Chapter 7
Targeting the Cell Cycle and p53 in Combination with *BRAF*-Directed Therapy

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Abstract The incidence of melanoma continues to increase with the vast majority of skin cancer-related deaths attributable to melanoma. Historically, response rates for systemic treatments for metastatic melanoma were only 5–20% for chemotherapy, and the prognosis of patients with metastatic disease was extremely poor. The discovery of *BRAF* mutations in melanoma led to the development of *BRAF*-directed therapy which dramatically increased response rates. However, most patients treated with *BRAF* inhibitors eventually show disease recurrence, and it is now believed that combination therapies based upon a *BRAF* inhibitor backbone is the therapeutic future. Appropriate regulation of the cell cycle is critical for preventing progression to cancer, however the vast majority of melanomas harbor alterations in cell cycle and p53 regulatory pathways such as loss of CDKN2A and overexpression of CDK4, cyclin D1, MDM2 and MDM4. The alterations in these pathways appear to play critical roles in the development of melanoma and may represent potential therapeutic targets. Furthermore, some studies suggest that there is interaction between *BRAF*, key cell cycle proteins and the p53 pathway and that *BRAF* inhibitors may synergize with treatments that either enhance p53 function or inhibit CDK activity. Preclinical studies in melanoma have shown the potential efficacy of enhancing p53 function through inhibition of MDM2 or MDM4. Other studies have shown potential benefit in antagonizing CDK activity through use of small molecule inhibitors. However, targeting p53 and CDK function in melanoma is at an early stage and additional studies are needed particularly to understand the effects of combining these therapies with *BRAF* inhibition. Furthermore, clinical trials testing these therapeutic combinations specifically in melanoma patients are also needed to determine if the results of preclinical studies can be translated into beneficial effects in humans.

Keywords Melanoma · *BRAF0* · Cell cycle · Cyclin dependent kinase · p53 · MDM2 · MDM4 · CDKN2A · p14 · p16

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7.1 Introduction

The incidence of melanoma continues to increase at a rate of 2–5 % per year with an estimated 76,250 new cases diagnosed in 2012 [120]. The magnitude of this disease is further exacerbated by the fact that although melanoma represents 5 % of skin cancers, it is the cause of 80 % of skin cancer-related deaths [93]. The majority (70 %) of newly diagnosed patients present with thin melanoma (≤ 1 mm) and the prognosis for patients with only local disease is excellent [87, 46, 7]. However, approximately 4 % of patients present with distant metastatic melanoma and a subset of patients with localized melanoma eventually develop systemic metastases and have significantly worsened survival [7, 32].

In the past, systemic treatments for distant metastatic melanoma were generally ineffective with response rates of 5–20 % for chemotherapy, and the prognosis of patients with distant disease is dismal with median survival rates of less than 1 year [48, 7]. However, in 2011 a turning point was achieved in the fight against metastatic melanoma with the FDA approval of both anti-CTLA-4 antibody immunotherapy and *BRAF* targeted therapy. In patients treated with the anti-CTLA-4 antibody ipilimumab, an objective response was seen in 10.9 % of patients and median overall survival was significantly increased to 10 months [59]. Even more impressive were the results seen with targeted therapy using vemurafenib in *BRAF* mutated melanoma. In the pivotal phase III trial, 48 % of treated patients with metastatic melanoma had an objective response as determined in an interim analysis, although the vast majority of patients had some decrease in tumor size, and the median progression-free survival was significantly extended to 5.3 months compared with 1.6 months for patients treated with dacarbazine [26]. Despite the impressive results and dramatic response rates seen after vemurafenib therapy, nearly all of these patients eventually developed recurrent disease. The mechanisms by which melanomas acquire resistance to *BRAF* inhibitors is an active area of research, and it is now apparent that combination therapy based upon a *BRAF* inhibitor backbone is the therapeutic future for disseminated disease [124, 127, 122, 123]. At this juncture, the cellular pathways that need to be targeted in conjunction with mutant *BRAF* are still being determined.

Acquisition of a *BRAF* mutation is believed to be an early event in melanoma development as evidenced by the fact that over 80 % of nevi harbor a *BRAF* mutation [104, 75]. As a single hit, oncogenic *BRAF* drives melanocytes into senescence and it is known that additional genetic insults are required for melanomagenesis. Among the hits identified so far that contribute to melanoma development are alterations in regulatory pathways for p53 and the cell cycle [28, 33; 16, 63, 135]. In this chapter, we will review the important alterations in the cell cycle and p53 regulatory pathways implicated in melanoma initiation and progression and will discuss the potential for targeting these alterations in combination with oncogenic *BRAF*.

7.2 Cell Cycle

Cells divide through a systematic and precisely regulated process with the ultimate goal of producing viable daughter cells that each possesses a set of faithfully duplicated chromosomes (Fig. 7.1). The majority of cells exist in G0 phase of the cell cycle, which is also known as quiescence or senescence. In the quiescent state, cells no longer replicate but have the potential to re-enter the cell cycle, whereas senescence refers to a cellular response to various types of stress (e.g. DNA damage, oncogene activation, oxidative stress, etc.) in which a cell is primarily arrested in G1 phase and has irreversibly lost the capability to replicate [48, 4]. The ability of cells to enter senescence in response to oncogene activation is believed to be a potential barrier to tumorigenesis [4]. Upon receiving mitogenic signals, a cell leaves G0 phase and enters G1 phase in which there is growth in preparation for S phase. In S phase, DNA is replicated with high fidelity, and is followed by G2 phase where cells continue to grow and make final preparations for M phase where mitosis and later cytokinesis occur. Depending on the cellular and signaling milieu, cells may either return to G1 phase to continue dividing or enter G0 phase [49, 83, 112].

7.2.1 Regulation of the Cell Cycle: Cyclin Dependent Kinases

The cell cycle is tightly regulated by a series of serine/threonine kinases known as cyclin-dependent kinases (CDKs) that form heterodimers with regulatory cyclins [83, 112]. According to the "classical" model, each phase of the cell cycle is controlled by the cyclic expression and activation of specific cyclins and CDKs

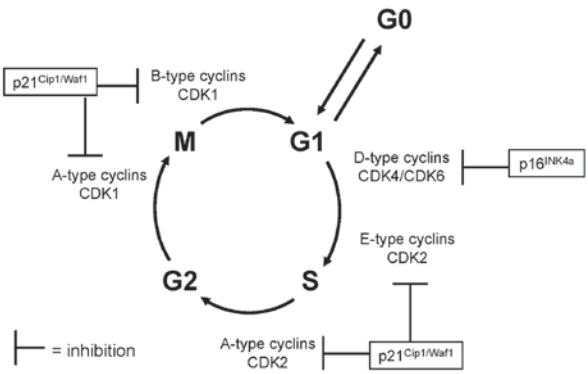

Fig. 7.1 Phases of the cell cycle. *G0* represents quiescent or senescent cells. Upon receiving mitogenic signals, cells enter *G1* phase and proceed through *S*, *G2* and *M* phases. Cells may then either re-enter *G1* phase to continue dividing or enter *G0* phase. Based on the "classical" model of the cell cycle, each phase is controlled by the expression of specific cyclins and cyclin-dependent kinases. In addition, cyclin-dependent kinase inhibitors such as $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ play important roles in helping to regulate the cell cycle. *CDK* cyclin-dependent kinase

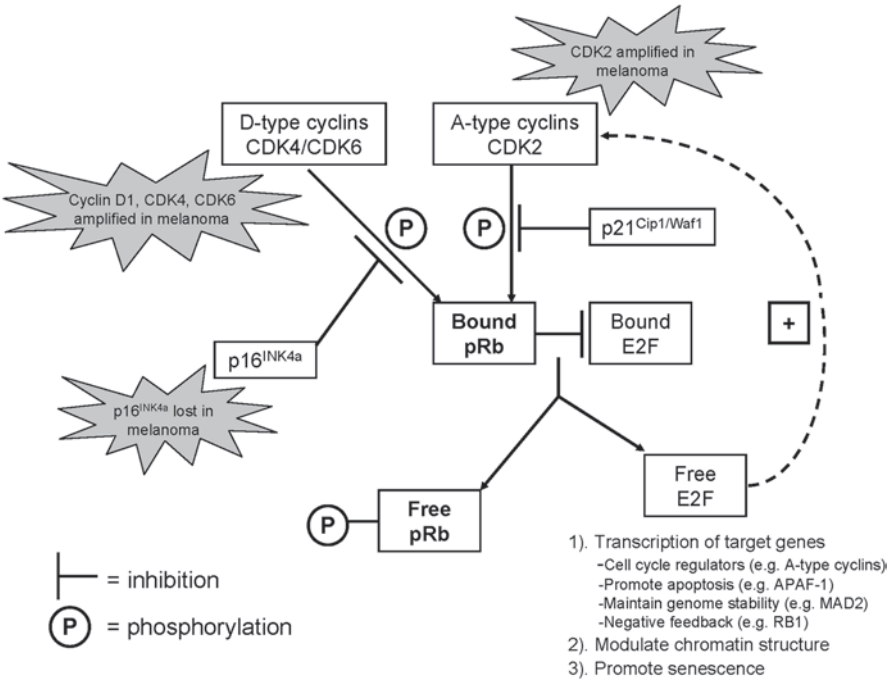

Fig. 7.2 The retinoblastoma protein (*pRb*) pathway. The transcription factor *E2F* is normally bound to and repressed by *pRb*. *E2F* plays a critical role in controlling the transcription of numerous genes involved in cell cycle regulation, apoptosis and maintaining genome stability. In addition, *E2F* is also involved in regulating chromatin structure and in promoting senescence. *CDK4* and *CDK6* that have been activated by *D-type cyclins* phosphorylate *pRb* which causes the release of *E2F* and transcription of *E2F* target genes. A positive feedback loop exists where *E2F*-mediated transcription leads to increased levels of *A-type cyclins* and eventual activation of *CDK2*. Activated *CDK2* then further phosphorylates *pRb* leading to release of additional *E2F* and passage through the "restriction point" of the cell cycle. In contrast, a negative feedback loop also exists where *E2F* activation leads to increased *pRb* levels, via transcription of the *RB1* gene, and sequestration of *E2F*. *CDK* inhibitors $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ play pivotal roles in regulating the *pRb* pathway by inhibiting *CDK4/CDK6* and cyclin-*CDK2/CDK1* complexes, respectively. In melanoma, prominent alterations in the *pRb* pathway are seen and include loss of $p16^{INK4a}$ and amplification of *cyclin D1*, *CDK2*, *CDK4* and *CDK6*. *CDK* cyclin-dependent kinase, *P* phosphorylation, *pRb* retinoblastoma protein

(Fig. 7.1). In response to mitogenic signals, D-type cyclins are expressed in early G1 phase and activate CDK4 and CDK6. Activated CDK4 and CDK6 then phosphorylate retinoblastoma protein (pRb) causing the release of transcription factor E2F, which is normally bound to and repressed by pRb (Fig. 7.2). This allows E2F to proceed with transcription of target genes including E-type and A-type cyclins. Expression of E-type cyclins during G1 phase activates CDK2, which then further phosphorylates pRb leading to amplification of E2F-mediated transcription. These steps ultimately result in G1 to S phase transition and passage through the "restriction point" at which point the cell has committed to cellular division. During S

phase, CDK2 associates with A-type cyclins to allow for progression from S to G2 phase. Eventually, CDK1 binds to A-type cyclins to initiate mitosis (G2 to M phase). A-type cyclins are degraded during mitosis and CDK1 then binds to B-type cyclins to complete mitosis.

7.2.2 Regulation of the Cell Cycle: Cyclin-Dependent Kinase Inhibitors

In addition to its regulation by cyclins, CDK activity is also regulated by two families of specific CDK inhibitors [22, 83, 112, 144]. The first family consists of the INK4 proteins ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$) that inhibit CDK4 and CDK6 during G1 phase and therefore primarily affect the pRb pathway [22]. In addition to its role in promoting cell cycle arrest, $p16^{INK4a}$ has also been associated with cellular aging and senescence particularly in melanocytes, however the exact role of $p16^{INK4a}$ in promoting cellular senescence is still debated [91, 53, 22, 46, 121]. The second family consists of the Cip/Kip family of proteins ($p21^{Cip1/Waf1}$, $p27^{Kip1}$, $p57^{Kip2}$) which inhibit CDK2 and CDK1 when complexed with E-type, A-type and/or B-type cyclins [144]. Inhibition of CDK2 leads to decreased pRb phosphorylation and sequestration of E2F. In addition, $p21^{Cip1/Waf1}$ further antagonizes pRb function by promoting proteosomal degradation of pRb [17]. Of note, levels of $p21^{Cip1/Waf1}$ are under the transcriptional control of activated p53 that utilizes $p21^{Cip1/Waf1}$ to arrest the cell cycle and to activate senescence pathways [140, 121, 94].

7.2.3 Regulation of the Cell Cycle: Retinoblastoma Protein Pathway

The retinoblastoma gene family consists of three members and encodes for the proteins pRb, p107 and p130 [56, 20, 29, 51]. Of these three proteins, pRb (encoded by the RB1 gene) has been extensively studied due to its key role in regulating the cell cycle and in functioning as a tumor suppressor gene. It is a 928 amino acid protein that consists of tandem cyclin fold regions separated by spacers and a C-terminal domain. These domains form a "pocket" which is the basis of pRb function. Targets that interact with the pRb pocket include E2F transcription factors and regulators of pRb, such as CDK-cyclin complexes. The affinity of the binding pocket is regulated by post-translational modifications, most commonly phosphorylation of serine and threonine residues in N-terminal and C-terminal domains and in spacer regions, which alter the conformation of the pocket and the binding affinity for specific targets.

CDK inhibitors such as $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ also play critical roles in regulating pRb function by directly inhibiting CDK4/CDK6 or inhibiting cyclin-CDK2/CDK1 complexes, respectively (Fig. 7.2) [56, 22, 20, 1, 29, 51, 144]. Feedback loops exist that also regulate pRb function [29]. Phosphorylation of pRb releases

E2F and allows for transcription of E-type and A-type cyclins that leads to further phosphorylation of pRb via CDK2. This positive feedback loop allows the cell to progress through the "restriction point" of the cell cycle. However, E2F that has been freed of pRb repression also initiates a negative feedback loop by promoting RB1 gene transcription. This results in an increase in pRb levels, sequestration of E2F and concomitant downregulation of E2F. Epigenetic signaling may also play a role in regulating pRb activity specifically through promoter hypermethylation and silencing of the RB1 gene [51].

Despite the key role played by pRb in regulating the G1 phase of the cell cycle, it is interesting to note that control of cell cycle arrest requires cooperation between pRb and p53 as shown by the fact that RB null mouse embryonic fibroblasts still transition from G1 to S phase but arrest in G2 phase under conditions of serum starvation due to upregulation of $p21^{Cip1/Waf1}$ via p53 [42]. Other studies have shown that combined heterozygous loss of pRb and p53 result in the development of a wider range of tumors compared with mice with heterozygous pRb loss alone [145]. Small cell lung cancer (SCLC) is induced in the lung epithelium of mice deficient in pRb and p53, while mice with CDKN2A loss and functional inactivation of pRb and p53 via loss of $p16^{INK4a}$ and $p19^{ARF}$ (mouse homologue of $p14^{ARF}$ in humans) respectively, develop sarcomas and lymphomas [115, 90]. Another study showed that over 75 % of melanoma cell lines had defects in both the p53 and pRb pathways [146]. These results highlight the importance of the interaction between the pRb and p53 pathways and the potential role of pRb in tumor initiation and the critical role played by p53 in acting as a failsafe cell cycle checkpoint [79, 82].

In addition to regulation of the cell cycle, pRb plays a role in several other related cellular functions. pRb is known to bind factors that regulate chromatin structure such as DNA methyltransferases, histone methyltransferases, histone demethylases and histone deacetylases [29, 51]. Through chromatin modification and interaction with E2F, pRb plays a fundamental role in regulating the transcription of an array of genes. In addition, pRb may play a role in promoting cellular senescence, through chromatin remodeling and formation of senescence associated heterochromatin foci, and in regulating apoptosis through E2F-1 which can transcribe genes necessary for apoptosis such as APAF-1 [29, 51, 121]. Another essential role of pRb that has been recently brought to light is its ability to help maintain genomic stability and to prevent aneuploidy [85]. Loss of pRb is associated with accumulation of DNA damage and with defects in the mitotic spindle, kinetochores and centrosomes. It is believed that dysregulation of E2F and its target genes such as MAD2 may help explain some of these mitotic defects.

7.3 p53 Pathway

Progression through the cell cycle is very tightly controlled with regulation mediated through a number of important checkpoints. Specifically, these checkpoints ensure that a cell is ready for the subsequent phases of the cell cycle by preventing the

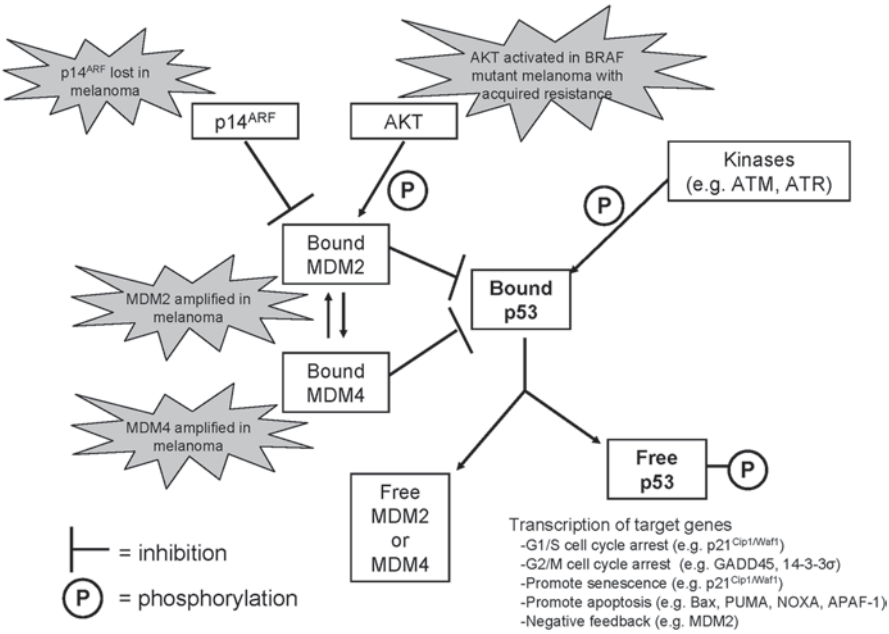

Fig. 7.3 The *p53* pathway. The cell cycle checkpoint protein *p53* mediates the transcription of a wide array of genes that are involved in senescence ($p21^{Cip1/Waf1}$), apoptosis (*Bax, PUMA, NOXA* and *APAF-1*) and cell cycle arrest, both at *G1/S* and at *G2/M* via the actions of $p21^{Cip1/Waf1}$, *GADD45* and *14-3-3σ*. A negative feedback loop also exists where activated *p53* induces the transcription of its negative regulator *MDM2*. Regulation of *p53* involves several proteins. Normally, *p53* levels are kept low through binding to *MDM2*. In addition, *MDM4* can also bind either to *p53* and directly inhibit the activity of *p53* or to *MDM2* and modify the inhibitory effects of *MDM2* on *p53*. In response to cellular stresses, kinases such as *ATM* or *ATR* phosphorylate *p53* and activate *p53* through release of *MDM2* or *MDM4*. Furthermore, *MDM2* activity is regulated by $p14^{ARF}$ and by other kinases such as *AKT*. Binding of $p14^{ARF}$ to *MDM2* results in decreased binding of *MDM2* with *p53* and ultimately in *p53* activation. In contrast, *AKT* phosphorylates *MDM2* leading to increased binding of *MDM2* with *p53* and downregulation of *p53* activity. In *melanoma*, prominent alterations in the *p53* pathway are seen and include loss of $p14^{ARF}$ and amplification of *MDM2* and *MDM4*. Furthermore, activation of AKT is also seen in *BRAF* mutant melanoma that has acquired resistance to *BRAF*-directed therapy. *CDK* cyclin-dependent kinase, *P* phosphorylation

propagation of DNA damage. One of the most recognized and well-known checkpoint is the p53 pathway. In response to DNA damage and/or cellular stress, p53 can either arrest cells at the G1/S phase and G2/M phase transitions to allow time for repair or alternatively activate senescence or apoptotic pathways in response to irreversible damage.

The TP53 gene encodes for the 393 amino acid protein known as p53 [132, 140, 94]. Structurally, p53 consists of an N-terminal transactivation domain, followed by a proline-rich domain, a DNA binding domain, which binds to sequence-specific p53 response elements, a tetramerization domain and a C-terminal domain. The activity of p53 is regulated through several types of post-translational modifications such as phosphorylation of serine and threonine residues in the N-terminal

transactivation domain and acetylation, methylation, ubiquitination, neddylation and sumoylation of C-terminal lysine residues.

In addition, protein-protein interactions are critical in regulating p53 activity (Fig. 7.3). Several important p53 regulators include MDM2 (also known as HDM2 in humans), MDM4 (also known as MDMX or HDMX in humans) and $p14^{ARF}$ [132]. MDM2 is part of the RING finger family of E3 ubiquitin-ligases. MDM2 binds to p53 and functions to inhibit the transcriptional activity of p53, to export p53 to the cytoplasm and to target p53 for proteosomal degradation through the action of an E2 ubiquitin-conjugating enzyme which is bound to the C-terminal RING finger domain of MDM2 [86, 61]. The importance of MDM2 in normal cellular function is highlighted by the fact that MDM2 knockouts are lethal in mouse embryos [97]. Normally, p53 is kept at low levels through binding to MDM2. However, cellular stress induces kinases to phosphorylate p53 at its N-terminal transactivation domain, leading to release of MDM2 and activation of p53. In contrast, $p14^{ARF}$ binds to MDM2 and prevents its binding to p53 with the net result of also increasing p53 activity. Other kinases, such as AKT, can upregulate MDM2 through phosphorylation, thereby decreasing p53 function [5]. There is also a negative feedback loop in which p53 promotes the transcription of MDM2, resulting in increased MDM2 levels and in downregulation of p53 [132].

Another negative regulator of p53 is MDM4. Although MDM4 and MDM2 are similar, MDM4 lacks ubiquitin-ligase activity [86, 84]. However, MDM4 does form heterodimers with MDM2 and is able to modify the ubiquitin-ligase activity of MDM2. MDM4 functions similarly to MDM2 and inhibits p53 activity by binding to the transactivation domain of p53. MDM4 null mice also die *in utero* but at a different time point from MDM2 knockout mice [101]. These results and additional studies suggest that the functions of MDM2 and MDM4 are not overlapping and are instead complementary [132]. It is believed that MDM2 primarily functions by degrading p53 while MDM4 inhibits p53 activity, however these hypotheses are still a matter of debate.

The activity of p53 is enhanced in response to various cellular stresses, such as DNA damage, hypoxia, metabolic stress, heat shock, and oncogene activation [132, 140]. This occurs due to increased stability and post-translational modifications of p53, ultimately leading to a cascade of potential responses such as cell-cycle arrest, DNA repair, apoptosis and senescence. Specifically in the setting of DNA damage, p53 halts progression of the cell cycle to either allow time for DNA repair or activate apoptotic or senescence pathways in the setting of irreparable damage. ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) kinases are activated in response to specific types of DNA damage and activate Chk2 and Chk1 kinases, respectively [94]. All four of these activated proteins can then phosphorylate and activate p53 leading to cell cycle arrest or apoptosis.

Many of p53's functions extend from its ability to regulate the expression of a wide array of genes. For G1 cell cycle arrest, p53 induces the expression of $p21^{Cip1/Waf1}$ resulting in inhibition of cyclin/CDK complexes and sequestration of E2F by pRb [1]. The induction of $p21^{Cip1/Waf1}$ also can stimulate senescence pathways leading to irreversible cell arrest [121, 144]. The product of other genes that are

regulated by p53 can induce a G2 arrest and include GADD45 and 14-3-3σ [57, 149]. In response to irreparable stress, p53 can also promote cell death through the induction of various proapoptotic factors such as Bax, PUMA, NOXA and APAF-1 [132, 140].

7.4 Abnormal p53 and Cell Cycle Regulation in Melanoma

Over the past several decades, it has become evident that most melanomas harbor alterations in regulatory pathways of the cell cycle and p53 and that these alterations play a prominent role in the development of melanoma.

7.4.1 Alterations in CDKN2A

The CDKN2A locus comprises 4 exons (1α, 1β, 2 and 3) and through alternative splicing creates two different proteins, $p16^{INK4a}$ (exons 1α, 2 and 3) and $p14^{ARF}$ (exons 1β and 2) [106]. Both of these proteins play important roles in regulating the cell cycle, and in addition, both $p16^{INK4a}$ and $p14^{ARF}$ have been implicated in promoting senescence [22, 54, 121]. The tumor suppressive effects of CDKN2A are manifested by *in vivo* studies where homozygous CDKN2A knockout mice develop spontaneous tumors particularly sarcomas and lymphomas, and by the finding of CDKN2A mutations and deletions in various cancers [67, 99,115, 111]. Most alterations of CDKN2A appear to predominantly affect $p16^{INK4a}$ with either preservation or inactivation of $p14^{ARF}$ [116]. Inactivation of $p16^{INK4a}$ has been found in many human cancers, and $p16^{INK4a}$ specific knockout mice readily developed tumors, including sarcoma, lymphoma and melanoma [117, 116].

The discovery of germline CDKN2A mutations in cases of familial melanoma highlighted the prominent role of cell cycle dysregulation in the development of melanoma [62]. In approximately 10% of melanoma cases, a family history of melanoma is seen, and from these familial cases, several high penetrance genetic loci have been determined that confer a high-risk for the development of melanoma [103]. Two of these loci map to CDKN2A on chromosome 9p21 with approximately 20–40% of familial melanoma cases having germline mutations in CDKN2A. Most of these CDKN2A germline mutations occur in exons 1α and 2 which encode for $p16^{INK4a}$, but in most cases deletions were found that also affected $p14^{ARF}$ [135]. Taken together, all of these studies suggest that $p16^{INK4a}$ is a true melanoma susceptibility gene. However, although much rarer than $p16^{INK4a}$ mutations, germline mutations specifically affecting $p14^{ARF}$ have also been found suggesting a separate tumor suppressor role [108, 58].

Melanomas also appear prone to somatic alterations in CDKN2A with the vast majority of melanoma cell lines demonstrating loss of CDKN2A [10, 25, 143]. It has

been further shown that CDKN2A knockout mice with activating *HRAS* mutations develop melanomas while murine cell lines cultured from spontaneous melanomas demonstrate loss of $p16^{INK4a}$ and $p19^{ARF}$ [118, 88]. Furthermore, approximately 70% of melanoma cell lines and melanoma samples from 44% of patients with metastatic disease were found to harbor mutations in CDKN2A with inactivating deletions representing the most common type of mutation [95, 143, 129, 52, 146]. The vast majority of CDKN2A mutations primarily affect $p16^{INK4a}$ with promoter silencing via methylation and deletions in $p16^{INK4a}$ representing most of these defects [129, 116, 11, 22]. Mice with specific knockout of $p16^{INK4a}$ develop melanoma although at a lower frequency compared with other tumor types [117]. Melanoma was also seen in mice with activating *KRAS* mutation and loss of $p16^{INK4a}$, particularly when this occurred in mice that showed a concurrent loss of p53 expression [96]. Transgenic mice with activating *HRAS* mutation in conjunction with $p16^{INK4a}$ deficiency readily develop melanomas, while mice with activating *NRAS* mutations and INK4a deficiency developed melanomas in > 90% of cases [118, 3].

Alterations in CDKN2A that preserve $p14^{ARF}$ function but specifically inactivate $p16^{INK4a}$ (exon 1α) are rare in cancers and are most commonly due to promoter hypermethylation [11, 100]. However, studies suggest that $p14^{ARF}$ has a distinct role in tumor suppression. Knockout studies in mice show that $p19^{ARF}$ null phenotypes are prone to the development of various tumors including sarcoma, lymphoma and lung cancer [119]. However, a role for melanomagenesis was suggested by the finding in familial melanoma cases of germline mutations in CDKN2A that specifically affected $p14^{ARF}$ [108, 58]. Furthermore, various types of transgenic mice with knockout of $p19^{ARF}$ were shown to develop melanoma [70, 68, 118, 54). In another study, 2 of 5 human melanoma cell lines demonstrated CDKN2A deletions that specifically affected $p14^{ARF}$ while preserving $p16^{INK4a}$ [73]. These lines of evidence suggest that in melanoma, $p14^{ARF}$ has a separate and important tumor suppressor role that is separate from $p16^{INK4a}$.

7.4.2 Alterations in the p53 Axis

Germline mutations in TP53 are seen in patients with Li-Fraumeni syndrome; a group of individuals with increased susceptibility to sarcomas, breast cancers, brain and adrenal tumors [50]. Somatic mutations in TP53 are very common in cancers with up to 50% of solid tumors found to harbor TP53 mutations [18] In the remaining half of tumors without TP53 mutations, alterations in other elements of the p53 pathway are often seen. For instance, approximately 10% of cancers have amplification of MDM2 while MDM4 is amplified in approximately 10–20% of cancers [132].

The important role of the p53 pathway in the development of melanoma has been demonstrated through animal modeling studies. Transgenic mice with activating *HRAS* mutation and either heterozygous or homozygous p53 loss developed melanomas at higher rates (two of 17 Tyr-RAS $p53^{+/-}$ and seven of 27 Tyr-RAS $p53^{-/-}$ mice) and at shorter latency (65 and 17 weeks, respectively) compared with

mice homozygous for wild-type p53 (one of 49 Tyr-RAS p532009$^{-/-}$ mice after 1 year) [9]. In another model, zebrafish with activating *NRAS* mutations developed hyperpigmentation, but melanoma developed in zebrafish that had both activating *NRAS* mutations and loss of p53 [37]. In addition, the tumor suppressive function of p53 in melanoma may be particularly dependent upon p21$^{Cip1/Waf1}$ [131].

However, in sharp contrast to what is seen in other cancers, TP53 mutations in melanoma are relatively uncommon and seen in <15% of primary tumors [28, 33, 63, 132]. Instead functional loss of the p53 pathway is relatively common and achieved not by actual loss or mutation in p53 itself, but instead by dysregulation of other components of the p53 pathway (Fig. 7.3). Studies have reported transcriptional inactivation of p53 or of its target genes in melanoma cell lines that have wild-type p53 and in tumor samples from melanoma metastases [6, 60]. In addition, inactivating mutations affecting p14^{ARF} are seen in familial melanoma cases and in some melanoma cell lines, and studies in transgenic mice with activating RAS mutations and p19^{ARF} loss also highlight the dysfunction of the p53 pathway in melanoma.

Other alterations in p53 regulators have also been found in melanoma. Overexpression of MDM2 protein was seen in 50% of human melanoma tumors in one study, although MDM2 gene amplification was seen in only one of 100 cases (1%), while a second study showed that two of 53 (3.8%) human melanoma samples had MDM2 gene amplification although increased MDM2 protein expression was again seen in several cases without gene amplification [105, 98]. Furthermore, in contrast to what is seen in melanocytes, melanoma cells appear to rely on MDM2 in order to suppress p53 activity and escape senescence [139]. Another negative regulator of p53 is MDM4 which has been shown to be overexpressed in 65% of melanoma specimens [43]. In this same study, MDM4 overexpression in transgenic mice with activating *NRAS* mutation and wild-type p53 was associated with the development of melanomas in all cases. In another mouse model study using activating *HRAS* mutation and the carcinogen 7,12-dimethylbenz-alpha-anthracene (DMBA), mice that were heterozygous for functional MDM4 showed increased survival and decreased melanoma growth [131]. *In vitro*, MDM4 knockdown inhibited melanoma cell growth while MDM4 expression protected melanoma cells from p53 mediated apoptosis [43]. These studies further exemplify how the p53 pathway is dysregulated in melanoma and also shed light into potential targets for therapy.

7.4.3 Alterations in the Retinoblastoma Protein Axis

Mutations in pRb play a prominent role in the initiation of retinoblastoma, osteosarcoma and SCLC [20, 29, 51]. Germline mutations in the RB1 gene are associated with hereditary retinoblastoma which is inherited in an autosomal dominant fashion [69]. Hereditary retinoblastoma manifests primarily as an ocular tumor, and although many of these patients achieve long-term cures with 5-year survival rates of over 90%, these patients have a 20-fold increased risk of developing secondary tumors. Most often (40–60%) the secondary tumors are sarcomas although much of the sarcoma risk is attributable to radiation exposure during treatment. However,

long-term survivors with germline RB1 mutations also appear susceptible to the development of melanoma which is likely due to loss of heterozygosity [132].

In melanoma, somatic mutations in pRb have not been well studied but appear to be infrequently found in melanoma cell lines [10, 147]. However, alterations in other parts of the pRb pathway are seen in greater frequency in melanoma (Fig. 7.2). The majority of melanomas have alterations in CDKN2A, which usually affect the $p16^{INK4a}$ locus [41, 72]. Knockout of $p16^{INK4a}$ in mice is associated with the development of melanoma, although at a lower frequency compared with other tumor types [117, 118, 3, 96].

Another component of the pRb pathway that is often altered in melanoma is the cell cycle kinase CDK4. Several melanoma prone families have been found to harbor autosomal dominant germline mutations in the CDK4 gene on chromosome 12q14 [103]. These families all have mutations at codon 24 in which arginine is changed to either cysteine or histadine. This amino acid change abrogates the ability of $p16^{INK4a}$ to bind to and inhibit the function of CDK4. CDK4 mutations can occur in the absence of $p16^{INK4a}$ mutations in these familial melanoma cases suggesting that these mutations are mutually exclusive. Amplification of CDK4 with preservation of CDKN2A expression has also been found in a small subset of melanoma tissue samples [98]. Mouse models also support a role for CDK4 in the development of melanoma. Transgenic mice with an activating *HRAS* mutation, wild-type $p16^{INK4a}$ and an R24C *CDK4* mutation developed melanoma in 58% of cases, with the incidence increasing to 83% following ultraviolet (UV) radiation treatment [55]. A second study showed that mice with wild-type $p16^{INK4a}$ and R24C *CDK4* mutation also developed melanomas after topical treatment with DMBA [128].

Amplification of other elements of the pRb pathway has also been reported in melanoma. In mice with activating *HRAS* mutation and $p19^{ARF}$ loss, UV radiation promoted the development of melanoma, and CDK6 amplification was found in approximately half of these tumors [68]. CDK2 amplification has also been found in melanoma in some studies, and melanoma cells seem particularly dependent on CDK2 for proliferation [130, 44, 38]. Amplification of cyclin D1, which binds to and activates CDK4 and CDK6, has been found in approximately 10% of melanomas particularly in the acral lentiginous histologic subtype (44%) and in tumors with *BRAF* mutation [114, 14, 15, 126]. Knockdown of cyclin D1 in melanoma cells reduced cell proliferation by 97% *in vitro*, decreased tumor growth in a mouse xenograft model and was associated with apoptosis [114]. Taken together, all of these studies demonstrate that multiple areas in the pRb pathway can become dysfunctional in melanoma and that these alterations could serve as potential targets for therapy.

7.5 Interaction of BRAF with p53 and Cell Cycle Pathways in Melanoma

Approximately 50% of melanoma have activating *BRAF* mutations, most commonly the V600E mutation [71]. Interestingly, *BRAF* mutations are also seen in approximately 80% of benign nevi suggesting that mutations in *BRAF* are an early event in

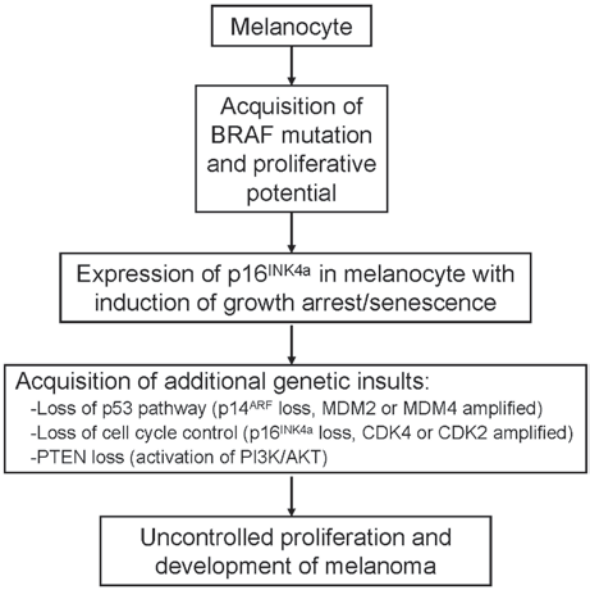

Fig. 7.4 A model for melanomagenesis and potential therapeutic targets. The development of melanoma requires multiple sequential genetic insults. *BRAF* mutation is an early event and found in the vast majority of nevi and melanocytes. Furthermore, co-expression of $p16^{INK4a}$ and markers of senescence are also found in melanocytes with *BRAF* mutations. It is believed that the proliferative influences of *BRAF* mutations in melanocytes are countered by $p16^{INK4a}$ expression and induction of senescence. Additional mutations are eventually acquired such as loss of $p16^{INK4a}$ and/or $p14^{ARF}$, *CDK* amplification, *MDM2* or *MDM4* amplification, *RAS* mutation or *PTEN* loss. These additional genetic insults lead to uncontrolled proliferation and the development of melanoma. However, these additional alterations and the interaction of *BRAF* with cell cycle and *p53* pathways provide potential therapeutic targets in conjunction with *BRAF*-directed therapy

the development of melanoma [104, 74]. Nevi appear to represent growth-arrested melanocytes since congenital nevi stain positively for the senescence-associated marker acidic β-galactosidase [91, 53, 92]. *In vitro* studies have demonstrated that the expression of $BRAF^{V600E}$ in melanocytes causes cell cycle arrest that is associated with $p16^{INK4a}$ expression [91]. It is believed that the expression of $p16^{INK4a}$ may protect the melanocyte from the proliferative influences of *BRAF* mutations by promoting senescence (Fig. 7.4) [33].

However, the presence of *BRAF* mutations alone is insufficient to transform melanocytes into melanoma and additional genetic alterations are required (Fig. 7.4). Specifically, alterations in components of the p53 pathway ($p14^{ARF}$, MDM2 and MDM4) and/or in components of the pRb pathway ($p16^{INK4a}$, CDK4 and CDK2) may cooperate with *BRAF* mutations to allow for the development of melanoma. Furthermore, activation of other pathways, as exemplified by increased PI3K/AKT activity through PTEN loss, may also interact with mutated *BRAF* to prevent senescence in melanocytes and to promote tumor growth [141]. This interaction was highlighted in a zebrafish study where the combination of

BRAF mutation in the background of p53 deficiency induced the development of melanoma while the presence of *BRAF* mutation alone was associated with the development of only benign nevi [102]. In another study, melanocytes within human skin tissue were transfected and then xenografted onto immunodeficient mice [30]. Melanoma was seen when either activating RAS or PI3K mutations were combined with hTERT expression and inhibition of the p53 or pRb pathways, whereas only benign nevi were seen with *BRAF* mutation. The loss of CDKN2A ($p16^{INK4a}$ and $p14^{ARF}$) in association with *BRAF* mutation appears to be the most frequent combination of mutations in melanoma and can promote the formation of tumors [34, 52]. This was shown in a study where mice that had $BRAF^{V600E}$ mutations and were deficient in $p16^{INK4a}$ showed a higher percentage of cases that developed melanoma, more cases of multiple tumors and shorter latency when compared with mice with wild-type $p16^{INK4a}$ [35]. In human melanocytes with $BRAF^{V600E}$ mutation, knockout of p53 enhanced proliferation, created lesions that resembled melanoma in situ and was associated with loss of the RB1 locus [148].

It is evident that the development of melanoma is associated with derangements in regulation of the cell cycle and p53 pathways. Loss of $p16^{INK4a}$ appears especially important in melanomagenesis by circumventing the senescence response in melanocytes that develop activating *BRAF* mutations. Furthermore, alterations in cell cycle pathways may also diminish therapeutic responses. For instance, melanomas that overexpress CDK4 and cyclin D1 show intrinsic resistant to *BRAF* inhibition [14, 126]. One alternative cellular pathway utilized by melanomas to overcome *BRAF* inhibition therapy is activation of the PI3K/AKT pathway [39]. MDM2 is upregulated via phosphorylation by activated AKT thereby inhibiting p53 function, and it is conceivable that increased PI3K/AKT activity in melanoma cells resistant to *BRAF* inhibition may play a role in promoting proliferation in these resistant cells [141]. In melanoma, there is obvious interaction amongst these pathways providing the potential for combining *BRAF* inhibitor treatment with therapies aimed at targeting these alterations in cell cycle and p53 regulation.

7.6 Therapeutic Potential of BRAF Inhibition in Combination with Modulation of p53 or Cell Cycle Pathways

BRAF mutations in melanoma have been successfully targeted through single agent treatment, but despite the dramatic responses initially seen, recurrences inevitably develop. *BRAF* mutant melanoma readily develops resistance and utilizes alternative cellular pathways to overcome *BRAF* inhibition, highlighting the need for additional therapeutic targets for combination therapy. The ubiquitous alterations in cell cycle and p53 pathways in melanoma and the interaction of *BRAF* with these pathways provide new potential therapeutic targets that can be combined with *BRAF* inhibition.

One promising area of cancer therapy research is looking into ways to reactive p53 function in tumors with inactivating p53 mutations [18]. However, p53 mutations are only seen in 10–15% of melanoma, but for this small subset, agents such as PRIMA-1, which help to stabilize the protein folding of p53, may help to reactivate mutated p53 and restore its function. *In vitro*, PRIMA-1 has been shown to promote p53-dependent apoptosis and inhibit growth in melanoma cells [8]. Much more common in melanoma is the functional loss of p53 caused by alterations in p53 regulatory pathways, and ongoing research is looking into ways to enhance the function of wild-type p53. MDM2 is overexpressed in many melanomas and serves as an attractive therapeutic target to increase p53 activity [142]. The nutlin class of inhibitors is derived from cis-imidazoline compounds and functions to displace MDM2 from p53 thereby increasing p53 activity [137]. In one study, melanocytes and melanoma cells were treated with the MDM-2 specific antagonist nutlin-3, and at doses <20 μM, cell cycle arrest was seen while doses >20 μM promoted apoptosis [131]. Based on these results, it was proposed that nutlin-3 primarily functioned to decrease melanoma growth by promoting cell cycle arrest. A second study demonstrated that restoration of p53 function in melanoma cells through use of nutlin-3 decreased cell viability in a dose-dependent manner [64]. More importantly, 27 of 51 (53%) melanoma cell lines tested had a *BRAF* mutation and wild-type p53, and the combination of nutlin-3 and MEK inhibition (U0126) appeared to synergistically decrease growth in 60% of melanomas. The effects of MDM2 antagonism are dependent upon the presence of functional p53, and the greatest effect was seen in cells that had *BRAF* mutation and wild-type p53.

Whereas some studies have demonstrated that melanoma cells treated with nutlin-3 exhibited either minimal apoptosis or favored cell cycle arrest, work from our lab demonstrated that treatment of melanoma cells with nutlin-3 induced p53-dependent apoptosis while Ji et al. showed that inhibition of both MDM2 (nutlin-3) and MEK (U0126 and AZD6244) in *BRAF* mutated melanoma promoted apoptosis [125, 131, 136, 64]. It could be extrapolated based on these studies that inhibition of mutated *BRAF* instead of MEK in combination with nutlin-3 therapy would produce similar effects, however this would need to be validated. A major potential issue in MDM2 inhibition therapy is the negative feedback loop between MDM2 and p53 [133, 142]. Activation of p53 via nutlin-3 would theoretically later increase MDM2 levels and diminish p53 activity. Despite this potential caveat, the MDM2 antagonist RO5045337 (RG7112) has been used in patients with MDM2-amplified liposarcoma and based on best RECIST response, resulted in a partial response in 1 patient, stable disease in14 patients and progressive disease in five patients [107]. Furthermore, this preliminary study demonstrated that after treatment with RG7112, there was an increase in p53 levels and a decrease in cell proliferation as determined by Ki-67 staining in tumor samples. RO5045337 has also been tested in clinical trials for both solid and hematologic malignancies (NCT00559533, NCT00623870)* and is currently being tested in soft tissue sarcoma in conjunction with doxorubicin (NCT01605526) and as an extension study (NCT01677780) in patients previously treated with RO5045337 [64]. Other MDM2 antagonists such as RO5503781 and thioureidobutyronitrile

* NCT: National Clinical Trial Identifier. Please refer to www.clinicaltrials.gov for additional information on individual clinical trials.

(Kevetrin) are also being tested in clinical trials (NCT01462175, NCT01664000), but the results of all of the aforementioned trials are still pending [75].

Other studies have shown that MDM2 overexpression was seen infrequently in melanoma, however MDM4 protein levels were upregulated in the majority of melanoma specimens regardless of *BRAF* mutational status, thus making MDM4 an appealing therapeutic target [43]. Gembarska et al. demonstrated the key role played by MDM4 in promoting the development of melanoma *in vivo* and in allowing for cell proliferation and protection against apoptosis *in vitr*o. Importantly, inhibition of MDM4 using the α-helical peptide SAH-p53-8, a compound that has high specificity for MDM4 and disrupts the binding of p53 with MDM4, appeared to synergize with *BRAF* inhibition in melanoma cells and also decreased cell viability in melanoma cells that had developed resistance to *BRAF* inhibition [12, 13]. SAH-p53-8 was also tested in uveal melanoma cell lines and inhibited growth in cells that overexpressed MDM4 and to a lesser extent in cells that overexpressed MDM2 [76]. Although MDM2 targeted therapy appears to inhibit melanomas that overexpress MDM2, MDM2-specific agents such as nutlin-3 appear to have little effect on melanomas that overexpress MDM4. This highlights the fact that tumor genotyping will play a critical role for determining specific oncogenic alterations (e.g. overexpression of MDM2 versus MDM4) so that targeted therapies can be fashioned on a case by case basis.

Inhibitors of several other targets that interact with p53 have also been described. Glycogen synthase kinase-3β (GSK-3β) regulates glycogen metabolism but is also involved in cell migration, proliferation, apoptosis and regulation of p53 [45, 65]. In one study, GSK-3β inhibition using the organometallic inhibitor DW1/2 promoted apoptosis in melanoma cells through a p53-dependent mechanism that involved downregulation of MDM2 and MDM4 [125]. The combination of the MDM2 inhibitor MI-319 and sorafenib appeared to have a GSK-3β-dependent cytotoxic effect in some melanoma cells lines [81]. Other studies have looked at BH3 mimetics in combination with MEK inhibition (U0126) and have shown a p53-dependent synergistic cytotoxicity in melanoma cells [138]. Theoretically, upregulating the function of $p14^{ARF}$ and $p21^{Cip1/Waf1}$ could also enhance p53 function. In one study, B16 mouse melanoma cells were transfected with retrovirus containing $p19^{ARF}$ and were subsequently treated with nutlin-3 [89]. The combination of direct MDM2 inhibition via nutlin-3 and indirect MDM2 inhibition through $p19^{ARF}$ expression resulted in enhanced p53 activity and decreased B16 cell viability *in vitro* and *in vivo*. TBX2 downregulates the expression of both $p14^{ARF}$ and $p21^{Cip1/Waf1}$, and interestingly it is overexpressed in melanoma [47]. TBX2 inhibition appears to promote senescence and may serve as a potential therapeutic target to augment p53 function. However, the role of the above-mentioned targets in relation to *BRAF* status has not been fully assessed and requires further study.

Alterations in the pRb axis, primarily due to upregulation of CDK4 either through loss of $p16^{INK4a}$ or amplification of either CDK4 or cyclin D1, are readily seen in melanoma. Furthermore, melanoma cells appear especially dependent on CDK2 for growth [38]. Taken together, these results suggest that direct small molecule CDK inhibition would serve as an attractive therapeutic option. Numerous types of CDK

inhibitors have been developed and used in clinical trials including broad-range first generation inhibitors such as flavopiridol and roscovitine and second generation specific inhibitors such as PD-0332991 and PHA-848125 [36, 78, 31, 21]. Although preclinical results appeared promising, the results of clinical trials using small-molecule CDK inhibitors for the treatment of various solid tumors have generally been disappointing. One of the issues was that first generation compounds lacked specificity and inhibited several CDKs thereby limiting efficacy and causing off-target effects and toxicity. For instance, flavopiridol inhibits CDK1, 2, 4, and 7 while roscovitine inhibits CDK1, 2, 5 and 7 [31]. New second generation compounds have been developed that are more selective and potent as exemplified by PD-0332991 which inhibits CDK4 and 6 and by PHA-848125 which is a potent CDK2 inhibitor although it also is capable of inhibiting CDK1, 4 and 7 [21]. Clinical experience with the second-generation CDK inhibitors has been relatively limited and the results of most trials are pending. However, the preliminary results of one phase II clinical trial were recently reported (NCT00721409) and showed promising results [40]. Post-menopausal women with estrogen receptor-positive/HER2 negative advanced breast cancer who were treated with PD-0332991 and aromatase inhibitor letrozole had a significant increase in progression-free survival to 26.2 months compared with 7.5 months for patients treated with letrozole alone. Another potential issue is that studies have shown that only CDK1 is essential for cell cycle progression, which is contrary to the "classical" model of the cell cycle where sequential expression of several CDKs is required [83, 112]. Specifically, the loss of other CDKs can either be compensated by CDK1 or the loss of a specific CDK is detrimental only in specialized cells types such as hematopoietic cells and cardiomyocytes. Therefore, inhibition of CDKs outside of CDK1 may have limited effect or benefit.

Studies evaluating CDK inhibition for melanoma are very limited and are even more limited in evaluating the combination of *BRAF* and CDK inhibition. In two studies, CDK activity was inhibited in *BRAF* mutant melanoma by upregulating $p16^{INK4a}$ activity. In both of these studies, siRNA knockdown of *BRAF* along with expression of $p16^{INK4a}$ in melanoma cells harboring *BRAF* mutations significantly inhibited cell growth, and in one study there was also a significant increase in apoptosis [110, 150]. In another set of studies, CDK activity was inhibited by antagonizing cyclin D1 [113, 114]. *In vitro* cyclin D1 antisense treatment in melanoma cells induced apoptosis, while *in vivo* cyclin D1 antisense therapy along with transfection with wild-type p53 led to tumor shrinkage and to a complete response in 57% of cases. Flavopiridol was tested in $p16^{INK4a}$ positive and $p16^{INK4a}$ negative melanoma cells and caused a dose-dependent growth inhibition, although the IC_{50} for $p16^{INK4a}$ positive melanoma was higher [109]. Furthermore, a dose-dependent increase in apoptosis was seen in both $p16^{INK4a}$ positive and $p16^{INK4a}$ negative melanoma cells treated with flavopiridol. A study utilizing melanoma cultures in a 3D skin reconstruction model demonstrated that treatment with roscovitine was associated with decreased cell growth and survival and increased apoptosis in melanoma cells but not in melanocytes [95]. In another study, melanoma cells were treated with the CDK inhibitor SCH 727965 which decreased cell proliferation at a dose as low as 0.5 μM, promoted apoptosis and slowed tumor growth in a mouse xenograft

model [2]. Caporali et al. tested the second-generation CDK inhibitor PHA-848125 in melanoma cells and demonstrated G1 arrest and growth inhibition with IC_{50} values ranging from 0.123 to 0.680 μM [23]. Treatment with PHA-848125 was also associated with increased $p21^{Cip1/Waf1}$ expression, decreased pRb phosphorylation at the CDK2 and CDK4 sites and differential expression of genes involved in cell cycle control [23, 24]. Another CDK inhibitor, P276-00, has shown efficacy *in vitro* in decreasing cell proliferation and colony formation in several cancer cell lines including melanoma [66]. The CDK inhibitors roscovitine and DRB were used in combination with nutlin-3 to treat melanoma cells [27]. This combination appeared to show additive effects on inhibiting cell growth and synergy in inducing p53-dependent apoptosis. Recently, a study was presented in which a *BRAF* mutant melanoma cell line (SKMel 28) was treated with *BRAF* inhibitor PLX-4720 and CDK inhibitors roscovitine and olomoucine [134]. The combination of CDK inhibitor and *BRAF* inhibition led to downregulation of MAP3K8 and PRKD3 survival pathways, decreased pRb phosphorylation and ultimately to decreased cell viability. Li et al. treated melanoma cells with MEK inhibitor PD98059 and CDK4 inhibitor 219476 and demonstrated a decrease in cell viability and a significant increase in apoptosis in cells treated with both agents [80].

Based on these encouraging preclinical results, several clinical trials were developed to evaluate CDK inhibitor therapy in patients with melanoma. SCH 727965 is being evaluated in stage IV and unresectable stage III melanoma patients (NCT01026324, NCT00937937), while PD-0332991 is being tested against various solid tumors, including recurrent and stage IV melanomas (NCT01037790). P276-00 is being evaluated as treatment for stage IV and unresectable stage III melanomas that express cyclin D1 (NCT00835419). The results of most of these trials are pending, however preliminary results were reported for NCT00937937 (SWOG S0826) in which no responses were seen in 65 evaluable patients [77]. Stable disease was seen in 22% of patients, and the 1-year overall survival rate was 36%. Similar results from a phase II study using flavopiridol to treat metastatic melanoma patients were also reported with no objective responses seen in 16 evaluable patients [19]. However, no definitive conclusions can be made since these results are either preliminary or were based on a small number of patients, and the therapeutic effect of CDK inhibition requires more rigorous evaluation. In addition, the effects of *BRAF* mutation status and use of *BRAF* inhibition along with CDK inhibition also need further study and the first of these combinations is underway with the BRAF/MEK inhibitor doublet, encorafenib and binimetinib, in combination with LEE011 (NCT01543698).

7.7 Conclusions

Melanoma represents a prime model for developing targeted therapy due to the well-validated identification of oncogenic “drivers” that promote tumor proliferation. However, despite the initial success of single agent *BRAF* targeted therapy, it

is evident that single agent treatment ultimately fails due to cellular adaptation and the development of resistance. It is now believed that combination therapy, which targets multiple cellular pathways, is the key to overcoming or sidestepping the development of treatment resistance. The fact that the vast majority of melanomas harbor alterations in regulators of the cell cycle and p53 makes these pathways extremely attractive targets for therapeutic intervention. Furthermore, preclinical studies suggest that therapies aimed at melanoma with *BRAF* mutation and wild-type p53, which represents the most common genotype, may have synergistic results when *BRAF* directed therapy is combined with treatments that either enhance p53 function or inhibit CDK activity. However, the use of agents that modify p53 and CDK function in melanoma is at an early stage and further studies are needed. In particular, melanoma specific clinical trials testing *BRAF* inhibition in combination with MDM2/MDM4 inhibitors and/or CDK inhibitors are needed to determine the efficacy of this treatment combination in humans. These questions highlight the fact that it is an extraordinarily exciting time in the field of melanoma research as our knowledge about melanoma biology continues to expand. Additional work in this area will undoubtedly lead to the development of new agents for the treatment of metastatic melanoma thereby further improving the prospects for melanoma patients.

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