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# Epigenetic regulation in human melanoma: past and future

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**Abbreviations:** 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; α-MSHm, α-melanocyte stimulating hormone; ACE, angiotensin converting enzyme; *ANCR*, anti-differentiation non-coding RNA; *ANRIL*, antisense noncoding RNA in *INK4* locus; ASK1, apoptosis signal-regulating kinase 1; ATRA, all-trans retinoic acid; *BANCR*, BRAF-activated non-coding RNA; BCL-2, B-cell lymphoma 2; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; BRG1, ATP-dependent helicase SMARCA4; CAF-1, chromatin assembly factor-1; CBX7, chromobox homolog 7; *CCND1*, cyclin D1; Cdc6, cell division cycle 6; CD28, cluster of differentiation 28; CDK, cyclin-dependent kinase; *CDKN2A/B*, cyclin-dependent kinase inhibitor 2A/B; ceRNA, competitive endogenous RNAs; CHD8, chromodomain-helicase DNA-binding protein 8; CREB, cAMP response element-binding protein; *CUDR*, cancer upregulated drug resistant; DNMT, DNA methyltransferase; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; EZH2, enhancer of zeste homolog 2; GPCRs, G-protein coupled receptors; GSK3a, glycogen synthase kinase 3 α; GWAS, genome-wide association study; HDAC, histone deacetylase; *HOTAIR*, HOX antisense intergenic RNA; IAP, inhibitor of apoptosis; IFN, interferon, interleukin 23; IDH2, isocitrate dehydrogenase; Jak/STAT, Janus kinase/signal transducer and activator of transcription; JNK, Jun N-terminal kinase; lncRNA, long ncRNA; MAFG, v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog G; *MALAT1*, metastasis-associated lung adenocarcinoma transcript 1; MAPK, mitogen-activated protein kinase; MC1R, melanocortin-1 receptor; MeCP2, methyl CpG binding protein 2; MGMT, $O^6$-methylguanine-DNA methyltransferase; MIF, macrophage migration inhibitory factor; MITF, microphthalmia-associated transcription factor; miRNA, micro RNA; ncRNA, non-coding RNA; MRE, miRNA recognition element; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NOD, nucleotide-binding and oligomerization domain; $p14^{ARF}$, p14 alternative reading frame; $p16^{INK4a}$, p16 inhibitor of CDK4; PBX, pre-B-cell leukemia homeobox; PEDF, pigment epithelium derived factor; PI3K, phosphatidylinositol-4, 5-bisphosphate 3-kinase; *PIB5PA*, phosphatidylinositol-4, 5-biphosphate 5-phosphatase A; PKA, protein kinase A; pRB, retinoblastoma protein; PRC, polycomb repressor complex; PSF, PTB associated splicing factor; PTB, polypyrimidine tract-binding; PTEN, phosphatase and tensin homolog; *RARB*, retinoic acid receptor-β2; *RASSF1A*, Ras association domain family 1A; SETDB1, SET Domain, bifurcated 1; snoRNA, small nucleolar RNA; SPRY4, Sprouty 4; STAU1, Staufen1; *SWI/SNF*, SWItch/Sucrose Non-Fermentable; TCR, T-cell receptor; *TET*, ten eleven translocase; *TGF* β, transforming growth factor β; *TINCR*, tissue differentiation-inducing non-protein coding RNA; *TOR*, target of rapamycin; TP53, tumor protein *53; TRAF6*, *TNF* receptor-associated factor *6; UCA1,* urothelial carcinoma-associated 1

The development and progression of melanoma have been attributed to independent or combined genetic and epigenetic events. There has been remarkable progress in understanding melanoma pathogenesis in terms of genetic alterations. However, recent studies have revealed a complex involvement of epigenetic mechanisms in the regulation of gene expression, including methylation, chromatin modification and remodeling, and the diverse activities of non-coding RNAs. The roles of gene methylation and miRNAs have been relatively well studied in melanoma, but other studies have shown that changes in chromatin status and in the differential expression of long non-coding RNAs can lead to altered regulation of key genes. Taken together, they affect the functioning of signaling pathways that influence each other, intersect, and form networks in which local perturbations disturb the activity of the whole system. Here, we focus on how epigenetic events intertwine with these pathways and contribute to the molecular pathogenesis of melanoma.

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## Introduction

Melanoma, a malignant tumor of melanocytes, is considered to be the most aggressive of all skin cancers.[1] The genesis and progression of melanoma arise from complex changes in multiple signaling pathways that control cell proliferation and the ability to evade cell death processes. Aberrant behavior of key signaling pathways, such as RAS/RAF/MAPK, JNK, PI3K/Akt, Jak/STAT, and MITF (**Fig. 1A and B**), can affect cell cycle

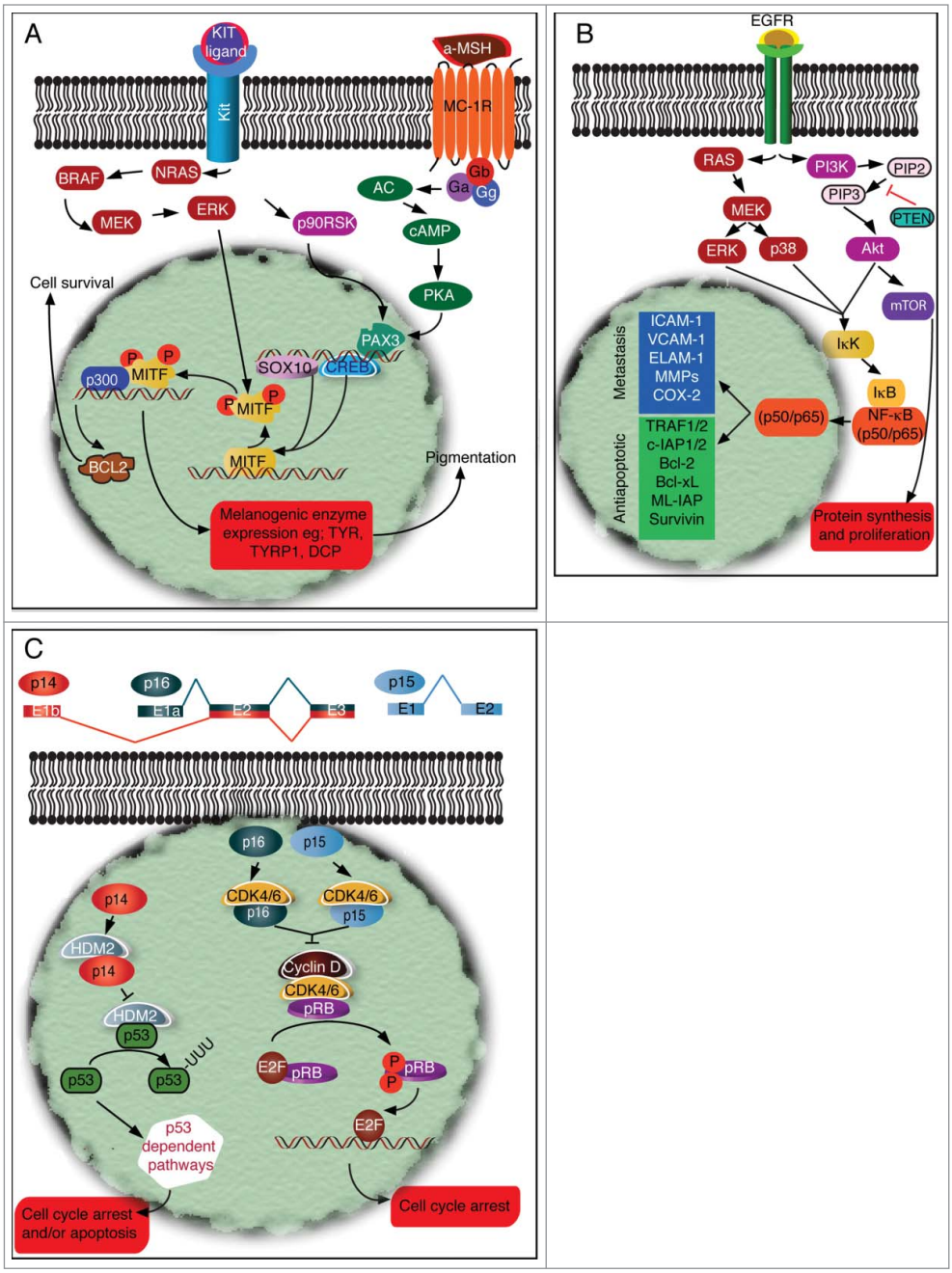


**Figure 1.** Schematic of pathways that play important roles in melanocyte and melanoma development. (**A**) Schematic of melanocyte differentiation through the MITF axis. KIT receptor and kit ligand are essential for melanocyte development. NRAS, BRAF and MITF are activated by the KIT receptor. The expression of the MITF transcription factor is regulated by α-MSH that binds to MC1R. MITF is phosphorylated by ERK. Activation of MITF controls expression of genes that help regulate melanocyte proliferation, differentiation, pigmentation and survival. Mutant *MITF*, *NRAS*, *BRAF* and *KIT* are known melanoma oncogenes. (**B**) Schematic of the EGFR signaling pathway. Signaling is activated by a ligand binding to EGFR receptor that leads to its dimerization. Downstream pathways through RAS and PI3K are activated. RAS signaling occurs via MEK, ERK and p38; PI3K via PIP3 and AKT. Both pathways regulate cellular functions such as metastasis and apoptosis which are vital for melanoma progression. Mutations in *EGFR*, *RAS*, *RAF*, *PTEN* and *PI3K* occur in melanoma. (**C**) Diagram showing the *CDKN2A/B* locus and its signaling pathway. The top panel illustrates the genomic organization of the *CDKN2A/B* locus. *CDKN2A* encodes for 2 proteins, $p14^{ARF}$ and $p16^{INK4a}$, which have identical DNA sequence in exons 2 and 3, while their first exons (E1a and E1b) are different. These proteins have different open reading frames and act in separate pathways. *CDKN2B* is located upstream of *CDKN2A* and encodes p15. $p16^{INK4a}$ and p15 are inhibitors of CDK4 and CDK6, which phosphorylate pRB, leading to progression from G1 to S phase. $p14^{ARF}$ acts as an inhibitor for HDM2 which regulates p53. The suppression of $p16^{INK4A}$ at this locus is the most common event reported in melanoma.

progression and apoptosis control, contributing eventually to the development of melanoma.[2] Causes of aberrant behavior include alteration of DNA sequence (genetic) and alteration of gene expression (epigenetic regulation). The development of effective treatment options, as well as improved diagnosis and prognosis, therefore requires greater understanding of the genetic and epigenetic changes that underlie melanoma development.

Genetic predisposition is a known risk factor associated with melanoma and accounts for 10% of melanoma cases.[3] *CDKN2A* located at chromosome 9p was the first gene locus linked to familial melanoma and codes for 2 tumor suppressor proteins, $p14^{ARF}$ and $p16^{INK4A}$.[4] $p14^{ARF}$ restricts cell proliferation through stabilization of p53, which in turn induces cyclin-dependent kinase inhibitor p21. $p16^{INK4A}$, on the other hand, controls cell proliferation by inhibiting the association of cyclin-dependent kinases 4 and 6 (CDK4/6) and cyclin D1 (CCND1).[4] *CDKN2A* mutations are the most frequent genetic events underlying familial melanoma susceptibility and have been reported in the germline of 8% to 57% of familial melanoma cases (reviewed in[5]). In addition to familial disposition, somatic mutations in key genes pose as considerable risk factors for melanoma.[5] *BRAF* is the gene most frequently mutated (50–70%) in melanoma, as demonstrated by genome wide-sequencing programs, with $BRAF^{V600E}$ being the most common mutation and generally found in benign nevi, which represent a precursor in melanomagenesis.[6]

In addition to the several well-documented gene mutations that have been associated with development of melanoma,[7] considerable attention is being focused on

the participation of epigenetic events. The interplay between epigenetic events affects the regulation of transcriptional and/or translational activities. The epigenetic events involved in initiation and progression of melanoma may be aberrant methylation of the promoter regions, histone modification, chromatin remodeling, and the positioning of nucleosomes.[8]

Additional epigenetic phenomena described more recently involve regulation of gene expression by non-coding RNAs (ncRNAs).[9] ncRNAs (small and long) are a new class of regulatory molecules, the differential expression of which is associated with normal physiological and diseased conditions, including cancer.[10] These ncRNAs are therefore suspected to play crucial roles in the pathogenesis of melanoma as well.

This review will focus on how these epigenetic events either act as triggers to initiate melanoma or promote further progression of the disease.

## Emergence of Melanoma

**Figure 1A** summarizes the normal pathways involved in melanogenesis. In response to UV exposure, melanocytes initiate melanogenesis, which is primarily regulated by microphthalmia-associated transcription factor (MITF). G-protein coupled receptors (GPCRs), which include the melanocortin-1 receptor (MC1R), play a crucial role in melanocyte development, proliferation, and differentiation. Activation of the MC1R by the α-melanocyte stimulating hormone (α-MSH) leads to the activation of the cAMP signaling pathway and of *MITF* expression, which in turn promotes differentiation and increases the transcription of genes underlying melanin synthesis.[11] MITF contributes to melanocyte survival by increasing the expression of *BCL-2*, a key antiapoptotic factor.[12]

Intermittent intense UV exposure is considered to be an important etiological factor for melanoma. Recently, 2 studies reported that UV exposure aids in metastatic progression through alternative pathways. The first pathway involves an inflammatory response induced by keratinocyte damage. UV-induced neutrophil activity stimulated angiogenesis and promoted the ability of melanoma cells to migrate toward the endothelial cells.[13] A second pathway acts through $BRAF^{V600E}$, which is not a UV signature mutation, but $BRAF^{V600E}$-expressing melanocytes are susceptible to melanomagenesis through UV-induced mutation of *TP53*, a tumor suppressor gene.[14]

## Epigenetic Events Involved in the Development of Melanoma

Epigenetic changes, as mentioned earlier, include the aberrant methylation of DNA at cytosine (5mC), 5-hydroxymethylcytosine (5hmC), histone modifications, ncRNA expression, chromatin remodeling, and nucleosome positioning.[15] Of these, aberrant DNA methylation and histone modifications have been most intensively studied.[16] Characterization of epigenetic changes that initiate and promote human melanoma development may identify biomarkers that could be used for prevention, early detection, treatment, and monitoring of the progression of this malignancy.[17]

## DNA Methylation

DNA hypermethylation of CpG islands at promoter sites is believed to contribute to tumorigenesis through transcriptional silencing of tumor suppressor genes.[17] Hypermethylation of specific tumor suppressor genes, including those involved in cell cycle regulation, cell signaling, transcription, DNA repair, and apoptosis, has been consistently reported in melanoma (**Table 1**).[18,19] More recent studies have shown the methylation of gene bodies, and suggested that this correlates positively with transcription.[20] Despite our expanding knowledge of DNA methylation, future studies investigating the mechanisms involved in gene regulation in promoter regions as well as in gene bodies remain priorities for melanoma research.

Analysis of melanoma cell lines by gene expression microarrays has identified a large cohort of hypermethylated genes.[17] However, how the hypermethylated status of these genes contributes to the pathogenesis of melanoma remains largely unknown. Though gene transfer and RNA interference techniques are being employed to understand the roles of these genes,[18] no study to date has been able to establish a direct relation between the hypermethylated status of these genes and development of melanoma.

The effects of gene hypomethylation have been less studied but the phenomenon is common (**Table 2**). Lian et al.[21] have shown that 5mC is converted to 5hmC by the ten eleven translocase (TET) family of dioxygenase enzymes in melanoma, and they functionally characterized this novel epigenetic marker and its impact on melanoma progression. A high level of 5hmC was identified as a distinctive epigenetic signature for melanocytes and nevi, whereas its abundance decreases in primary and metastatic melanoma. This pattern suggested that loss of 5hmC in melanoma could be used as a diagnostic or prognostic marker in patients. Downregulation of TET-family enzymes, with the most dramatic decrease in TET2, was detected in melanoma as compared to nevi.[21,22] 5hmC is the most abundant intermediate of active DNA demethylation and acts as a positive transcriptional regulator in normal development and cancer. The study of molecular mechanisms underlying the global loss of 5hmC through altered TET family and isocitrate dehydrogenase (IDH2) activities remains to be unraveled in melanoma.

Described below are some of the most frequently reported and best characterized hypermethylated genes.

### *RAR*-β2 (retinoic acid receptor-β2)

In malignant melanoma the frequencies of aberrant methylation and loss of expression of *RAR*-β2 (*RARB*) have been reported to be as high as 70%.[23] The product of this tumor suppressor gene mediates growth inhibition by *all-trans* retinoic acid (ATRA).[24] *RARB* is suppressed also in various other

**Table 1.** List of genes hypermethylated in melanoma

| Gene | Gene Description | Relevance to melanoma | Ref |
|---|---|---|---|
| *APC*[*] | Adenomatous Polyposis Coli | Reduced expression increases cell proliferation without compromising invasive capacity | 104 |
| *ASC/PYCARD*[*] | PYD, an N terminal PYRIN-domain, and CARD, a C-terminal caspase-recruitment domain | Expression inhibits tumorigenesis by reducing IKKα/β phosphorylation and inhibiting NF-κB activity | 105 |
| *AS3MT* | Arsenic (+3 Oxidation State) Methyltransferase | Unknown | 19 |
| *ADCY4* | Adenylate Cyclase 4 | Unknown | 19 |
| *AKR7L* | Aldo-Keto Reductase Family 7-Like | Unknown | 19 |
| *AK3* | Adenylate Kinase 3 | Unknown | 19 |
| *BRF1* | BRF1, RNA Polymerase III Transcription Initiation Factor 90 KDa subunit | Unknown | 19 |
| *BST2* | Bone Marrow Stromal Cell Antigen 2 | Unknown | 106 |
| *COL11A1*[#] | Collagen, Type XI, Alpha 1 | Promotes tumor aggressiveness via TGF-β1-MMP3; part of a 12 gene signature for melanoma diagnosis; associated with focal adhesion | 19 |
| *CMTM2* | CKLF-Like MARVEL Transmembrane Domain Containing 2 | Unknown | 19 |
| *CCKBR* | Cholecystokinin B Receptor | Unknown | 19 |
| *Caspase 8*[*] | Apoptosis-Related Cysteine Peptidase | Linked to cadmium-stimulated cell growth and inhibition of cell death pathways | 107 |
| *CDH1*[*] | E-Cadherin | A cell adhesion molecule; loss correlates with high tumor grade and poor prognosis | 108<br>109 |
| *CDKN2A*[*] | p16 | Arrests cell cycle in G1 by inhibiting CDK4 and CKD6 and activating pRB | 110 |
| *CDKN2B* | p15 | Unknown | 111 |
| *CDKN1C*[#] | p57 | Arrests cell cycle in G1 by inhibiting G1 cyclin-CDK complexes; expressed in proliferative melanocytes; possible role in melanomagenesis | 106<br>112 |
| *CDH8* | Cadherin 8 | Unknown | 106 |
| *CIITA-PIV* | Class II, Major Histocompatibility Complex Transactivator, Promoter IV | Acts on IFNγ pathway | 109 |
| *COL1A2*[#] | Collagen, Type I, Alpha 2 | Loss may compromise tissue integrity | 106<br>19 |
| *CYP1B1* | Cytochrome P450, Family 1, Subfamily B, Polypeptide 1 | Unknown | 106 |
| *CXCR4* | Chemokine (C-X-C motif) Receptor 4 | Unknown | 113 |
| *DLL3* | Delta-Like 3 | Unknown | 19 |
| *DDIT4L*[#] | DNA-Damage-Inducible Transcript 4-like | Loss results in depression of cell growth | 19 |
| *DAL1* | Erythrocyte Membrane Protein Band 4.1-like 3 | Unknown | 106 |
| *DAPK*[#] | Death Associated Protein Kinase | Methylation higher in metastases | 23 |
| *DNAJC15* | DNAJ (Hsp40) Homolog, Subfamily C, Member 15 | Unknown | 106 |
| *DPPIV*[#] | DiPeptidyl Peptidase IV | Serine protease involved in cancer progression; decline in serum activity in melanoma patients compared to controls | 114<br>115 |
| *FRZB*[*] | Frizzled-Related Protein | A metastasis suppressor; inhibits Wnt5a signaling | 116,117 |
| *GDF15* | Growth Differentiation factor 15 | Unknown | 106 |
| *GATA4* | GATA Binding Protein 4 | Unknown | 26 |
| *GPX7* | Glutathione Peroxidase 7 | Unknown | 19 |
| *HOXB13* | Homeobox B13 | Unknown | 106 |
| *HSP11* | Heat Shock protein H11 | Unknown | 118 |
| *HMW-MAA* | Human High Molecular Weight Melanoma Associated Antigen | Unknown | 119 |
| *HLA-DOA* | Major Histocompatibility Complex, Class II, DO Alpha | Unknown | 19 |
| *HSPB6* | Heat Shock Protein, Alpha-Crystallin-Related, B6 | Unknown | 19 |
| *HPSE2* | Heparanase 2 | Unknown | 19 |
| *HOXA7* | Homeobox A7 | Unknown | 19 |
| *ISG15* | ISG15 Ubiquitin-Like Modifier | Unknown | 19 |
| *IL34* | Interleukin 34 | Unknown | 19 |
| *IGFBP4* | Insulin-Like Growth Factor Binding Protein 4 | Unknown | 19 |
| *KCNK4* | Potassium Channel, Subfamily K, Member 4 | Unknown | 116 |
| *KCNK6* | Potassium Channel Subfamily K Member 6 | Unknown | 19 |
| *LOX* | Lysyl Oxidase | Unknown | 109 |
| *LRRC1* | Leucine Rich Repeat Containing 1 | Unknown | 106 |

(*Continued on next page*)

**Table 1.** List of genes hypermethylated in melanoma *(Continued)*

| Gene | Gene Description | Relevance to melanoma | Ref |
|---|---|---|---|
| *LXN*[*] | Latexin | Inhibition of cell proliferation; alters stem cell-like properties of melanoma cells | 106 120 |
| *LYNX1* | Ly6/Neurotoxin 1 | Unknown | 19 |
| *MFAP2* | Microfibrillar-Associated Protein 2 | Unknown | 106 |
| *MGMT*[#] | O-6-Methylguanine-DNA Methyltransferase | Repairs damage caused by Temozolomide; renders cancer cells resistant | 108 23 121 |
| *MINT 17* | Methylated-in-Tumor 17 | Unknown | 26 |
| *MINT 31* | Methylated-in-Tumor 31 | Unknown | 26 |
| *MT1G* | Metallothionein 1G | Unknown | 19 |
| *MTSS1L* | Metastasis Suppressor 1-Like | Unknown | 19 |
| *MIB2* | Mindbomb E3 Ubiquitin Protein Ligase 2 | Unknown | 122 |
| *NPM2* | Nucleophosmin/Nucleoplasmin 2 | Unknown | 19 |
| *NAP1L5* | Nucleosome Assembly Protein 1-Like 5 | Unknown | 19 |
| *NELF* | NMDA receptor synaptonuclear signaling and neuronal migration factor | Unknown | 19 |
| *NEFH* | Neurofilament, Heavy Polypeptide | Unknown | 19 |
| *NPR2* | Natriuretic Peptide Receptor 2 | Unknown | 116 |
| *PCSK* | Proprotein Convertase, Subtilisin/Kexin-type | Unknown | 106 |
| *PRDX2* | Peroxiredoxin-2 | Unknown | 29 |
| *PTGS2* | Prostaglandin-Endoperoxidase Synthase 2 | Unknown | 106 |
| *PDE9a* | Phosphodiesterase 9A | Unknown | 19 |
| *PCDHGA9* | Protocadherin Gamma-A9 | Unknown | 19 |
| *PACS2* | Phosphofurin Acidic Cluster Sorting Protein 2 | Unknown | 19 |
| *PCDHGC4* | Protocadherin Gamma-C 4 | Unknown | 19 |
| *QPCT* | Glutaminyl-Peptide Cyclotransferase | Unknown | 106 |
| *RAR-b2*[*] | Retinoic Acid Receptor-b2 | Tumor suppressor gene; mediates growth inhibition by ATRA | 108 23 24 |
| *RASSF1A*[*] | RAS Association Domain Family Member 1 | Upregulates ASK1, which activates p38 MAPK; induces apoptosis via mitochondrial pathway | 108 26 27 |
| *RUNX3*[#] | Runt-Related Transcription Factor 3 | Upregulates TSP-1 expression levels | 108 123 |
| *RIN3* | Ras and Rab Interactor 3 | Unknown | 19 |
| *RAB33A* | Ras-Related Protein Rab-33A | Unknown | 19 |
| *RAB31* | Ras-Related Protein Rab-31 | Unknown | 19 |
| *RASIP1* | Ras-Interacting Protein 1 | Unknown | 19 |
| *RCBTB2* | Regulator Of Chromosome Condensation And BTB Domain-Containing Protein 2 | Unknown | 19 |
| *SOCS1*[*] | Suppression of Cytokine Signaling 1 | Attenuates cytokine-induced effects; blocks G1/S and M phases; associates with CDH1 | 19 26 124 |
| *SOCS2* | Suppression of Cytokine Signaling 2 | Attenuates cytokine-induced effects | 109 |
| *SYK* | Spleen Tyrosine Kinase | Unknown | 106 |
| *SOCS3*[*] | Suppression of Cytokine signaling 3 | Inhibits IL-17/Stat3 pathway; suppresses tumor growth in murine models | 125 126 |
| *SCN4B* | Sodium Channel Subunit Beta-4 | Unknown | 19 |
| *SLC30A2* | Solute Carrier Family 30 Member 2 | Unknown | 19 |
| *SERPINF1* | Serpin Peptidase Inhibitor, Clade F | Unknown | 19 |
| *TERC* | Telomerase RNA Component | Unknown | 108 |
| *TFPI-2* | Tissue Factor Pathway Inhibitor 2 | Unknown | 26 |
| *TNFRSF10C (DcR1)* | Tumor Necrosis Factor Receptor Superfamily, 10C | Decoy receptor that protects cells from TRAIL-mediated apoptosis | 109 |
| *TNFRSF10D (DcR2)* | Tumor Necrosis Factor Receptor Superfamily, 10D | Decoy receptor that protects cells from TRAIL-mediated apoptosis | 109 |
| *TPM1* | Tropomyosin-1 | Control of actin-mediated cell motility | 109 |
| *THBS1*[*] | Thrombospondin-1 | Mediates cell-to-cell and cell-to-matrix interactions important for platelet aggregation and angiogenesis | 127 |
| *TIMP3*[*] | Tissue Inhibitor Of Metalloproteinase 3 | Dominant negative regulator of angiogenesis | 109 128 |

(*Continued on next page*)

**Table 1.** List of genes hypermethylated in melanoma *(Continued)*

| Gene | Gene Description | Relevance to melanoma | Ref |
|---|---|---|---|
| *TM*[#] | Thrombomodulin | Downregulation associated with transformation and progression | 129 |
| *TNK1* | Tyrosine- Kinase Non-Receptor 1 | Unknown | 19 |
| *THRA* | Thyroid Hormone Receptor, Alpha | Unknown | 19 |
| *TRIP6* | Thyroid Hormone Receptor Interactor 6 | Unknown | 16 |
| *VPS18* | Vacuolar Protein Sorting-18 homolog | Unknown | 19 |
| *WIF1*[*] | WNT Inhibitory Factor | Wnt pathway antagonist implicated in cellular proliferation | 26 |
| *WFDC1* | WAP Four-Disulfide Core Domain 1 | Unknown | 106 |
| *ZNF132* | Zinc Finger Protein 132 | Unknown | 19 |
| *ZNF154* | Zinc Finger Protein 154 | Unknown | 19 |
| *ZBTB47* | Zinc Finger And BTB Domain Containing 47 | Unknown | 19 |
| *ZFYVE28* | Zinc Finger FYVE Domain-Containing 28 | Unknown | 19 |

*Function validated in melanoma; [#] Function proposed in melanoma.

human cancers.[25] Many melanoma cells are resistant to the anti-proliferative effects of ATRA, and positive correlations between the anti-proliferative activity of ATRA and expression of *RARB* have been confirmed. However, no strict correlation was found between the methylation status of the *RARB* gene and its expression in melanoma cell lines. Hypermethylation of *RARB* was predominantly found in a cell line that was derived from vertical phase melanoma.[24] This study proposed that *RARB* expression was silenced through other mechanisms, such as histone hypoacetylation.[24] This indicates that silencing mechanisms of many genes may switch during the progression of melanoma.

### *RASSF1A*

Ras association domain family 1A (*RASSF1A*) is methylated in 55% of melanoma specimens.[23] The degree of methylation of *RASSF1A* varies with tumor stage as hypermethylated *RASSF1A* is found in stage IV, but not in stage I and II melanoma. This

**Table 2.** List of genes hypomethylated in melanoma

| Gene | Gene Description | Relevance to melanoma | Ref |
|---|---|---|---|
| *CD2* | Cluster of Differentiation 2 | Higher levels related to lower recurrence rate and improved overall survival | 116, 130 |
| *CARD15* | Nucleotide-Binding Oligomerization Domain Containing 2 | Unknown | 116 |
| *COL19A1* | Collagen, Type XIX, Alpha 1 | Unknown | 19 |
| *DDX26B* | DEAD/H (Asp-Glu-Ala- Asp/His) Box Polypeptide 26B | Unknown | 19 |
| *EMR3* | Egf-Like Module-Containing Mucin-Like Hormone Receptor 3 | Unknown | 116 |
| *EVI2A* | Ecotropic Viral Integration site 2A | Unknown | 116 |
| *GAGE 1–6* | G antigen 1–6 | Unknown | 131 |
| *GPR89A* | G Protein-Coupled Receptor 89A | Unknown | 19 |
| *HLA-DP1* | Major Histocompatibility Complex, Class II, DP Alpha 1 | Unknown | 116 |
| *IFNG* | Interferon Gamma | Unknown | 116 |
| *IL2* | Interleukin 2 | High levels linked to better survival | 116, 132 |
| *ITK* | IL2-Inducible T-Cell Kinase | Unknown | 116 |
| *KLK10* | Kallikrein-Related Peptidase | Unknown | 116 |
| *LAT* | Linker for Activation Of T cells | Unknown | 116 |
| *LARP7* | La Ribonucleoprotein Domain Family, Member 7 | Unknown | 19 |
| *MPO* | Myeloperoxidase | Unknown | 116 |
| *MAGE-A1* | Melanoma Antigen Family A, 1 | Unknown | 131 |
| *MAGE-A2* | Melanoma Antigen Family A, 2 | Unknown | 131 |
| *MAGE-A4* | Melanoma Antigen Family A, 4 | Unknown | 131 |
| *MAGE-A6* | Melanoma Antigen Family A, 6 | Unknown | 131 |
| *NY-ESO-1* | New York Esophageal Squamous Cell Carcinoma 1 | Unknown | 133 |
| *NIPBL* | Nipped-B Homolog (Drosophila) | Unknown | 19 |
| *p15* | Cyclin-Dependent Kinase Inhibitor 2B | Unknown | 134 |
| *PRAME* | Preferentially Expressed Antigen In Melanoma | Unknown | 131 |
| *PSCA* | Prostate Stem Cell Antigen | Unknown | 116 |
| *PTHLH* | Parathyroid Hormone-Like Hormone | Unknown | 116 |
| *PTHR1* | Parathyroid Hormone 1 Receptor | Unknown | 116 |
| *POLA1* | Polymerase (DNA Directed), Alpha 1, Catalytic Subunit | Unknown | 19 |
| *SSX 1–5* | Synovial Sarcoma, breakpoint 1–5 | Unknown | 131 |
| *TNFSF8* | Tumor Necrosis Factor (Ligand) Superfamily, Member 8 | Unknown | 116 |
| *TAF1* | TAF1 RNA Polymerase II, TATA Box Binding Protein (TBP)-Associated Factor | Unknown | 19 |

suggests that *RASSF1A* might be used as a marker of progression and prognosis in malignant melanoma.[26] The role of this gene as a human tumor suppressor, and how it contributes to melanoma development, have been elucidated. RASSF1A upregulates ASK1, which in turn activates p38 MAPK. This alters the expression of multiple components of the mitochondrion-dependent apoptosis pathway to induce apoptosis.[27] Silencing of *RASSF1A* expression through promoter methylation contributes to melanoma by suppressing apoptosis.

### *CDKN2A/INK4A/ARF*

One of the most well-studied epigenetic markers implicated in melanoma pathogenesis is hypermethylation of the *INK4A* promoter. The *INK4A* product arrests the cell cycle in G1 phase by inhibiting the cyclin D-dependent kinases CDK4 and CKD6, thereby activating the tumor suppressive effects of the retinoblastoma protein (pRB) (**Fig. 1C**). Hypermethylation of *INK4A*[28] is apparent in 10–20% of vertical phase melanomas and is associated with both increased Ki-67 index and reduced patient survival.[28] Epigenetic silencing of *ARF* through hypermethylation leads to loss of p53-mediated apoptosis and to melanoma progression.[29] van der Velden[30] reported hypermethylation of the *INK4A* promoter in 32% of primary uveal melanomas and 50% of uveal melanoma cell lines, while in many cases *ARF* was not affected.[30] Straume et al.[28] reported loss of p16 protein expression by hypermethylation of the *CDKN2A* promoter in 19% of primary cutaneous melanomas and in 33% of metastases.[28]

### *MGMT*

The gene encoding $O^6$-methylguanine-DNA methyltransferase (*MGMT*) is located at 10q26. Epigenetic inactivation of *MGMT* through promoter hypermethylation has been reported in 34% of melanoma specimens.[23] Primary and metastatic melanoma were compared in order to identify differences in *MGMT* methylation status, but no such differences were found.[31] This could be explained by the finding in various types of cancer that histone 3 lysine 9 (H3K9) dimethylation and MeCP2 binding are common and essential for *MGMT* silencing regardless of DNA methylation status at the promoter CpG island.[32] This emphasizes that functional characterization of hypermethylated genes identified in melanoma is essential and that the methylation status of a gene may not necessarily serve as a suitable marker for tracking the progression of melanoma.

A genome-wide methylation study in $BRAF^{V600E}$-mutant melanoma cells identified numerous functionally important genes that manifest altered methylation and expression. Knockdown of $BRAF^{V600E}$ reduced expression of *DNMT1*. It was proposed that $BRAF^{V600E}$ promotes gene hypermethylation by upregulating DNMT1.[33] A similar study in colorectal cancers confirmed that a $BRAF^{V600E}$-directed pathway was responsible for aberrant CpG island hypermethylation.[34] $BRAF^{V600E}$ promoted transcriptional silencing through increased ERK-directed phosphorylation of the transcriptional repressor MAFG, which reduced its polyubiquitination and proteasomal degradation and increased its binding to DNA. MAFG recruited a co-repressor complex that includes BACH1, CHD8, and DNMT3B, leading to promoter hypermethylation and transcriptional silencing.[36] It is not known whether this $BRAF^{V600E}$-driven CpG island hypermethylation pathway operates in melanoma, but it could explain the association of $BRAF^{V600E}$ and *PTEN* silencing in metastatic melanoma.[35,36]

These studies provide evidence that genetic and epigenetic events are interlinked and contribute to initiation and progression of melanoma.

## Histone Modification

The close association between aberrant DNA methylation and histone modification is well established.[37] Investigation of histone modifications in melanoma, therefore, would facilitate interpretation of the available DNA methylation data. However, the lack of well-established and robust assays has made it difficult to characterize histone modifications.[38] Aberrant acetylation of histones, in particular hypoacetylation, is thought to influence the pathobiology of melanoma by disrupting the same pathways as are affected by mutations and CpG island hypermethylation.[39] In melanoma, gene expression profiles revealed loss of expression of tumor suppressor genes through reversible deacetylation of lysine residues in local histones by histone deacetylases (HDACs).[40] *CDKN1A* is one such tumor suppressor gene, and expression of its product, $p21^{cip1}$, was upregulated following inhibition of histone deacetylase. This indicates that aberrant histone deacetylation leads to loss of tumor suppressor mechanisms in melanoma.

Histone hypoacetylation has also been linked to the downregulation of certain pro-apoptotic proteins like Bim, Bax, and Bak, which belong to the BCL-2 family.[41] A recent study revealed that phosphatidylinositol-4,5-biphosphate 5-phosphatase A (*PIB5PA*) has a tumor suppressive role and is commonly downregulated in melanoma. Its overexpression blocks PI3K/Akt signaling, inhibits proliferation and reduces survival of melanoma cells *in vitro.* Downregulation of *PIB5PA*, found in a proportion of melanomas, was due to histone hypoacetylation mediated by histone deacetylases through binding to the transcription factor Sp1 at the *PIB5PA* gene promoter.[42] HDAC inhibitors are being considered for the therapy of melanoma despite limited data available on posttranslational modifications of histones.[43]

The histone methyltransferase SET Domain, Bifurcated 1 (SETDB1) is upregulated in melanoma and accelerates tumor development in zebrafish melanoma models harboring the $BRAF^{V600E}$ mutation. SETDB1 catalyzes the trimethylation of histone H3K9 and thereby promotes the repression of target genes.[44] Unlike $BRAF^{V600E}$, which is present in both melanoma and benign nevi,[45] SETDB1 protein is elevated in melanoma but not in benign nevi or normal melanocytes.[44] This indicates that an unknown trigger may lead to the upregulation of SETDB1. The genes that are targeted by elevated levels of SETDB1 remain unknown. This study provides further evidence that genetic mutation interacts with epigenetic events during the progression of melanoma.

## Chromatin Remodeling

Histone modifications are closely associated with the function of polycomb group (PcG) proteins, which are transcriptional repressors.[46] This association leads to structural changes in the organization of the chromatin that regulate gene expression. PcG proteins function through the formation of the polycomb repressor complexes PRC1 and PRC2, both of which are implicated in tumor development. Enhancer of zeste homolog 2 (EZH2) is the H3K27 methyltransferase catalytic subunit of PRC2, and plays a role in the pathogenesis of melanoma. The protein levels of EZH2 increase from benign nevi to melanoma. Depletion of EZH2 in melanoma cells leads to the removal of histone deacetylases from, and normalizes the acetylation of, the *CDKN1A* locus, and restores apoptosis.[47] Increased expression of *EZH2* is tightly associated with uncontrolled proliferation in melanoma. Key pathways, such as RAS/RAF/MEK, AKT, and E2F1, involved in melanoma biology, also regulate EZH2 activity. Knockdown of $BRAF^{V600E}$ reduced *EZH2* expression levels, suggesting that deregulated BRAF activity contributes to the abnormal overexpression of *EZH2* seen in melanoma.[33] High levels of EZH2 were associated with increased Ki-67 index, thicker primary melanomas, and increased invasion.[48] One of the key genes that EZH2 targets is *CDKN2A*[49], which is hypermethylated frequently in melanoma.[50] *EZH2* is regulated by E2F1, a transcription factor that acts downstream of the *CDKN2A* product $p16^{INK4A}$. Upregulation of E2F1 leads to increased levels of EZH2 that represses Bim, a pro-apoptotic factor.[49] In summary, aberrant BRAF signaling and increased E2F1 activity could lead to high expression of *EZH2* resulting in increased DNA methylation and silencing of tumor suppressor genes (*CDKN2A* and *CDKN1A*).

ATP-dependent chromatin-remodeling enzymes found in multiprotein complexes also alter chromatin structure non-covalently (reviewed in[51]). These complexes have been sub-classified into different families and their different cellular functions are summarized in Wang et al.[51] SWI/SNF complexes are an example of such a family and consist of ATP-dependent chromatin remodeling enzymes; deregulation of this complex has been linked to the development of melanoma.[52]

BRG1, a SWI/SNF complex subunit, promotes survival of melanoma cells exposed to UV-radiation through stable activation of ML-IAP, a potent inhibitor of apoptosis and a MITF target gene (**Fig. 1A**).[53] De la Serna[54] suggested that MITF recruits SWI/SNF complexes to melanocyte-specific promoters, where chromatin remodeling takes place and gene expression is activated.[54] BRG1 was found to remodel chromatin on the ML-IAP promoter and to facilitate MITF and coactivator binding. Expression of ML-IAP is associated with increased histone acetylation though recruitment of histone acetyltransferases and decreased levels of histone methylation marks through decreased recruitment of EZH2. Thus, this mechanism promotes pro-survival function of MITF by remodeling chromatin structure.[53]

Chromatin assembly factor-1 (CAF-1), a trimeric protein complex formed by the p48, p60, and p150 subunits, promotes histone incorporation into chromatin and acts in strict association with both the S-phase and DNA repair processes. Overexpression of p60 subunit has been shown to be a novel proliferation and prognostic marker in melanoma.[55]

## Regulatory Role of Non-Coding RNA (ncRNA) in Melanoma

A remaining question is how these epigenetic marks are targeted to these genes. Based on the evidence accumulated over the last decade, ncRNAs have been added to the growing list of gene-regulatory effector molecules[9] that contribute toward epigenetic regulation of gene expression and their deregulation is associated with the development of cancer, including melanoma.[10] ncRNAs are classified into 2 broad categories based on their size: small ncRNA (<200 bp) and long ncRNA (lncRNA, >200 bp). Small ncRNAs are further classified into micro RNA (miRNA), piwi-interacting RNA (piRNA), small nucleolar RNA (snoRNA), and many others with yet uncharacterized functions.[9] Among the different types of small ncRNAs mentioned, miRNAs are the best-studied class in melanoma. Many miRNAs have been identified and were shown to play a role in the progression of melanoma.

## Role of miRNA in Melanoma

Significant progress has been made in identifying miRNAs and characterizing their specific functions in skin morphogenesis and normal regulation. miRNAs known to play a role in normal skin development are summarized in **Table 3**.

Extensive reports indicating the roles of various miRNAs in melanomagenesis have been published, and a list of miRNAs and their targets is shown in **Table 4**. miRNAs can act as oncomiRNAs or tumor suppressive miRNAs. Regulation of miRNA is associated with several hallmarks of melanoma pathogenesis, such as promotion of proliferative signaling (e.g., miR-137,

**Table 3.** List of miRNAs known to be involved in normal skin development

| miRNA | Function | Target Gene | Ref |
|---|---|---|---|
| miR-203 | Reduces proliferative potential of terminally differentiating keratinocytes | *TP63* | 135 |
| miR-34a/c | Possess anti-proliferative potential and induce cell cycle arrest, senescence and/or apoptosis | *SIRT1* | 136 |
| miR-125b | Repressor of stem cell differentiation | *Blimp1* | 137 |
| miR-200/ miR-205 | Maintains proliferation of progenitor cells and inhibits EMT | *ZEB1 & ZEB2* | 138, 139, 140 |

**Table 4.** miRNA regulated in melanoma with respect to affected hallmarks of cancer capabilities

| miRNA | Function | Target Gene | Ref |
|---|---|---|---|
| miR-221# | Sustaining proliferative signaling | *CDKN1B, c-Kit* | 141,142 |
| miR-15b# | | *BCL2* | 143 |
| miR-149# | | *GSK3a* | 144 |
| miR-506–514# | | *HOXB7, PBX* | 145 |
| miR-137# | | *MITF* | 146 |
| miR-193b* | | *CCND1* | 147 |
| miR-148* | | *MITF* | 148 |
| miR-18b* | Resisting cell death | *MDM2* | 149 |
| miR-26a* | | *SMAD1* | 150 |
| miR-205* | Enabling replicative immortality | *E2F1, E2F5* | 151 |
| miR-34a* | | *CDK6* | 152 |
| miR-203* | | *E2F3* | 153 |
| miR-34a/c* | | *c-Met* | 154 |
| miR-214# | Activating invasion and metastasis | *ITGA3, MET* | 155 |
| miR30b/ 30d# | | *GALNT7* | 156 |
| miR-182# | | *MITF, FOXO3* | 157 |
| let-7a* | | *ITGB3* | 158 |
| miR-126* | | *ADAM9, MMP7* | 159 |
| miR-145* | | *FSCN1* | 160 |
| miR-137* | | *EZH2* | 161 |
| miR-18b* | | *MDM2* | 149 |
| miR-34a/c* | | *c-Met* | 154 |
| miR-211* | | *BRN2* | 162 |
| miR-9* | | *NK-kB, Snail* | 163 |
| miR-31* | Cell migration and invasion | *EZH2* | 57 |
| miR-101* | Melanocyte differentiation, cell cycle progression, proliferation and invasion | *MITF, EZH2* | 59 |
| miR-200c# | Cell proliferation and migratory capacity as well as drug resistance | *BMI-1, ABCG2, ABCG5 and MDR1* | 164 |
| miR-99a* | Cell proliferation | *mTOR* | 165 |
| miR-449a# | Cell cycle exit and epidermis differentiation | *HDAC-1* | 166 |
| miR-29* | Suppress tumorigeneis by changing the methylation status of DNA | *DNMT3A/B* | 167 |

# Upregulation; * Downregulation.

miR-221), resistance to cell death (miR-18b and miR26a), replicative immortality (e.g., miR-205, miR-203), and invasion or metastasis (miR-214, let-7a).[56] miR-31 is located at chromosome 9p21.3, which is often deleted in melanoma. It acts as a tumor suppressor in melanoma by negatively regulating the expression of *EZH2* and other oncogenes. EZH2 may epigenetically regulate the expression of miR-31 in a mutually antagonistic feedback loop. DNA methylation at the promoter region of miR-31 has been shown in a melanoma cell line.[57] It has also been reported that miR-101[58,59] and miR-31[57] both negatively regulate *EZH2* and aid cancer progression. These observations provide insight into the functional interactions of mRNA, miRNA, chromatin modifying complexes, and DNA methylation and point to a new era of research in complex regulatory networks in melanoma. Some miRNAs may serve as markers to discriminate between benign and malignant cells.[60]

## lncRNA Mediated Epigenetic Regulation of Gene Expression

lncRNAs, like miRNAs, play crucial roles in epigenetic control, with diverse modes of action and functional consequences. Therefore, it is likely that aberrant expression of lncRNAs would contribute to melanoma development as it does with other cancer types.[9]

Some of the mechanisms by which lncRNAs perform these functions are summarized in **Figure 2**. lncRNAs act as transcriptional regulators by recruiting histone modifying complexes (e.g., PRC) to target loci in *cis* or *trans* mode (**Fig. 2A**).[61] As a consequence of this, the target loci are either activated (**Fig. 2B and C**) or silenced (**Fig. 2A**) depending on the histone marks.[61] This is the most common mechanism employed by lncRNAs to exercise control over gene regulation. An alternative mode of action involves the binding of regulatory proteins by lncRNAs, thereby inhibiting transcription of protein coding genes (**Fig. 2D**).[62] Another class of lncRNA influences splicing patterns via physical interactions with an alternative splicing regulator (**Fig. 2E**).[63] Many lncRNAs are known to host snoRNAs, called sno-lncRNA. The functions of many of the host lncRNAs are not known, although some of these are associated with modulation of splicing pattern (**Fig. 4F and G**).[63] snoRNAs themselves are involved in modification of rRNAs, although the targets of many of these remain to be identified. Linear or circular lncRNAs function as miRNA decoys and sequester miRNAs from their target mRNAs (**Fig. 4H**).[64,65] It has also been proposed that pseudogene transcripts with high homology to mRNAs can act as miRNA decoys and act as competitive endogenous RNAs (ceRNA) to regulate translation, since they have common miRNA recognition

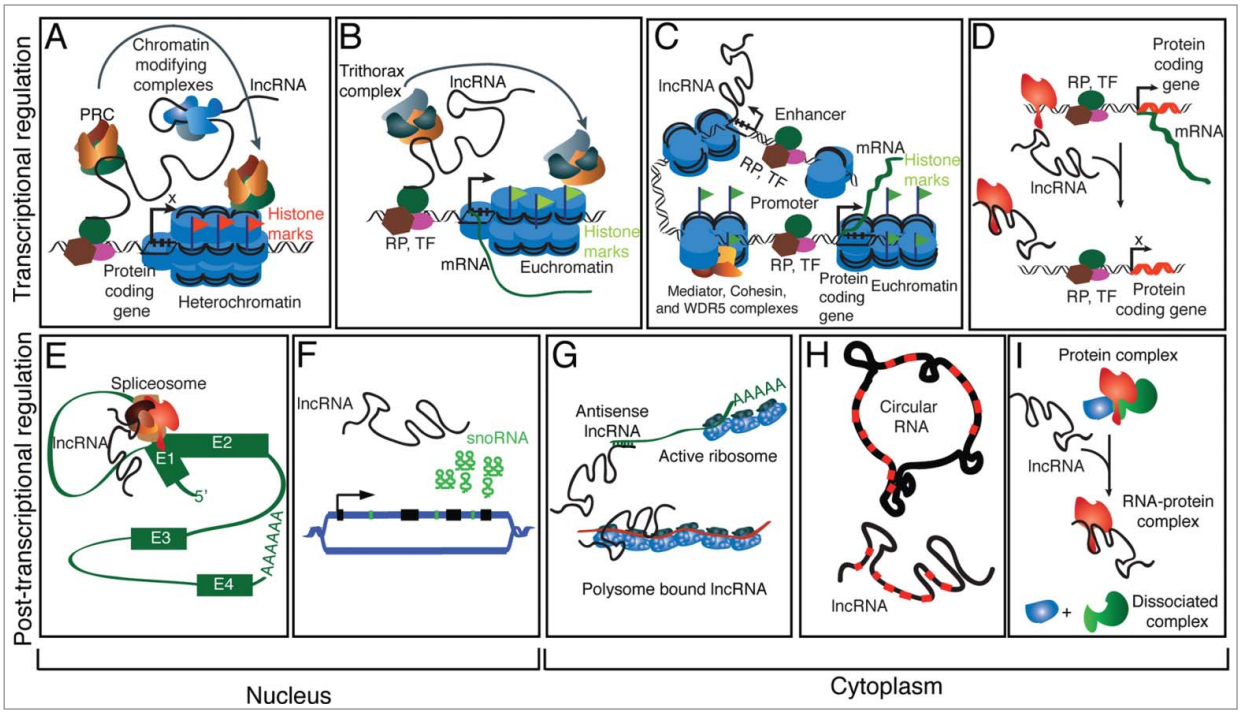


**Figure 2.** Schematic illustrating different functions proposed for lncRNAs. A-D indicate functions regulating transcription, while E-I show posttranscriptional regulatory mechanisms. (**A**) lncRNAs can suppress transcription by interacting with PRCs or other chromatin modifying proteins. This leads to heterochromatin formation and gene suppression. (**B**) Trithorax complexes interact with lncRNA and induce transcription. Chromatin is retained in its euchromatin, actively transcribed state. (**C**) lncRNAs may be transcribed at enhancer regions, and establish and maintain enhancer-promoter looping and gene induction. (**D**) lncRNAs, e.g., those with decoy function, may bind to transcription factors and suppress their activities, leading to diverse changes in cells. (**E**) lncRNAs regulate alternative splicing by interacting with the spliceosomal machinery or mRNA. (**F**) Intronic regions of many lncRNAs encode snoRNAs. The processed lncRNA may be exported to the cytoplasm and perform roles as yet undefined. The snoRNAs remain in the nucleus. (**G**) Many lncRNAs are located in the cytoplasm and most of them are associated with polysomes. (**H**) lncRNAs, either as linear or as circular molecules, may sequester and inactivate miRNAs or mRNAs. The functions of many ribosome-associated lncRNAs are not known; but antisense lncRNAs, such as *UCHL1AS*, regulate the translation of their associated mRNAs. (**I**) Decoy lncRNAs, present in the cytoplasm, may bind to proteins and regulate their functions.

elements (MREs).[66] Many lncRNAs are associated with ribosomes and it is speculated that they help in maintaining the ribosome complex, thereby stabilizing the translational machinery (**Fig. 4G**).[67] Some lncRNAs influence the stability of protein complexes by interacting with RNA-binding domains of components of those complexes (**Fig. 4I**).[68]

## lncRNAs Involved in Regulation of Normal Skin Homeostasis

Functional studies using mammalian skin as a model system have revealed that lncRNAs control normal tissue homeostasis as well as transitions to melanoma.[10] Summarized below are some lncRNAs that are associated with maintaining normal homeostasis in skin.

### Anti-differentiation non-coding RNA (*ANCR*)

In an attempt to identify transcripts altered during transition from a progenitor to a differentiated cell population, a combination of high-throughput RNA-seq and high-resolution tiling arrays identified *ANCR,* which maintains keratinocyte progenitors in their non-differentiated state.[69,70] *ANCR* is located on human chromosome 4 and produces an 855 bp transcript. This locus consists of 3 exons and harbors a miRNA (miR4449) and a snoRNA (SNORA26) in introns 1 and 2, respectively.[69]

*ANCR* is downregulated during the differentiation of progenitor-containing populations. It represses a substantial portion of the epidermal differentiation program, both *in vitro* and in regenerated organotypic human epidermal tissue. *ANCR* depletion led to enhanced expression of genes associated with differentiation in the progenitor-containing epidermal basal layer, a compartment where expression of differentiation proteins is normally never found.[69] These data demonstrated a functional requirement for *ANCR* in maintaining the undifferentiated state that is characteristic of progenitor cells.

The molecular mechanisms by which this lncRNA mediates suppression of epidermal differentiation have been investigated. Decreased levels of *ANCR* have been reported in the case of osteoblast differentiation by Zhu et al.[70] This study indicated that *ANCR* is involved in maintaining the undifferentiated state of osteoblasts, as with epidermis[69]. Zhu et al.[70] found that *ANCR* recruits EZH2 which catalyzes formation of H3K27me3 in the *Runx2* gene promoter. Runx2, a transcription factor required for

osteoblast differentiation, is not expressed and osteoblast differentiation is attenuated.[70]

The mechanism of action of *ANCR* in melanoma has yet to be studied, although it is known that the interplay between keratinocytes and melanoma cells affects the invasiveness of melanoma.[71] The expression pattern and role of *ANCR*, as well as the miRNA and snoRNA derived from it, needs further investigation.

**Tissue differentiation-inducing non-protein coding RNA (*TINCR*)**

Transcriptome sequencing of progenitor and differentiating human keratinocytes revealed another lncRNA *TINCR* which was highly induced during differentiation. The locus is located on human chromosome 19, between *SAFB2* and *ZNRF4*, and produces a 3.7 kb RNA transcript.[72] Knockdown studies using siRNA against *TINCR* showed its requirement for maintaining high mRNA abundance of key differentiation genes, such as filaggrin and loricrin, which are responsible for epidermal barrier function.[72]

Genome-scale RNA interactome analysis revealed that *TINCR* is associated with a range of different mRNAs involved in epidermal differentiation. Human protein microarray analysis also identified *TINCR*-binding proteins of relevance to epidermal differentiation control, including Staufen1 (STAU1) protein. STAU1-deficient tissue showed impaired epidermal differentiation, as was seen with *TINCR* depletion. Gene set enrichment analysis (GSEA) performed using siRNAs specific for STAU1 and *TINCR* showed that the set of transcripts that was suppressed overlapped markedly with the keratinocyte differentiation signature indicating that *TINCR,* together with STAU1, acts to maintain stability of RNAs associated with the differentiated phenotype.[73] These studies indicate the importance of *TINCR* as an inducible lncRNA required to stabilize mRNAs required for differentiation.

The interaction between keratinocytes and melanocytes is of prime importance for epidermal homeostasis, and growth of melanocytes is strictly regulated by keratinocytes. Initiation of melanoma has therefore been thought of as a consequence of the initial escape of melanocytes from the growth control exerted by keratinocytes, leading to benign melanocytic lesions.[71] Therefore, increased expression of *ANCR* and decreased expression of *TINCR* may lead to maintenance of keratinocyte progenitors in undifferentiated states and, consequently, to melanomagenesis. This indicates that a delicate balance needs to be maintained in the expression levels of *ANCR* and *TINCR* to secure the optimum effect of keratinocytes upon melanocytes. Any association between these 2 lncRNAs should be investigated.

## lncRNAs and Their Implicated Role in Melanoma

Several lncRNAs have been shown to have potential roles in the transition of normal melanocytes to melanoma.[10] Summarized below are some lncRNAs with putative or confirmed roles in the development of melanoma.

**BRAF-activated non-coding RNA (*BANCR*)**

RNA-seq analysis identified *BANCR*, a 4-exon transcript of 693 bp that is highly induced by $BRAF^{V600E}$ in melanocytes. It is located on chromosome 9 and is overexpressed in human melanomas. *BANCR* was identified as a potential regulator of melanoma cell migration as profound migration defects were observed following *BANCR* depletion.[74] The mechanism by which *BANCR* regulates gene expression remains to be identified.

A recent study confirmed the contribution of *BANCR* to the proliferation of melanoma cells and that expression of *BANCR* increased with tumor stage. This study also demonstrated that *BANCR* can promote melanoma proliferation by activating the ERK1/2 and JNK MAPK pathways both *in vitro* and *in vivo*. This link between *BANCR* and the MAPK pathways points to a novel mechanism in the regulation of melanoma proliferation.[75] In a previous section, it was mentioned that $BRAF^{V600E}$ was associated with increased *EZH2* expression and H3K27 trimethylation of tumor suppressor genes. It will be interesting to see if there is any correlation between the expression of *BANCR* and *EZH2* or poor prognosis.

**HOX transcript antisense RNA (*HOTAIR*)**

This lncRNA originates from the *HOXC* cluster and acts in *trans* to regulate transcription of the *HOXD* cluster.[76] There is growing evidence that *HOTAIR* has pro-metastasis activity in several cancer types like breast,[77] pancreatic[78], and hepatocellular carcinoma.[79] Recently, the expression of 6 well-documented lncRNAs associated with metastasis was evaluated in primary melanoma and matched lymph node metastases. *HOTAIR* ranks among the 6 lncRNAs most consistently expressed in metastases compared to matched primary tumors.[80] Knockdown of *HOTAIR* inhibited the motility and invasiveness of melanoma cells, with decreased degradation of extracellular matrix.[80] Another study by Tian et al.[81] found no statistical difference in expression levels of *HOTAIR* lncRNA between melanoma and adjacent normal tissue. This observation was attributed to the inclusion criteria of the study that restricted samples only to superficial spreading and nodular melanomas.[81]

*HOTAIR* recruits PRC2 to specific target genes, leading to H3K27 trimethylation and epigenetic silencing of metastatic suppressor genes.[82] Further mechanistic investigation into the regulation of metastasis by *HOTAIR* is necessary. In breast cancer cells, *HOTAIR* may indirectly increase expression of *STAT3*. *HOTAIR* suppresses expression of *HOXD* which produces miR-7, which inhibits expression of the histone methyltransferase SETDB1, required for *STAT3* transcription.[83] A corroborating report indicates that *SETDB1* is recurrently amplified in melanoma and accelerates tumor development in zebrafish melanoma models harboring the common $BRAF^{V600E}$ mutation.[84] Reports of pro-metastatic activity in multiple pre-clinical model systems, support the hypothesis that this lncRNA is a potential target for melanoma metastasis therapy.

***SPRY4-IT1***

*SPRY4-IT1* is derived from an intron of the Sprouty 4 (*SPRY4*) gene and is predicted to contain several long hairpins in

its secondary structure. This lncRNA was identified by Khaitan et al.,[84] who compared lncRNAs in melanoma cell lines, melanocytes, and keratinocytes using an lncRNA microarray.[84] *SPRY4-IT1* was found to be elevated in the melanoma cell lines. Knockdown of this lncRNA caused defects in cell growth and differentiation, and elevated apoptosis rates in melanoma cell lines.[84]

Molecular mechanisms by which *SPRY4-IT1* affects melanoma progression require further investigation. RNA-FISH analysis showed that this lncRNA is predominantly localized in the cytoplasm of melanoma cells[67], and an association with polysomes has been demonstrated.[85] *SPRY4-IT1* associates with, and reduces the abundance of, the lipid phosphatase lipin 2 and may suppress apoptosis arising from lipid metabolism and lipotoxicity.[85] SPRY4 is an inhibitor of the MAPK signaling pathway and may have a tumor suppressor role.[84] *SPRY4-IT1* is located within an intron of *SPRY4*, and these genes have concordant expression profiles[84], although both of them are transcriptionally and functionally independent.[85] A recent study in non-small cell lung cancer (NSCLC) has found evidence that *SPRY4-IT1* controls epithelial-mesenchymal transition (EMT) through regulation of E-cadherin and vimentin expression leading to cell proliferation and metastasis.[86]

***Llme23***

The mouse lncRNA *VL30–1* binds to polypyrimidine tract-binding (PTB) protein associated splicing factor (PSF) and inhibits PSF tumor suppression function in mouse.[87] Since PSF protein is highly conserved from humans to mice, Wu et al.[87] employed RNA-SELEX affinity chromatography to select human PSF-binding lncRNAs from the nuclear RNA repertoire of the human melanoma line YUSAC. This study identified a novel 1,600 base lncRNA which was termed *Llme23*.[87] Gel-shift, UV-crosslinking assays and RNA immunoprecipitation further verified that *Llme23* bound PSF proteins. *Llme23* was also found to be exclusively expressed in human melanoma lines. Significant growth defects following *Llme23* knock out suggested that *Llme23* plays an oncogenic role in human melanoma.[87]

PSF promotes tumor suppression by binding to the promoter of the proto-oncogene *Rab23*, which encodes a RAS-related small GTPase. *VL30–1* inhibits this function in mouse when it binds to the RNA-binding domain of PSF. Identification of a conserved PSF-targeting sequence embedded in the promoter region of the human *Rab23* gene suggested that *Rab23* might be a target for PSF in human cells. Concordant expression of *Rab23* and *Llme23* was reported, indicating that the activation of the *Rab23* proto-oncogene is involved in the oncogenic role of PSF-binding *Llme23*.[87] Taken together, these studies provide evidence that *Llme23* is involved in the etiology of human melanoma.

**Antisense non-coding RNA in INK4 locus (*ANRIL*)**

Sequence-tagged site (STS) real-time PCR-based gene dose mapping of the entire *INK4/ARF* locus in a melanoma-neural system tumor (NST) family revealed an antisense lncRNA *ANRIL*.[88] *ANRIL* consists of 19 exons, spans a region of 126.3 kb and is transcribed as a 3,834-bp lncRNA in the antisense orientation relative to the *p15/CDKN2B-p16/CDKN2A-p14/ARF* gene cluster. Several isoforms of *ANRIL* have been reported, including various short and long isoforms, and a recently discovered circular isoform.[89] Different exons of *ANRIL* are differentially expressed in melanoma cell lines, and there is evidence for the existence of circular *ANRIL* in some of these cells. This discovery suggested that alternative splicing modifies *ANRIL* structure.[89] This mechanism has been studied in atherosclerosis, and further work is required to characterize this mechanism in melanoma. Interestingly, GWAS identified a single nucleotide variant rs1011970 (intron 9 of the *ANRIL* isoform with 19 exons) that is associated with melanoma risk, but only for the variant T-allele homozygote. This polymorphism was also associated by GWAS with breast cancer risk.[90] These results strongly suggest that *ANRIL* is involved in the etiology of melanoma.

A *cis*-acting silencing mechanism, mediated by specific *ANRIL* transcripts, was proposed to negatively regulate *CDKN2A/2B* expression via chromatin remodeling.[68,91] *ANRIL* associates with PRC1 by RNA-binding domains of CBX7, a component required for repression of gene transcription, and thereby represses *CDKN2A/B* gene activity by H3K27 methylation. Competitive inhibition of *ANRIL* binding by expression of an antisense sequence impairs CBX7-mediated repression of the *CDKN2A* locus and causes a concomitant shortening of cellular life span. Several RNA loop structures formed by the *ANRIL* transcript specifically bind CBX7, and at least one of them participates in CBX7 recognition of H3K27. CBX7 recognition of H3K27 is required for the monoubiquitination of histone H2A lysine 119 (H2A-K119), which in turn results in maintenance of repression in the locus.[68] Binding of SUZ12 (a PRC2 component) results in transcriptional repression of *CDKN2B* and influences cell proliferation or prevents premature cell senescence.[92] In a recent study, *ANRIL* was found to be upregulated in gastric cancer relative to non-tumor tissue, and could therefore serve as an independent predictor for overall survival in gastric cancer (GC).[93]

Regulation of the *CDKN2A/B* locus by *ANRIL* indicates that it has a major role in controlling cell proliferation[94] and also facilitates cell proliferation after DNA damage repair (DDR).[95] *ANRIL* is induced by the E2F1 transcription factor in an ATM-dependent manner after DNA damage. In this case, elevated expression levels of *ANRIL* in later stages of DDR suppress *CDKN2A/B* expression.[95] *ANRIL* is involved in progression of GC also through induction by E2F1. *ANRIL*-mediated growth promotion in GC is partially due to epigenetic suppression of miR-99a and miR-449a in *trans* (controlling the mTOR and CDK6/E2F1 pathways) by binding to PRC2, thus forming a positive feedback loop that promotes GC cell proliferation.[93] High miR-449a expression reduces HDAC expression and consequently inhibits cell proliferation, while downregulation of miR-449 is associated with cell growth. A study found miR-449a downregulated in melanomas of older patients compared to melanomas of young adult melanomas.[96] This indicates that *ANRIL* might promote progression of melanoma through a similar process. *ANRIL* also regulates key genes of glucose and fatty acid metabolism[97] and, since it is regulated by interferon-gamma-*STAT1* signaling, it is predicted to have possible roles in inflammatory responses.[98]

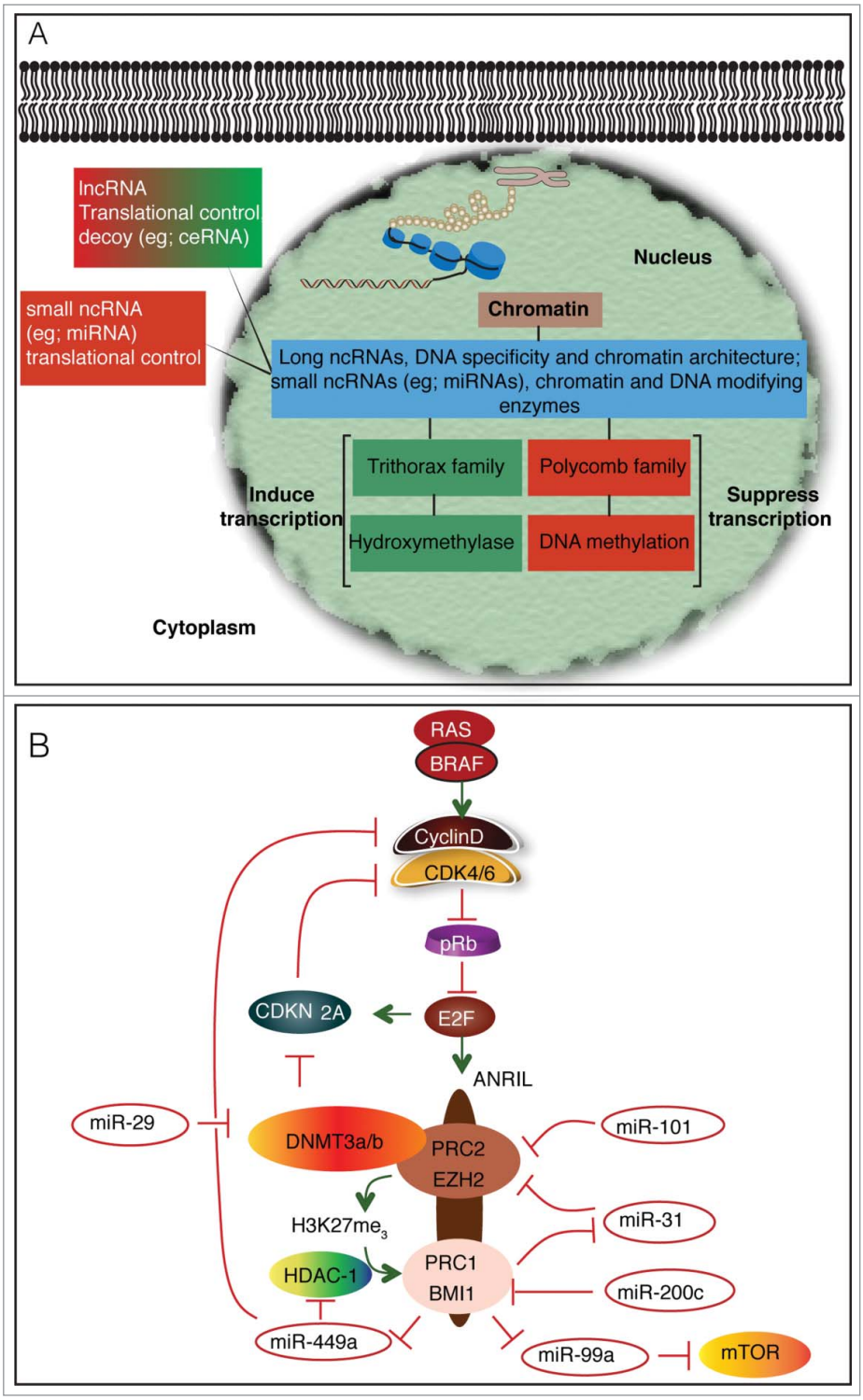


**Figure 3.** Epigenetic regulators as central components in melanoma signaling. (**A**) Epigenetic networks. Chromatin modifications are integral to gene regulation at the transcriptional level and are guided by lncRNAs acting as specific sequence identifiers or scaffolds. PRC and trithorax complexes respectively suppress (red) and induce (green) gene expression. Chromatin-modifying enzymes are also regulated by miRNA. DNA methylation and demethylation are late events in DNA modification. In the cytoplasm, lncRNAs can regulate gene expression by acting as decoys or by undefined mechanisms involving ribosome interaction. miRNAs also act as key regulatory molecules in the cytoplasm. Each of these transcripts can be regulated through epigenetic events and contributes to feedback regulatory loops. (**B**) Example of an epigenetic intertwine in the melanoma signaling pathway. The lncRNA *ANRIL* may be a transcriptional target of oncogenic receptor tyrosine kinase-NRAS-BRAF signaling. *ANRIL* may recruit PRC2 and PCR1 to reduce the expression of tumor suppressor miR-449a and miR-99a. Other miRNAs counteract the actions of PRC2-associated EZH2 (miR-101) and DNMT3 (miR-29), and of PRC1-associated BMI1 (miR-200c). EZH2 and miR-31 engage in mutual suppression.

Given the role of *CDKN2A* as a tumor suppressor[99] and the fact that it was discovered in case of familial melanoma, the role of *ANRIL* in melanoma needs to be clarified. It was found that carriers of T-allele polymorphism rs3088440 of *CDKN2A* (3' UTR of *CDKN2A*) had an elevated melanoma risk. This variant tagged a total of 6 SNPs, of which 3 were found to be located in the intergenic region and the others in intron 1 of *CDKN2A*, the 3' UTR of *CDKN2B* and intron 3 of *ANRIL*.[90] Further elucidation of *ANRIL* as regards to its function and the mechanism by which it controls the *INK4a-ARF-INK4b* locus will help a great deal in understanding its role in melanoma. *ANRIL* has potential as a therapeutic target, or a diagnostic marker for early detection of melanoma.

### Urothelial carcinoma-associated 1 (*UCA1*)

This lncRNA was originally identified in bladder transitional cell carcinoma and the entire sequence consists of 3 exons 1.4 kb in length. As it is highly expressed in bladder transitional cell carcinoma, it was suggested to serve as a biomarker for the diagnosis of bladder cancer.[100] Subsequently, another isoform (2.2 kb) was identified by a different group as cancer upregulated drug resistant (*CUDR*) gene in a doxorubicin-resistant subline of human squamous carcinoma A431 cells. *UCA1* also promotes breast cancer cell growth both *in vitro* and *in vivo*, in addition to its role in embryonic development.[101] In a recent study that investigated the roles of 6 cancer-related lncRNAs in paired melanoma and

adjacent normal tissues, elevated expression of *UCA1* in melanomas was reported, especially at advanced stages.[81] Knockdown of *UCA1* suppressed migration of melanoma cells *in vitro*, suggesting that *UCA1* might contribute to tumor dissemination.[81]

Functional studies carried out to determine the mechanism of action of this lncRNA have revealed that *UCA1* negatively regulates *p27* (a tumor suppressor gene) in breast cancer.[101] Phosphorylated heterogeneous nuclear ribonucleoprotein 1 (hnRP1) found in cytoplasm forms a complex with *UCA1* and increases *UCA1* stability. hnRP1 enhances translation of *p27* mRNA by interacting with its 5'-untranslated region, and the interaction of *UCA1* with hnRNP1 suppresses the p27 protein level by competitive inhibition.[101] However, the mechanisms by which *UCA1* promotes melanoma progression remain to be identified.

#### Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*)

This lncRNA is also known as nuclear-enriched transcript 2 *(NEAT2)*. It was discovered as a prognostic marker for lung cancer metastasis but has been linked to several other human tumor types.[102] *MALAT1* is highly expressed in melanoma compared to adjacent normal tissues.[81] Knockdown studies showing an effect of *MALAT1* on migration of melanoma cells suggest that *MALAT1* may promote melanoma spread as with *UCA1*.[81]

## Conclusion

Despite developments in chemotherapy, the prognosis of metastatic melanoma remains poor and resistance to therapy remains a challenge. Genetic risk factors associated with the etiology of melanoma have been well characterized. However, epigenetic factors, which are also associated with the pathogenesis of melanoma, present many open-ended questions. High throughput gene expression studies have helped identify candidate genes that are thought to be aberrantly regulated through methylation or histone modifications. These genes, however, have not been validated and their specific roles have yet to be characterized. Since epigenetic phenomena such as DNA methylation/demethylation, histone modifications and chromatin remodeling are interlinked, it is important to understand both the molecular mechanisms involved and the chronological order connecting them (**Fig. 3**). Epigenetic alterations may promote genetic mutations and genomic rearrangements in cancer, although the mechanisms involved are yet to be elucidated. The vast amount of data published in the last decade indicates how epigenetic processes result in the differential expression of key genes in different types of cancer. A recent study by De Raedt et al.[103] has identified genetic alterations of *SUZ12* and *EED* in melanoma. Deletion of *SUZ12* leads to loss of H3K27me3 and a consequential increase in H3K27Ac, which recruits bromodomain proteins and induces transcriptional activity. Therefore, further investigation of bromodomain inhibitors as therapy in these tumors is needed. Epigenetic events at the early stages of neoplasia may contribute to the transformation of stem cells into cancer cells, and to the facility with which phenotypic switches occur in cancer. Understanding these mechanisms will be a great achievement and provide extensive resources for future diagnosis and therapy. Recent research into the molecular biology of ncRNAs has not only revealed their versatility but has added to the complexity of how epigenetic events coordinate with one another. Several high throughput studies have identified miRNA and lncRNA species that are associated with melanoma. The tissue specificities of miRNAs and lncRNAs make them good candidates for use as markers for early diagnosis of melanoma. In addition, both classes of ncRNAs may provide specific targets for treatment of melanoma. However, in order to achieve this, further scrutiny of these regulatory phenomena at the molecular level is required. A better understanding of the mechanisms by which DNA methylation/demethylation, chromatin remodeling, and ncRNAs affect cell proliferation and differentiation and melanoma progression will facilitate the development of therapeutic strategies. lncRNAs may act as scaffolds or may aid the binding of chromatin modifying complexes, such as PRC or trithorax, to target site(s) to regulate gene expression. It is unknown whether lncRNAs have ribozyme activity and catalyze reactions in the same manner as rRNA. The potential interactions of miRNA with lncRNAs in regulating chromatin and DNA modifying enzymes[57] adds further layers of complexity to the system. Many chromatin marks are reversible and transformations depend on guidance by ncRNA(s) and chromatin modifying complexes. DNA methylation normally happens post-chromatin-modification, and was considered to be a permanent mark on DNA for silencing genes, although demethylation is common in melanoma (**Fig. 3**). The explosion of data in the last decade has provided a peek into the existence of the multilayer complexity in gene expression and regulation that is a goldmine for basic research, biomarker discovery, and therapeutic options.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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