7 Altered Signal Transduction Pathways in Melanoma

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7.1 Introduction

Signal transduction pathways are central to all cellular biological processes, as they provide the link between extracellular or intracellular stimuli and an array of regulatory proteins, including protein kinases, ubiquitin ligases, and transcription factors. Given this, it is not surprising that signal transduction pathways are often deregulated in cancer. Indeed, melanoma is a paradigm for rewired signaling because most critical mutations discovered in this tumor type are centered around relatively few major signaling cues, the most significant of which are the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) pathways. Both pathways contain regulatory components with catalytic activities, making them the preferred targets for therapy. Here, we summarize our current understanding of the major deregulated signaling pathways in melanoma and the implications of such deregulation for tumor biology.

7.1.1 Extracellular Receptors

Among the receptors reported to be deregulated in melanoma are numerous membrane-bound G protein-coupled receptors and receptor tyrosine kinases (RTKs), including MC1R (melanocortin 1 receptor), c-Kit (mast/stem cell growth factor receptor), c-Met (hepatocyte growth factor receptor), IGFR (insulin-like

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growth factor receptor), and Frizzled (WNT receptor). Deregulation of other RTKs, including AXL, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), and vascular endothelial growth factor receptor (VEGFR), has also been implicated in the resistance of melanomas to certain treatments such as BRAF inhibitors (BRAFi) (Fargnoli et al. 2010; Landi et al. 2006; Mattei et al. 1994; Topcu-Yilmaz et al. 2010; van Ginkel et al. 2004).

7.1.1.1 MC1R

MC1R is a melanocyte-specific G protein-coupled receptor that binds to α-melanocyte-stimulating hormone (α-MSH, Fig. 7.1). MC1R–α-MSH interactions play a central role in the regulation of both pigmentation, by inducing generation of eumelanin and cAMP, and melanocyte proliferation (Hunt et al. 1995; Mountjoy et al. 1992; Robinson and Healy 2002; Suzuki et al. 1996).

MC1R exhibits genetic variance, with mutations at the hot spot residues R151C, R160W, and D294H being the most significant. The mutations reduce receptor function and result in a phenotype of fair, freckled skin and red hair (Kadekaro et al. 2003). Stimulation of MC1R by α-MSH normally potentiates p16INK4A tumor suppressor activity after UV exposure (Pavey et al. 2002); these specific mutations are associated with reduced UV-induced DNA damage repair efficiency and increased melanoma risk (Scott et al. 2002; Song et al. 2009). In contrast, overexpression of MC1R variants has been shown to render cells insensitive to α-MSH-mediated suppression of cell proliferation (Robinson and Healy 2002), suggesting that polymorphic variants of MC1R may enhance melanoma susceptibility or progression by attenuating p16INK4A function, at least in part.

Although MC1R is neither genetically nor epigenetically silenced (Kim et al. 2008a), expression of the functionally impaired variants compromises receptor activity and correlates with increased melanoma risk (Landi et al. 2006). Carriers of MC1R variants who have mutations in CDK2NA also have a higher melanoma risk (Fargnoli et al. 2010). Notably, germline mutations of MC1R are associated with an increased incidence of BRAF mutations in melanoma (Landi et al. 2006). Likewise, inactivation of MC1R in the $Braf^{V600E}$:$Pten^{-/-}$ mouse melanoma model increases the incidence of melanoma independently of UV radiation (Mitra et al. 2012).

Mutation of G proteins themselves, in particular the α-subunit of G(q) (GNAQ), may induce alterations in early melanoma lesions (Kusters-Vandevelde et al. 2010; Lamba et al. 2010; Van Raamsdonk et al. 2009). GNAQ is mutated within a RAS-like domain at position Q209L, which renders the protein constitutively active and amplifies PKC and MAPK signaling. Accordingly, overexpression of the GNAQ Q209L mutant is sufficient to confer anchorage independence and increase the tumorigenicity of immortalized melanocytes.

7.1.1.2 Receptor Tyrosine Kinases

Many cell surface receptors for growth factors, hormones, and cytokines are RTKs. Ligand binding activates the intrinsic RTK enzymatic activity, often by autophosphorylation, with subsequent phosphorylation of tyrosine residues on many substrates, including PLCγ, PI3K, and MAPK, which drive cell proliferation

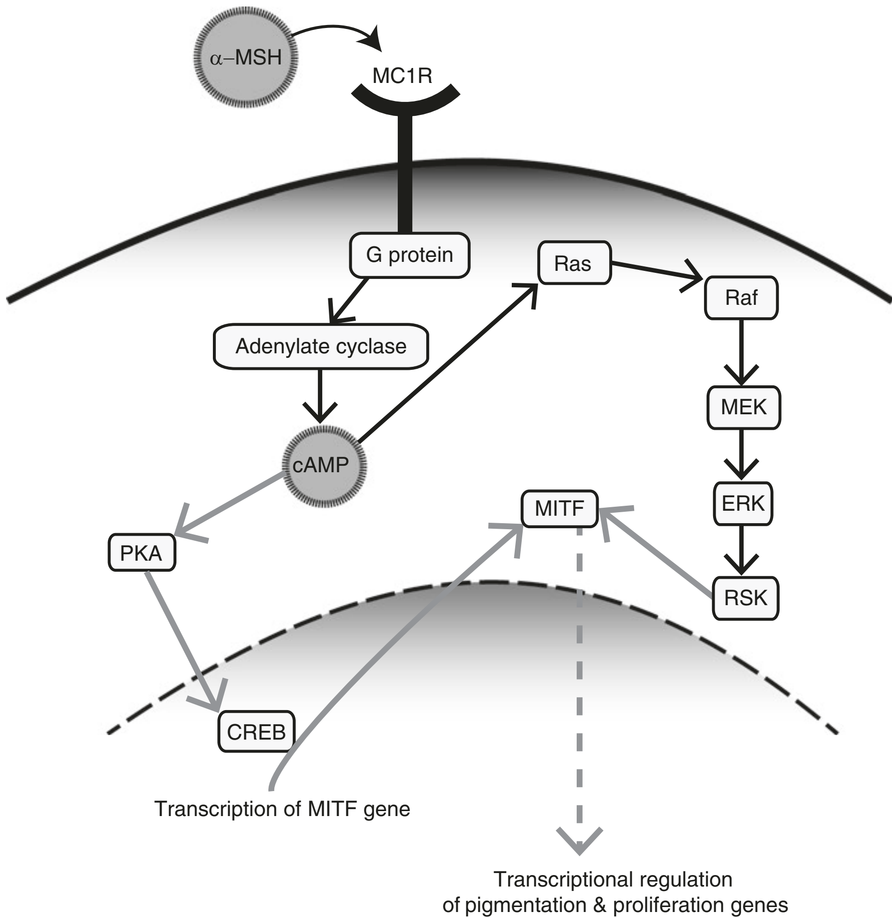

Fig. 7.1 α-MSH and MC1R receptor signaling. Binding of α-melanocyte stimulating hormone (*α-MSH*) to its cognate receptor melanocortin-1 receptor (*MC1R*) activates G protein-coupled adenylate cyclase and increases cytoplasmic cAMP levels. cAMP initiates a cascade that sequentially activates protein kinase A (*PKA*), CREB, and transcription of MITF. In parallel, cAMP activates the RAS–RAF–MAPK–RSK cascade, which results in MITF activation. MITF then modulates the transcription of downstream pigmentation and proliferation genes. Branches of this signaling pathway that are upregulated in melanoma are indicated in *black*. The *dashed arrow* indicates modulation of transcriptional programming by MITF to favor tumorigenesis

differentiation, survival, and cell migration. Among the growth factor RTKs deregulated in melanoma are AXL, EGFR, c-Kit, c-Met, and IGFR, which bind to growth arrest-specific 6 (Gas6), epidermal growth factor (EGF), stem cell factor (SCF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF), respectively (Fig. 7.2). Ligand binding by these receptors activates the downstream RAS–RAF–MEK–MAPK and PI3K–AKT signaling pathways independently of any existing MAPK pathway mutations and promotes cell survival and proliferation. Changes in

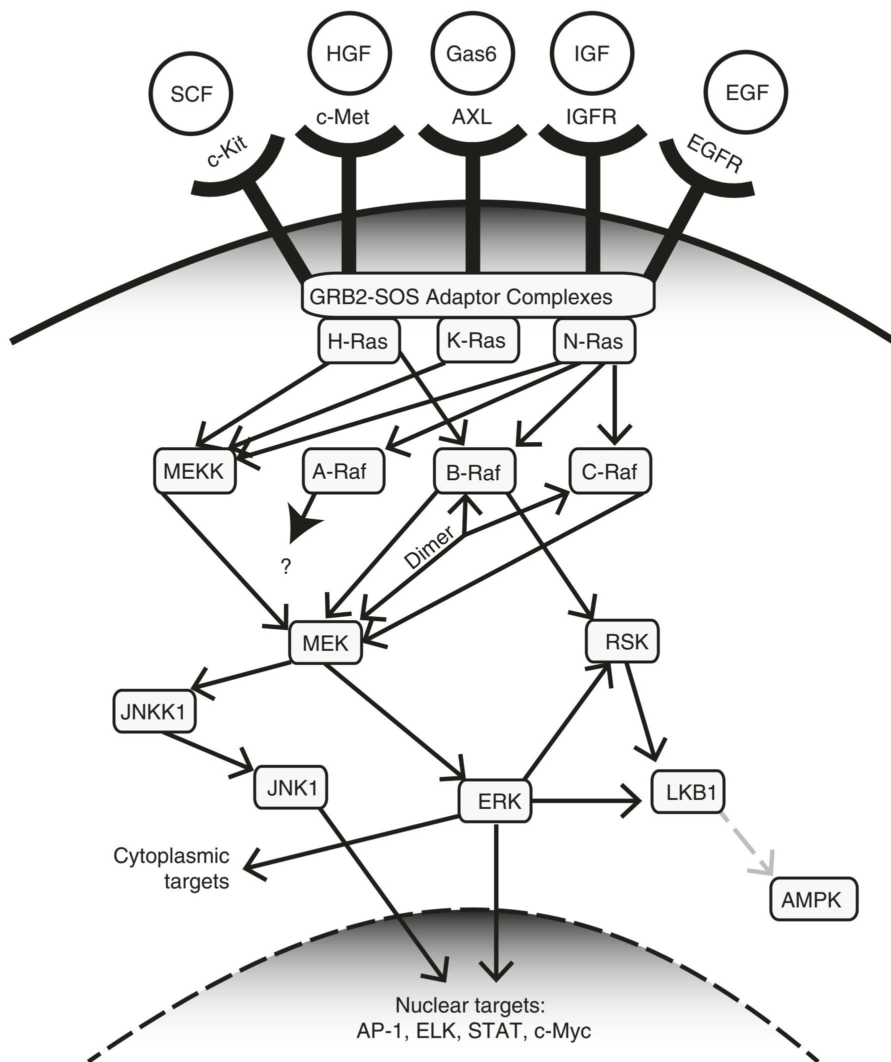

Fig. 7.2 Growth factor receptors and MAPK signaling. Membrane-bound growth factor receptors (*c-Kit, c-Met, IGFR, AXL, EGFR*) generally signal inwards through adaptor complexes containing proteins, such as GRB-SOS, which recruit the RAS family members HRAS, KRAS, or NRAS for activation. In melanoma, NRAS is the most commonly mutated protein and plays a predominant role in activation of the downstream effector kinases, RAF and MEKK. The RAF family of effector kinases includes A-, B-, and CRAF. Although signaling through ARAF has been less studied, in melanoma, it appears that BRAF- and CRAF-mediated signaling predominate. BRAF and CRAF activate downstream MEKK, MEK, and RSK. MEK activation leads to further downstream activation of JNKK1–JNK1 and ERK, as well as their cytoplasmic or nuclear transcriptional targets. Together, BRAF and ERK activate RSK to suppress LKB1, which would otherwise activate AMPK. In melanoma, this arm of AMPK activation, which normally regulates cell growth and survival, is downregulated (*gray dashed arrow*). Branches of the MAPK pathway that are upregulated in melanoma appear in *black*

the expression of these receptors have been implicated in several tumor types; in melanoma, altered expression has not been observed uniformly but may be associated with specific phases of tumor development.

7.1.1.3 AXL

AXL RTK is ubiquitously expressed in epithelial, mesenchymal, and hematopoietic tumors, and in the corresponding non-transformed cells. AXL was discovered in patients with chronic myeloproliferative disorder, and has since been implicated in the proliferation and drug resistance of numerous cancers, including melanoma (Paccez et al. 2014).

AXL is upregulated in ocular melanomas and melanoma cell lines, where it promotes cell proliferation and confers a survival advantage under serum starvation conditions (van Ginkel et al. 2004). Increased AXL expression is also found in both NRAS- or BRAF-mutated tumors, although the incidence is higher in NRAS mutant melanomas. Of interest, AXL expression inversely correlates with melanoma differentiation and microphthalmia-associated transcription factor (MITF) expression (Muller et al. 2014), and the combination of low MITF and high AXL expression is associated with a highly invasive phenotype. Pharmacological AXL inhibitors attenuate but do not abolish the invasive phenotype (Sensi et al. 2011), indicating a role for other RTKs and/or signaling pathways in the invasive phenotype. In response to chemotherapy, a subset of tumor cells that exhibit a senescence phenotype show elevated production and secretion of cytokines (Ohanna et al. 2011), with concomitant activation of STAT3 and AXL and increased migration and invasion properties in surrounding cells (Ohanna et al. 2013).

Lastly, a major clinical obstacle in melanoma therapy is the acquisition of resistance to either BRAFi or MEKi. The mechanisms underlying such resistance include upregulation of AXL, among many RTKs, which coincides with low levels of MITF and activation of NF-kB and JAK1 (Konieczkowski et al. 2014). Accordingly, treatment of resistant melanomas with a pharmacological inhibitor of AXL augments the effect of the BRAFi or MEKi and enhances cell death (Muller et al. 2014).

7.1.1.4 EGFR

EGFR is a transmembrane receptor for members of the EGF family of growth factors. Increased expression or mutation of the EGFR gene is commonly seen in a number of tumor types, including colorectal, breast, and non-small cell lung cancers, where it is thought to serve as an oncogenic driver.

EGFR upregulation has been implicated as a mechanism of therapy resistance for several tumor types, including melanoma. Consistent with this, co-administration of BRAFi and EGFRi appears to attenuate ERK activity and sensitizes resistant melanomas to BRAFi or MEKi inhibition (Girotti et al. 2013). The resistance of $BRAF^{V600E}$ mutant colorectal cancer to BRAFi therapy has also been associated with high EGFR expression, which enables reactivation of ERK via RAS and CRAF to bypass BRAF inhibition (Corcoran et al. 2012).

Elevated EGFR expression, as observed in resistant melanoma, has been linked to SOX10 and MITF expression (Ji et al. 2015). Furthermore, EGFR upregulation is often

accompanied by upregulation of other RTKs that are associated with SOX10 and TGF-β signaling, including platelet-derived growth factor receptor β (Sun et al. 2014).

Interestingly, SOX10 was also found to regulate the expression of the ubiquitin ligase RNF125, which controls JAK1 stability through ubiquitin–proteasome-dependent degradation. BRAFi-resistant melanomas exhibit downregulation of SOX10 and concomitant inhibition of RNF125 expression. Consequently, JAK1 stability and availability increase, and the expression of several RTKs, including EGFR and AXL, is stimulated. As might be expected, inhibition of JAK1 effectively reduces the expression of RTKs and overcomes the resistance of melanomas to BRAFi (Kim et al. 2015).

7.1.1.5 c-Kit

c-Kit expression is apparent in early or radial growth phase melanomas. Although the penetrance appears to be low, a c-Kit-activating mutation, L576P, has been reported in melanoma (Antonescu et al. 2007; Rivera et al. 2008; Willmore-Payne et al. 2006). Interestingly, however, downregulation of c-Kit expression is associated with melanoma progression (Giehl et al. 2007; Janku et al. 2005; Montone et al. 1997; Natali et al. 1992). These observations suggest that upregulation of c-Kit and its ligand SCF may be required to establish the primary lesions, but that continued expression of c-Kit is not needed for invasion and metastasis. The disparity of mutations in or altered expression of c-Kit among different melanomas was initially overlooked in clinical trials of c-Kit-specific inhibitors. In more recent trials, clinical efficacy has been observed when patient cohorts harboring c-Kit mutations were treated with the highly selective pharmacological inhibitor, Gleevec (Terheyden et al. 2010). How inhibition of c-Kit contributes to melanoma progression remains an important topic for investigation. The c-Kit ligand SCF is a keratinocyte-secreted factor, and it has been proposed that downregulation of c-Kit in melanoma cells may allow them to escape SCF-induced cell death. Indeed, *in vitro* studies have shown that re-expression of c-Kit in metastatic melanoma sensitizes the cells to SCF-mediated apoptosis and reduces their tumorigenic and metastatic potential in vivo (Bar-Eli 1997; Huang et al. 1996; Willmore-Payne et al. 2005).

Although the mechanism by which c-Kit is downregulated during melanoma progression remains unclear, a recent study found that downregulation may be epigenetically linked to expression of microRNAs (miRNAs) (see also Chap. 6), specifically miR-221 and miR-222, which were shown to suppress expression of both c-Kit and p27Kip (Felicetti et al. 2008).

7.1.1.6 c-Met

c-Met-dependent signaling is amplified in melanoma, although genetic mutations or modifications that result in aberrant activation of c-Met do not appear to be common. Two c-Met mutations, N948S and R988C, have been identified in melanoma cell lines and tumor tissues and shown to activate c-Met signaling through several downstream effectors, including MITF, tyrosinase, and AKT and its effectors (Chin et al. 2006; Puri et al. 2007). However, c-Met upregulation has also been observed in melanoma, particularly in the later stages of disease (Natali et al. 1993). This has been suggested to play a role in metastasis, especially in the liver (Rusciano et al. 1995). Genetic amplification and activation of c-Met concomitant with Src

activation has been reported in BRAFi-resistant melanoma cells; accordingly, genetic or pharmacological inhibition of c-Met attenuated the proliferation and invasion of the BRAFi-resistant cells (Vergani et al. 2011).

c-Met upregulation can be induced by a number of mechanisms. One is via MITF, which is induced by MC1R–α-MSH signaling, as mentioned above (Rouzaud et al. 2006; Rusciano et al. 1999). Indeed, impaired MC1R function, which is frequently observed in melanoma, is indicative of deregulated c-Met at both the genetic and protein levels.

The ubiquitin ligase skeletotrophin is another protein implicated in the regulation of c-Met. In melanoma, expression of skeletotrophin is lost due to increased SNAIL-mediated transcriptional repression. Re-expression of skeletotrophin impairs the invasive capacity of melanoma cells *in vitro*, and this correlates with a reduction in c-Met mRNA transcripts (Takeuchi et al. 2006). MicroRNAs have been shown to contribute to increased c-Met levels in melanoma. miR-34a is normally expressed in melanocytes but is downregulated in melanoma. Here too, re-expression of miR-34a *in vitro* reduces c-Met expression and suppresses the growth, migration, and invasive capacities of melanoma cells (Yan et al. 2009).

7.1.1.7 IGFR1

IGF1R is another growth factor receptor that is upregulated in progressively malignant melanoma (Mallikarjuna et al. 2006). In early melanoma lesions, IGF1R appears to enhance cellular growth and survival by promoting MAPK- and ß-catenin-dependent signaling pathways; however, IGF1R-dependent stimulation of these two pathways may be dispensable in later stage melanomas where other oncogenes are constitutively activated (Satyamoorthy et al. 2001).

Upregulation of IGF1R is associated with both malignant progression and resistance to apoptotic stimuli. Antisense-mediated inhibition of IGF1R is sufficient to inhibit the growth of mouse melanoma cells in nude mice (Resnicoff et al. 1994), and monoclonal antibody-targeted inhibition of IGF1R in human melanoma cells similarly inhibits their growth and invasion in xenograft mouse models (Maloney et al. 2003). Furthermore, disruption of IGF1R can sensitize melanoma cells to TRAIL-induced apoptosis (Karasic et al. 2010) and increase radiosensitivity of melanoma by impairing the ATM-mediated DNA damage response (Macaulay et al. 2001). Moreover, inhibition of IGF1R is sufficient to suppress growth of human melanomas harboring the $BRAF^{V600E}$ mutation (discussed further below), indicating that IGF1R inhibition can override signaling events that circumvent the known IGF1R effector, the RAS-MAPK signaling axis (Yeh et al. 2006). High levels of IGF1R have been reported in BRAFi-resistant cells, and have been implicated in upregulation of the PI3K pathway (Villanueva et al. 2010).

7.2 Cellular Kinases and Transcription Factors

7.2.1 WNT–ß-catenin

The WNT–ß-catenin signaling pathway plays an important regulatory role in melanocyte development and is deregulated in melanoma (see also Chap. 5). WNT is a

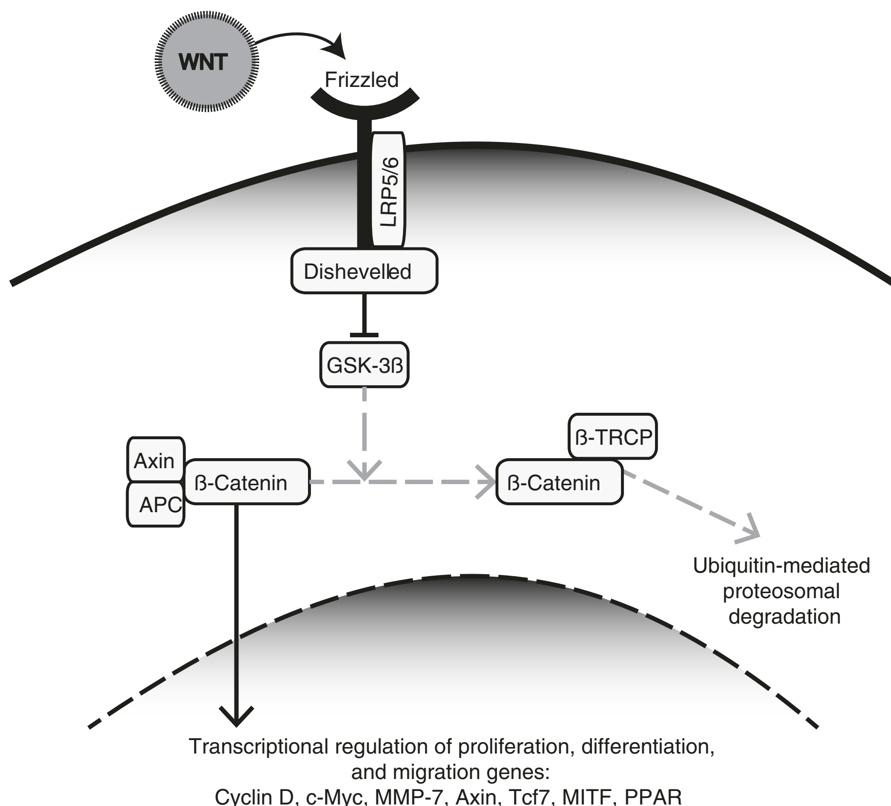

Fig. 7.3 WNT signaling pathway. Binding of WNT to its cognate receptor Frizzled and co-receptor LRP5/6 results in inactivation of GSK3ß via Dishevelled. Inactivation of Dishevelled stabilizes ß-catenin by releasing it from its degradation complex, which includes Axin and APC. Under non-stimulated conditions, ß-catenin is bound by ß-TRCP, which facilitates its ubiquitination and subsequent proteasomal degradation. Stabilized ß-catenin is then imported into the nucleus to facilitate transcriptional regulation of proliferation, differentiation, and migration genes. Branches of this signaling pathway that are upregulated in melanoma are indicated in *black*; downregulated branches are indicated by *dashed gray arrows*

secreted ligand for the membrane receptor, Frizzled (Fig. 7.3), and WNT binding activates the cytoplasmic Frizzled effector, Dishevelled. Consequently, Dishevelled inhibits GSK3ß–Axin–APC-mediated degradation of ß-catenin, stabilizing its levels and allowing nuclear import to execute its transcriptional functions. Activation of the WNT–ß-catenin pathway facilitates ß-catenin-mediated upregulation of MITF, which promotes melanocyte differentiation and development (Dorsky et al. 2000; Takeda et al. 2000). MITF can itself bind to ß-catenin, thereby tilting the transcriptional activity towards MITF targets and generating a positive feedback loop (Schepsky et al. 2006).

As expected, melanomas harboring activating ß-catenin mutations also have increased MITF levels (Doglioni et al. 2003). In turn, MITF upregulation has been

shown to increase multivesicular body synthesis and consequently WNT signaling, allowing the cells to enter a proliferative stage (Ploper et al. 2015). Interestingly, ß-catenin also upregulates the transcription factor Brn-2, which transcriptionally represses Mitf and simultaneously enhances invasive melanoma behavior. Brn-2 expression also characterizes distinct subsets of MITF-negative melanoma cells (Goodall et al. 2008). The implications of MITF heterogeneity within the same and different tumors is the subject of intense investigation.

WNT–ß-catenin signaling is upregulated in melanoma, and although only ~3 % of melanoma biopsies harbor ß-catenin mutations, ~30 % of human melanomas exhibit increased nuclear localization of ß-catenin (Larue and Delmas 2006). Increased WNT signaling directly upregulates Mitf and Brn-2 expression, with concomitant suppression of p16INK4A transcription; these changes act in concert to drive melanoma growth and proliferation (Delmas et al. 2007; Goodall et al. 2004a; Widlund et al. 2002). Nuclear localization of ß-catenin is observed in melanomas carrying mutant phosphatase and tensin homolog (PTEN) phosphatase and has been implicated in melanomagenesis by cooperating with $NRAS^{Q61K}$ to increase caveolin-dependent transcriptional activity. This effectively bypasses the senescence phenotype elicited by mutant NRAS alone and promotes metastasis, in part by internalization of E-cadherin (Conde-Perez et al. 2015).

Interestingly, the functional role of WNT signaling in melanoma development varies with the specific WNT isoform. WNT3, an activator of the canonical WNT signaling axis, is anti-tumorigenic and its expression correlates with primary/nevi lesions and decreased proliferation of tumor models *in vitro* and *in vivo*. Furthermore, WNT3 expression correlates with upregulation of genes controlling melanocyte development and differentiation, including *Axin*, *Tcf7*, and *Mitf* (Chien et al. 2009). In contrast, WNT5A, which activates the non-canonical WNT signaling axis, appears to antagonize the transcriptional effects of WNT3A. Indeed, WNT5A is pro-tumorigenic, cooperating with other signaling pathways (e.g., PKC) to enhance the metastatic and invasive behavior of melanoma cells, which most likely occurs through its known function in the re-distribution of adhesion receptors (Weeraratna et al. 2002; Witze et al. 2008). Consistent with this, WNT5A-positive melanoma tumors appear to exhibit increased invasiveness and decreased proliferation compared with WNT5A-negative but MITF- and Melan-A-positive tumors, supporting a model of proliferative vs invasive phenotype switching during tumor progression (Eichhoff et al. 2010). Of note, increased WNT5A expression was also seen in tumors with acquired BRAFi resistance, and its inhibition re-sensitized tumors to BRAFi and reduced their proliferation, in part via attenuation of p-AKT activity (Anastas et al. 2014).

Melanomas with activated WNT–β-catenin signaling have a strong immunosuppressive effect on dendritic cells and cytotoxic T lymphocytes, mediated by increased IL-10 secretion and reduced IFN-γ secretion by the T cells. In this regard, it is interesting to note that activated WNT–β-catenin signaling has been linked with resistance to immunotherapy (Spranger et al. 2015; Yaguchi et al. 2012).

7.2.2 MAPK Signaling Axis

Alterations in the expression or activity of AXL, EGFR, MC1R, c-Kit, c-Met, IGFR, and WNT are examples of the most external layers of perturbed signaling cues that promote melanoma formation and progression. Several of the signaling pathways downstream of these receptors are themselves deregulated in melanoma.

The majority of melanomas display deregulated MAPK signaling due to mutations in the NRAS or BRAF genes. As a consequence, the downstream kinases and transcription factors are rendered constitutively active, regardless of aberrations upstream of NRAS or BRAF. In this section, we review our current understanding of alterations in the MAPK signaling axis and the implications for melanoma development or progression.

The MAPK pathway is coupled to upstream membrane receptors by the RAS family of small G proteins; HRAS, KRAS, and NRAS (Bos 1989; Dhillon et al. 2007). In non-transformed cells, the RAS proteins are responsive to activation by c-Kit, c-Met, IGFR, and WNT, for example, and transduce activating signals through interplay with the RAF family of effector serine/threonine kinases; ARAF, BRAF, and CRAF. Signals are then transduced by sequential activation of a cascade of MAP kinases: MEK, MEKK, and finally, ERK (Fig. 7.2). Of these proteins, NRAS and BRAF are the most commonly mutated in melanoma, with ~15 % and more than 50 % of melanomas harboring NRAS and BRAF mutations, respectively (Davies et al. 2002; Fecher et al. 2007). Further downstream, MEK mutations have also been reported, particularly in the context of acquired resistance to chemotherapy. For instance, MEK1 mutations occur at low incidence overall, but they are most frequently reported following BRAFi therapy and confer resistance to MEKi and BRAFi (Emery et al. 2009; Murugan et al. 2009).

While most melanoma-associated mutations in NRAS occur at amino acid 61, BRAF deregulation is attributed to mutations at several hotspots, most prominently V600E, resulting in a constitutively active kinase (Wan et al. 2004). Indeed, the catalytic activity of $BRAF^{V600E}$ was calculated to be ~10-fold higher than that of wild-type BRAF (Brummer et al. 2006). While mutant BRAF and NRAS share some downstream effectors, most notably ERK, they each also activate unique downstream components. As illustrated in Fig. 7.2, BRAF activity also affects MEK–ERK kinases and RSK. These kinases in turn suppress the activity of the LKB1–AMPK signaling pathway, thereby promoting melanoma proliferation (Esteve-Puig et al. 2009; Zheng et al. 2009). As a result of their unique contributions to signaling, BRAF and NRAS mutants have distinct characteristics with respect to melanoma development and progression.

As noted above, ERK is the downstream kinase most commonly affected by NRAS and BRAF mutations in melanoma, and constitutive or super-activation of ERK perturbs critical regulators of cellular behavior. For example, $BRAF^{V600E}$ antagonizes apoptosis via ERK-dependent inhibition of the apoptotic proteins Bad, Bim, and PUMA, and upregulation of anti-apoptotic proteins such as Mcl-1 (Jiang et al. 2008; Sheridan et al. 2008; Wang et al. 2007b). Enhanced ERK activation also alters cell cycle control and proliferation by suppressing the negative cell cycle

regulator p27/Kip1 (Kortylewski et al. 2001) and, importantly, by modulating the expression of melanocyte MITF isoforms and inducing the M-MITF 6a isoform (Primot et al. 2010; Wellbrock et al. 2008). MAPK/ERK activation can further enhance the proliferative capacity of melanoma cells by promoting upregulation of other transcription factors such as c-Jun and Brn-2, either by increasing their stability (c-Jun) or increasing their expression (Brn-2) (Goodall et al. 2004a, b; Lopez-Bergami et al. 2007). Mutant BRAF-mediated ERK signaling also impinges on invasive cellular behaviors resulting from changes in growth or invasion regulatory proteins such as Plexin B or matrix metalloproteinase-1 (Argast et al. 2009; Huntington et al. 2004). Indeed, activation of the RAS–RAF–MAPK–ERK pathway has been implicated in immune evasion by modulating the production of immunosuppressive cytokines such as IL-6, IL-10, and VEGF by melanoma cells (Sumimoto et al. 2006).

Constitutive upregulation of ERK signaling results in rewiring of signaling pathways, a common occurrence in many tumors, including melanoma. For example, rewired ERK signaling causes constitutive activation of c-Jun via two complementary pathways. ERK-mediated upregulation of RSK–CREB increases c-Jun transcription, whereas ERK-mediated phosphorylation of GSK3ß inhibits its ability to phosphorylate c-Jun on residue 243, which is required for targeting of c-Jun for ubiquitination and degradation by FBW7 (Nateri et al. 2004; Wei et al. 2005), thereby resulting in increased c-Jun stability (Lopez-Bergami et al. 2007). In turn, c-Jun induces transcription of a large set of targets genes that include cell cycle regulators such as Cyclin D as well as components of other signaling pathways. One is the PKC adaptor protein RACK1, which potentiates both PKC and JNK signaling. As a result, RACK1–PKC signaling increases JNK activity and further activates its substrates, including c-Jun, thus enforcing a feed-forward signaling pathway. Another c-Jun transcriptional target implicated in melanoma development is the kinase PDK1, which activates AKT. The c-Jun-mediated increase in PDK1 transcription, and thus activation of both the AKT and PKC pathways. Inhibition of c-Jun effectively attenuates melanoma development in a xenograft mouse model, and this can be rescued by re-expression of PDK1 (Lopez-Bergami et al. 2010). Thus, ERK signaling causes activation of the JNK, PKC, PDK1, and AKT pathways, representing a paradigm for rewired signaling.

Constitutive activation of MAPK itself is sufficient for transformation of immortalized melanocytes through elevation of angiogenic and invasive behavior secondary to upregulation of VEGF and MMP-2 (Govindarajan et al. 2003). However, synergistic crosstalk between upregulated MAPK signaling and other major signaling axes (e.g., PI3K–AKT–MTOR), can further promote additional tumorigenic behaviors such as the enhanced proliferation observed in uveal melanoma (Babchia et al. 2010).

Upregulated or constitutive activation of the MAPK signaling cascade correlates with poor clinical outcome (Houben et al. 2004), which is commonly attributed to activating mutations at different branch points along the signaling pathway. Thus, HRAS and KRAS mutations appear to correlate with benign Spitz nevi and primary lesions, whereas NRAS is most frequently mutated in primary and metastatic

melanoma and is characteristic of chronically sun-exposed lesions (Ball et al. 1994; Jafari et al. 1995; Jiveskog et al. 1998; Shukla et al. 1989; van Dijk et al. 2005; van Elsas et al. 1995). Immediately downstream of the RAS proteins are the RAF kinases, of which BRAF is the most frequently mutated (specifically $BRAF^{V600E}$) in melanoma. The more dominant oncogenic role of BRAF compared with ARAF and CRAF is most likely due to its higher kinase activity (Emuss et al. 2005; Lee et al. 2005). Although BRAF germline mutations have been reported, they are not common in familial melanoma (Lang et al. 2003), suggesting that BRAF mutations occur during melanoma development. Interestingly, $BRAF^{V600E}$ and NRAS mutations appear to be mutually exclusive in melanoma; a phenomenon that may be influenced by type and site of origin of the melanoma tumor. For example, BRAF mutation does not appear to correlate with the degree of sun exposure, as is the case for NRAS mutations (Davies et al. 2002). Interestingly, NRAS mutations induce a switch in the dominant usage of RAF isoforms from BRAF to CRAF (Dumaz et al. 2006).

Mutant BRAF is also found in congenital nevi and other non-malignant lesions, where it is associated with upregulation of senescence markers such as senescence-associated ß-galactosidase (SA-ß-gal) and mosaic p16INK4A induction (Michaloglou et al. 2005). Indeed, mutations of NRAS and BRAF (V600E) alone can promote cellular senescence *in vitro*, which not only illustrates oncogene-induced senescence but also highlights the need for other oncogenic events to drive tumor progression (see also Chaps. 4, 5, and 11). Nevertheless, ERK activity does not always correlate with BRAF activation, most likely due to variations in the functional status of ERK phosphatases. It has been proposed that some ERK phosphatases might be less active in malignant than non-malignant cells, although it is not yet clear whether and how ERK phosphatases might be deregulated in melanoma. In this regard, BRAF mutation alone is insufficient to transform melanocytes, and secondary mutations that confer uncontrolled cell cycle progression are required. The notion that BRAF acts cooperatively to induce melanoma development is supported by observations in zebrafish, where mutant BRAF promotes nevi development but additional oncogenic changes, such as loss of p53, can promote progression to invasive lesions (Patton et al. 2005).

Other secondary mutations that can support uncontrolled proliferation include p16INK4A and p19INK4D (see cell cycle section). Although it is unclear what role p16INK4A may play in BRAF-driven senescence, loss of p16INK4A can facilitate melanoma tumor formation driven by mutant RAS (Ackermann et al. 2005; Chin et al. 1997). Interestingly, cooperative stabilization of ß-catenin results in silencing of p16INK4A, and in combination with mutant NRAS, this is sufficient to promote melanoma progression (Delmas et al. 2007). Additional signaling mechanisms that contribute to oncogene-induced senescence and earlier barriers to melanoma progression continue to be identified. For example, early oncogene-induced activation of the ER stress-activated unfolded protein response was found to halt tumorigenesis independently of conventional senescence mechanisms (Denoyelle et al. 2006).

Although epigenetic perturbations that promote activation of these pathways remain largely obscure, the mutational status of melanoma tumors is known to correlate with some characteristic epigenetic profiles. For example, melanomas with BRAF mutations exhibit changes in several miRNAs, such as downregulation of

miR-193a, miR-338, and miR-565, and upregulation of miR-191 (Caramuta et al. 2010). A further level of complexity is suggested by the finding that pseudogene transcripts can act as false miRNA "decoy" targets (a.k.a., competitive endogenous RNAs), thereby absorbing and nullifying the function of miRNAs targeting specific endogenous transcripts (Chen 2010; Poliseno et al. 2010; Karreth et al. 2015). Such regulation has been demonstrated for PTEN and BRAF and may effectively alter the expression of other genes that are central to the control of melanoma development. As increasing effort is devoted to sequencing the melanoma genome, a wealth of pseudogenes contributing to different stages of melanoma biology are expected to be identified (Pleasance et al. 2010).

7.2.3 PTEN–PI3K–AKT

The phosphatidylinositol-3-kinase–AKT (PI3K–AKT) pathway is also frequently deregulated in melanoma (Inoue-Narita et al. 2008; Robertson 2005), although the mechanisms underlying the deregulation of many components remain largely elusive. Like the MAPK pathway, the PI3K pathway is an effector signaling cascade positioned downstream of the membrane receptors described earlier, including c-Met and IGFR1. PI3K converts phosphatidylinositol-4,5 bisphosphate (PIP2), located on the cytoplasmic face of the plasma membrane, into the secondary lipid signaling molecule, phosphatidylinositol-3,4,5 trisphosphate (PIP3). In turn, PIP3 activates the downstream effector AKT/protein kinase B. The three members of the AKT family of serine/threonine kinases (AKT1, AKT2, and AKT3) have well-characterized pro-survival functions (Datta et al. 1999; Madhunapantula and Robertson 2009) (Fig. 7.4), with AKT3 appearing to be the isoform most affected in melanoma.

One mechanism by which AKT signaling is enhanced is via deregulation of PIP2 processing by perturbation of inositol polyphosphate 4-phosphatase type II (Gewinner et al. 2009). However, upregulation of AKT activity in melanoma can largely be attributed to deregulation of its negative regulator, PTEN (Parmiter et al. 1988). Although loss of PTEN protein is prevalent in melanoma (Chudnovsky et al. 2005), deregulation by mutation accounts for only a small fraction of melanomas with deregulated PTEN. Accordingly, while PTEN is commonly mutated in melanoma cell lines, such genetic mutations are rare in actual tumor samples, particularly those of metastatic grade (Goel et al. 2006; Pollock et al. 2002; Wu et al. 2003). These observations indicate that downregulation or loss of PTEN in patient's tumors results from additional transcriptional and post-translational modifications, with the latter being the most common. Although not shown yet in melanoma, the ubiquitin ligase NEDD-4, which targets PTEN for destruction, is upregulated in numerous cancer types, including gastric and colorectal cancers (Kim et al. 2008b; Trotman et al. 2007; Wang et al. 2007a). Oncogenic deregulation of PTEN also occurs via Fyn-related kinase (FRK, previously known as RAK), which is overexpressed in melanoma and numerous other cancers. FRK phosphorylates PTEN, thereby abrogating its interaction with NEDD-4 and increasing its availability (Brauer and Tyner 2009; Yim et al. 2009).

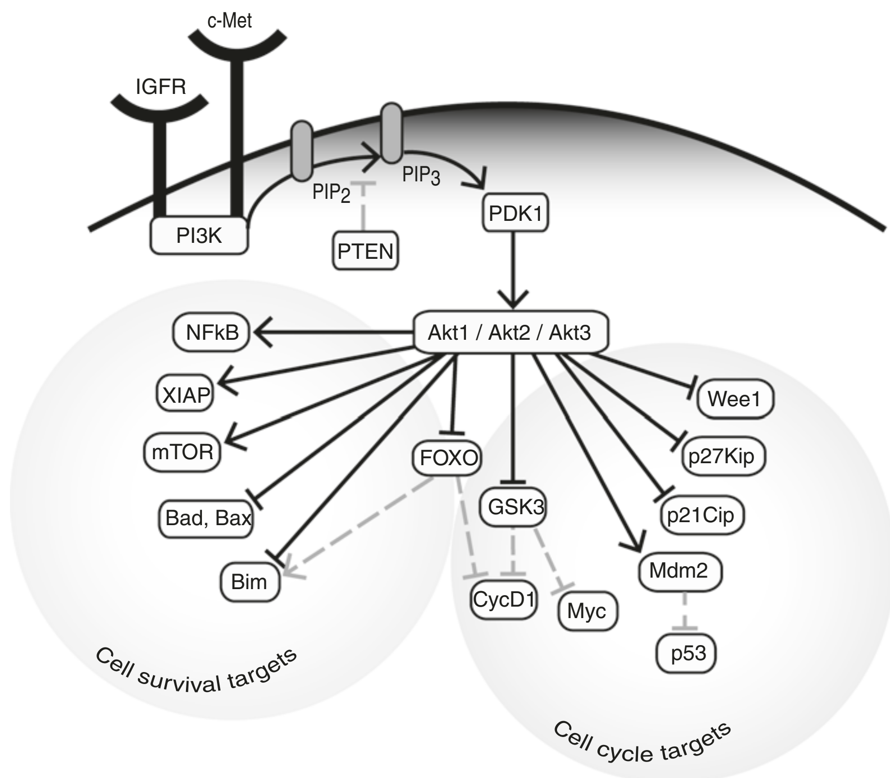

Fig. 7.4 AKT signaling. Various membrane-bound growth factor receptors (c-Met and IGFR) signal inwards via PI3K, which phosphorylates PIP_2 to produce the secondary messenger molecule PIP_3. PIP_3 then functions to activate PDK1, which in turn activates AKT family kinases (*AKT1*, *AKT2*, and *AKT3*). AKT is a widely recognized pro-survival effector kinase that acts by upregulating or activating numerous cell survival-related proteins, such as NF-kB, XIAP, and mTOR, and inhibiting cell death-related proteins such as Bad, Bax, and Bim (see also Chap. 10). AKT also inhibits transcription factors, such as FOXO, which contribute to cell death or cell cycle arrest. AKT promotes cell cycle progression by inhibiting cell cycle inhibitors, including Wee1, p21Kip, p21Cip, and p53 (via activation of *Mdm2*), and relieving Cyclin D1 and Myc from suppression by GSK3. Branches of the AKT pathway that are upregulated in melanoma appear in *black*; downregulated branches appear in *dashed gray*

Other forms of epigenetic silencing of PTEN include promoter methylation, which is observed in up to 62% of patients with metastatic melanoma (Mirmohammadsadegh et al. 2006). In effect, loss of PTEN promotes an excess of PIP3 and activation of AKT and its downstream targets, resulting in increased growth and survival of melanoma. Notably, activated AKT which is affected by deregulated PTEN is inversely correlated with a positive clinical outcome for melanoma patients (Dai et al. 2005).

In addition to loss of PTEN, direct changes to AKT family members can modulate the PI3K pathway in melanoma. Of the three AKT isoforms, AKT3 is specifically and significantly upregulated in sporadic melanoma tumors, particularly those

of metastatic grade (Robertson 2005; Stahl et al. 2004). Although AKT3 upregulation has mainly been attributed directly to an increase in genomic copy number, a recent report has identified a novel activating mutation of AKT3 (E17K) in some melanoma cases (Davies et al. 2008). Targeted siRNA-mediated silencing of AKT3 is sufficient to suppress melanoma progression and induce cell death, emphasizing the oncogenic potential of deregulated AKT3 activation.

AKT signaling affects numerous cellular process: it influences cell cycle dynamics through regulation of the G1/S phase regulator Cyclin D3 (Spofford et al. 2006); affects cell growth, metabolism, and proliferation via control of VEGF expression and interplay with mTOR and the TORC1 and TORC2 complexes (Bhaskar and Hay 2007; Govindarajan et al. 2007; Levine et al. 2006); contributes to invasive behavior by NF-kB-mediated regulation of matrix metalloproteinase-2 and -9 (Kim et al. 2001); and suppresses apoptosis by inhibiting the expression of pro-apoptotic proteins such as Bad and caspase-9 (Cardone et al. 1998; Datta et al. 1997). Each of these AKT effects can be attenuated or suppressed by the antagonistic function of PTEN. Importantly, deregulation of the ERK–c-Jun signaling axis in melanoma leads to c-Jun-mediated transcriptional upregulation of PDK1, further enhancing AKT activation (Lopez-Bergami et al. 2010). PDK1 has also been demonstrated to make critical AKT-independent contributions to tumorigenesis via activation of its substrate SGK3/CISK; this has been shown for breast cancer, among others (Vasudevan et al. 2009).

Recent studies using genetic melanoma models have substantiated the role of PDK1 in the development and progression of melanoma. Thus, melanocyte-specific inactivation of PDK1 in the Braf^{V600E}::Pten$^{-/-}$ mouse model delayed the formation of tumors and largely abolished the metastatic lesions commonly seen in this model. Consistent with these findings, examination of melanoma tissue microarrays revealed upregulation of PDK1 in primary melanomas compared with nevi (Scortegagna et al. 2014). Further dissection of the pathways underlying the PDK1 effects on melanoma development identified a role for the PDK1 substrate SGK3. Indeed, inhibition of SGK3 partially phenocopied the changes seen upon PDK1 inhibition. Interestingly, a synthetic lethal screen for kinases that may synergize with PDK1 in eliciting these effects identified PI3K, suggesting that concerted inhibition of the PI3K–PDK1 axis alone may suffice to inhibit growth of BRAF-mutant melanomas (Scortegagna et al. 2014, 2015).

7.3 Cell Cycle Regulation

Malignant melanoma cells are highly proliferative and often exhibit genomic instability (Hazan et al. 2002; Henrique et al. 2000; Satoh et al. 2000; Soyer 1991; Steinbeck et al. 1996; Urso et al. 1992). Such an aggressive proliferative state results from the specific expansion of transformed cells with imbalanced signal transduction favoring proliferation while deregulating normal replicative senescence and apoptotic signaling (Bennett 2008). Accordingly, the stringent cell cycle regulatory mechanisms that govern cell proliferation in normal skin tissues are frequently impaired during melanoma development. For instance, proper function of the G1/S checkpoint that restricts cell cycle progression is often lost in melanoma (Sauroja

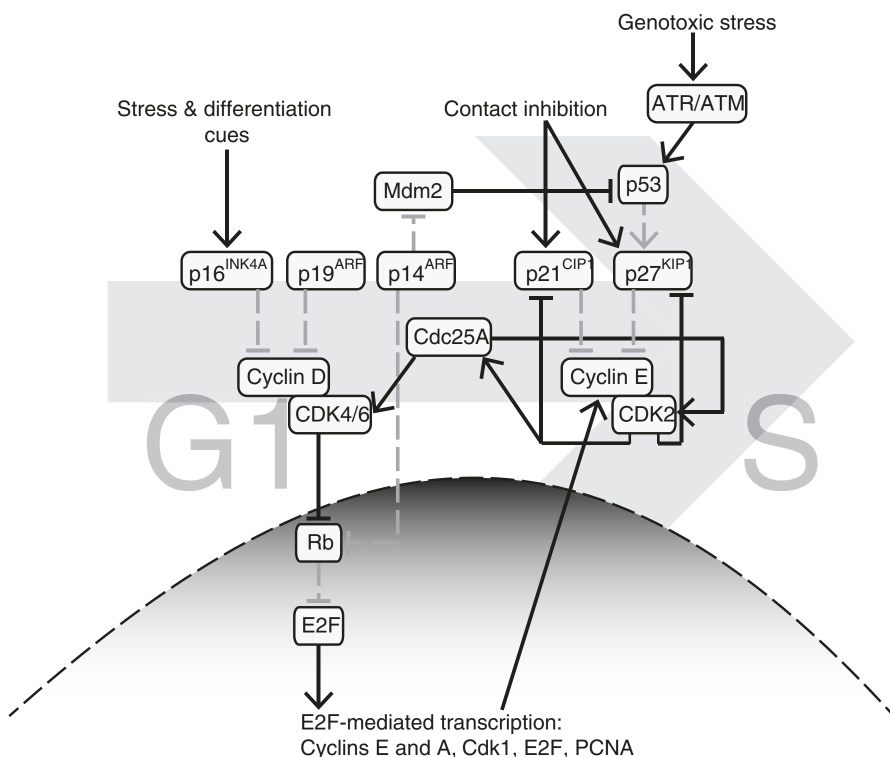

Fig. 7.5 Cell cycle control. In melanoma, loss of CDK2NA compromises major branch points in the regulatory pathways governing G1/S progression. Loss of p16INK4A, p19ARF, and p14ARF causes deregulation of Cyclin D/CDK4/6 and subsequent deregulation of Rb and p53 function, thereby promoting enhanced E2F transcriptional activity and cell cycle progression. Loss of function of other cell cycle inhibitors, such as p21CIP1 and p27KIP1, results in enhanced Cyclin E/CDK2 activity, which positively feeds back on itself and on CDK4/6 via CDC25. Branches of the cell cycle regulation that are upregulated in melanoma appear in *black*; downregulated branches appear in *dashed gray*

et al. 2000). Similar perturbations in signaling can be traced to specific familial mutations or epigenetic dysregulation that result in the downregulation of tumor suppressor genes that negatively regulate the cell cycle or the upregulation of oncogenic genes that promote cell cycle progression.

Patients afflicted with familial melanoma commonly exhibit conserved mutations in 9p21, a chromosomal locus associated with deregulation of cell cycle control. The 9p21 locus comprises the CDK2NA gene, which encodes p16INK4A and its alternate reading frames p14ARF and p19ARF. These proteins negatively regulate cell cycle progression and contribute to senescence through their control of cell cycle-promoting proteins such as Cyclin D and E and the transcription factor E2F1 (Fig. 7.5) (Bandyopadhyay and Medrano 2000; Ranade et al. 1995). Furthermore, p16INK4A binds to and inhibits the cell cycle-promoting kinase CDK4, with concomitant effects

on Rb and its control of E2F in the cell cycle. Germline mutations in CDK2NA have been reported in melanoma (Koh et al. 1995; Ranade et al. 1995) and result in amplified CDK4-mediated signaling, thereby perturbing normal cell cycle control. The increased CDK4 activity observed in melanoma can also result from mutations in the CDK4 gene (Chudnovsky et al. 2005). These perturbations impair proper cell cycle control and appropriate entry of melanocytes into senescence (Bandyopadhyay and Medrano 2000; Haferkamp et al. 2008; Rane et al. 2002).

Mutational perturbation of the alternate reading frame gene product p14ARF also promotes cellular proliferation. Indeed, mutation of ARF has been demonstrated to synergize with RAS mutations in promoting melanoma tumor development (Ha et al. 2007). p14ARF normally contributes to p53 function by targeting and suppressing the p53 negative regulator, Mdm2. Thus, although mutations in p53 are relatively rare in melanoma, its activity can be downregulated by the increased Mdm2 levels induced by mutational silencing of ARF (Freedberg et al. 2008). Of note, there have been few reports on upregulation of Mdm2 or downregulation of p53 expression or activity in melanoma (Bardeesy et al. 2001) (see also Chap. 4). Hence, the precise contribution of p53 to melanoma development remains unclear. Among the possibilities currently being explored is that p53 is partially inactivated, impairing its ability to control cell cycle arrest or apoptotic cues in melanoma.

Enhanced proliferation of melanoma cells can also be elicited by alterations in other negative regulators of cell cycle progression, including Rb. Notably, Rb is silenced in melanoma (Yang et al. 2005) as a result of nonsense mutations or of inactivating phosphorylation of the translated protein (Bartkova et al. 1996; Brantley and Harbour 2000). Loss of Rb function can also contribute to abrogation of melanocyte senescence (Haferkamp et al. 2008).

In addition to genetic mutations, alterations in the epigenetic regulation of core cell cycle and proliferation genes also contribute to melanoma development and progression. Direct modification of chromatin structure, such as by aberrant promoter hypermethylation, results in the CDK2NA silencing reported in multiple melanoma types (Straume et al. 2002; van der Velden et al. 2001). CDKN2A silencing can also be achieved via upregulation of repressor proteins. One example in melanoma is overexpression of the CDKN2A transcriptional repressor Id1 (Healey et al. 2010). Suppression of p16INK4A in melanoma is also mediated by ß-catenin (Delmas et al. 2007). The histone methyltransferase EZH2 has been implicated in the epigenetic repression of the CDK2NA locus and is upregulated in melanoma via a non-canonical NF-kB pathway. Inhibition of this pathway promotes senescence by inducing re-expression of p16INK and p21 (De Donatis et al. 2016).

Recent studies have highlighted the role played by miRNAs in the epigenetic control of melanoma progression (Jukic et al. 2010). Several miRNAs that suppress proliferation are downregulated during melanoma progression, including miR-let-7 and miR-34a. miR-let-7 targets numerous cell cycle proteins, including Cyclin D1/D3/A, and is expressed at lower levels in melanoma compared with nevi (Schultz et al. 2008). Expression of miR-34a, a transcriptional target of p53, is sufficient to induce G1 arrest/senescence and can act as a tumor suppressor by targeting c-Met. However, in melanoma, miR-34a is silenced by aberrant CpG promoter methylation

(Lodygin et al. 2008), which derepresses the cell cycle proteins Rb, CDC2, and E2F3, among others (Satzger et al. 2010; Yan et al. 2009). Deregulation of other miRNAs, including miR-210 and miR-15b, have been demonstrated to promote melanoma tumorigenesis (Satzger et al. 2010; Zhang et al. 2009) (see also the section on miRNAs and melanoma).

7.4 Therapeutic Targets

The MAPK–MEK–ERK and PTEN–P13K–AKT pathways are recognized to be critical determinants of melanoma development and progression, and an intensive effort is under way to develop inhibitors of components of these pathways (Madhunapantula and Robertson 2009; Meier et al. 2005; Russo et al. 2009). A series of specific inhibitors of $BRAF^{V600E}$ showed impressive results in early clinical trials (Kumar et al. 2004). However, the initial success was tempered by the high incidence of therapy-resistant tumors, limiting the effectiveness of these specific drugs (Flaherty et al. 2010). In recent years, extensive work on the molecular basis for this resistance has pointed to diverse mechanisms, most of which cause amplification of the downstream MAPK signaling pathway and bypass the effects of the BRAFi. One emerging approach to overcome resistance is the use of immunotherapeutic drugs that do not directly target the tumor per se, but instead unleash the anti-tumor immune response. Among these therapies are antibodies to CTLA-4 and PD-1, which overcome the drug-resistant tumor phenotype by blocking inhibitory immune checkpoints. These agents have recently been assessed as first-line therapy or as combination therapies with selective BRAFi or MEKi (Larkin et al. 2015; Menzies and Long 2013).

7.4.1 Overcoming Resistance to Targeted Therapies

To date, the use of single agents as first-line therapy has demonstrated only limited clinical efficacy. This disappointing outcome has been attributed to the unexpected plasticity of tumors, as reflected by their ability to adapt to harsh growth conditions and become resistant to initially effective drugs. The mechanisms for achieving resistance largely center on signal transduction pathways that have been rewired, either by genetic mutation or alteration in epigenetic control (Smalley et al. 2009; Emery et al. 2009).

Although BRAF inhibitors suppress tumor growth, the effect is transient, and the tumor cells eventually develop one or more “salvage” mechanisms that bypass BRAF or MEK inhibition. Such mechanisms include upregulation of activated CRAF activity (Gollob et al. 2006; Hatzivassiliou et al. 2010; Kaplan et al. 2011; Montagut et al. 2008; Paraiso et al. 2010; Tsai et al. 2008; Wellbrock and Hurlstone 2010); mutation of NRAS, which leads to CRAF activation and bypasses BRAF inhibition (Nazarian et al. 2010); upregulation of COT, which activates ERK in a MEK-dependent and RAF-independent manner (Johannessen et al. 2010); and upregulation of RTKs (Nazarian et al. 2010) (see section 7.1.1.2 on RTKs).

Melanomas may also develop resistance to BRAF-targeting therapies via upregulation of AKT3-dependent mechanisms (Shao and Aplin 2010), which is consistent with the reported cooperation between mutant BRAF and active AKT (Dankort et al. 2009). Additionally, resistance to BRAFi can be achieved by alternative splicing of BRAF (Wellbrock et al. 2004), which results in a protein lacking the RAS-binding domain due to a silent mutation in intron 8 (Salton et al. 2015). This BRAF isoform dimerizes even in the presence of low levels of RAS, conferring drug resistance through reactivation of the ERK pathway.

Combined targeting of MEK and BRAF results in additive and synergistic effects on progression-free survival of melanoma patients, with a 67 % response rate and 93 % overall survival at 6 months (Flaherty et al. 2012a; Flaherty et al. 2012b). Nevertheless, resistance to MEKi emerges through the same mechanisms seen in BRAFi resistance, including increased CRAF activity and mutation of both NRAS and MEK1 (Greger et al. 2012).

Most tumor resistance mechanisms result in increased activity of the translation initiation complex, which supports the translation of cancer-driving genes such as oncogenes and cell cycle/DNA damage response genes. Hence, partial disruption of the eIF4F complex is a potential therapeutic strategy for drug-resistant tumors, including melanoma. In support of this approach, recent studies have demonstrated efficacy in overcoming melanoma resistance to therapy by targeting eIF4F with silvestrol and several flavaglines (Boussemart et al. 2014), and by targeting a key component of the eIF4F complex, eIF4G1, with the small molecule SBI-756 (Feng et al. 2015).

7.4.2 Immunotherapy

A great deal of effort has been devoted to the use of immune-based therapies to overcome drug resistance in melanoma (Hu-Lieskovan et al. 2014; Vanneman and Dranoff 2012), but the success rate has been low and pronounced toxicity has been observed in most cases. The notion that the immune system could be manipulated to enable a global attack on tumors was initially met with skepticism, largely due to fears that uncontrolled activation would lead to autoimmunity. However, the pioneering work of Drs. Allison and Honjo introduced the immune checkpoint molecules, CTLA-4 and PD-1, respectively, as new paradigms for cancer immunotherapy (Leach et al. 1996; Okazaki et al. 2013; Peggs et al. 2006). Targeting of CTLA-4 circumvents downregulation of T-cell proliferation, whereas PD-1 blockade is likely to affect both activation of T cells and the direct anti-tumor activity of effector T cells.

Clinical trials with anti-CTLA-4 antibodies have shown unexpected success, with an overall response rate of about 20 %, albeit with notable toxicity (Attia et al. 2005; Hodi et al. 2010). Clinical trials with anti-PD-1 antibodies have achieved greater response rates (30–40 %) and significant increases in patient survival (Topalian et al. 2014). More recently, combination therapy with anti-CTLA-4 and anti-PD-1 achieved about 60 % response rate and 79 % 2-year survival rate (Topalian

et al. 2014). Other ongoing work includes the evaluation of combination therapies of immune checkpoint blockers with BRAFi or MEKi, which have shown promising results in pre-clinical experiments (Hu-Lieskovan et al. 2015).

7.5 Epilogue

Our understanding of the mechanisms underlying the development, progression, and drug resistance of melanoma has increased significantly in recent years. At the same time, we have come to appreciate that a major obstacle to achieving sustained therapeutic responses is the innate plasticity of tumor cells, which allows them to adapt to harsh conditions, withstand therapy, and acquire metastatic ability. This means that the rewired signaling observed in tumor cells could be further changed by the tumor microenvironment or by stress imposed by the chemotherapeutic drugs. Thus, we must divert from our current approach to cancer therapy—more intense targeting of a mutated pathway—to find new therapeutic modalities. These include fine-tuning the immune checkpoint machinery to enable a concerted immune attack on the tumor, and targeting the central mechanisms that provide a global advantage to the tumors. Among the latter mechanisms are the translation initiation complex and the unfolded protein response, which are cardinal nodes for tumor-driving genes and may offer a more global approach to targeting the plastic tumor. These molecular hubs have already garnered attention and we may expect an exciting new cadre of modulators to reach clinical evaluation in the coming years.

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