Chapter 2

# The Emerging Epigenetic Landscape in Melanoma

Robert A. Rollins, Kimberly H. Kim and
Cheng-Chung Tsao

Additional information is available at the end of the chapter

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**Abstract**

Melanoma is the deadliest form of skin cancer. The disease is driven by molecular alterations in oncogenic signaling pathways, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K). Activating mutations in oncogenes, such as *BRAF* and *NRAS*, and inactivating mutations in tumor suppressors genes, such as *PTEN*, promote this disease by altering cellular processes involved in growth, survival, and migration. Therapies targeting critical nodes in these pathways have demonstrated efficacy in clinical trials, but their therapeutic potential has been limited by the rapid onset of drug resistance. Durable therapeutic responses have also been observed in patients receiving immunotherapy. However, this activity appears to be confined to a subset of patients, and combinations with targeted therapies have raised safety concerns. Accumulating evidence strongly suggests that the pathogenesis of melanoma is also shaped by the aberrant activity of epigenetic factors that regulate gene expression through the modification of DNA and chromatin. This chapter provides a comprehensive review of the epigenetic alterations in melanoma and highlights the roles played by specific chromatin regulators during disease progression. We also discuss the clinical utility of both first and second generation epigenetic therapies in the melanoma setting, placing emphasis on the potential to overcome resistance to targeted therapies and to serve as priming agents for immunotherapies.

**Keywords:** melanoma, epigenetics, chromatin structure, epigenetic therapy

## 1. Introduction

Melanoma is the most deadly form of skin cancer. While melanoma patients represent a small percentage (~1%) of the total number of skin cancer cases, this aggressive disease is responsible for the vast majority of skin cancer deaths [1]. The incidence of melanoma has been

rising steadily for several decades, with a 1.4% increase in the number of new cases each year for the last 10 years. The overall 5-year survival rate is >90%, due in large part to early detection and the ability to surgically excise localized cancer cells. However, for patients with metastatic melanoma, the 5-year survival rate drops dramatically to ~17%. In 2016, it is estimated that there will be 76,380 new melanoma cases and 10,130 melanoma-related deaths, underscoring the need for therapeutic strategies to treat this disease [2].

Melanoma arises from the malignant transformation of melanocytes in the epidermal layer of the skin. During embryonic development, neural crest cells migrate from the neural tube to the skin where they give rise to melanocytes [3]. The transformation of melanocytes to melanoma is driven by oncogenic signaling pathways that are triggered by genetic and environmental factors. Metastatic melanoma cells are highly invasive and display stem cell–like properties that are characteristic of their neural crest progenitors, making them extremely aggressive and difficult to treat [4].

Therapeutic intervention in melanoma has historically focused on targeting nodes in the MAPK pathway [5]. Activating oncogenic mutations in *BRAF* and *NRAS* have been identified in 40–60% and 15–20% of melanoma patients, respectively, leading to constitutive pathway signaling that promotes cell proliferation and survival [6, 7]. While NRAS has proven extremely difficult to target pharmacologically, potent and selective small molecule inhibitors of BRAF (vemurafenib and dabrafenib) and MEK (trametinib), a downstream signaling kinase, have been approved by the FDA for the treatment of patients with metastatic melanoma harboring $BRAF^{V600}$ mutations [8–12]. While response rates in the clinic have been impressive, resistance develops quickly and in some cases these agents have been shown to exacerbate the aggressive nature of the disease due to the paradoxical activation of the MAPK pathway in cells harboring wild-type BRAF [13, 14]. The combination of BRAF and MEK inhibitors has demonstrated improved rates of progression-free survival, however, these combinations are still prone to resistance, thereby limiting the long-term survival in melanoma patients harboring $BRAFV^{600}$ mutations [15–17]. More recently, durable clinical responses have been observed following treatment with antibodies that target immune checkpoint molecules, such as PD-1 (pembrolizumab and nivolumab) and CTLA-4 (ipilimumab) [18–20]. However, the benefit of these therapies appears to be limited to smaller subsets of the overall patient population and there are potential safety concerns around the use of these agents in combination with inhibitors of the MAPK pathway [21, 22].

While recent clinical advances provide much needed hope for melanoma patients, there is a clear need to understand additional mechanisms that contribute to the pathology of this disease. To this end, emerging data has demonstrated the importance of aberrant epigenetic regulation during melanoma growth, metastasis, and drug resistance. In addition to contributing to a more thorough understanding of melanoma pathogenesis, these studies have revealed potential drug targets implicated in the regulation of chromatin structure and gene expression [23–25].

## 2. Epigenetic changes as a hallmark of cancer

Epigenetics is defined as heritable changes in gene expression that occur in the absence of alteration in the DNA sequence (i.e. mutations) [26]. Epigenetic regulators are enzymes and proteins that covalently modify or bind to DNA and histones to alter chromatin structure and function. These proteins are divided into three broad classes: writers, erasers, and readers. Writers and erasers are enzymes that add and remove covalent modifications, such as acetylation, methylation, and phosphorylation, while reader molecules recognize these marks and serve as downstream effector molecules [27, 28]. The combination of DNA and histone modifications and DNA- and histone-binding proteins creates an epigenetic code that governs genome-wide transcriptional networks. Epigenetic mechanisms drive gene expression programs that regulate a multitude of cellular processes, including differentiation, proliferation, pluripotency, cell migration/motility, cell signaling, and immune recognition/response [29].

The advent of next-generation sequencing (NGS) and epigenome mapping technologies has facilitated the systematic evaluation of cancer genomes. These studies have revealed a remarkably high frequency of genetic alterations in genes encoding epigenetic regulators [30, 31]. For example, genes encoding subunits of the SWI/SNF chromatin remodeling complex are mutated in 20% of all tumors, making it the second highest mutational frequency behind *TP53* [32]. In concordance with the spectrum of genetic lesions, genome-wide analysis of DNA and chromatin structure has revealed alterations in the normal patterns of DNA methylation and histone modifications [33, 34]. These epigenetic abnormalities reprogram cancer cells by altering transcriptional programs and influencing cell fate decisions and cellular identity [35]. Based on the multitude of biological pathways influenced by epigenetic reprogramming in tumors, it has been suggested that defects in epigenetic control contribute to all of the classical hallmarks of cancer [29].

## 3. Epigenetic alterations in melanoma

### 3.1. DNA methylation

In mammalian genomes, DNA methylation occurs almost exclusively in the context of 5′-CpG dinucleotides (CpGs). Hypermethylation of CpG island promoters is a common event in cancer and results in the aberrant silencing of tumor suppressor genes [36, 37]. Paradoxically, tumors are also characterized by DNA hypomethylation, primarily at repetitive DNA sequences, transposable elements, and some single-copy genes [38]. The global loss of DNA methylation is thought to promote tumorigenesis by several mechanisms, including the creation of genomic instability, the reactivation of latent retrotransposons, and the potential activation of proto-oncogenes [39].

Aberrant DNA methylation is a hallmark of malignant melanoma [40]. Hypermethylation has been observed at key tumor suppressor genes, such as *p16/INK4A*, *p14/ARF*, *RASSF1A*, and

*RARβ2*, and a CpG island methylator phenotype has been correlated with disease progression [40, 41]. Interestingly, the loss of methylation at repetitive elements and the hypomethylation-induced expression of cancer-testis antigens, such as *MAGE*, have also been described as markers of poor prognosis, highlighting the complexity of tumor-associated DNA methylation patterns [40, 42]. Multiple studies have also described links between DNA methylation abnormalities and $BRAF^{V600E}$-mediated signaling [43, 44]. Genome-wide epigenomic profiling of metastatic melanoma tumors has identified subgroups of patients with distinct DNA methylation patterns that correlate with specific proliferative and immune gene expression signatures and various clinical outcomes [45, 46]. In addition to the potential use as clinical biomarkers, the data suggests that the reversion of DNA methylation patterns may provide a therapeutic benefit in melanoma patients.

### 3.2. Histone modifications

In addition to DNA methylation, posttranslational modifications on histone tails provide another layer of epigenetic regulation. Histone deacetylases (HDAC) are highly expressed in melanoma cells and altered histone acetylation has been linked to the downregulation of tumor suppressor genes, such as *p14/ARF* and *p16/INK4a* [25, 47, 48]. In addition to changes in histone acetylation, genomic, proteomic, and immunohistochemical approaches have also identified aberrant histone methylation patterns [49–51]. The advent of genome-wide chromatin immunoprecipitation has also uncovered global redistribution of histone marks, such as methylation on lysine 27 of histone H3 (H3K27me) [52, 53]. These observations suggest that histone modifications cooperate with DNA methylation to reprogram gene expression patterns during melanoma progression. They also point toward underlying defects in the enzymes and proteins that regulate these epigenetic mechanisms.

## 4. Genetic landscape of epigenetic regulators in melanoma

The emergence of NGS has proven to be a powerful tool in identifying oncogenic driver mutations. In 2012, two independent studies reported whole-genome sequencing data from 121 and 147 primary melanoma tumors, respectively [54, 55]. In addition to confirming a high frequency of oncogenic mutations in the *BRAF* and *NRAS* genes, these studies identified loss-of-function mutations in the SWI/SNF components *ARID2*, *ARID1A*, *ARID1B*, and *SMARCA4* as well as hot-spot mutations in the histone methyltransferase EZH2 [54, 55]. Identical *EZH2 Y641* mutations had previously been identified in germinal center diffuse large B cell lymphoma (DLBCL) and follicular lymphoma (FL) where they have been shown to result in gain-of-function activity [56, 57].

The high frequency of mutations in epigenetic regulators was recently confirmed in an NGS analysis of 38 treatment-naive melanoma samples [23]. Targeted sequencing of 275 known cancer genes revealed mutations in genes encoding known epigenetic regulators, including histone methyltransferases (*MLL2*, *SETD2*), chromatin remodeling factors (*ARID1B*, *ARID2*), and DNA demethylases (*TET2*). Interestingly, 92.1% of the patient melanoma samples

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harbored at least one mutation in an epigenetic regulator and UVB-signature mutations were found more commonly among epigenetic genes.

Analysis of publicly available data from The Cancer Genome Atlas (TCGA) confirms the high frequency of genetic lesions in epigenetic regulators [58] (**Figure 1**). These genetic alterations are often coincident with mutations in the prominent melanoma oncogenes *BRAF* and *NRAS*, suggesting that epigenetic reprogramming may modulate key oncogenic signaling pathways. Close inspection of the TCGA data also provides important clues to the functional relationships between various epigenetic regulators. For example, mutations in genes from related families, such as the histone methyltransferases *MLL* (*KMT2A*) and *MLL2* (*KMT2D*) or protein complexes, such as SWI/SNF, are often mutually exclusive (**Figure 1**). This suggests functional redundancies that may be important to melanoma biology. The data also reveals that a subset of melanomas harbor genomic amplifications that contain epigenetic genes, such as the histone methyltransferases *EZH2* and *SETDB1* (**Figure 1**). Overall, the high frequency of genetic alterations that impact chromatin regulators implicates epigenetic regulation as a driving force behind melanoma pathogenesis.

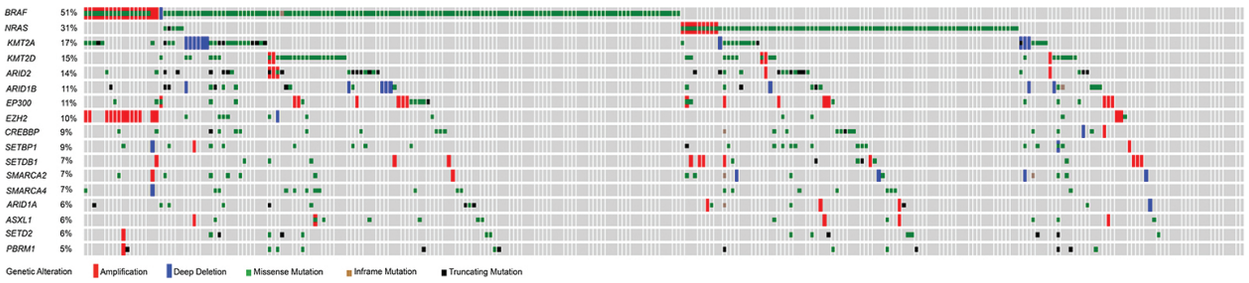


**Figure 1.** Prominent genetic alterations in epigenetic regulator genes in cutaneous melanoma. The oncogenes BRAF and NRAS are altered in 51% and 31% of the 278 melanoma tumor samples, respectively. Many of these tumors also harbor additional mutations in genes that encode for epigenetic factors. This figure highlights epigenetic genes that are altered in >5% of melanoma tumors. Data is publicly available courtesy of Memorial Sloan-Kettering Cancer Center's cBioPortal for Cancer Genomics (http://www.cbioportal.org).

## 5. Novel epigenetic drug targets in melanoma

The discovery of widespread epigenetic alterations in melanoma has led to the premise that melanoma patients would derive therapeutic benefit from therapies that reprogram cancer-specific gene expression patterns [25, 59]. To this end, first generation epigenetic therapies, such as DNA hypomethylating agents and histone deacetylase inhibitors, have made their way into melanoma clinical trials, primarily in combination with other therapeutic agents. Unfortunately, these early molecules lack selectivity and have demonstrated limited single-agent clinical activity outside of hematological malignancies, highlighting the need for therapies that target alternative epigenetic mechanisms and cellular pathways. The combination of genome-wide analyses and targeted genetic approaches has uncovered potential drug targets representing multiple classes of epigenetic regulators, including histone methyltransferases, histone

demethylases, histone ubiquitin ligases, and epigenetic readers (**Table 1**). In addition, the prevalence of inactivating mutations in histone modifying enzymes and chromatin remodeling factors suggests the potential for identifying targetable vulnerabilities in the context of specific genetic backgrounds.

| Target | Enzyme class | Therapeutic rationale in melanoma | References |
|---|---|---|---|
| **EZH2** | Histone Methyltransferase | Amplified and overexpressed gain-of-function mutations identified in 3% of patients. Genetic and pharmacological inhibition impairs tumor growth and metastasis | [53, 54, 70, 75, 77–79] |
| **SETDB1** | Histone Methyltransferase | Amplified and overexpressed. Accelerates tumor progression in zebrafish melanoma model | [86] |
| **JARID1B** | Histone Demethylase | Required for continuous tumor growth. Potential cancer stem cell marker. Potential role in mediating drug resistance | [96–98] |
| **JMJD3** | Histone Demethylase | Promotes growth and metastasis of melanoma cells. Modulates tumor microenvironment through NF-κB and BMP signaling | [105] |
| **RNF2** | Histone Ubiquitin Ligase | Overexpressed in melanoma. Part of gene signature that correlates with melanoma invasion. Dual role in tumor growth and invasion | [107–109] |
| **BRD4** | Bromodomain | Amplified and overexpressed. Genetic and pharmacological inhibition impairs tumor growth Potentiates Ras-driven transcription in *NF1*-mutant tumors | [119–123] |

**Table 1.** Novel epigenetic drug targets in melanoma.

### 5.1. EZH2

Polycomb group (PcG) proteins are regulators of chromatin structure that play essential roles in transcriptional control during development [60, 61]. The histone methyltransferase EZH2 is the catalytic subunit of polycomb repressor complex 2 (PRC2), a conserved multiprotein complex that represses gene expression by methylating lysine 27 on histone H3 [62–64]. In the context of PRC2, EZH2 methyltransferase activity plays a key role in a number of biological processes, including cellular differentiation, X-inactivation, and stem cell pluripotency [65].

In addition to its normal roles in development, accumulating evidence supports an oncogenic role for EZH2 in the initiation and progression of a variety of cancers [66–68]. Overexpression of EZH2 has been observed in a wide range of tumor types, and elevated expression is often correlated with aggressive disease and poor prognosis [69, 70]. More recently, oncogenic gain-of-function mutations in EZH2 have been identified in DLBCL and FL that alter its enzymatic activity, resulting in elevated levels of H3K27me3 [56, 57]. Moreover, genetic and pharmacological inhibition of EZH2 enzymatic activity in both wild-type and mutant settings has been shown to inhibit cell proliferation and regress tumor growth, further validating EZH2 as a potential cancer target [71–73].

Several lines of evidence suggest that aberrant EZH2 activity plays a role in melanoma pathogenesis and progression. EZH2 expression has been shown to increase incrementally during the progression from benign nevi to malignant tumor [74]. To this end, EZH2 is genetically amplified in melanoma patient samples, and elevated expression has been shown to correlate with aggressive disease and poor survival [70, 75]. In addition, genetic depletion of EZH2 in human melanoma cells has been shown to inhibit cell proliferation *in vitro* and *in vivo* by inducing p21-/CDKN1A-mediated cellular senescence [76]. More recently, it was demonstrated that conditional ablation of *Ezh2* in a melanoma mouse model inhibited tumor growth and abolished metastasis without affecting normal melanocytes. Importantly, these effects were mimicked by pharmacological inhibition of EZH2 confirming the importance of EZH2 catalytic activity [77]. In addition to genetic amplification, whole-exome sequencing analysis has also identified previously characterized $EZH2^{Y641}$ gain-of-function mutations in melanoma tumors [54, 55]. While inhibition of EZH2 in melanoma cell lines harboring $EZH2^{Y641}$ mutations has been shown to inhibit cell proliferation and induce apoptosis, a study examining the growth of EZH2 mutant cells in three-dimensional assays also uncovered important roles in cell motility and migration that are independent of cell proliferation [78, 79]. The alterations in cell proliferation and motility are consistent with the proliferative and metastatic phenotypes reported following inhibition of wild-type EZH2 in mouse melanoma models [77]. Interestingly, $EZH2^{Y641}$ mutations appear to be coincident with $BRAF^{V600}$ mutations, suggesting an important link between epigenetic alterations and the MAPK pathway signaling [80]. This is supported by a recent finding that the combination of $Braf^{V600E}$ and $Ezh2^{Y641F}$ mutations accelerated disease progression in a melanoma mouse model [53].

### 5.2. SETDB1

SET domain bifurcated 1, or SETDB1, is a histone methyltransferase that mediates trimethylation of lysine 9 on histone H3 (H3K9me3) [81]. SETDB1 has been shown to be involved in the transcriptional silencing of both euchromatic genes and retro-elements [81–84]. The mechanism of transcriptional repression by SETDB1 may involve the DNA methylation machinery as SETDB1 was shown to be recruited to chromatin by the methyl-CpG-binding protein MBD1 to silence tumor suppressor genes, such as *RASSF1A* and *p53BP2* [83, 85]. In 2011, the *SETDB1* gene, located on chromosome band 1q21, was found to be amplified in several tumor types, including melanoma [86]. In the same study, expression of SETDB1 accelerated tumor progression in a zebrafish melanoma model harboring an oncogenic $BRAF^{V600E}$ mutation [86]. Chromatin immunoprecipitation coupled to gene expression analysis confirmed that increased levels of SETDB1 correlated with aberrant silencing of key genes involved in development [86].

On heels of the zebrafish study, there has been surprisingly little data in mammalian systems linking SETDB1 to melanoma progression. However, in addition to melanoma, SETDB1 is also focally amplified in non–small cell lung cancer, small cell cancer, ovarian cancer, hepatocellular carcinoma, prostate cancer, and breast cancer [86–91]. Emerging data in these settings suggests that elevated expression of SETDB1 may provide tumor cells with a growth advantage. For example, depletion of SETDB1 by siRNA or shRNA in *SETDB1*-amplified breast, liver,

prostate, and lung cancer cells has been shown to inhibit proliferation *in vitro* and *in vivo*, indicating that these cells require elevated SETDB1 for their growth [87–89, 91]. In addition, inhibition of SETDB1 has been shown to negatively impact cell migration and invasion, suggesting that the role of SETDB1 may extend beyond the regulation of cell proliferation [88].

While the pathways that trigger cancer-specific overexpression of SETDB1 remain poorly elucidated, recent data has begun to shed light on the downstream mechanisms by which SETDB1 facilitates tumor growth in specific genetic contexts. SETDB1 has been shown to regulate the stability of tumor suppressors p53 and p53-related p63 [89, 91]. In 2015, Fei et al. reported the molecular interplay between SETDB1 and the well-known hotspot gain-of-function *TP53* R249S mutation. In this study, the authors demonstrated that SETDB1 catalyzes the demethylation of p53K370 and prevents the degradation of p53 by MDM2. More importantly, they found that inactivation of SETDB1 in HCC cell lines harboring R249S mutation suppresses cell growth, suggesting that *TP53* mutational status renders cancer cells dependent on SETDB1 activity. While reliance on the tumor suppressive capacity of p53 is profoundly emphasized by its near universal malfunction in all cancers and *TP53* is the most altered gene in cancer, accumulating evidence indicates that many mutant p53 isoforms can exert additional oncogenic activity by a gain-of-function mechanism [92–94]. With the recent observation that 19% of melanoma tumor harbor mutations in the *TP53* gene, the interplay between SETDB1 and p53 may suggest a therapeutic strategy for targeting melanoma patients with *TP53* mutations [54].

### 5.3. JARID1B

One of the main characteristics of melanoma is intratumor heterogeneity, as different subpopulations of cancer cells are found across patient samples. JARID1B (KDM5B) is a member of the highly conserved family of Jumonji proteins and is responsible for demethylation of methylated lysine 4 of histone H3 [95]. While highly expressed in benign nevi, JARID1B expression is restricted to ~5–10% of the total cell population in aggressive and metastatic melanomas [96]. Notably, even within highly proliferative melanomas, a JARID1B-positive subpopulation was present in a slow-cycling state [97]. Although not required for tumor initiation, elegant studies by Roesch et al. have demonstrated that JARID1B is required for the continuous tumor growth. While knockdown of JARID1B induces an initial burst of tumor growth, this is quickly exhausted [97]. Follow-on studies have shown that the JARID1B-positive subpopulation is intrinsically resistant to chemo- and targeted therapies [98]. While self-renewal and drug resistance are characteristics of stem-like cells, expression of JARID1B does not follow the classical hierarchical cancer stem cell model [97]. Overall, these studies provide valuable insight regarding the mechanisms of tumor heterogeneity and suggest the possibility of targeting JARID1B as a strategy for overcoming drug resistance in melanoma.

### 5.4. JMJD3

JMJD3 is a histone demethylase that is responsible for the removal of the trimethyl group from the H3K27 [99]. Several studies have shown that JMJD3 may play a dual role in human cancers, functioning as either a tumor suppressor or an oncogene depending on the cell type and

cellular context [100–104]. Recently, an oncogenic role for JMJD3 was elucidated in melanoma [105]. Contrary to previous reports showing an antiproliferative effect of JMJD3 in different cancer types, JMJD3 promoted melanoma tumor growth and metastasis by modulating intrinsic cellular properties as well as the tumor microenvironment through PI3K signaling. Importantly, JMJD3 activity in melanoma cells was responsible for the transcriptional activation of target genes in the NF-κB and BMP signaling pathways [105]. Although additional work will need to be done to fully understand the mechanisms by which JMJD3-mediated modulation of H3K27 methylation promotes melanoma, this study provides the initial evidence linking epigenetic regulation by JMJD3 to melanoma progression and metastasis.

### 5.5. RNF2

Ubiquitination of histone tails is emerging as an important epigenetic modification in cancer. RNF2, an E3 ubiquitin ligase, is a core component of the polycomb repressive complex 1 (PRC1). In the context of PRC1, RNF2 promotes gene silencing by monoubiquitinating lysine 119 on histone H2A (H2AK119ub) [106]. RNF2 is overexpressed in multiple cancers and it is also part of an 18 gene signature that correlates with melanoma invasion [107]. Through a series of genetic studies in mouse and human systems, Rai et al. recently demonstrated that RNF2 plays a dual role in melanoma progression, regulating both tumorigenic and invasive potential [108]. Importantly, the proinvasive function of RNF2 was shown to require its E3 ligase activity, while its ability to promote tumor growth was independent of this catalytic function [108]. The TGF-β pathway is a key regulator of cancer cell invasion and metastasis. RNF2 potentiates TGF-β signaling by monoubiquitinating H2AK119 at the promoter of the LTBP2 gene, leading to the transcriptional repression of this negative regulator of the TGF-β pathway [108]. Given that the vast majority of melanoma deaths stem from metastatic disease, this mechanistic insight may provide an opportunity for future therapeutic intervention with catalytic inhibitors of RNF2. Interestingly, the noncatalytic function of RNF2 also provides important insight to potential therapies. MEK1-dependent phosphorylation of RNF2 leads to the formation of alternative complexes containing the histone demethylase KDM6A and the histone acetyltransferase EP300, which activate downstream target genes such as *CCND2* [108].This suggests the possibility of using RNF2 inhibitors in combination with either MEK or EP300 inhibitors to target both tumor growth and metastasis [109].

### 5.6. BRD4

In addition to the numerous chromatin modifying proteins and complexes, epigenetic readers also constitute key components of the mechanisms by which gene expression is regulated. First identified in members of the SWI/SNF and mediator complexes [110–112], bromodomains are found in many transcription factors and developmental regulators that control gene expression through histone modification and chromatin remodeling [113–115]. In addition to playing important roles in cell-cycle control during normal development, several bromodomain-containing proteins have also been implicated in cancer [116, 117]. The bromodomain and extraterminal (BET) protein family member BRD4 has gained considerable attention due to its aberrant activity in multiple cancer indications and its ability

to drive expression of key oncogenes, such as *MYC* and *BCL2* [118]. BRD4 was found to be amplified or overexpressed in melanoma cell lines and primary tumors, and stable gene knockdown caused a significant reduction in tumor growth with significant impact on key cell-cycle genes [119]. In agreement with this genetic data, pharmacological inhibition of BRD4 has been shown to impair melanoma cell growth both *in vitro* and *in vivo*, further validating this epigenetic reader as a cancer target in this disease [119–121]. In addition, BET bromodomain inhibitors have been shown to selectively inhibit uveal melanoma cells harboring *Gnaq/11* mutations in a Myc-independent manner, suggesting a precision medicine strategy for therapeutic intervention [122]. Melanomas are also characterized by loss-of-function mutations in the gene encoding the Ras GTPase-activating protein *NF1* [123]. It was recently reported that the combined loss of *NF1* and the PRC2 component *SUZ12* amplifies RAS-driven transcription, and that this effect is mediated by BRD4 recruitment to H3K27Ac at downstream target genes [123]. This study also highlighted the therapeutic potential of simultaneously targeting BRD4 and the MAPK pathway.

### 5.7. Synthetic lethal strategies

SWI/SNF is evolutionarily conserved, ATP-dependent chromatin remodeling complex. Recent genome-wide sequencing approaches have revealed that subunits of SWI/SNF are recurrently mutated across many human cancers, including melanoma [32, 124, 125]. Although the roles of SWI/SNF complex in cancer are still poorly understood, studies indicate that SWI/SNF complexes may be master regulators of genes involved in cellular differentiation and that perturbations in the activity and stoichiometry of this complex promote tumorigenesis [126–128]. In melanoma, loss-of-function mutations have been identified in several genes encoding SWI/SNF components, including *ARID2*, *ARID1A*, *ARID1B*, *SMARCA2*, and *SMARCA4* [23, 54, 55]. Investigation into these deficiencies has begun to reveal tumor-specific referred dependencies that may represent druggable targets. For example, recent studies have identified putative synthetic lethal relationships in *SMARCA4*- and *ARID1A*-deficient tumors, where proliferation of the mutant cells depends on the activity of the closely related paralogs *SMARCA2* and *ARID1B* [129–132]. While similar dependencies have yet to be demonstrated in melanoma tumors harboring SWI/SNF mutations, these observations have at least opened up the possibility of treating melanoma patients with genetically defined mutations in the SWI/SNF complex. Recently, this concept of epigenetic synthetic lethality has been extended to *CBP*/*EP300*-deficient cancers [133]. EP300 and CBP are closely related chromatin modifying proteins facilitating acetylation of lysine residues on histones H3 and H4 [134, 135]. Genome-wide sequencing studies have revealed that multiple types of human cancers, including melanoma, harbor loss-of-function mutations in both *CBP* and *EP300* [136–139]. A recent study has identified EP300 as a key target in *CBP*-deficient cancer cells, describing another example of a paralog targeting strategy that specifically exploits human cancers harboring loss-of-function *CBP* mutations [133]. Given the frequency and mutually exclusive nature of *CBP* and *EP300* mutations in melanoma, the paralog targeting approach may provide an additional opportunity for targeting these epigenetically defined subsets of melanoma patients.

## 6. Clinical strategies for the treatment of melanoma with epigenetic inhibitors

### 6.1. First-generation epigenetic inhibitors: DNA hypomethylating agents and HDAC inhibitors

The initial foray into epigenetic therapy focused on the development of small molecule inhibitors to DNA methyltransferase (DNMT) and histone deacetylase enzymes, and these molecules are being explored as therapeutic modalities in multiple cancer types, including melanoma. However, treatment of solid tumors with these agents continues to be a challenge, with approvals in the clinic being limited to a subset of hematological malignancies [140]. In recent years, treatment paradigms have shifted toward the use of lower, transient doses that favor modulation of DNA and chromatin structure over general cytotoxicities [141]. Treatment strategies in melanoma are currently focused on drug combinations that override resistance mechanisms and potentiate antitumor immune responses (**Table 2**) [24].

| Clinical trial name | Class of epigenetic therapy | Identifier | Phase |
|---|---|---|---|
| Study to Determine Efficacy and Safety of CC-486 with Nab-Paclitaxel in Patients with Chemotherapy Naïve Metastatic Melanoma | DNA Hypomethylating Agent | NCT01933061 | II |
| Treatment of Resistant Disease Using Decitabine Combined with Vemurafenib Plus Cobimetinib | DNA Hypomethylating Agent | NCT01876641 | I/II |
| Combination of Decitabine and Temozolomide in the Treatment of Patients with Metastatic Melanoma | DNA Hypomethylating Agent | NCT00715793 | I/II |
| Parallel Trial of Decitabine and Peg-Interferon in Melanoma: Phase II Portion | DNA Hypomethylating Agent | NCT02605473 | II |
| Parallel Trial of Decitabine and Peg-Interferon in Melanoma: Phase I Portion | DNA Hypomethylating Agent | NCT00791271 | I |
| Azacitidine and Interferon Alfa in Treating Patients with Metastatic Melanoma | DNA Hypomethylating Agent | NCT00398450 | I |
| Phase I/II Trial of Valproic Acid and Karenitecin for Melanoma | DNA Hypomethylating Agent | NCT00358319 | I/II |
| Azacitidine and Recombinant Interferon Alfa-2B in Treating Patients with Stage III or IV Melanoma | DNA Hypomethylating Agent | NCT00217542 | I |
| Decitabine in Treating Patients with Melanoma or Other Advanced Cancer | DNA Hypomethylating Agent | NCT00002980 | I |
| Treatment of Resistant Metastatic Melanoma using | DNA Hypomethylating | NCT00925132 | I/II |

| Clinical trial name | Class of epigenetic therapy | Identifier | Phase |
|---|---|---|---|
| Decitabine, Temozoomide and Panobinostat | Agent, HDAC inhibitor | | |
| Ph1b/2 Dose Escalation Study of Entinostat with Pembrolizumab in NSCLC with Expansion Cohorts in NSCLC and Melanoma | HDAC Inhibitor | NCT02437136 | I/II |
| Phase I of Histone Deacetylase Inhibitor Panobinostat with Ipilimumab with Unresectable II/IV melanoma | HDAC Inhibitor | NCT02032810 | I |
| Vorinostat in Treating Patients with Metastatic Melanoma of the Eye | HDAC Inhibitor | NCT01587352 | II |
| Proteasome inhibitor NPI-0052 and Vorinostat in Patients with NSCLC, pancreatic cancer, melanoma or lymphoma | HDAC Inhibitor | NCT00667082 | I |
| Vorinostat in Treating Patients with Metastatic or Unresectable Melanoma | HDAC Inhibitor | NCT00121225 | II |
| Safety and Efficacy of a New Chemotherapy Agent to Treat Metastatic Melanoma | HDAC Inhibitor | NCT00185302 | II |
| MS-275 in Treating Patients with Advanced Solid Tumors or Lymphoma | HDAC Inhibitor | NCT00020579 | I |

**Table 2.** Melanoma clinical trials with epigenetic therapies.

### *6.1.1. Overcoming resistance to chemo- and targeted therapies*

As stated earlier, rapid onset of drug resistance is a major impediment to targeted melanoma therapies. The observation that drug resistance is often accompanied by changes in chromatin structure and gene expression suggests the possibility of reversing this process through epigenetic therapy. To this end, increased expression of HDACs has been shown to mediate drug resistance in melanoma, and acquired resistance to vemurafenib in *BRAF*-mutant melanoma cells can be overcome when this agent is used in combination with HDAC inhibitors [142]. Epigenetic therapies are also being explored as a strategy for overcoming resistance to chemotherapy. For example, sequential treatment with the DNA hypomethylating agent decitabine and the HDAC inhibitor panobinostat is currently being explored in combination with temozolomide, a DNA alkylating agent, in metastatic melanoma (**Table 2**) [143]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has shown promise in melanoma clinical trials; however, its utility has been limited by intrinsic and acquired resistance. The combination of TRAIL and the HDAC inhibitor entinostat was shown to override TRAIL resistance and induce cell death [144]. Resistance to interferon-based immunotherapy has been postulated to result from epigenetic silencing of interferon response genes. Treatment of melanoma cell lines with DNMT inhibitors has been shown to upregulate interferon response

genes, such as *DR4* and *XAF1*, and augment the antiproliferative effects of interferon-alpha and interferon-beta [145, 146]. These examples highlight the potential use of epigenetic therapy to overcome resistance mechanisms that limit the efficacy of current melanoma therapies.

#### *6.1.2. Combinations with immunotherapy*

Durable survival benefits have been achieved in melanoma patients treated with immune checkpoint inhibitors [18–20]. Data in extensively treated patients with advanced non–small cell lung cancer suggests that this effect can be enhanced by combined epigenetic therapy. In this study, a subset of patients pretreated with a low-dose combination of the DNMT inhibitor 5-azacytidine and the HDAC inhibitor entinostat had major objective responses following subsequent treatment with immune checkpoint inhibitors [147]. Further mechanistic studies suggest that these agents may prime cancer cells to immunotherapy treatment by modulating immune response pathways [148, 149]. Intriguingly, this effect does not appear to be mediated through direct regulation of immune response genes, but rather by the upregulation of endogenous retroviruses that trigger viral immune response pathways [150, 151]. This observation has led to the hypothesis that epigenetic therapy activates innate immune response pathways by inducing a state of viral mimicry. In support of this premise, a retrospective analysis of RNA-seq data from melanoma patients treated with the immune checkpoint inhibitor anti-CTLA-4 revealed high levels of a viral defense signature in patients that correlated with long-term therapeutic benefit. Moreover, the authors went on to demonstrate that treatment with low-dose 5-azacytidine potentiates the antitumor activity of anti-CTLA-4 antibodies in a mouse melanoma model [150]. Epigenetic mechanisms also promote immune evasion by downregulating the expression of cell surface receptors and antigens required for immune recognition. Treatment of melanoma cell lines with DNMT and HDAC inhibitors induces the expression of tumor-associated antigens, costimulatory molecules, and MHC class I molecules, which unmask the tumor cell to allow T-cell-mediated responses [152–154]. In addition, epigenetic therapy has been shown to alter immunogenicity in melanoma cells by upregulating the expression of PD-L1, the ligand for the immune checkpoint molecule PD-1. In immunocompetent mice, the combination of HDAC inhibitor and PD-1 blockade inhibited tumor growth and significantly improved survival [155]. Taken together, these data highlight the potential for first-generation epigenetic drugs to augment the activity of immunotherapies in melanoma patients.

### 6.2. The emergence and promise of second-generation epigenetic therapies

The next generation of targeted epigenetic therapies has recently made its way into the clinic. In general, these agents are more selective than their first-generation counterparts and target a broader range of epigenetic mechanisms. Small molecule inhibitors of EZH2 are currently in phase I/II trials for the treatment of genetically defined tumors harboring mutations in *EZH2*, *INI1*, and *SMARCA4* [156]. The first of these agents, EPZ-6438, has demonstrated robust signs of clinical activity that extend beyond the original precision medicine hypothesis [157]. Preclinical data in both *EZH2* mutant and wild-type melanoma models demonstrating that EZH2 inhibitors negatively impact tumor growth and metastasis suggests that EZH2 inhibition

will also be a promising strategy for treating melanoma patients [77, 79]. In addition to potential single agent studies, several lines of evidence support the pursuit of combination trials with current therapies. Recent studies in mice have uncovered a role for EZH2 in the maintenance of T regulatory (Treg) cell identity during cellular activation [158, 159]. In addition, EZH2 promotes immune evasion and suppression by directly repressing the expression of chemokines and cell surface antigens [75, 160, 161]. In both of these cases, EZH2 inhibition is likely to revert this immunosuppressive environment, rendering the tumor susceptible to immunotherapies. There is also the potential for using EZH2 inhibitors in combination with DNA methyltransferase inhibitors and/or HDAC inhibitors as these agents have demonstrated the ability to upregulate immune response pathways and synergize with immune checkpoint blockade therapy in non–small cell lung cancer [147, 148].

BET inhibitors modulate gene expression by disrupting the interaction between BET family bromodomains and acetylated lysine residues. Several BET inhibitors are currently being evaluated in the phase I/II clinical trials in multiple indications, and clinical proof of concept has recently been reported for OTX015/MK-8628 in patients with NUT midline carcinomas harboring the oncogenic BRD4-NUT translocation [162, 163]. This data highlights the potential for clinical application in additional indications. Treatment of melanoma cell lines with BET inhibitors leads to the rapid downregulation of cell-cycle genes and induces robust antiproliferative effects *in vitro* and *in vivo* [119, 120]. In addition, the combination of BET and HDAC inhibitors synergistically induces Bim-dependent apoptosis in melanoma cell lines and downregulates components of the AKT and YAP signaling pathways [164]. Most recently, important data has emerged around the role of BRD4 in drug resistance. In breast cancer cells that are intrinsically resistant to PI3K inhibitors, BRD4 participates in activation of upstream receptor tyrosine kinases to induce a feedback activation loop. The combination of the BET inhibitor JQ1 and the PI3K inhibitor GDC-0941 was able to overcome this resistance mechanism and inhibit tumor growth in multiple cancer models, suggesting a broad role for BRD4 in drug resistance [165]. Along these lines, it was also discovered that drug tolerant leukemia cells require BRD4 to maintain expression of proliferative and antiapoptotic genes, such as *MYC* and *BCL2* [166]. Treatment of this drug-resistant population with the BET inhibitor JQ1 resulted in downregulation of BRD4 target genes and induction of apoptosis. As stated earlier, the primary limitation of targeted therapies in melanoma is the rapid onset of acquired resistance. Given the emerging roles of BRD4 in this process, it is tempting to think that BET inhibitors could be used as a therapeutic strategy to overcome MAPK pathway-mediated drug resistance in melanoma patients. In addition, the ability of BET bromodomain inhibitors to modulate differentiation status and inflammatory functions of T cells warrants further investigation of their potential use in combination with targeted immunotherapies [167, 168].

While histone methyltransferase and BET bromodomain inhibitors continue to be evaluated in the clinic, there is increasing emphasis on the discovery of pharmacological inhibitors targeting other classes of epigenetic enzymes and chromatin regulators [169]. Catalytic inhibitors of the demethylases JMJD3 and JARID1B and the ubiquitin ligase RNF2 have been reported in the literature, and further optimization and preclinical evaluation in cancer models are underway [104, 170, 171]. In addition, potent inhibitors of bromodomains outside the well-

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characterized BET family have also recently been identified. These novel molecules are being pursued as alternative approaches to targeting epigenetic enzymes whose catalytic domains have been historically difficult to drug, such as the histone acetyltransferases and ATPase/helicases [172, 173]. The continued development of second-generation epigenetic therapies and the exploration of their use as single agents as well as in combination with targeted and immunotherapies is likely to have a significant impact on future treatment options for patients with advanced or metastatic melanoma.

## 7. Conclusions

It is becoming increasingly clear that epigenetic reprogramming is a hallmark of melanoma. In addition to changes in DNA methylation and histone acetylation, the advent of genome-wide whole-exome sequencing from patient samples has revealed a high incidence of genetic alterations in genes from key families of epigenetic regulators. The identification of gene amplifications and activating mutations, in addition to gene deletions and inactivating mutations, indicates that an individual epigenetic regulator may play either an oncogenic or tumor suppressive role depending on the genetic background or stage of the disease. Importantly, further interrogation in human and mouse cancer models has led to the identification of several proteins that appear to play important roles in melanoma growth, metastasis, and stem cell renewal, suggesting that they may be bona fide cancer targets. DNA hypomethylating agents and histone deacetylase inhibitors, the first generation of epigenetic therapies, continue to be evaluated in clinical trials, primarily in combination with chemo-, targeted, and immunotherapies. The second generation of epigenetic inhibitors are highly selective and target novel epigenetic mechanisms that regulate multiple facets of cancer biology, including cell proliferation, cell migration, metastasis, stem cell renewal, drug resistance, and immune regulation. While it remains to be seen if these epigenetic targets are oncogenic drivers in the strict sense, they may cooperate with other oncogenes (for example, *BRAF*) to fine tune the transcriptional landscape in melanoma cells to promote tumorigenesis, confer drug resistance and evade immune responses. As they continue to make their way into the clinic, this next generation of novel epigenetic therapies will provide opportunities for multiple levels of therapeutic intervention for melanoma patients. Moreover, further exploration of the evolving melanoma landscape will continue to uncover novel epigenetic mechanisms and guide the future generations of epigenetic therapy.

## Author details

Robert A. Rollins*, Kimberly H. Kim and Cheng-Chung Tsao

*Address all correspondence to: robert.rollins@pfizer.com

Pfizer, Inc, Oncology Research & Development, Pearl River, New York, USA

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