MET signaling may enhance melanoma growth and metastasis. For example, HGF exposure may promote increased melanocytic cell mobility (Damm et al. 2010), and HIF-1α may promote MET-dependent invasion and vasculogenic mimicry in melanoma cells (Comito et al. 2011). Thus, MET activation may serve to augment rather than "drive" melanomagenesis and progression per se.

A small-molecule inhibitor of c-MET (SU11274) is now available for potential clinical use. In one preclinical analysis, melanoma lines that exhibited constitutive c-MET stimulation in the absence of *MET* alterations underwent decreased proliferation and an increase in apoptosis when exposed to SU11274. In a xenograft model, the compound also demonstrated significant anti-tumor activity (Kenessey et al. 2010). Currently, the role of anti-c-MET treatment in melanoma remains a theoretical promise at best.

Ephrin receptors Ephrin receptors constitute the largest family of RTKs in the kinome. Physiologically, ephrin ligands are membrane-bound, so forward (through Eph receptors) and reverse (through ephrin molecules) signaling can occur upon cell–cell interaction (Genander and Frisen 2010). One specific Eph member, EphA2, has been directly implicated in tumor formation. Published studies report an increased expression of EphA2 in multiple cancer types, including melanoma (Easty et al. 1999; Seftor et al. 2002; Hendrix et al. 2003; Kinch and Carles-Kinch 2003; Miyazaki et al. 2003; Herath et al. 2006; Genander and Frisen 2010). Furthermore, the *EPHA2* locus maps to chromosomal region 1p36, which is a region frequently altered in melanoma (Sulman et al. 1997). Mechanistically, EphA2 appears to participate in cross-talk between other cancer signaling circuits, such as the RAS–phosphatidylinositol 3-kinase (PI3K)–AKT and RAS–MAPK pathways (Menges and McCance 2008). Recent investigations revealed that some melanomas are, in fact, "addicted" to EphA2 (Udayakumar et al. 2011). shRNA-mediated silencing of EphA2 led to a rapid apoptotic response along with tumor suppression in a xenograft model, while ectopic expression of EphA2 led to enhanced colony formation and migration. Interestingly, EphA2 appears to be essential for UV-mediated apoptosis, and acute introduction of EphA2 into normal and immortalized cells also elicits an apoptotic response (Zhang et al. 2008). These findings suggest that Eph receptors play a complex role in melanocytes and melanomas. It is possible that early death purges the genetically susceptible population, thereby leaving more aggressive tumor cells that come to depend on EphA2 signaling for sustenance; this is compatible with an "overdose/addiction" model whereby oncogene stress, which is a common physiological response, serves as a selection pressure for tumorigenic recruits.

Although mutagenic activation of *EPHA2* is not commonly observed (Udayakumar et al. 2011), other Eph receptors have been shown to be mutated in melanoma. In particular, multiple lesions have been observed in *EPHA6*, *EPHA10*, *EPHB1*, *EPHB2*, and *EPHB6* (Prickett et al. 2009). Given the various molecular systems that are impacted by these receptors, functional classification of the Eph receptors into oncogenes or tumor suppressors is not yet possible without further study.

ERBB4 In a sweeping analysis of the tyrosine kinome, Prickett et al. (2009) screened the coding exons of 86 protein tyrosine kinases and identified 99 nonsynonymous somatic mutations. Most prominent among these was *ERBB4*, which was mutated in 19% of melanoma cases. The missense mutations were oncogenic in several in vitro assays, such as NIH-3T3 transformation and soft agar growth; a recent genotyping effort (Dutton-Regester et al. 2012) points to a possible low-prevalence ERBB4(E452K) hot spot. Inhibition of ERBB4 by lapatinib also led to apoptosis in *ERBB4*-mutated cells. Given the broad-based nature of this kinome screen, it is unlikely that common forms of melanoma harbor high rates of recurrent activating mutations in any single RTK. It is possible that melanoma cells have bypassed the need for more upstream signaling, given the high rate of oncogenic changes in downstream molecules such as NRAS and BRAF (see below).

Glutamate receptor dysfunction

GRMs Although RTKs represent attractive biological targets for oncogenic activation, other surface receptors may promote melanomagenesis in unsuspecting ways. It is intriguing that several neurotransmitter receptors have now been shown to participate in melanoma pathogenesis. Since melanocytes are derived embryologically from neural crest cells, it is possible that a shared mechanistic circuitry between neural and melanocyte descendants permits melanoma cells privileged access to neurophysiological molecules. The earliest hints that glutamate receptors contribute to melanoma formation were derived from mouse studies (Pollock et al. 2003a) in which aberrant *Grm1* expression was associated with a melanoma-prone phenotype. GRM1 is expressed in human melanoma specimens but not benign melanocytic nevi. This surface molecule is a member of the metabotropic glutamate receptor family, which is comprised of G-protein-coupled receptors (GPCRs) that activate phospholipase C upon ligand binding. More recently, Choi et al. (2011) showed that mice in which metabotropic glutamate receptor 5 (mGluR5) expression was driven by the *Trp1* promoter also developed murine melanomas with high penetrance. Furthermore, mGluR5 expression could be documented in human melanomas and may provide an oncogenic signal through ERK (Choi et al. 2011).

The role of metabotropic glutamate receptors remained circumstantial until mutations were uncovered in another metabotropic glutamate receptor, *GRM3*, during a GPCR family-wide screen of human melanoma specimens (Prickett et al. 2011). Four of these mutations were subjected to functional analysis (p.Gly561Glu, p.Ser610Leu, p.Glu767Lys, and p.Glu870Lys) and found to stimulate MEK1/2 in the presence of agonist and melanoma migration even in the absence of agonists. Melanoma cells with GRM3 variants were also more sensitive to MEK inhibition by AZD6244. On the surface, activated GRM3 appears to be an accessory to MAPK signaling in melanomas. However, since BRAF or NRAS is often mutated in melanoma cells (see below), the precise contribution of GRM3 to this pathway is unclear. It is known that even among BRAF-mutated melanomas, there is a range of primary sensitivity to BRAF and MEK inhibitors (Flaherty et al. 2010; Chapman et al. 2011). Thus, accessory MAPK signal flux through proteins such as GRM3 may play a compensatory role.

GRIN2A Through an unbiased exome-wide sequencing effort, mutations in *GRIN2A* have also been discovered (Wei et al. 2011). Unlike GRM3, GRIN2A is an ionotropic glutamate-gated ion channel that binds N-methyl-D-aspartate (NMDA). This ligand-gated channel is permeable to cations, including Ca^{2+}. Mutations in *GRIN2A* was found in 34 out of 135 melanoma samples (25.2%).

Unlike GRM3, however, there were multiple missense and several nonsense mutations scattered along *GRIN2A*, suggesting that this gene is not a canonical oncogene in melanoma. Functional validation of GRIN2A as an oncogene has yet to be performed.

Small G proteins, including RAS

The *RAS* family of small G proteins serves to transduce signals triggered by extracellular growth factors (Ji et al. 2012a). Unlike other solid tumors, activating *RAS* mutations occur in a relatively small fraction of melanomas (~10%–15%), with a higher frequency noted in amelanotic nodular melanoma subtypes (for review, see Chin et al. 1998). Among *RAS* genes, melanoma mutations are most common in *NRAS*, which is also thought to be mutated in the majority of congenital nevi (Papp et al. 1999) but rarely in dysplastic nevi (Albino et al. 1989; Jafari et al. 1995; Papp et al. 1999). *HRAS* mutation has been associated with Spitz nevi, based on both genomic amplifications and mutations (Bastian et al. 2000). *KRAS* and *HRAS* mutations have both been reported in ~2% of melanomas (http://www.sanger.ac.uk/genetics/CGP/cosmic).

Interestingly, differences between HRAS and NRAS have also been noted in relation to the consequences of their transgenic targeting to melanocytes. Whereas activated HRAS together with loss-of-function mutations in *Cdkn2a* and/or *Trp53* produce nonmetastatic melanomas in mice (Chin et al. 1997; Bardeesy et al. 2001; Sharpless et al. 2003), activated *NRAS* together with *Cdkn2a* deficiency produce melanomas with major metastatic propensity to both lymph nodes and distant sites (Ackermann et al. 2005).

Since oncogenic RAS proteins were among the first oncogenes described in humans, potent pharmacological inhibitors of RAS proteins have been a source of aggressive development. Farnesyl transferase inhibitors (FTIs; e.g., R115777/tibifarnib) were first deployed as selective RAS inhibitors, given the drug's ability to interfere with the requisite lipid modification of RAS (James et al. 1993); despite initial enthusiasm, FTIs have not fared well in clinical trials (Caponigro et al. 2003). One hypothesis for this failure is that FTIs impair other farnesylated proteins, which then lead to dose-limiting toxicities. Alternatively, RAS proteins may use geranylgeranyltransferases, thereby bypassing the block imposed by FTIs (Whyte et al. 1997). More recently, S-*trans*, *trans*-farnesylthiosalicylic acid (FTS) was developed to mimic the C-terminal farnesylcysteine (Weisz et al. 1999), thereby competing with the active, GTP-bound forms of RAS for specific binding sites on the cellular membrane (Aharonson et al. 1998). FTS appears to be effective in inhibiting melanoma growth both in vitro and in animal models (Jansen et al. 1999; Smalley and Eisen 2002). Clinical studies in pancreatic cancer also showed some possible survival benefits (Johnson et al. 2009; Laheru et al. 2009), although the efficacy of FTS as a single agent in melanoma awaits clinical testing.

GNAQ The story behind *GNAQ*'s involvement in melanoma unfolded through an exchange between developmental biology and cancer genetics. In a forward genetic screen, Barsh's laboratory (Van Raamsdonk et al. 2004) identified hypermorphic mutations in *GNAQ* and *GNA11* as causative of diffuse hyperpigmentation and dermal melanocytosis in mice. Among comparable human lesions, blue nevi represent a benign proliferation of dermal melanocytes. Given the phenotypical overlap, analyses of *GNAQ* and *GNA11* were performed in a collection of benign and malignant melanocytic tumors, and *GNAQ* mutations were found in 83% of blue nevi ($N = 29$), 50% of "malignant blue nevi" ($N = 2$), and 46% of uveal melanomas ($N = 48$) (Van Raamsdonk et al. 2009). Like other oncogenes, a single missense change (p.Q209L) accounts for all identified GNAQ mutations. This GNAQ variant fully activates the MAPK pathway and is oncogenic in both in vitro and in vivo assays. In a follow-up study (Van Raamsdonk et al. 2010), somatic mutations in exon 5 (affecting Q209) and exon 4 (affecting R183) of both GNA11 and GNAQ were seen in a mutually exclusive pattern. GNA11 mutations are present in 7% of blue nevi, 32% of primary uveal melanomas, and 57% of uveal melanoma metastases, while GNAQ alterations were present in 55% of blue nevi, 45% of uveal melanomas, and 22% of uveal melanoma metastases. Both GNAQ and GNA11 mutations activate the MAPK pathway. The epistatic relationship between GNAQ and GNA11 implies that both fulfill overlapping functions in melanocytes or that the presence of both mutations creates a synthetic-lethal condition. There is also early evidence that GNA11 alterations may be more predictive of metastases and thus may represent a potentially crucial therapeutic target (Van Raamsdonk et al. 2010).

Other members of the heterotrimeric G-protein family, which includes GNAQ and GNA11, have also been screened in unselected metastatic melanomas (Cardenas-Navia et al. 2010). No other recurrent alterations were detected, although the overall nonsynonymous somatic mutation rate was 17.5%.

BRAF activation—the heart of melanoma oncogenesis

The discovery of oncogenic *BRAF* mutations in melanoma (Davies et al. 2002) stands as one of the most powerful affirmations of the transformative potential of systematic cancer genome characterization. In hindsight, it is all the more remarkable that BRAF mutations were discovered by Stratton and colleagues (Davies et al. 2002) using a discovery set of only 15 tumor/normal pairs—only one of which was a melanoma sample! Since that seminal discovery, *BRAF* mutations—most commonly a valine-to-glutamic acid substitution at codon 600—have been observed in ~50% of melanomas (Maldonado et al. 2003; Pollock et al. 2003b; Uribe et al. 2003; Daniotti et al. 2004; Kumar et al. 2004; Shinozaki et al. 2004; Libra et al. 2005) and to a lesser extent in other cancers (Ciampi and Nikiforov 2005; Young et al. 2005). *BRAF* mutations also occur at high frequencies (>80%) in melanocytic nevi (Pollock et al. 2003b; Yazdi et al. 2003; Kumar et al. 2004; Saldanha et al. 2004), suggesting that these somatic alterations occur early in melanomagenesis. Interestingly, in-

dividuals with germline *BRAF* mutations develop cardio–facio–cutaneous syndrome but do not exhibit increased cancer risk (Niihori et al. 2006; Rodriguez-Viciana et al. 2006)—nor do they harbor the V600E mutation that is so prevalent in melanoma, colorectal cancer, and thyroid cancer.

Although it is tempting to speculate that the BRAF(V600E) mutation is induced by UV damage, the T → A transversion that converts the valine to glutamic acid at amino acid 600 (V600E) is not part of the "classic" UV-induced mutational signature (Daya-Grosjean et al. 1995). Nonetheless, a durable epidemiological relationship between *BRAF* mutations and sun exposure has been noted. In particular, *BRAF* mutations are much more common in melanomas arising on intermittent sun-exposed (solar elastosis of arms, trunk, etc.) than in acral melanomas—which arise on less sun-exposed glabrous skin—and mucosal melanomas (e.g., gastrointestinal, vaginal origin, etc.) (Maldonado et al. 2003; Edwards et al. 2004; Curtin et al. 2005; Bauer et al. 2011). Strikingly, BRAF mutations are absent in uveal melanoma (Cohen et al. 2003; Cruz et al. 2003; Edmunds et al. 2003; Rimoldi et al. 2003; Weber et al. 2003). Conceivably, the substitution that undergirds the BRAF(V600E) mutation may reflect a secondary effect of UV damage, such as the generation of reactive oxygen species. Toward this end, recent results suggest that melanocytic cells may be deficient in repair of oxidative DNA damage (Wang et al. 2010). Alternatively, this event may arise as a result of "nonclassic" DNA lesions induced by UV (for review, see Besaratinia and Pfeifer 2008).

Given that melanocytic nevi rarely progress into melanoma, it stands to reason that BRAF(V600E)-induced checkpoint mechanisms may produce a senescence-like state in the absence of additional genetic or molecular events that promote tumorigenesis. Toward this end, congenital nevi stain positively for senescence-associated acidic β-galactosidase (SA-β-Gal) (Michaloglou et al. 2005), and BRAF(V600E) expression in primary human melanocytes induces cell cycle arrest. Thus, oncogene-induced senescence (OIS) appears to constrain progression of premalignant melanocytic lesions (Sharpless and DePinho 2005). Senescent melanocytes exhibit a mosaic p16-staining pattern, suggesting that melanocytic senescence is not invariably dependent on p16 up-regulation (Michaloglou et al. 2005).

While BRAF(V600E) mutation proved insufficient to transform human melanocytes by itself, multiple lines of evidence showed that dysregulated MAPK activation was necessary for melanoma cell viability in this setting. Suppression of oncogenic BRAF by RNAi-mediated knockdown resulted in markedly reduced cell growth, diminished ERK phosphorylation, and induction of apoptosis in some instances (Hingorani et al. 2003; Karasarides et al. 2004; Wellbrock et al. 2004). BRAF knockdown also reduced tumor formation in murine xenograft models (Hoeflich et al. 2006). Furthermore, selective small-molecule RAF inhibitors potently suppressed the growth of BRAF mutant melanoma cell lines but had little effect on melanoma cells that lacked these mutations (Joseph et al. 2010). Thus, the presence of BRAF mutations conferred a stringent tumor dependency on MAPK signaling in general and ectopic BRAF activation in particular.

Several groups have identified genes whose protein products may drive oncogenesis together with BRAF in melanoma. The master melanocyte regulator MITF was found to cooperate with BRAF in melanoma tumor formation in vitro (Garraway et al. 2005) and in vivo (Jane-Valbuena et al. 2010). In zebrafish, BRAF activation alone resulted in benign nevus formation, while malignant transformation requires concurrent loss of p53 (Patton et al. 2005). Expression of the BRAF(V600E) allele alone in TERT-immortalized RB–p53 mutant human melanocytes produced only junctional moles in a human/mouse skin graft model, in contrast to activated *NRAS* or *PI3K p110a* mutants, which generated invasive melanoma lesions (Chudnovsky et al. 2005). Two very similar *Braf(V600E)* murine models have emerged, confirming the tumorigenic potential of the mutated *Braf* allele (Dankort et al. 2009; Dhomen et al. 2009). While both models capitalize on a Tyr-cre-inducible "knock-in" of the oncogenic change (i.e., V600E), the phenotypic details differ in subtle but important ways. The Dankort model (Dankort et al. 2009) provided evidence for cooperativity between *Braf* activation and *Pten* loss—an observation first made in human melanoma cell lines (Tsao et al. 2004). Furthermore, they found that *Braf(V600E)* expression alone in skin melanocytes led to widespread benign melanocytic neoplasia but very few, if any, frank melanomas. This is consistent with the idea that BRAF activation alone results in senescence and nevi. In contrast, the Dhomen model (Dhomen et al. 2009) generated a 60%–70% rate of melanoma tumor formation with *Braf(V600E)* expression alone, although abrogation of *Cdkn2a* enhanced tumor multiplicity and shortened the latency period. Interestingly, loss of *Cdkn2a* did not constrain nevus development, suggesting that senescence from *Braf(V600E)* is independent of *Cdkn2a*. The animal models offer substantive proof that BRAF(V600E) mediates melanoma growth in various melanocytic systems.

Biochemical and biophysical studies have recently unraveled the mechanism by which RAF molecules become catalytically primed to phosphorylate MEK. RAF enzymatic activity appears to be triggered by a specific "side-to-side" mode of dimerization as either dimers between two RAF proteins or heterodimers with the RAF-related pseudo-kinase KSR (kinase suppressor of RAS). This side-to-side mechanism is essential for signaling by oncogenic BRAF mutants (Rajakulendran et al. 2009) and is also a critical determinant of resistance to anti-BRAF therapies (see below).

Sorafenib, a type II kinase inhibitor, was the first RAF inhibitor subjected to clinical trial. Sorafenib inhibits several kinases, including BRAF, CRAF, and the VEGF and PDGF RTKs (Wilhelm et al. 2004). After failing to demonstrate a survival benefit in a phase III trial of patients with advanced melanoma (Hauschild et al. 2009), sorafenib has now been largely abandoned in melanoma, certainly as a single agent. Whittaker et al. (2010) provided evidence against the dogma that sorafenib's effects were primarily against BRAF. They engineered cells with a BRAF "gate-

keeper" mutation (i.e., p.T529N) that rendered the BRAF(T529N/V600E) cells resistant to sorafenib in vitro and yet sensitive to sorafenib in vivo, a paradox that does not exist with more selective BRAF inhibitors (SBIs) such as PLX4720 (see below). It is thus possible that sorafenib possesses BRAF-independent cellular effects and may also explain why sorafenib causes toxicity at a lower dose than needed for MEK inhibition (Whittaker et al. 2010).

SBIs are now available in both clinical and research settings. The first of these SBIs, vemurafenib, is 10-fold more selective for mutated BRAF compared with its wild-type counterpart (Tsai et al. 2008), although its effectiveness against CRAF (IC_{50} = 48 nM) in vitro is often overlooked (Poulikakos et al. 2010). The clinical efficacy of vemurafenib in the metastatic setting has now been thoroughly validated in clinical trials (Flaherty et al. 2009, 2010; Chapman et al. 2011). A total of 675 patients with documented BRAF(V600E) mutations enrolled in BRIM-3 (the pivotal phase III study). Patients received either dacarbazine or vemurafenib. The rates of OS were 84% in the vemurafenib (N = 336) group and 64% in the dacarbazine (N = 336) group (hazard ratio = 0.37; P < 0.001). This translated to an estimated median PFS of 1.6 mo in the dacarbazine group and 5.3 mo in the vemurafenib group. The compelling outcomes of BRIM-3 led to the approval of vemurafenib by the Food and Drug Administration (FDA) in 2011.

There are several other BRAF inhibitors that are currently undergoing clinical testing (Smalley and Flaherty 2009). GSK2118436 is another promising BRAF inhibitor (Kefford et al. 2010), with potential benefits for patients with brain metastasis. In wild-type BRAF tumors, CRAF may also be an effective therapeutic target (Garnett et al. 2005; Gray-Schopfer et al. 2005), especially since CRAF appears to play a key role in NRAS-mutated melanomas (Dumaz et al. 2006). Broader-spectrum pan-RAF inhibitors may be developed for NRAS-mutated and NRAS/BRAF dual wild-type tumors.

Despite vemurafenib's success as an SBI, several sentinel questions have also surfaced as a result of deeper investigations: (1) How do SBIs paradoxically stimulate MEK–ERK signaling in BRAF wild-type (e.g., RAS-mutated) cells? (2) How do BRAF-mutated cells eventually escape SBI suppression? As alluded to above, RAF activation involves a side-to-side dimerization, and two models that invoke this mechanism have emerged to answer the first question (Heidorn et al. 2010; Poulikakos et al. 2010).

In response to growth factor receptor engagement or in the face of an oncogenic mutation, RAS mobilizes to the plasma membrane and induces homodimers and heterodimers of BRAF and CRAF, which then leads to MEK phosphorylation and activation. In BRAF(V600E) cells, MEK is largely phosphorylated by the constitutively active BRAF, and consequently, an SBI abrogates nearly all downstream MEK–ERK signaling. So why is there paradoxical activation of MAPK signaling in BRAF wild-type cells? In one model, low concentrations of any RAF inhibitor leads to inactivation of a single monomer in the RAF dimer, thereby "transactivating" its partner RAF molecule and triggering MAPK signaling (Poulikakos et al. 2010); increasing levels of the RAF inhibitor inhibits both RAF partners, and all signaling is thus abrogated. In the Heidorn model (Heidorn et al. 2010), wild-type BRAF translocates to the cell membrane upon SBI binding, dimerizes with CRAF, and further stimulates CRAF signaling. A pan-RAF inhibitor suppresses both BRAF and CRAF, thereby effectively shutting down all MAPK signaling. Gatekeeper CRAF mutations [e.g., CRAF(T421N)] that interfere with the binding of RAF inhibitor to CRAF could restore signaling. The bottom line is that RAS-mutated cells are stimulated by a SBI (Fig. 3), which could

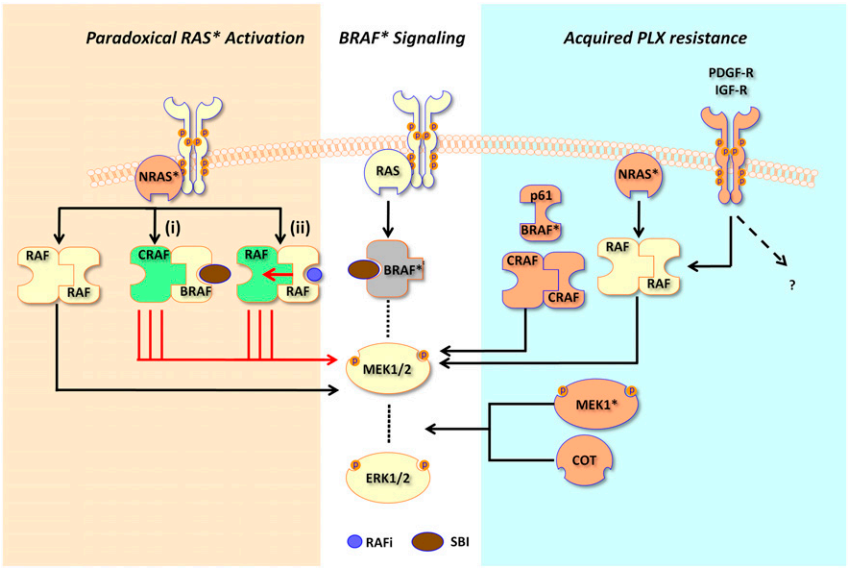

Figure 3. Pharmacological mechanisms underlying selective BRAF inhibition. In cells dependent on BRAF(V600E) (BRAF*) signaling, binding of a SBI such as PLX4720/4032 leads to attenuation of downstream MEK1/2 and ERK1/2 signaling. In RAS*-dependent cells (tan background shading), MEK and ERK are paradoxically stimulated by RAF inhibitors. In one model (i), binding of SBIs to BRAF leads to binding between BRAF/CRAF and increased stimulation of CRAF by RAS*, since BRAF activity is inhibited by the SBI. In another model (ii), RAS* mediates dimerization between RAF partners; when one RAF molecule within the dimer is inhibited by a RAF inhibitor (RAFi), there is transactivation of the uninhibited partner, thereby stimulating downstream signaling. In cells with acquired SBI resistance, several mechanisms have been described. *BRAF* gene amplification, a BRAF(V600E) splice variant (p61BRAF*), MEK1 mutation (MEK1*), secondary NRAS activation (NRAS*), and stimulation of PDGF-R or IGF-R have all been observed in tumors samples. Overexpression of CRAF and COT1 has been shown to confer resistance in functional screens.

explain poor response to vemurafenib among patients with BRAF wild-type tumors and the observed HRAS-driven squamous cell carcinomas (SCCs) that develop while on vemurafenib (Su et al. 2012) and other first-generation RAF inhibitors. Thus, the use of vemurafenib requires absolute genetic precision in order to avoid acceleration of disease and potentially untoward side effects.

How do cells escape SBI inhibition? Multiple mechanisms have now come to light to explain vemurafenib resistance (Fig. 3). The acquisition of a new NRAS or MEK1 mutation or the up-regulation of PDGFRβ or IGFR results in reactivation of MAPK signaling and acquired resistance to vemurafenib (Nazarian et al. 2010; Villanueva 2010; Wagle et al. 2011). Ectopic expression of both CRAF and COT/TPL2/MAP3K8 was also associated with greater resistance to PLX4720, a tool compound of vemurafenib (Johannessen et al. 2010). Most recently, a 61-kDa variant form of BRAF(V600E) [p61BRAF(V600E)], which lacks the RAS-binding domain, was found to enhance RAF dimerization as compared with full-length BRAF(V600E) (Poulikakos et al. 2011). Ectopic p61BRAF(V600E) expression creates constitutive ERK signaling that is unaltered by the inhibitor. Splice variants that lack the RAS-binding domain were also detected in six out of 19 tumors from patients with acquired resistance to vemurafenib. BRAF amplification has also been recently reported to be associated with vemurafenib resistance (Shi et al. 2012). It is clear at this moment that vemurafenib creates a high selection pressure for survival, and any genetic or epigenetic mechanism that allows for reactivation or bypass of ERK signaling will likely induce resistance. One clinically untenable situation is the emergence of multiple distinct resistance mechanisms within different metastatic deposits, thereby rendering a uniform secondary therapeutic attack nearly impossible.

Downstream MAPK effectors

Recent whole-exome sequencing approaches have yielded other MAPK pathway components that are also mutated in addition to NRAS and BRAF. In the first study from Australia, investigators performed exome-wide sequencing of eight melanoma lines along with matched normal germline DNA. In this screen, isolated mutations in MAP3K5 and MAP3K9 were identified (Stark et al. 2011). A subsequently expanded screen found that eight out of 85 additional melanoma cell lines harbored nonsynonymous changes in MAP3K5, and 13 out of 85 cell lines had mutations in MAP3K9. Functional analysis of several MAPK3K5 alterations (p.Glu663Lys, c.G1987A, and p.Ile780Phe) and MAP3K9 changes (p.Trp333* and p.Lys171Ala) led to a loss of kinase function. In a parallel set of studies, these investigators also found that suppression of MAP3K9 activity also contributed to temozolomide resistance in melanoma. Undertaking a similar approach, a Swiss group (Nikolaev et al. 2011) also performed whole-exome sequencing of seven metastatic melanoma specimens and identified two samples with somatic mutations in MEK1 and MEK2. In a subsequent screen of MEK1 and MEK2 in 127 additional melanomas, 10 (8%) samples harbored mutations in either MAP2K1 or MAP2K2, although the presence of these mutations did not correlate with BRAF mutation status. Although it is possible that the MEK1/2 mutations activate ERK, the presence of these alterations in the face of oncogenic BRAF(V600E) lesions suggests that other signaling effects may be occurring. Initial evidence has also accrued to suggest that at least some MEK1/2 mutations may also confer resistance to RAF inhibition (Wagle et al. 2011).

Although MEK mutations have only recently been described in melanoma, MEK inhibition has long been recognized as an attractive therapeutic approach in tumors with BRAF(V600E) mutations. Before potent and selective RAF inhibitors were widely available, MEK inhibitors were found to exhibit exquisite potency against BRAF(V600E) melanomas (Solit et al. 2006). Accordingly, multiple small-molecule MEK inhibitors are in development. Early clinical trials of first-generation MEK inhibitors were confounded by suboptimal potency and pharmacodynamic parameters (e.g., CI-1040) (Rinehart et al. 2004). In hindsight, then, it is perhaps not surprising that these inhibitors showed only moderate effects in phase I trials of a limited number of patients with BRAF (V600E) melanomas (Adjei et al. 2008). However, new MEK inhibitors have shown clinical promise both as single agents and in combination with RAF inhibitors (Gray-Schopfer et al. 2007; Infante et al. 2010; Gilmartin et al. 2011). Thus, MEK inhibition may ultimately find a role in the treatment of BRAF mutant melanoma.

PTEN, negative regulator of the PI3K–AKT pathway

The PI3K pathway is often dysregulated in melanoma. The *PTEN* tumor suppressor gene encodes a lipid and protein phosphatase that regulates cell growth and survival through PI3K/AKT signaling. PTEN negatively regulates signal transduction that uses phosphatidylinositol phosphate (PIP_3) as a cytosolic second messenger. Upon cell surface receptor (e.g., RTK) activation, growth factors augment intracellular PIP_3 levels, which triggers downstream events that typically converge on phosphorylation of the serine/threonine kinase AKT. AKT is a well-known oncogene that sends many downstream signals to promote cell growth and survival. In melanoma, elevated phospho-AKT levels may correlate adversely with patient survival (Dai et al. 2005).

PTEN/PI3K pathway genomic alterations are considerably less prevalent than MAPK pathway alterations in melanoma; however, recent results may suggest a larger diversity of genetic events affecting this pathway—at least indirectly—than was initially appreciated. Since PI3K itself is rarely mutated in melanoma (Omholt et al. 2006) in contrast to other malignancies, the PTEN tumor suppressor gene has emerged as the dominant genetic target in this pathway. The *PTEN* locus is situated on chromosome 10q, which undergoes frequent hemizygous deletion and LOH in melanoma (Bastian 2003; Wu et al. 2003). In melanoma, allelic loss or altered expression of PTEN comprises 20% and 40% of melanoma tumors, respectively (Tsao et al. 1998b, 2003; Pollock et al. 2002;

Mikhail et al. 2005; Slipicevic et al. 2005; Goel et al. 2006). Somatic point mutations and homozygous deletions are uncommon (Lin et al. 2008), although focal deletions affecting the *PTEN* locus may be more common than initially appreciated, based on recent genome sequencing studies (Berger et al. 2012). This same study, along with an earlier whole-genome study of a single melanoma cell line (Pleasance et al. 2010), also found recurrent *MAGI2* mutations. *MAGI2* encodes a protein known to interact with and stabilize PTEN (Tolkacheva et al. 2001; Vazquez et al. 2001); thus, MAGI2 disruption may offer an additional means of PTEN dysregulation in melanoma. More recently, Karreth et al. (2011) described a competitive endogenous RNA (ceRNA), designated ZEB2, which modulates PTEN levels and whose attenuation leads to increased PI3K/AKT pathway signaling. Phosphatase-independent function, such as direct binding and transactivation of p53 (Tang and Eng 2006), may also contribute to its melanoma-suppressive effects. Forced expression of PTEN in PTEN-deficient melanoma cells suppresses AKT phosphorylation, cell growth, and several other tumorigenic phenotypes (Robertson et al. 1998; Stewart et al. 2002; Stahl et al. 2003; for review, see Robertson 2005).

Several genetic studies have noted that hemizygous deletions spanning chromosome 10q (which includes the *PTEN* locus) occur with high frequency in BRAF mutant melanomas (Bastian 2003; Lin et al. 2008). In contrast, *PTEN* deletions are less common in NRAS mutant melanomas. These observations suggested that BRAF and PTEN may cooperate in melanoma tumorigenesis (Tsao et al. 2004). Indeed, this notion has been borne out in genetically engineered mouse models in which melanocytes were engineered to undergo *PTEN* inactivation in the setting of BRAF(V600E) expression (Dankort et al. 2009). The melanomas that form are often highly aggressive, but their growth can be inhibited by combined MEK/target of rapamycin (TOR) inhibition (Dankort et al. 2009). In one mouse model, the mechanism of metastasis appears to involve β-catenin signaling (Damsky et al. 2011). Together, these observations suggest that cooperation between dysregulated MAPK and PI3K signaling (governed by BRAF mutation and PTEN deficiency) may comprise an oncogenic driver for a substantial subset of melanomas.

Aberrant AKT activation can also promote melanocyte transformation (Chudnovsky et al. 2005), as predicted from the aforementioned studies. Some lines of evidence point to a possible role for AKT3 in this process. For example, copy gains spanning *AKT3* have been observed in melanoma, and AKT3 activation may be common (Stahl et al. 2004). Similarly, point mutations in AKT3 have occasionally been reported (Davies et al. 2008). Despite these observations, the role of AKT as a melanoma oncogene remains incompletely understood. For example, AKT1 activation inhibits migration and invasion in some cancer models (Yoeli-Lerner et al. 2005), including MDA-MB435, a cell line previously annotated as a breast cancer model but subsequently found by genetic studies to be melanoma-derived (Ross et al. 2000; Garraway et al. 2005). Thus, while the general importance of PTEN/PI3K dysregulation in at least a subset of melanoma seems well-established, the contribution of AKT as a downstream effector remains a topic of active investigation.

There is substantial evidence that the mammalian TOR (mTOR) pathway acts as a major effector of the AKT oncogenic signal downstream from PI3K activation. Thus far, however, TOR inhibitors have proved mostly ineffective in melanoma, at least as single agents (Margolin et al. 2004; Xu et al. 2004). However, no trial has been completed that evaluates TOR inhibitors in combination with the selective RAF and MEK inhibitors that are either FDA-approved or in clinical trials. Conceivably, such trials might be further enhanced by the concomitant incorporation of genomic profiling to identify patients whose tumors harbor genetic alterations predicted to dysregulate PTEN/PI3K signaling.

Apoptosis regulators in melanoma

An abundance of mechanistic studies indicated that the capacity to undergo efficient apoptosis may predict sensitivity to anti-cancer therapeutics (Johnstone et al. 2002). The melanocyte lineage uses several mechanisms that appear to diminish the propensity to activate an apoptotic program. Synthesis of eumelanin provides UV protection. Yet melanocytes and melanoma cells exhibit greater protection against UV-induced apoptosis than adjacent keratinocytes, suggesting that additional survival signals may protect the melanocyte lineage against cell death (for review, see Soengas and Lowe 2003). Melanomas commonly resist induction of apoptosis (Glinsky et al. 1997) and appear to be protected by multiple mechanisms, which include activation of the MAPK and PI3K/AKT pathways (Wada and Penninger 2004; Kharas and Fruman 2005). In melanoma, the MAPK pathway antagonizes apoptosis via multiple mechanisms that include suppression of Smac/DIABLO release from mitochondria (Zhang et al. 2003), expression of anti-apoptotic BCL2 through MITF (McGill et al. 2002), suppression of the proapoptotic protein BAD (Eisenmann et al. 2003), and phosphorylation-sensitive degradation of proapoptotic BIM (Cartlidge et al. 2008). While a fully integrated understanding of apoptosis regulation remains to be completed for melanoma, it is likely that such information will be mechanistically informative regarding therapeutic efficacy and resistance for the new generation of melanoma treatments.

BCL2 has long been known to be expressed in both melanocytes and melanoma cells (Plettenberg et al. 1995). Its expression has been seen to be up-regulated by a variety of growth factors, including KIT ligand (SCF) (Zhai et al. 1996; von Willebrand et al. 2005), NRAS (Borner et al. 1999), and MITF (McGill et al. 2002). BCL2 expression has been correlated with several poor prognostic features that include presence of ulceration and patient survival (Leiter et al. 2000; Ilmonen et al. 2005). Genetic deletion of *BCL2* in the mouse germline results in a striking hair-graying phenotype that was found to result from abrupt death of hair follicle melanocyte stem cells at approximately postnatal day 8 (Nishimura et al. 2005). Other anti-apoptotic

BCL2 family members, such as BCL-XL and MCL1, may also contribute to melanoma survival and drug resistance (Tron et al. 1995; Selzer et al. 1998; Skvara et al. 2005).

Attempts to therapeutically target BCL2 were carried out through the use of Genasense (G3139; Genta, Inc.), which was an antisense oligonucleotide targeting *BCL2* mRNA (Badros et al. 2005; Marcucci et al. 2005; O'Brien et al. 2005; Tolcher et al. 2005). Despite encouraging data from phase II studies in prostate cancer and myeloma (Badros et al. 2005; Tolcher et al. 2005), phase III data in advanced melanoma patients did not demonstrate a significant survival advantage, resulting in lack of FDA approval for melanoma (FDA summary report, http://www.fda.gov/ohrms/dockets/ac/04/slides/4037S1_02_FDA-Kane-Yang%20.ppt). While these data were not encouraging for the antisense targeting of BCL2, it remains plausible that BCL2 targeting may offer therapeutic benefit in subsets of melanoma patients or that targeting of BCL2 may be particularly advantageous in a combinatorial context with other agents for whom treatment resistance might be mediated by BCL2.

NEDD9, a melanoma metastasis gene identified by cross-species comparison

The NEDD9 gene was discovered as a metastasis gene for melanoma using a novel strategy that used genetically engineered mouse models in order to discover new oncogenes (Kim et al. 2006). A comparison of parental melanomas that were not metastatic, relative to a metastatic variant in an inducible system (Chin et al. 1999) using genome-wide high-resolution array comparative genomic hybridization, was employed and revealed genomic amplification that correlated with acquired metastatic potential. The genomic region in mice was found to be syntenic to human 6p24-25, a genomic region known to undergo gains in copy number within ~36% of human metastatic, but not primary, melanomas (Bastian et al. 1998; Namiki et al. 2005). The 6p gain in human melanomas typically involves a vast genomic region, making identification of individual driver oncogenes extremely difficult. In contrast, the focal and recurrent amplifications seen in the mouse model permitted identification of the NEDD9 locus. At the protein level, NEDD9 expression was found to correlate with progression in human melanomas (Kim et al. 2006). Loss- and gain-of-function analyses further validated NEDD9's functional role in progression and metastasis. NEDD9 was seen to localize to dynamic focal contacts at the periphery of the cell, where it interacts with focal adhesion kinase to mediate invasive behavior. These data suggest the possibility that suppression of this signaling complex may inhibit progression from early melanoma to late stage disease. It will be of value to determine whether NEDD9 elevations are prognostically meaningful in predicting future disease progression.

GOLPH3—a new class of oncoprotein identified by genomics

Another approach toward gene discovery leveraged extensive comparative analyses of array genomic hybridization profiles from multiple tumor types with the goal of prioritizing common copy number alterations that are more likely to be oncogenically relevant (Scott et al. 2009). This analysis identified frequent amplifications at 5p13, and fluorescence in situ hybridization on tumor tissue microarrays (TMAs) confirmed significant amplification frequencies ranging from 24% to 56% in several tumor types that included breast, colorectal, and non-small-cell lung cancer, among others. Detailed mapping of focally amplified specimens delimited an informative minimal common region of gain comprised of four genes (*GOLPH3*, *MTMR12*, *ZFR*, and *SUB1*). Subsequent functional analyses of these genes identified *GOLPH3*, which encodes a highly conserved 34-kDa protein first identified through proteomic characterizations of the Golgi apparatus (Scott and Chin 2010), as one gene that is likely targeted in cancers with 5p13 gain. Parallel studies using *Saccharomyces cerevisiae* coupled with confirmatory biochemical and functional assays in human cell systems established that Golph3 physically interacts with the Vps35 subunit of the retromer protein-recycling complex (Scott et al. 2009). In addition, those studies also revealed that *GOLPH3* expression modulates signaling through the mTOR, a serine/threonine protein kinase that is a critical oncogenic effector in the RTK–PI3K pathway that integrates input from multiple signaling pathways to control cell growth, proliferation, and survival (Guertin and Sabatini 2007).

Additional expression analyses indicated that 5p13 copy number correlated with increased phosphorylation of the mTOR substrate p70 S6 kinase in non-small-cell lung cancer specimens, which further linked Golph3 function to mTOR activity. When transplanted into mice, human tumor cell lines overexpressing *GOLPH3* not only developed tumors faster than control, but also were significantly more sensitive to rapamycin, a potent inhibitor of mTOR (Sabatini 2006), providing pharmacological proof that the protumorigenic activity of *GOLPH3* is mediated through mTOR signaling. These results also raise the possibility that *GOLPH3* expression level or copy number status may serve as a predictive biomarker for sensitivity to mTOR inhibitors, especially for advanced cancers driven by aberrant RTK–PI3K signaling that remain refractory to standard chemotherapy regimens.

MITF—melanocyte master regulator

The identification of MITF as a melanoma oncogene came about through an integrated analysis of genomic copy number gain (amplification) with matched mRNA expression analysis (Garraway et al. 2005), while loss of function was already known to affect survival of the entire melanocyte lineage (Hodgkinson et al. 1993). In assessing MITF's potential role as a melanoma oncogene, it was also found that enforced overexpression of MITF participated actively in conferring a transformed phenotype when introduced together with BRAF(V600E) into immortalized human melanocytes (Garraway et al. 2005). Furthermore, survival analysis indicated that MITF amplification was associated with worsened 5-year survival.

Genomic sequencing of sporadic melanomas has provided evidence for the occurrence of scattered somatic point mutations within the MITF gene (Cronin et al. 2009), consistent with the possibility of their participating as oncogenic variants of MITF. While additional functional evidence for such activity will be of great interest, recently, a coding variant was discovered in studies seeking to identify familial melanoma genes. Two independent studies identified the same variant, MITF(E318K), which largely cosegregates with affected individuals in instances of familial melanoma, as well as a small fraction of sporadic melanomas and renal cell carcinomas (see above) (Bertolotto et al. 2011; Yokoyama et al. 2011). The point mutation occurs at a site that had been previously identified as a target for SUMO modification on MITF (Miller et al. 2005). Modification by SUMOylation on MITF had been shown to modestly suppress MITF activity on certain transcriptional target genes. Thus the MITF(E318K) variant was predicted to represent a gain-of-function allele for MITF, consistent with MITF's role as a melanoma oncogene. Interestingly, clinical analysis of affected individuals carrying the germline variant suggested a statistically significant incidence of nonblue eye color (Yokoyama et al. 2011). Since MITF is known to transcriptionally regulate pigment gene expression, for which stronger gene expression is predicted to produce darker pigmentation, these observations are further consistent with the MITF(E318K) variant representing gain-of-function biological activity for MITF. Thus far, the transcriptional targets of MITF that mediate its oncogenic activity, as distinct from its pigmentation activity, remain to be fully elucidated.

Multiple genes besides MITF are known to be essential to melanocyte lineage survival. Certain ones are known to function as upstream regulators of MITF expression. These include Pax3 and SOX10, factors whose germline mutation shares clinical pigmentary features with germline Mitf mutation in humans (for review, see Price and Fisher 2001). Additional regulators of MITF that are important for melanocyte survival are growth factor receptor genes and the pathways that they regulate. These factors include endothelin 3, endothelin receptor B, SCF, c-KIT, and components of their signaling pathways, which include RAS, RAF, GNAQ/11, and others. One biochemical consequence of MAPK pathway activation is the direct phosphorylation of MITF by ERK/MAPK on Ser 73, an effect that was shown to enhance molecular recruitment of the coactivator p300 to MITF (Hemesath et al. 1998; Price et al. 1998). Another consequence of this MITF phosphorylation is ubiquitination and proteolysis of MITF (Wu et al. 2000; Xu et al. 2000), an effect recently shown to be antagonized by the deubiquitinase USP13 (Zhao et al. 2011). Of note, bacterial artificial chromosomes containing Ser 73-to-alanine mutation of MITF were capable of rescuing coat color/pigmentation of Mitf-deficient mice (Bauer et al. 2009). It will be interesting to determine whether other biological activities of MITF, such as its roles in melanomagenesis, are affected by the MAPK phosphorylation at Ser 73. This question is important because a very high fraction of human melanomas contain mutations that produce constitutive overactivity of the MAPK pathway. MITF undergoes several other post-translational modifications, which include phosphorylation by p38 kinase (Mansky et al. 2002) and phosphorylation by glycogen synthase kinase 3b (Takeda et al. 2000). The MITF genomic organization is complex, in that a series of upstream promoters can be alternatively spliced onto common downstream exons, most of which contain common coding sequences. The most 3′ (downstream) of the promoter/exon 1 units is the melanocyte-specific M-MITF promoter (Fuse et al. 1996), whose expression is controlled by a combination of factors that include WNT, cAMP/CREB, SOX10, and PAX3.

MITF's DNA-binding domain is characterized by the basic helix–loop–helix/leucine zipper (bHLHzip) DNA-binding/dimerization motifs (Hemesath et al. 1994). This motif structure, while homologous to those found in the MYC oncoprotein family, confers heterodimerization capabilities with three other transcription factors—TFEB, TFE3, and TFEC (the MiT family)—but not with MYC oncoproteins. Genetic studies in mice by Steingrimsson et al. (2002) demonstrated genetic redundancy between MITF and TFE3 within the osteoclast lineage—a feature that provides genetic support to the concept that members of the MiT family share considerable biochemical activities in vivo.

Another MiT family-associated human malignancy is clear cell sarcoma (also referred to as melanoma of soft parts). This tumor expresses multiple melanocytic markers, is often pigmented (Granter et al. 2001), and has been known to contain a diagnostic translocation that fuses the *EWS* gene to the *ATF1* gene (Fujimura et al. 1996). The resulting EWS–ATF1 fusion protein mimicks the activity of CREB, thus stimulating uncontrolled expression of MITF within clear cell sarcoma (Davis et al. 2006)—and imitating MITF's oncogenic role seen in melanoma.

A large list of transcriptional target genes for MITF has accrued in recent years. While a complete discussion of these genes is beyond the scope of this review, it is notable that numerous pigmentation-related genes appear to be direct transcriptional targets of MITF. From its initial molecular characterization, it was rapidly recognized that MITF protein binds and transcriptionally activates gene expression at consensus DNA-binding elements containing the consensus sequence CA[C/T]GTG, a sequence known to be present in the promoters of most or all pigmentation-related genes (Bentley et al. 1994; Hemesath et al. 1994; Yasumoto et al. 1994). Some of these melanocytic genes include Pmel17/silver/gp100 (which encodes the melanoma diagnostic epitope HMB45) (Halaban et al. 1996; Baxter and Pavan 2003; Du et al. 2003) and MelanA/Mart1 (Du et al. 2003). HMB45, Mart1, and MITF are now commonly used as immunohistochemical stains of the melanocytic lineage for melanoma diagnosis. Many additional MITF target genes have been identified, with some notable ones including Melastatin (*TRPM1*) (Miller et al. 2004; Zhiqi et al. 2004), *AIM1* (ocular albinism 4 gene) (Du and Fisher 2002), Ocular albinism 1 gene (*OA1*) (Vetrini et al. 2004), *VMD2* (Esumi

et al. 2004), *HIF1α* (Busca et al. 2005), and Plasminogen activator inhibitor-1 (Murakami et al. 2006); a variety of mast cell genes including Prostaglandin D2; multiple mast cell proteases; adhesion molecules; and others (Ito et al. 2004; Morii et al. 2004; Takeda et al. 2006).

Another interesting set of MITF target genes includes those involved in cell cycle regulation. This list includes the CDK inhibitors (CDKi) $p16^{INK4a}$ (Loercher et al. 2005) and p21 (Carreira et al. 2005). In addition, the cell cycle kinase CDK2 was found to be a direct transcriptional target of MITF selectively within the melanocyte lineage (Du et al. 2004). The *CDK2* genomic locus was found to reside directly adjacent to the melanocyte-specific *pmel17/gp100* locus. Via an enhancer element containing the consensus MITF-binding sequence, MITF was seen to coregulate these genes, with CDK2 expression being uniquely regulated by MITF within melanocytes (its expression is constitutively regulated in other lineages). *Slug* has also been suggested to be a direct transcriptional target of MITF (Sanchez-Martin et al. 2002), a notable finding because Waardenburg syndrome type 2 patients were also identified to carry deletions of Slug (either germline or somatic). Slug has also been shown to contribute invasive behavior to melanocytes (Gupta et al. 2005). c-Met, the RTK activated by the ligand HGF, was also identified as a direct transcriptional target of MITF (McGill et al. 2006). It is anticipated that the coming years will provide a comprehensive picture of target genes regulated by MITF in melanocytes and melanoma cells through the use of genome-wide technologies such as ChIP-seq (chromatin immunoprecipitation in combination with deep sequencing) and RNA-seq (RNA sequencing) (Strub et al. 2011). Moreover, in all cases, it is virtually certain that additional transcription factors beyond MITF play similarly important roles in controlling target gene expression and perhaps function as partners with MITF to control specific biological pathways (such as pigmentation). A key mechanistic question involves the means by which MITF induces melanomagenesis on the one hand versus differentiation/pigmentation on the other. It is possible that such activities relate to altered protein levels, distinct post-translational modifications, or other contextual features within the melanocyte.

Cell cycle dysregulation as a melanoma therapeutic target

Deletion, mutation, or silencing of *CDKN2A* is common in melanoma (Chin 2003) and, in combination with evidence for CDK2 dependency (Du et al. 2004), suggests that the RB pathway should represent a potentially "druggable" dependency in melanoma cells. Activation of CDK2 and CDK4/6 results in RB hyperphosphorylation in melanoma cells (Halaban 1999). In the case of CDK2, there appears to be a lineage-selective melanoma dependency because multiple nonmelanomas and normal tissues are resistant to CDK2 suppression (Tetsu and McCormick 2003; Du et al. 2004). However, selective CDK2 antagonists have not yet appeared, and less-selective CDK2 inhibitors (such as flavopiridol, which antagonizes CDK2, CDK1, CDK4, and CDK7) are the types of small molecules that have been clinically tested for a variety of malignancies. Numerous additional CDKi are in various stages of development currently.

Circumventing p53 DNA damage signaling

Inactivation of the p53 pathway appears to be a rite of passage for virtually all tumor cells. While most human solid tumors neutralize this tumor-suppressive pathway at the level of p53 itself (e.g., mutations within the *TP53*-coding region), melanoma provides a notable exception to this rule. In a recent meta-analytical survey of 645 uncultured melanoma specimens, there were only 85 (13.2%) *TP53* mutations reported. Interestingly, more than half of these ($N = 43$) were UV signature changes (Hocker and Tsao 2007), suggesting that *TP53* is, in fact, a melanoma-promoting target of UV. With the recognition that p14ARF (an exon 1β-specific transcript of *CDKN2A*) down-regulates HDM2 (Kamijo et al. 1998; Pomerantz et al. 1998; Stott et al. 1998; Zhang et al. 1998), it became clear that functional inactivation of p53 could be achieved by the melanoma cell through *CDKN2A*/p14ARF loss. In fact, concomitant deletion of the *CDKN2A* locus does occur in the face of activating *CDK4* mutations (Yang et al. 2005), suggesting that abrogation of the RB pathway through a CDK4-mediated mechanism is still insufficient and that secondary inactivation of p53 is needed and accomplished through abrogation of p14ARF. If direct *TP53* mutagenesis and p14ARF represent two possible mechanisms for p53 pathway injury, one would expect *HDM2* amplification as a third potential mechanism; indeed, *HDM2* amplification has been described in 3%–5% of human melanomas (Muthusamy et al. 2006).

Since p53 is only rarely mutated but only functionally constrained by HDM2, restoration of p53 function through small-molecule antagonists of HDM2 represents a viable therapeutic approach. Ji et al. (2012b) recently demonstrated that melanomas can be suppressed by nutlin-3, a canonical HDM2 antagonist, in a *TP53*-dependent fashion. Moreover, synergy with MAPK pathway inhibition suggests that optimization may be possible with future development (Ji et al. 2012b).

Conclusion: future perspectives on melanoma

The recent approval of ipilimumab and vemurafenib marks 2011 as a historical year for melanoma therapeutics. However, rather than celebrating the extraordinary knowledge that has been uncovered in the last 20 years, perhaps it is also appropriate to conclude with the most pressing and challenging questions in melanoma today.

(1) Is there a melanoma-initiating cell? The existence of a rare melanoma stem cell population was suggested by early studies that identified a CD20-enriched subpopulation of cells from melanoma lines or metastases that grew as nonadherent spheroids when cultured in human embryonic stem cell-like medium and that could be induced to differentiate toward multiple lineages,

including melanocyte, adipocyte, osteoblast, and chondrocyte, suggesting the presence of such subpopulations with the stem cell phenotype (Fang et al. 2005). Separately, Frank et al. (2005) isolated a small subpopulation of melanoma cells that were enriched for expression of both ABCB5 and CD133. In xenograft experiments, the melanoma-initiating cell frequencies for unsegregated and ABCB5$^+$ populations were one in 1,090,336 versus one in 158,170, respectively, suggesting that the marker enriched for a more tumorigenic population of cells; i.e., stem cells. Furthermore, selective depletion of ABCB5$^+$ melanoma cells using anti-ABCB5 antibodies also led to inhibition of tumor formation (Schatton et al. 2008). However, no less than a few months after this initial publication, Sean Morrison's group (Quintana et al. 2008) showed that, under the proper experimental context, up to one in four melanoma cells could initiate tumors, thereby challenging the notion that there is a defined cellular hierarchy in melanoma tumor initiation. Other putative markers of melanoma-initiating cells have also been proposed, including CD271 (Boiko et al. 2010). Amidst conflicting results, varied technical approaches, and nonuniform definitions of a "cancer stem cell," this debate will likely continue.

(2) Why is resistance to anti-BRAF therapies so rampant? One of the greatest challenges in melanoma therapeutics is ironic juxtaposition of rapid and widespread response to vemurafenib with the often rapid and fatal relapses that occur months to years later. The systematic layer-by-layer unpeeling of mechanisms suggests that single-agent molecular approaches in melanoma may not be ultimately curative but that cocktails and combination targeted/immunotherapy will be needed.

(3) How are patients with BRAF wild-type and NRAS-mutated tumors to be treated? A tremendous amount of effort exists for optimizing anti-BRAF treatments despite a large need among patients with BRAF wild-type tumors. With a significant set of recurrent melanomas having been shown to harbor activating NRAS mutations, the next generation of melanoma treatments will have to contend with RAS-mediated reactivation of MAPK and non-MAPK mechanisms. Approaches including the targeting of MEK and the PI3K pathway have recently been incorporated into clinical trials and will undoubtedly be vigorously tested in the coming months.

(4) Is it possible to cripple "master regulators"? While traditionally considered nondruggable, the increasing recognition that transcription factors (e.g., MITF) may play oncogenic roles in melanoma has driven efforts to devise alternative approaches to target this protein class. One such approach involves the use of peptide to block transcription factor activities. In melanoma, ATF2 has been targeted in this fashion with impressive preclinical results (Bhoumik et al. 2002, 2004). These observations are particularly intriguing given evidence that ATF2 nuclear localization correlates with prognosis in human melanoma specimens (Berger et al. 2003). Small molecules that block transcription factor dimerization represents another approach and are currently being developed for the inhibition of MYC–MAX interaction (Prochownik and Vogt 2010). An additional strategy involves elucidating the critical downstream targets of oncogenic transcription factors, some of which may prove to be more amenable to small-molecule compound inhibition. Attempts to target the MITF pathway are being increasingly scrutinized, balancing the challenges of antagonizing a noncatalytic protein against the benefits of inhibiting a lineage-selective master survival factor.

(5) How do we maximally leverage the advances in immunotherapy with those in targeted therapeutics? In parallel evolution, progress in our understanding of tumor immunology and immunotherapy has led to equally exciting and novel approaches to melanoma treatment using immune-based modifiers (for review, see Mellman et al. 2011; Sharma et al. 2011). Although a full exploration of immunological treatments is beyond the scope of this review, there are scientific and clinical considerations to suggest combinations of molecularly based and immunologically driven approaches. For instance, suppression of BRAF(V600E) by PLX induces the expression of melanocyte differentiation antigens, raising the possibility that concurrent treatment with SBIs may in fact improve the efficacy of immunological targeting (Boni et al. 2010).

The unprecedented propagation of melanoma genetics, genomics, and biological understanding justifies an optimistic view of its future therapeutic outlook. Undoubtedly, the road ahead will remain stunted with challenges and occasional setbacks; however, salient tumor dependencies that underlie definable melanoma subtypes have begun to emerge. In partnership with versatile in vitro and in vivo model systems, increasingly rigorous target validation (for maintenance) and rational drug development efforts, and judicious use of correlative "-omics" studies for robust biomarkers, melanoma promises to provide an instructive framework for progress against even the most brittle of human malignancies.

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