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# Genetics and genomics of melanoma

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## Abstract

The rapidly increasing incidence of melanoma, coupled with its highly aggressive metastatic nature, is of urgent concern. In order to design rational therapies, it is of critical importance to identify the genetic determinants that drive melanoma formation and progression. To date, signaling cascades emanating from the EGF receptor, c-MET and other receptors are known to be altered in melanoma. Important mutations in signaling molecules, such as BRAF and N-RAS, have been identified. In this review, some of the major genetic alterations and signaling pathways involved in melanoma will be discussed. Given the great deal of genetic heterogeneity observed in melanoma, it is likely that many more genetic determinants exist. Through the use of powerful genomic technologies, it is now possible to identify these additional genetic alterations in melanoma. A critical step in this analysis will be culling bystanders from functionally important drivers, as this will highlight genetic elements that will be promising therapeutic targets. Such technologies and the important points to consider in understanding the genetics of melanoma will be reviewed.

### Keywords

array-comparative genomic hybridization; BRAF; CDKN2A; genomics; MAPK; metastasis; N-RAS; PI3K; receptor tyrosine kinase

## Clinical presentation of melanoma

Melanoma is the most aggressive form of skin cancer, surpassing most solid tumors in terms of propensity to metastasize. Based on the National Cancer Institute estimates, the lifetime risk of a person in the USA developing melanoma of the skin is an astounding 1 in 55 and, in the Western world, cases of melanoma have doubled in the last 20 years [1,2]. Meanwhile, the risk of invasive melanoma in the USA has increased almost tenfold in the last 50 years [3]. So, while the incidence of many cancers has decreased or is leveling off, the occurrence of melanoma is still on the rise. Since the risk of developing melanoma may be correlated with UV exposure given an individual's genetic background, and as sun exposure trends change



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worldwide, the personal and economic toll on society from melanoma is likely to increase. This will be exacerbated by the fact that there are few therapeutic options for metastatic melanoma, a lethal end-stage progression of the disease. Therefore, it will be of critical importance for the scientific and healthcare community to develop diagnostic and therapeutic strategies to manage and treat melanoma. This will require a much more comprehensive understanding of the genetic basis of melanoma than currently exists.

While a description of melanoma has been noted as far back as the 5th Century BC, much remains to be elucidated about the causal events in melanoma pathogenesis. Cutaneous melanoma arises from uncontrolled proliferation of melanocytes, melanin-producing cells located in the basal layer of the epidermis [4]. By distributing pigment from melanosomes to keratinocytes present in the skin, melanocytes serve a protective role against UV radiation for the skin. Melanoma can also occur in the eye (ocular), meninges and digestive tract. Clinically, four subtypes exist. Lentigo maligna is associated with chronic sun-exposed areas of the body, such as the face. Acral lentiginous melanoma is found on non-sun-exposed regions, such as the palms, nail beds and soles of the feet. Nodular melanoma is a raised nodule that may or may not have an associated superficial spreading component. The most common subtype – particularly among young adults in the USA – is superficial spreading melanoma [1], which generally occurs on areas of the body with intermittent sun exposure, such as the trunk and proximal extremities.

For chronic sun-exposure sites, lentigo maligna may be the most common subtype [5]. In an analysis of a military population in Texas (USA) assumed to have high sun exposure, most patients presented with lentigo maligna [6]. This has clinical relevancy, since sun exposure might vary from population to population, thereby altering the predominant melanoma subtype.

Melanoma progression can begin with the development of either dysplastic or benign nevi (common acquired or congenital) [4]. These can then progress to the radial growth phase, in which the growth expands laterally but remains localized to the epidermis. At this phase, cells are still dependent on growth factors, and are not anchorage independent or tumorigenic. Progression to the vertical growth phase is hallmarked by invasion into the dermis, subcutaneous tissue and upper epidermis. In the vertical growth phase, cells are no longer growth factor dependent, are anchorage independent and presage distal metastasis.

Clinical staging of melanoma progresses from an *in situ* growth to one increasing in thickness and vertical invasion, to regional lymph-node spread and, finally, to distal metastasis. Vertical invasion may be representative of the degree of progression and is often measured by the Breslow thickness, a measure of the thickness of the tumor from the upper layer of the epidermis to the innermost depth of invasion [7]. The Breslow thickness remains the most significant predictor of metastasis and prognosis. However, although most thin melanomas do not metastasize, a portion do, suggesting that there is a high-risk subgroup of melanomas among the generally low-risk thin melanomas [8]. This highlights the urgent need to develop prognostic molecular biomarkers that can complement gold-standard clinicopathological parameters in stratifying patients with a high risk of metastasis.

## Genetics of melanoma

To date, some of the genetic events underlying the development of melanoma have been characterized. These include alterations in receptor tyrosine kinase (RTK) function and the emanating signaling cascades or in intracellular regulatory molecules. These changes have been identified through genomic structure and sequence analysis, some of which are summarized below.

## CDKN2A

The complexion of an individual can suggest propensity for melanoma development. Light complexion, inability to tan or presentation of atypical moles or freckles correlates with increased risk for melanoma [9,10]. However, familial history of melanoma is also a strong predictor of melanoma development. A meta-analysis showed that first-degree relation to a person with melanoma increased one's risk 2.24-fold [11,12]. Such relationships led to the identification of 9p21 as an important locus in melanoma, with loss of heterozygosity or mutation at 9p21 occurring in melanomaprone families [13,14]. Within this region are the *CDKN2A* and *CDKN2B* genes. Interestingly, *CDKN2A* encodes for two tumor-suppressor proteins, INK4A ($p16^{INK4A}$) and ARF ($p14^{ARF}$ in humans and $p19^{ARF}$ in mice) [15].

INK4A ($p16^{INK4a}$) is a cyclin-dependent kinase inhibitor that activates the tumor-suppressor gene retinoblastoma (*RB*) via negative regulation of Cdk4/6 (Figure 1). This ultimately leads to progression through the G1/S transition. Tellingly, 25–40% of familial melanomas have mutations in the INK4A coding region [16,17]. Albeit rarely, alterations in *CDK4* and *RB* have also been associated with melanoma in humans [18–22].

Alternative reading frame is a negative regulator of the tumor suppressor p53, a functional relationship that explains the infrequent direct *p53* mutation in human melanoma in contrast to other solid tumors [4,23–25]. Genetic evidence in mice also supports this phenomenon. In Tyr-$HRAS^{V12}$ transgenic mice with cutaneous melanoma sustaining loss of heterozygosity of Trp53, *Arf* remains wild type; in Tyr-$HRAS^{V12}$ transgenic mice on *Ink4a/Arf*$^{-/-}$ background, p53 remains wild type [26,27].

### Receptor tyrosine kinases

Receptor tyrosine kinases are widely dysregulated in various cancers. In melanoma, alterations in the EGF receptor (EGFR), Met RTK (c-MET) and Kit receptor tyrosine kinase (c-KIT) result in changes to the associated signaling cascades (Figure 2).

The EGFR can be activated by EGF, TGF-α, amphiregulin and heparin-binding EGF. Such binding can activate the MAPK and PI3K signaling cascades. EGFR is located on chromosome 7 and copy number gains of chromosome 7 have been observed in the later stages of melanoma [28–30]. However, no focal amplifications have been detected in melanoma, making assignment of EGFR as the target of such genomic changes difficult. Although *EGFR* mutation has not been reported, it should be noted that systematic resequencing in a large number of melanoma samples has not been conducted. In the tetracycline-regulated H-$RAS^{V12}$-inducible mouse model of melanoma, transcriptome analysis revealed an EGFR signaling loop driven by RAS (from rat sarcoma virus) via the regulation of EGF family ligands [31]. When the inducible *RAS* allele is shut off in an explanted tumor, a sustained EGFR signal promotes survival through PI3K activation of AKT. Consistent with this, loss of EGFR inhibits RAS-driven tumorigenicity of melanoma cells [32–34]. This suggests that EGFR may be an alternate target for therapeutic intervention in RAS-driven melanoma.

The c-MET receptor is activated by the binding of its ligand, HGF [35]. The c-MET receptor is known to be expressed in melanocytes. Located at 7q33, copy number gains of this locus are considered to be a late-stage event in melanoma [28,36]. As with the EGFR, no focal amplifications or mutations of the *c-Met* gene have been identified in melanoma [15]. However, additional data suggest that *c-Met* is a critical regulator of melanoma progression. For example, *c-Met* expression is upregulated in metastatic melanoma [37]. This may be accounted for by the fact that while MET activation is predominantly paracrine, in melanoma, autocrine activation of MET has been described [38]. Interestingly, expression of *c-Met* in melanocytes drives metastasis rather than the initial steps of melanoma development [15]. Furthermore,

increased *c-Met* expression or activity promotes invasiveness and, in explant models, correlates with metastasis [39]. In genetically engineered HGF-mouse models, continuous expression of HGF drives a c-MET autocrine loop that promotes cutaneous and metastatic melanoma [40–42]. Interestingly, Ink4a/Arf deficiency enhanced melanoma development in HGF mice exposed to UVB [42]. In addition, *c-Met* has been identified as a target of the microphthalmia-associated transcription factor (discussed later), which is itself amplified in a subset of melanoma [43,44].

c-KIT is a RTK that binds to the stem-cell factor ligand. c-KIT activation can stimulate various signaling pathways, including RAS/ERK and PI3K. Oddly, c-KIT does not represent a typical RTK in melanoma, since immunohistochemical analysis has indicated a frequent loss of c-KIT with melanoma progression [45–47]. Furthermore, metastatic melanoma cells expressing *c-Kit* have a greater responsiveness to stem cell factor-mediated apoptosis [48]. However, a prosurvival and proliferation role for c-KIT has also been suggested. In mice or humans, loss or inhibition of c-KIT, respectively, results in loss of melanocytes [49,50]. c-KIT activation also promotes proliferation of cells with a melanocytic lineage [51]. Additionally, a study of human metastatic melanomas showed three out of 153 samples to have high c-KIT expression as detected by immunohistochemistry. Through high-resolution amplicon melting and DNA sequencing, it was found that a L576P mutation was present in three samples, with a loss of the normal allele [52,53]. The L576P mutation, found in exon 11, maps to the 5′ juxtamembrane domain, the region in which most *KIT* activating mutations are found [54,55]. The L576P mutation has also been found in other tumors (e.g., gastrointestinal stromal cell tumors) [55].

More recently, through copy number analysis and sequencing, a study found that 21% of mucosal, 11% of acral and 28% of chronic sun-damaged skin had alterations in *KIT* [56]. Recently, a screen for mutations in melanoma subtypes suggested that 23% of acral, 15% of mucosal and only 2% of cutaneous melanomas have *KIT* mutations [57]. This suggests that *KIT* status might define a subpopulation of melanomas. Interestingly, imatinib can inhibit KIT and has been effective in patients with gastrointestinal stromal tumors with *KIT* mutations [58–61]. Indeed, in a patient with rectal acral melanoma harboring an exon duplication in the juxtamembrane domain of *KIT*, imatinib was effective in reducing tumor volume by half in 4 weeks, with prolonged survival for 4 months [62]. In another case, a patient presenting with mucosal melanoma responded to imatinib and exhibited regression of anal nodules for up to 6 months [63]. These case reports emphasize the existence of genetically distinct melanoma subtypes and also the ability of genetic alterations to stratify patients into therapeutic subgroups.

## Signaling cascades

Two major signaling cascades emanate from the aforementioned RTKs, the RAS/RAF/MEK/ERK and the PI3K pathways (Figure 2). Growth-factor independence is a requisite for cancer cells, and dysregulation of receptor signaling in cancers can occur at various levels, from alterations at the receptor to changes in the intracellular signaling cascades [64]. While a growth factor may offer a degree of signaling stimulation, robust activation of signaling can arise from the activation of individual signaling molecules. Disruption in these signaling pathways may result in aberrant cell proliferation and/or apoptosis, and eventual tumor development.

The RAS/RAF/MEK/ERK pathway is activated by various receptors, including c-KIT, FGF receptor and c-MET. The RAS family of small G proteins is made up of N-RAS, H-RAS and K-RAS. Mutations in *N-RAS*, the most common in melanoma, are found in 33% of primary and 26% of metastatic tumors, and are correlated with sun exposure and nodular lesions [65, 66]. *N-RAS* mutations are rarely found in dysplastic nevi, one of the potential starting points for melanoma [65,67,68]. *H-RAS* point mutations and genomic 11p amplification have only been identified in Spitz nevi, a benign lesion that does not progress to melanoma [69]. It should

be noted, however, that some atypical Spitz nevi have been shown to metastasize to regional lymph nodes but whether *H-RAS* mutations correlate with metastasis is uncertain and, furthermore, the distinction of atypical Spitz nevi from melanoma is controversial [70,71]. No mutations of *K-RAS* have been described in melanoma. These data suggest that the RAS family members have distinct roles in melanoma. Consistent with this, while activated *H-RAS* expression on an *Ink4a, Arf* or *p53* mutation background promotes nonmetastatic melanoma in mice [26,27,72], *N-RAS* expression in *Ink4a/Arf* deficiency promotes metastasis of cutaneous melanoma with high penetrance and short latency [73].

The RAF family consists of ARAF, BRAF and CRAF. In a systematic screen for mutations, *BRAF* mutations were identified in a variety of tumor cell lines, with the highest occurrence in melanoma. Sequencing revealed up to 67% of melanoma tumors samples had a mutation in *BRAF*. An amino acid substitution (V600E) was, by far, the most common mutation both in cell lines and tumor samples [74]. Alterations in BRAF may be an early somatic event (see later) as germline mutations in *BRAF* are not commonly found in familial melanoma [75–77].

BRAF can regulate various aspects of cell survival. Activated BRAF promotes IκB degradation, while inhibition of BRAF sensitizes cells to apoptosis [78]. BRAF can also regulate cell growth by regulating $p27^{kip1}$ levels [79]. The majority of *BRAF* mutations occur in defined areas of biochemical function, with a single phosphomimetic substitution of V600E in the kinase activation domain being the most common [80]. Mutations in *BRAF* are found most frequently in melanomas at sites with intermittent UV exposure and only occur in approximately 11% of lentigo maligna melanomas arising from chronically sun-exposed areas [81–83]. *BRAF* mutation is associated with germline variants of the *MCR1* gene, which promotes melanin production [83,84]. The melanocortin receptor 1 (MC1R) is the receptor for α-melanocyte-stimulating hormone, which is stimulated by UV radiation, perhaps suggesting a degree of interplay between *BRAF* and UV exposure [85]. In this regard, it is interesting that *BRAF* mutation is most prevalent in the melanomas associated with intermittent sun exposure [81]. MC1R itself is also interesting, since MC1R variants are associated with fair skin, red hair and freckles; physical attributes that have been associated with melanoma risk [10,86, 87].

The role of BRAF in melanoma is complicated by the fact that *BRAF* mutations are also found in benign and dysplastic nevi [88–91]. These nevi often remain growth-arrested for their lifetime and rarely progress to melanoma. This may suggest that $BRAF^{V600E}$ promotes a checkpoint for malignant transformation. In fact, congenital nevi are positive for the senescence marker senescence-associated β-galactosidase and for $p16^{INK4A}$ [92]. Furthermore, while $BRAF^{V600E}$ cannot transform human melanocytes, it can transform murine melanocytes that are $p16^{INK4A}$ deficient [44,93] and can induce INK4A, senescence-associated β-galactosidase and cell cycle arrest in normal cells [15]. The BRAF effect, therefore, is an example of oncogene-induced senescence – a method by which premalignant lesions are inhibited from progressing [94]. Interestingly, INK4A expression is not present in 100% of BRAF oncogene-induced senescence cases, suggesting that another pathway aside from INK4A may exist [92]. BRAF-dependent oncogene-induced senescence may rely on signaling through IGF binding protein (*IGFBP7*) and other genes. A genome-wide shRNA screen for factors necessary for $BRAF^{V600E}$-mediated senescence identified *IGFBP7*. IGFBP7 was required for $BRAF^{V600E}$-induced apoptosis and senescence in melanocytes, and media containing IGFBP7 induced apoptosis in BRAF mutant melanoma cells lines. *In vivo*, recombinant IGFBP7 administration suppressed $BRAF^{V600E}$ tumors and in humans, benign nevi with $BRAF^{V600E}$ had high levels of IGFBP7, while $BRAF^{V600E}$ melanomas did not [95].

Additional evidence shows that BRAF is not sufficient for transformation of melanocytes. In zebra fish, BRAF activation forms benign nevi, but requires p53 deficiency to progress to full

melanoma [79]. Additionally, while N-RAS expression in telomerase reverse transcriptase (TERT)-immortalized *RB–p53* mutant human melanocytes produced invasive melanoma, $BRAF^{V600E}$ expression only produced junctional nevi, or a nevi between the dermis and epidermis [96]. Again, this demonstrates that BRAF requires the cooperation of other determinants to drive melanoma progression.

It is of note that $BRAF^{V600E}$ and *N-RAS* mutations are mutually exclusive in melanoma, and in fact, the occurrence of each mutation may be specific to certain subtypes of melanoma [74,97,98]. For example, *BRAF* mutations are associated with melanomas arising on body sites of intermittent sun exposure, while *N-RAS* mutations may be more common in sites of chronic sun damage [99]. This suggests that there are innate differences in the roles that BRAF and N-RAS may play in melanoma and this may be reflected in shifts in signaling. In BRAF mutant cells, BRAF is required for ERK signaling. However, in the context of *N-RAS* mutation, melanoma cells signal to ERK through CRAF after a concomitant alteration in cAMP signaling to CRAF (Figure 2) [100]. Furthermore, with supervised clustering, the gene-expression profile of primary melanomas with *BRAF* mutations was shown to be distinct from those with *N-RAS* mutations, although it must be noted that others have not found such differences in cell lines [101,102]. Interestingly, even the specific *BRAF* mutation may determine signaling. In non-V600E BRAF mutant melanoma cells, CRAF inhibition induced apoptosis, suggesting that, in these specific cells, CRAF is of consequence [103].

The ERK signaling pathway can regulate various molecules important for tumorigenesis (Figure 2). In wild-type BRAF or NRAS cells, ERK activation is quite low compared with mutant cells and can control proteins involved in extracellular adherence, cell motility and angiogenesis [102]. In melanoma cells, ERK can inhibit the cell cycle regulator $p27^{kip1}$ [104]. ERK can also alter *in vitro* invasion capability by regulating the production of matrix metalloproteinase -1 [105]. However, ERK can also regulate survival and senescence. In a microarray screen, Bcl-2 was found to be regulated by microphthalmia-associated transcription factor (MITF) in melanocytes and melanoma cells [106]. Therefore, a close examination of the phenotypic outcome of ERK activation in melanoma should be carried out [107].

The PI3K pathway is responsive to various extracellular signals, including those from integrins, extracellular matrix proteins, HGF and insulin-like growth factors. Upon receptor stimulation, PI3K generates the lipid second messenger, phosphoinositol-3,4,5 triphosphate ($PIP_3$). $PIP_3$ triggers the phosphorylation and activation of the serine/threonine kinase, AKT (Figure 2). Interestingly, patient survival is inversely related to phospho-AKT levels [108]. There are three AKT isoforms and each may have a distinct role in cancers, including melanoma. Of the three, DNA copy number gain of *AKT3* is observed in melanoma, and 40–60% of sporadic tumors may have AKT3 activation [109]. Additionally, AKT3 correlates well with melanoma progression and loss of AKT3-triggered apoptosis [109]. On the other hand, AKT1 activation inhibits the migration and invasion of various cell lines, including a melanoma cell line [44, 110,111]. Although AKT may have a role in melanoma, the extent to which AKT is a critical regulator of melanoma is yet to be determined.

By far, the majority of genetic alterations that do occur in the PI3K pathway in melanoma occur in the phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*) tumor suppressor. PTEN is a *bona fide* tumor suppressor. By dephosphorylating the D3 position of $PIP_3$, PTEN negatively regulates the PI3K pathway. Loss of heterozygosity of 10q, where the *PTEN* gene is located, has been observed in various cancers, including melanoma [112,113]. Loss of an allele or expression changes in PTEN occur in 20 and 40% of melanoma tumors, respectively, and somatic point mutations and homozygous deletions are rarely observed [98,114–116]. Expression of PTEN can inhibit AKT, promote apoptosis and inhibit growth, tumorigenicity

and metastatic potential of PTEN-deficient melanoma cells [117–120]. Somatic or germline mutations of *PTEN* promote tumor phenotypes in the mouse, including melanoma [121–125].

Phosphatase and tensin homologue deleted on chromosome 10 itself can be regulated by NEDD4-1-mediated polyubiquitination [126]. Interestingly, PTEN inactivation through NEDD4-1 enhances the efficiency of transformation of mouse fibroblasts with RAS expression and p53 deficiency [127].

The importance of both the ERK and PI3K signaling in tumor induction is also conserved in melanoma. In transgenic TP-Ras mice and in 3D melanoma cultures, inhibition of both pathways is required for suppression of cell growth [128,129]. While mutations in *PTEN* and *N-RAS* are generally discordant in melanoma, *BRAF* and *PTEN* mutations are found concurrently in 20% of melanomas [83,130].

## Genomic approach to melanoma

While various signaling molecules have been identified in melanoma, the genomic heterogeneity of melanoma suggests that many more genetic drivers remain to be discovered. The genetic heterogeneity is illustrated by array-comparative genomic hybridization (aCGH) analysis, which shows that different subtypes of melanoma have different copy number alteration profiles [83]. Furthermore, upon sequencing, it was found that *BRAF* mutations are not distributed equally but instead are predominantly found in melanomas without sun damage, rather than in chronic sun-exposed melanomas [83]. Such distinctions between different melanoma types may, in fact, indicate the predominance of a signaling pathway in a particular subgroup. A critical extension of this observation is that a melanoma subgroup defined by a molecular signature may respond favorably to a therapy specifically directed against that signature. Therefore, understanding the alterations present in melanoma will be critical for the development and application of therapies. These alterations will include changes in sequence (i.e., *BRAF*), copy number gains and losses (i.e., *CDKN2A*), epigenetic changes, alterations in non-coding miRNAs and changes in protein-coding RNA (Figure 3). This will define the melanoma atlas – the genomic, epigenomic and proteomic changes that define melanoma. Identification of such changes can be accomplished through the use of various technologies, including sequencing efforts, comparative genomic hybridizations, chromatin immmunoprecipitation plus microarray technology (ChIP–Chip) and various microarrays.

Sequence and copy number analyses have provided insight into the melanoma genetic profile. As discussed previously, sequencing efforts identified *BRAF* and *KIT* mutations as critical alterations in melanoma. As sequencing becomes less expensive, allowing for the sequencing of a greater number of tumor samples and a greater portion of the genome, it is expected that the identification of mutations of known and novel genes important in melanoma will increase dramatically. For copy number analysis, a powerful tool has been the use of array-based high-resolution comparative genomic hybridization (aCGH). Through comparison of samples (i.e., normal vs tumor), aCGH can identify regions of copy number gains or losses, which may contain genes that regulate tumor formation, development or progression. In an analysis of metastatic tumor samples grouped by unsupervized methods and matched against clinical outcome, a particular group correlated with poor prognosis; rather than being defined by metastatic site, this group was defined by similar patterns of chromosomal alterations [15]. This suggests that analysis of genomic alterations has biological validity. Another analysis demonstrates how aCGH might stratify melanoma patients based on risk. Over 400 copy number alterations were identified in metastatic lesions upon comparison of melanocytic lesions, primary and metastatic melanomas, melanoma cell lines and melanocytic nevi [15]. This comprised previously described events, including gain of 1q, 6p, 7 and loss of 6q, 9p and 10. The number of copy number alterations, which is, in metastatic melanoma, reflective of

genomic heterogeneity, was found to be significantly greater than that in primary melanomas. Using such comparisons, genes that may represent useful prognostic biomarkers for the clinical stratification of patients or serve as therapeutic targets might be identified.

As a result of such alterations (i.e., sequence and copy number), overall gene expression may be altered. While many gene-expression profiles have been based on cell line comparisons, more recent work has concentrated on primary human tumor samples. Such analyses have identified gene-expression signatures relevant to melanoma prognosis. Unsupervised hierarchieal clustering of 83 primary tumor samples identified 254 genes that defined the expression profile of patients with 4-year metastasis-free survival [131]. This data set showed differential expression between mutant and wild-type *BRAF* samples, suggesting that gene-expression analysis of primary tumor samples will give insight into the distinct genetic signatures of melanoma subtypes [101].

A specific example of the value of analyzing genetic aberrations is the identification of MITF as an important factor in melanoma progression. *MITF* amplification was detected in single nucleotide polymorphism analysis of a collection of cancer cell lines. In determining lineage-specific alterations, gain of 3p14–3p13 was identified in melanoma. Integration of the 3p amplified melanoma cell lines with expression data identified *MITF* as the gene showing the greatest change in expression and copy number gain in the 3p region [44]. Subsequent analysis showed *MITF* to be amplified in 10% of primary cutaneous and 15–20% of metastatic melanoma. Furthermore, *MITF* amplification was inversely related to 5-year survival [44]. However, such data cannot discriminate between driver and bystander effects; for that, functional studies are required. In TERT-immortalized melanocytes with $BRAF^{V600E}$ and RB and p53 deficiency, *MITF* expression drove transformation capability (e.g., soft agar colony formation). Loss of MITF also inhibited growth and survival in 3p13–14 amplified cell lines [44]. These *in vitro* studies indicate the existence of genetic subsets in which MITF functions, suggesting that additional molecular subclasses of melanoma may exist.

It must be noted that the role of MITF is not entirely clear-cut. In mouse melanocytes with $BRAF^{V600E}$, *MITF* expression reduced cell proliferation, while re-expression of *MITF in vivo* reduced tumor formation [132]. Follow-up by the same group showed that while short-term inhibition of ERK increased MITF levels in melanoma cell lines, longer term inhibition increased MITF levels [133]. It is possible that while some melanomas have MITF expression, some may not [134].

In cancer, tumor cells often demonstrate characteristics and signaling pathways of the developmental lineage of the tumor cell, and *MITF* is a perfect example of a lineage-specific gene in melanoma. MITF is a central regulator of normal melanocyte differentiation and survival. Indeed, disruption of the *MITF* gene results in graying of the coat color of mice and abnormal pigmentation in humans [135–137]. Tumors may use these pathways for their own benefit. In fact, BRAF may promote the selection of variants with amplified *MITF*. Therefore, MITF may promote the survival of both normal and neoplastic melanocytic cells. Other melanocyte signaling may also have a role in tumorigenesis, including c-KIT, Wnt and MC1R [84–86,138].

Epigenetic changes, such as DNA methylation and histone modification, are regulatory mechanisms in melanoma. Through the use of 5-Aza-2´-deoxycytidine to inhibit DNA methylation, it was found that expression of 0.8% of genes on a microarray were altered in melanoma cells lines compared with untreated controls [139]. Further analysis found that *HOXB13* and *SYK* expression increased in melanocytes with 5-Aza-2´-deoxycytidinetreatment. Overexpression of either gene reduced cell proliferation and decreased tumor size in a xenograft model [139]. Other genes, including the estrogen receptor

α, have differential methylation status in primary and metastatic melanoma [140]. Stratification of patients for treatment will require disease biomarkers, and analysis of DNA methylation state might be a usable biomarker. It has been demonstrated that the levels of methylated PTEN and estrogen receptor α DNA in the serum is increased in metastatic patients [140,141]. Furthermore, histone deacetylase inhibitors have inhibited growth of melanoma cells *in vitro* and in xenografts, suggesting that epigenetic changes are of importance in tumors [142]. Currently, epigenetic alterations can be analyzed through the use of various methodologies, including ChIP–Chip, restriction landmark genome scanning and modification of histones. As the technologies improve, it is likely that a greater number of epigenetic changes in melanoma will be identified.

miRNAs are noncoding RNAs that can regulate translation and stability of protein-coding RNAs. In some solid tumors, miRNAs have been found to target tumor suppressors and oncogenes [143]. Interestingly, melanoma can be segregated from other tumors types by patterns of miRNA expression [144]. Furthermore, miRNA copy numbers are altered in melanoma cell lines [145]. While not conclusive, such data suggest that miRNAs in melanoma may be a fruitful line of research.

As future genomic analyses will generate a wealth of data, the ability to cull bystanders from true genetic drivers of melanoma will be of critical importance. This may be carried out through integration of different data sets (i.e., copy number and sequencing), identification of evolutionarily conserved alterations (i.e., cross-species analysis) and cell-based analysis (Figure 3). In all cases, genes of interest must be validated through biological assays that determine the functional and clinicopathological relevance of an alteration. A greater understanding of the role of a genetic alteration in tumor development can be garnered, using model systems (i.e., mouse), genetic manipulation (i.e., gain-of-function and loss-of-function), functional assays (i.e., migration) and investigation of biochemical function (i.e., signaling). This must be placed in the context of the clinicopathological characteristics of the gene of interest, such as prevalence in tumor samples, occurrence in different histological or molecular subtypes and responsiveness of a tumor with the alteration to specific therapies. Together, such analyses will address three necessary questions: is the gene genetically targeted for deregulation? Does this alteration have a functional consequence? And, if so, what is the clinical relevance of such alterations?

Since tumor promoting alterations can occur at various levels, integrating data sets may allow for the identification of genes important in melanoma. For example, sequencing identified c-KIT activation in mucosal and acral melanoma, while aCGH revealed the amplification of the genomic region [56]. A gene altered by multiple mechanisms may represent a critical player in tumorigenesis, and must be altered by cancer. The Cancer Genome Atlas has begun to catalog alterations in glioblastoma multiforme. A similar effort on a melanoma cancer genome project will undoubtedly transform this field.

Since genes that are drivers for tumor development are likely to be conserved evolutionarily, comparing murine genomic profiles against human profiles can act as a filter to identify critical genomic events. This methodology of cross-species comparison was used successfully to identify *Nedd9* as a novel melanoma gene. Using a genetically engineered mouse model, metastatic tumors independent of inducible H-RAS status were isolated [146]. aCGH analysis of these tumor lines compared with nonmetastatic parental cells identified a recurrent focal amplification associated with metastatic progression. A syntenic amplification, observed in 36% of metastatic tumors, is found at 6p24–25 in humans. While the amplification of this region in humans quite wide, making gene identification difficult, comparison with the mouse amplification narrowed the region of interest. Further triangulation against expression data demonstrated *Nedd9* as is the most likely gene candidate. Not only did cross-species analysis

filter human genetic data, it also provided the logical framework for further *in vitro* characterization. Focal adhesion kinase was shown to be an important mediator of *Nedd9* function and important in *in vitro* invasion and attachment to matrigel. These *in vitro* assays are reflective of characteristics (i.e., cell motility) that are important for a tumor cell to attain in order to metastasize *in vivo*. Therefore, the metastatic phenotype of the amplification (e.g., *Nedd9*) led to directed *in vitro* tests of invasion.

It must be noted that the degree of genomic aberrations observed in melanoma in humans is much greater than that seen in mice, suggesting that while cross-species comparison may help to filter out genomic noise, inherent differences between species may limit the completeness of the investigation. However, engineering genomic instability onto a mouse model can provide DNA double-strand breaks and consequent copy number aberrations and translocation. The use of *Atm*−/−, Trp53-deficient mice with telomere dysfunction generated lymphomas with greater number of chromosomal aberrations than seen in lymphomas with intact telomeres [147]. Many of the copy number alterations that emerged in the murine genome were syntenic to human T-cell acute lymphoblastic leukemia/lymphoma. Even more interestingly, most of the minimal common regions from the murine model overlapped with minimal common regions from aCGH data sets of various other human tumor types, including melanoma. This suggests that murine models designed with genetic instability may provide a more humanized model with which to filter genomic noise in the human cancer genomes and, thereby, identify significant genomic aberrations.

Upon gene identification, the use of cell-based assays has provided insight into the role of different genes in melanoma. Commonly, gain-of-function (e.g., overexpression) and loss-of-function (e.g., siRNA) studies can shed light on the biological consequence occurring from the genetic alterations. Knowledge of a gene's role in a biological context (i.e., cell migration) and in signaling will be useful in deciding how a therapy might affect a tumor (i.e., limit invasiveness). However, cell-based analysis will be limited by the genetic background of any cell line and artifacts arising from time in culture. Even though 3D culture systems and xenograft models attempt to mimic the tumor *in vivo* environment, neither can fully recapitulate *in vivo* human tumors. Therefore, concurrently, extensive clinicopathological analysis must be performed in order to establish the relevance of any alteration in human cancers. That said, cell line-based experiments can provide an important starting point for mechanistic and phenotypic analyses.

## Expert commentary

With the rapid advances of high throughput genomic technologies, particularly the emerging next-generation sequencing technology, researchers will soon be able to define the complete somatic architecture of human melanoma through multidimensional genomic analyses of melanoma specimens. Such an atlas will enable not only identification of genetic events associated with melanoma but also comprehensive data for molecular subclassification that can enhance the clinicians' ability to stratify and personalize treatment of melanoma patients.

## Five-year view

Over the next 5 years, there will be great advances in the understanding of the genetic and epigenetic changes underlying melanoma genesis and progression. As technologies that allow high-throughput analysis of the genome develop at break-neck speed, the rate at which genetic alterations associated with melanoma are discovered will only increase. Efforts such as The Cancer Genome Atlas, will propagate the integration of different types of genomic aberrations found in melanoma. In parallel, methodologies to sift through the data and discover 'true' drivers of disease will become of critical importance. Interestingly, as the cost of genome sequencing drops, it may also become common to sequence each individual's tumor. However,

given the gross amount of aberrations found, the clinical relevancy of such data will have to be rigorously established.

Currently, the prognosis for patients with metastatic melanoma is very grim. Research over the next few years will shed light on the genetic determinants of metastasis, thereby allowing the therapeutic targeting of tumor cells that are about to, or have, metastasized. As the cases of melanoma increase worldwide, and since sun exposure is a cooperative factor in melanoma, research into the relationship between the molecular underpinnings of melanoma and UV radiation will be of critical importance. Through such research, the next 5 years may bring forth promising treatments for melanoma.

**Key issues**

- The incidence of melanoma has increased in the last 50 years. As patterns of sun exposure change, and given the potential link between UV radiation and melanoma, understanding the determinants of melanoma will be of critical importance.
- While localized melanoma is surgically excisable, therapies for metastatic melanoma have poor clinical efficacy. This has resulted in a grave prognosis for patients with metastatic melanoma.
- Genetic alterations in *CDKN2A*, *PTEN*, *BRAF* and *N-RAS* are common in melanoma.
- Various signaling pathways have been shown to be involved in melanoma, including the MAPK and PI3K pathways.
- Alterations in c-KIT, c-MET and EGF receptor copy numbers have also been observed in melanoma.
- Melanoma shows a high degree of heterogeneity, suggesting the existence of patient subgroups defined by specific genetic alterations. Understanding the genetic and molecular underpinnings of these subgroups will be necessary for the development of therapies.
- Analyses of genomic changes in melanoma (i.e., sequence, copy number, genomic structure and epigenetic) have identified key regulators of melanoma. More are likely to be identified.
- As a testament to the power of genomic analysis to identify genetic determinants in a disease, *Nedd9* and *MITF* were identified as critical regulators of melanoma. However, such work requires extensive biological validation.
- Of critical importance will be the culling of bystanders from genetic drivers of melanoma. This can be achieved through the integration of different data sets, cross-species analysis and cell line-based assays.
- Functional and clinicopathological validation of any gene or genetic element of interest must be performed rigorously to identify the critical regulators of melanoma initiation and progression.

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• of interest

•• of considerable interest


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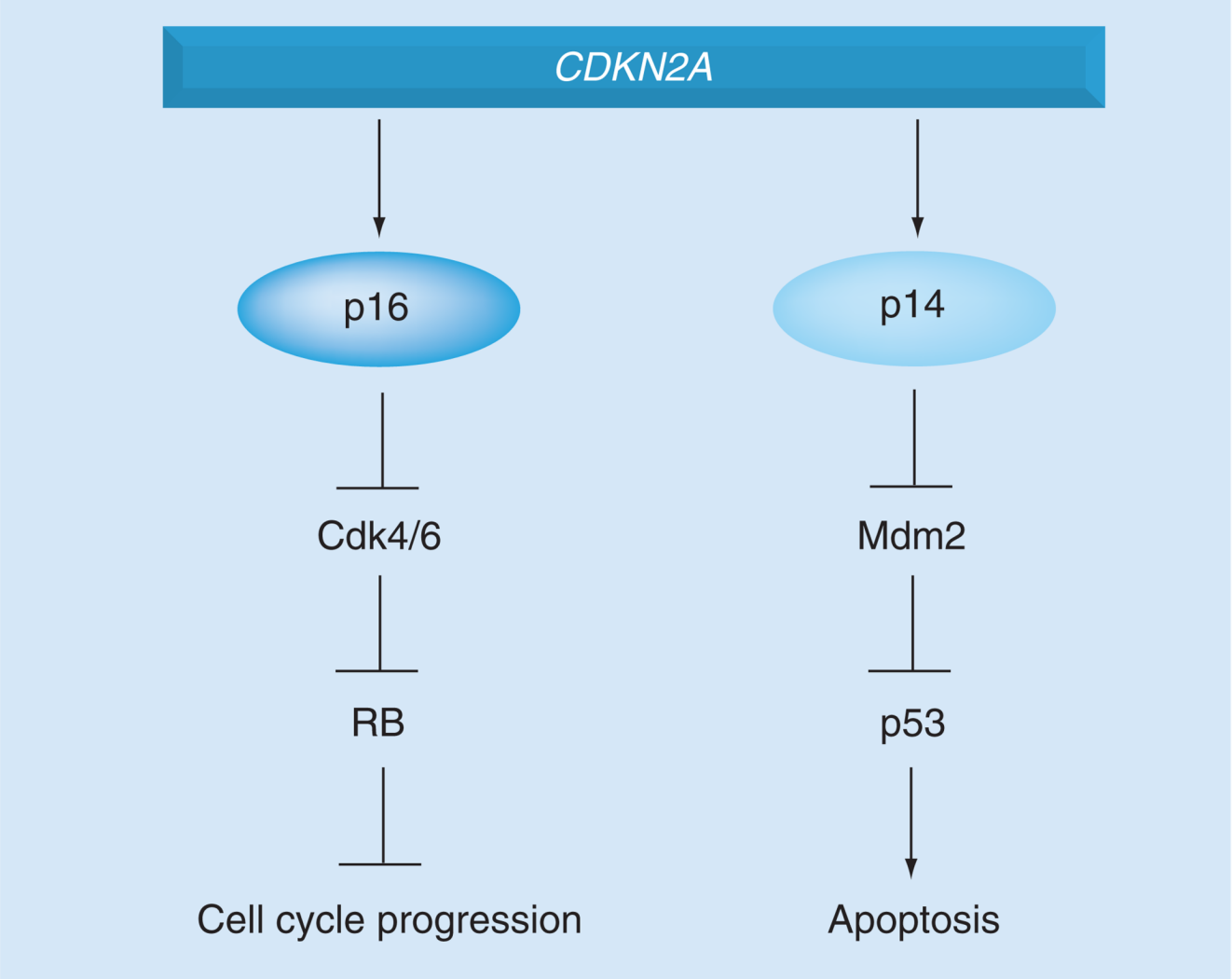


**Figure 1. CDKN2A encodes two important regulators of melanoma**

The *CDKN2A* gene encodes both $p16^{INK4A}$ and $p14^{ARF}$, which control the RB and p53 pathways, respectively. Germline alterations in *CDKN2A* are common events in familial melanoma. RB: Retinoblastoma.

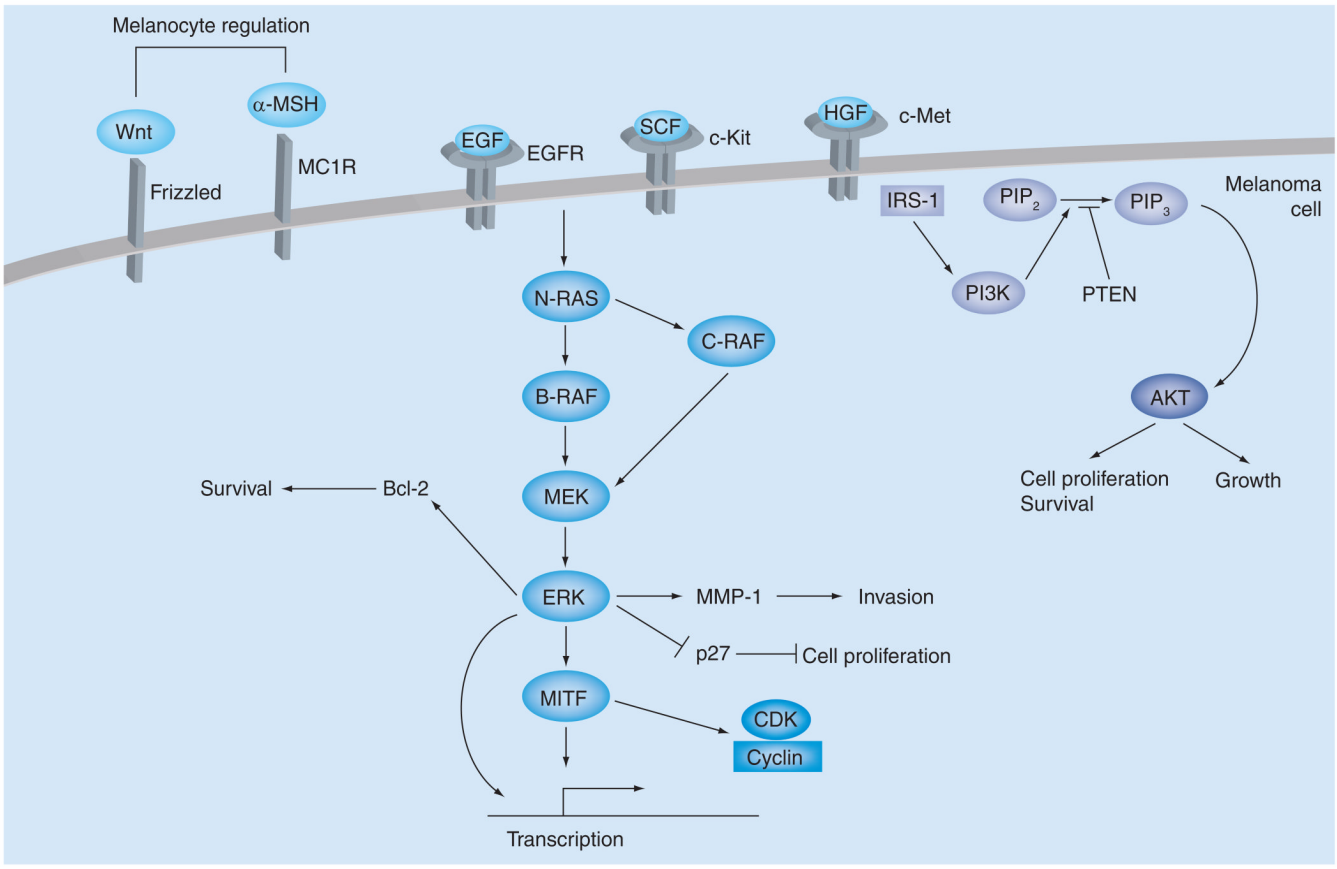


**Figure 2. Signaling pathways involved in melanoma**

Various receptors can activate the ERK and PI3K pathways, both important signaling cascades in melanoma that control cell proliferation and survival. In addition, signaling pathways important for melanocyte regulation may be co-opted in tumors.

EGFR: Endothelial growth factor receptor; MMP: Matrix metalloproteinase.

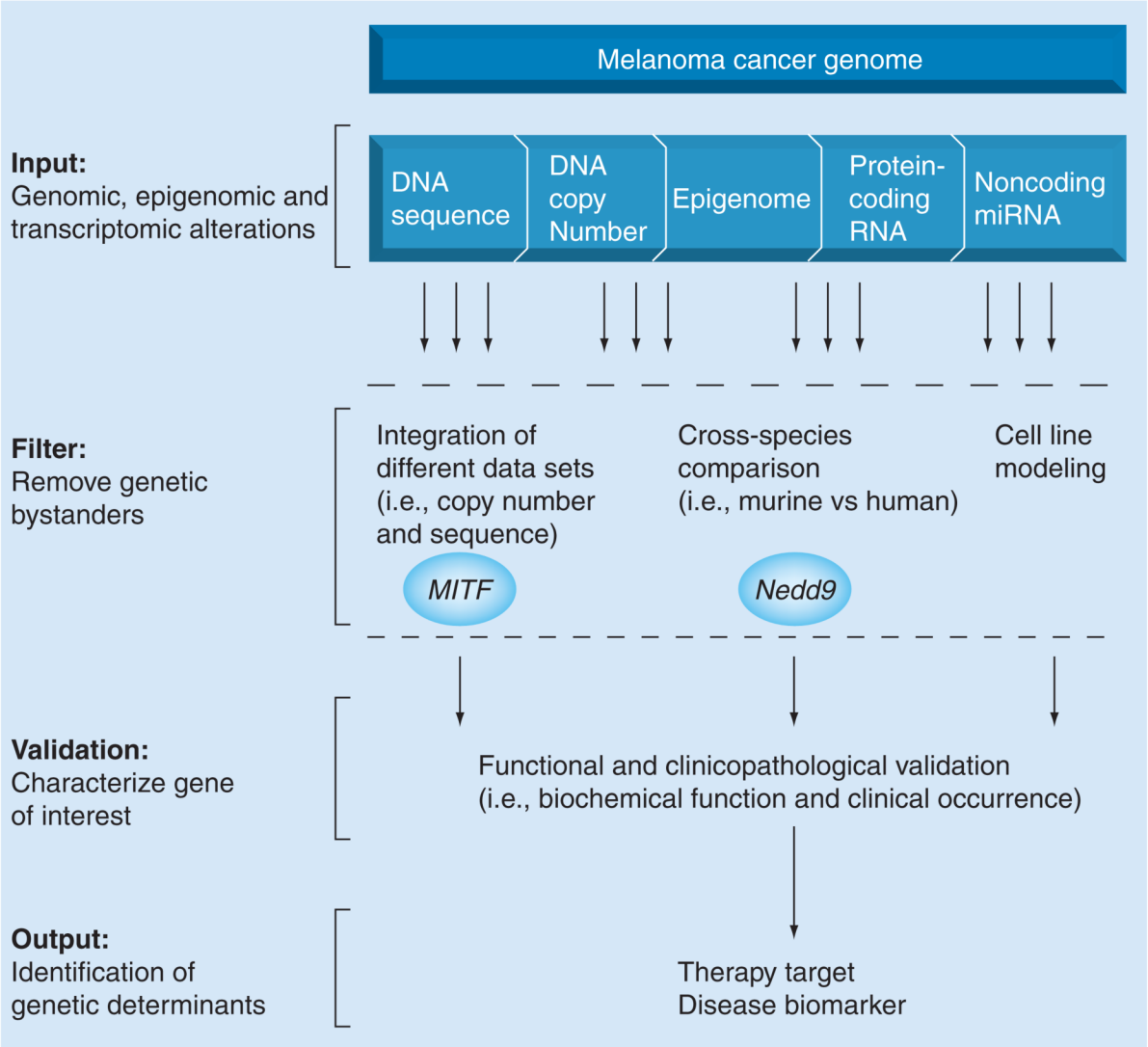


**Figure 3. Filtering of genomic alterations identifies genetic determinants of melanoma**

The melanoma cancer genome is composed of genomic, epigenomic and transcriptomic alterations found in melanoma. Bystanders may be culled from true genetic drivers of disease through integration of different data sets, cross-species comparison for evolutionarily conserved alterations and analysis in cell lines. These filtering methods have been used successfully to identify *MITF* and *Nedd9* as important regulators of melanoma. All genes of interest must undergo extensive functional and clinicopathological validation in order to identify genetic determinants of melanoma that may serve as therapeutic targets or disease biomarkers.