

# Chapter 2

## Melanoma Pathogenesis

Jennifer A. Lo and David E. Fisher

**Abstract** Melanoma is an aggressive and heterogeneous disease with respect to clinical behavior and underlying genomic lesions. Melanoma development is multifactorial, and increased susceptibility is associated with sun exposure, fair pigmentation, family history, and melanocytic nevi. Major advances in our understanding of its molecular pathogenesis include the identification of recurrent mutations and aberrations in key signaling and developmental pathways. BRAF is the most commonly affected gene, with BRAF(V600E) mutations found in half of all melanomas. The discovery and characterization of oncogenic mutations in the MAPK, RB, p53, and MITF pathways have set the stage for clinically meaningful progress in the melanoma field.

**Keywords** Melanoma · Melanocyte · Pigmentation · Nevus · BRAF · MAPK · MITF · NRAS

### 2.1 Melanocyte Biology

Melanomas arise from the malignant transformation of melanocytes. Melanocytes are the pigment producing cells of the skin and are derived from neural crest stem cells. Their development is modulated by the receptor tyrosine kinase c-KIT and microphthalmia-associated transcription factor (MITF), two genes that are mutated or amplified in many melanomas [1].

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Melanocytes can produce multiple types of pigment, most obviously dark brown eumelanin and reddish pheomelanin. Pro-pigmentation signaling is initiated by binding of  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) to the melanocortin 1 receptor (MC1R) on the melanocyte cell surface. MC1R is a seven-transmembrane G-protein-coupled receptor that activates adenylate cyclase, leading to increased intracellular cAMP levels and expression of MITF. MITF in turn induces transcription of pigment synthesis genes and production of melanin [2]. Although many loci are involved in human pigmentation, MC1R is a major determinant of pigmentation phenotype. MC1R polymorphisms involving single amino-acid substitutions can reduce MC1R signaling, resulting in impaired eumelanin production and a red hair/fair skin phenotype [3].

In addition to basal pigmentation, acquired pigmentation can occur in response to stimuli such as ultraviolet radiation (UVR). Eumelanin is the pigment that provides UVR attenuation in darkly pigmented skin. The tanning response to UVR has been shown to involve p53 activation in keratinocytes following UV-induced DNA damage, leading to p53-mediated POMC/MSH expression. Secreted MSH stimulates MC1R in neighboring melanocytes and produces cutaneous pigmentation [4].

## 2.2 Melanoma Risk Factors

Melanoma pathogenesis is driven by both environmental and genetic factors. Epidemiologic studies have linked melanoma to geographic location and sun (UV) exposure, which is believed to be the most important environmental risk factor. In particular, severe sunburns early in life are associated with the highest risk for melanoma [5, 6]. Melanomas occur more frequently on sun-exposed regions of the body. However, unlike most keratinocyte skin cancers which are known to be products of UVR, melanoma primary tumors are not restricted to sun-exposed skin. Although individuals with fair skin are more susceptible, melanomas also arise in darkly pigmented individuals, most often at acral or mucosal sites. These observations suggest that sun exposure does not account for all melanoma risk.

Increased melanoma susceptibility is associated with family history, fair pigmentation phenotypes, and higher numbers of melanocytic nevi. A family history of melanoma confers an estimated twofold increase in melanoma risk, and approximately 10% of melanoma patients have a family history of the disease [7]. Melanoma is considered familial if two first-degree relatives or three individuals in a family, irrespective of relationship, are diagnosed with melanoma. Familial melanoma is most often associated with dysregulation of cell cycle checkpoints due to mutations in cell cycle regulatory genes such as cyclin-dependent kinase 4 (CDK4) [8] and cyclin-dependent kinase inhibitor 2A (CDKN2A), which accounts for 40% of cases and is the most common high-penetrance melanoma susceptibility locus [9]. Germline CDKN2A mutations are responsible for familial atypical multiple mole melanoma (FAMM) syndrome, an autosomal dominant genodermatosis characterized by increased incidence of melanocytic nevi and melanoma, and elevated

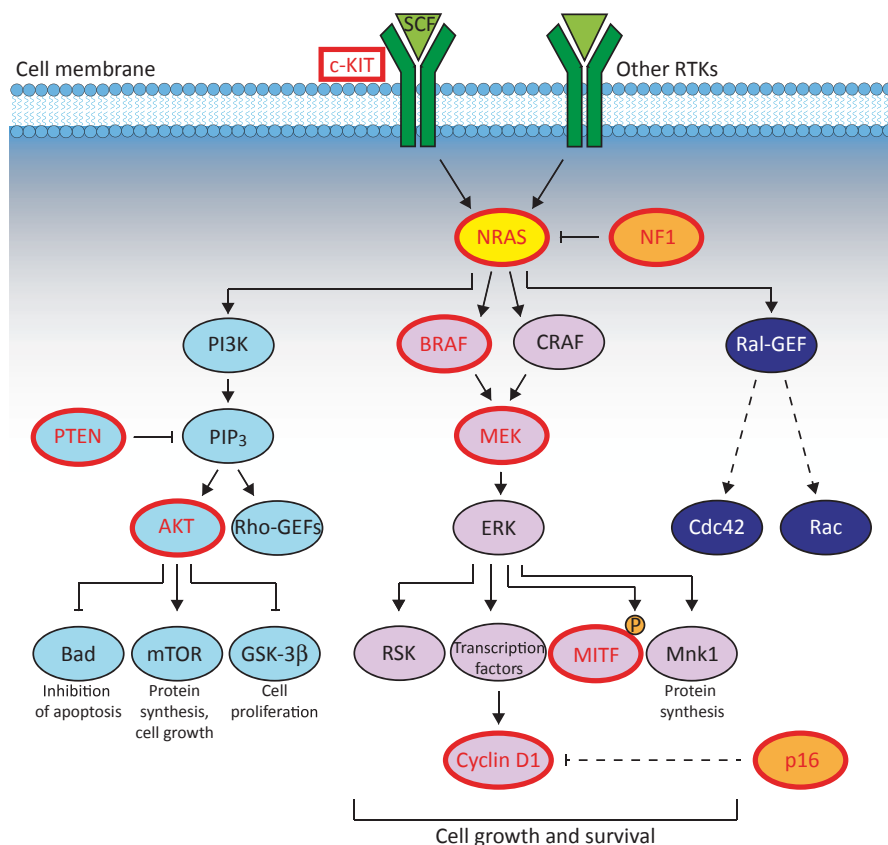
risk of other malignancies such as pancreatic cancer in some FAMM kindreds [10, 11]. A less common cause of familial melanoma is the recently reported E318K variant of MITF [12, 13]. This variant exhibited gain-of-function activity for MITF, which is a previously described amplified melanoma oncogene. Individuals carrying the allele exhibited elevated nevus counts and non-blue eye colors, together with increased melanoma risk. The risk was intermediate in nature in the general population (sporadic) and also segregated among many studied melanoma families in multiple continents. The E318K coding variant disrupts a sumoylation site on MITF [14], thereby inhibiting a functionally suppressive post-translational modification on MITF.

Heritable physical characteristics such as fair skin complexion, inability to tan, and blue eyes are associated with elevated melanoma susceptibility. Germline variants of pigmentation genes such as MC1R, agouti signaling protein (ASIP), and tyrosinase (TYR) confer low- or moderate-penetrance melanoma risk [15, 16]. Individuals with non-signaling variants of MC1R have the red hair/fair skin phenotype, characterized by fair pigmentation, freckling, and sun sensitivity, that is associated with the highest risk of melanoma of all pigmentation phenotypes [17]. MC1R coding variants are found in 80 % of individuals with red hair, less than 20 % of individuals with brown or black hair, and less than 4 % of individuals with a robust tanning response [3]. Comparison of melanomas in murine models of different pigmentation phenotypes has demonstrated that the synthesis pathway of the red pigment pheomelanin contributes to melanomagenesis via a UV-independent mechanism [18].

## 2.3 MAPK and PI3K Pathways

Oncogenic driver mutations have been identified in key signaling and developmental pathways that are involved in survival and proliferation of melanocytes. The most frequently observed recurrent mutations in melanoma occur within the mitogen-activated protein kinase (MAPK) signaling pathway, which promotes cell survival, cell cycle progression, and transformation (Fig. 2.1). In nonmalignant cells, the MAPK pathway is only activated in response to ligand binding to receptor tyrosine kinases or cytokine receptors. Stimulation of receptors leads to activation of RAS family members, monomeric G proteins that act as GTPase switch proteins. RAS-GTP promotes formation of signal-transduction complexes and activates a cascade of serine/threonine kinases culminating in activation of ERK, also known as MAPK. ERK is a serine/threonine kinase that can phosphorylate many targets such as transcription factors.

MAPK signaling is constitutively activated in almost all melanomas. The v-raf murine sarcoma viral oncogene homolog B1 (BRAF) is the dominant genetic target in this pathway, with 40–50 % of melanomas carrying a somatic mutation [19–22]. To a lesser extent, BRAF mutations are also observed in other cancers [23, 24]. BRAF is a serine/threonine kinase directly activated by RAS and is highly expressed in melanocytes, neuronal tissues, testis, and haematopoietic cells. Unlike



**Fig. 2.1 RAS signaling.** RAS family members are monomeric G proteins that are activated by receptor tyrosine kinases and signal through direct interaction with effector enzymes including phosphoinositide (PI) 3-kinases, RAF kinases, and Ral-guanine nucleotide exchange factors (*Ral-GEFs*). Although RAS mutations are less common in melanoma than other solid tumors, *NRAS* activating mutations are found in 10–20% of melanomas. Mitogen-activated protein (MAP) kinase signaling in response to RAF kinase activity promotes cell growth and survival, and the MAPK pathway is constitutively activated in almost all melanomas. *BRAF* is the most frequently mutated gene in melanoma, with activating lesions found in 40–50% of tumors. Melanoma oncogenes and tumor suppressors are labeled in red. Dotted lines represent omitted pathway components. *NF1* neurofibromatosis 1, *PTEN* phosphatase and tensin homolog, *PIP<sub>3</sub>* phosphatidylinositol-3,4,5-triphosphate, *mTOR* mammalian target of rapamycin, *GSK-3β* glycogen synthase kinase-3β, *RSK* ribosomal S6 kinase, *Mnk1* MAP kinase-interacting kinase 1, *Cdc42* cell division control protein 42 homolog

CRAF, which can participate in signaling events outside the MAPK pathway, BRAF's only known substrate is MEK/MAP2K. Phosphorylated MEK activates ERK by phosphorylation, leading to pro-growth and transforming effects that are critical in melanoma pathogenesis.

The most common BRAF mutation in melanoma, accounting for 90% of variants, is a valine to glutamic acid substitution at codon 600 (V600E) in exon 15 [25].

This mutation constitutively activates the kinase domain. Other oncogenic BRAF mutations are found elsewhere in exon 15 or in exon 11, and most of the over 100 rare non-V600E mutations described occur in the glycine-rich loop and activation segment of the kinase domain. Mutations in these regions indirectly activate BRAF by disrupting the normal intramolecular interactions which hold BRAF in an inactive conformation [26].

An alternate oncogenic mechanism in melanoma involves rare BRAF mutants with low kinase activity. Although no CRAF activating mutations have been reported in melanoma [25, 27, 28], mutations such as G469E and D594G produce a BRAF that directly activates CRAF but minimally phosphorylates MEK. Melanoma lines with these low-activity BRAF mutations are dependent on CRAF for survival [29].

BRAF(V600E) mutations are observed much more frequently in melanomas arising in intermittently sun-exposed skin regions than acral or mucosal melanomas, suggesting that BRAF mutations may be linked to sun exposure. However, the thymidine to adenine (T>A) transversion at position 1799 that is responsible for the V600E substitution is not a typical UV-signature DNA mutation. It is possible that the transversion could result from a “non-classic” UV-induced DNA lesion or from secondary effects of UVR exposure such as generation of reactive oxygen species [30].

Mutations that increase RAS activity also promote cell proliferation. In comparison to other solid tumors, RAS mutations occur with relatively low frequency in melanomas. Only 10–20% of melanomas, most often amelanotic nodular subtypes, carry an activating RAS mutation. NRAS is the most commonly affected RAS family member in melanoma [31, 32], and NRAS activating mutations [33, 34] primarily involve glycine 12, glycine 13, and glutamine 61 and trap NRAS in its active, GTP-bound conformation. While BRAF mutations activate only MAPK signaling, NRAS activating mutations simultaneously activate the MAPK and phosphatidylinositol 3-kinase (PI3K) pathways.

Although oncogenic mutations are usually not stand-alone events in melanoma, some are thought to be mutually exclusive. For example, NRAS and BRAF mutations almost never occur concomitantly [35, 36], suggesting that NRAS and BRAF have overlapping oncogenic activities and either is sufficient for constitutive activation of the MAPK pathway. Both BRAF and NRAS mutations are associated with poorer clinical prognosis. In the rare cases when both BRAF and NRAS mutations are present in melanoma, the BRAF mutation is not the classic V600E substitution [37]. “Acquired” (or selected) NRAS mutations have also been observed simultaneously with BRAF(V600E) in the context of melanomas which initially responded to BRAF-targeted therapy but subsequently became resistant [38].

PI3K signaling results in increased activation of the serine/threonine kinase AKT (also known as protein kinase B), which is a major mediator of cell survival through activation of targets such as mammalian target of rapamycin (mTOR) and inhibition of pro-apoptotic signals. While PI3K itself is rarely mutated in melanoma [39], constitutive activation of NRAS, amplification of AKT3, or loss of the phosphatase and tensin homolog (PTEN) tumor suppressor can lead to dysregulation of the PI3K

pathway. PTEN encodes a lipid and protein phosphatase that negatively regulates signaling pathways which use the cytosolic second messenger phosphatidylinositol-3,4,5-triphosphate (PIP<sub>3</sub>), such as the PI3K pathway. Lower levels of intracellular PIP<sub>3</sub> result in less downstream activating phosphorylation of AKT. Thus, loss of PTEN protein or function eliminates a mechanism of negative regulation of AKT and cell survival. Increased phospho-AKT levels are associated with poor melanoma prognosis [40].

PTEN can be lost upon chromosome 10q deletion. 50–60% of melanomas contain hemizygous deletions or point mutations in 10q, while 10% contain homozygous deletion [41]. Epigenetic silencing of PTEN has also been described [36, 42, 43]. Hemizygous PTEN deletions tend to occur with BRAF mutation [42, 44], suggesting that BRAF and PTEN can cooperate in melanomagenesis. This idea is supported by studies of a murine model of melanoma in the setting of BRAF(V600E) and PTEN inactivation [45].

Neoplastic transformation of melanocytes can give rise to benign nevi as well as malignant melanoma, and activating mutations in BRAF and NRAS are implicated in both. Activating BRAF mutations are found in 70–80% of dysplastic nevi [22, 46–48], while NRAS mutations are rare in dysplastic nevi [49, 50] but present in most congenital nevi [50]. Mutation of HRAS is associated with Spitz nevi [51]. The BRAF(V600E) mutation induces nevus formation, involving initial cell proliferation followed by oncogene-induced senescence likely due in part to accumulation of p16<sup>INK4A</sup> [52]. Mutation of p16<sup>INK4A</sup> in addition to BRAF leads to transformation of cells *in vitro*, and deletion of PTEN or p16<sup>INK4A</sup> results in the formation of invasive melanoma in BRAF(V600E) mice [53]. In zebrafish, concomitant BRAF(V600E) mutation and deletion of TP53 leads to the formation of invasive and metastatic melanoma [54].

Given the high incidence of BRAF mutations in nevi, mutation of BRAF was traditionally thought to be a founder event that preceded all other oncogenic events in BRAF mutant melanoma [46]. In this model, senescence induced by BRAF activation is overcome by cooperating genetic lesions such as loss of p16<sup>INK4A</sup> or PTEN. However, other evidence suggests that the order of melanocytic lesions and relationship between nevi and tumor may be more complex. Although BRAF mutations are found in most nevi and half of vertical growth and metastatic melanomas, they are rare in initial malignant lesions: only 10% of radial growth phase melanomas and 6% of *in situ* melanomas have mutant BRAF. In addition, many nevi and primary melanomas are polyclonal (contain both BRAF wild type and BRAF mutant cells) while metastatic melanomas are not polyclonal, suggesting that BRAF mutation might occur at later stages of melanomagenesis [25, 46, 55, 56]. In addition, recent data have suggested that a stereotypical mutation in the promoter of the enzyme telomerase reverse transcriptase (TERT) is found in both BRAF mutant or NRAS mutant melanomas, suggesting that it may be an earlier mutation event [57]. This mutation in the TERT promoter occurs at a frequency of approximately 70% in melanomas and is also found in many non-melanoma cancers [57]. Regardless of when BRAF lesions occur, activation of BRAF in invasive melanoma promotes cell growth and dependence on the MAPK signaling pathway [58].

Dysregulation of MAPK signaling in melanoma can alternatively be caused by overexpression or hyperactivation of growth factor receptors such as c-Met, KIT, and epidermal growth factor receptor (EGFR) [59–61]. Mutations in the tumor suppressor neurofibromatosis 1 (NF1), a negative regulator of Ras, were identified in 5 out of 21 tumors without BRAF or NRAS mutations [62]. In the context of BRAF(V600E), NF1 mutations dysregulate the MAPK and PI3K pathways, ultimately suppressing mutant BRAF-induced senescence and promoting melanoma development and proliferation [63]. In some melanomas, inactivating mutations have also been identified in the tumor suppressor neurofibromatosis 2 (NF2) [64]. Germline mutations in NF1 and NF2 are associated with hereditary neurofibromatosis. Recent whole-exome sequencing approaches have identified somatic mutations in downstream MAPK effectors such as MAP3K5, MAP3K9, MEK1, and MEK2 in melanomas [65, 66].

## 2.4 RB and p53 Pathways

The retinoblastoma (RB) and TP53 tumor suppressor pathways are dysregulated in many sporadic and familial melanomas, and all known inherited high-risk melanoma susceptibility loci are genes in the RB pathway. However, loss or lesions of RB and TP53 occur much less frequently in sporadic melanomas than in most other solid tumors. Instead, genetic alterations in CDKN2A can eliminate upstream signaling in these pathways in melanoma. The CDKN2A locus at chromosome 9p21 encodes four exons, and alternative splicing yields two distinct tumor suppressors that share a common second exon: p16<sup>INK4a</sup> and p14<sup>ARF</sup> [67]. Mutations in p16<sup>INK4a</sup> functionally inactivate the RB pathway while mutations in p14<sup>ARF</sup> functionally inactivate the p53 pathway. The most common CDKN2A lesions in melanoma are point mutations, which are found as germline lesions in 25–40% of familial melanomas and as sporadic alterations in 10% of non-familial melanomas [67]. CDKN2A point mutations are also associated with dysplastic nevi. As with PTEN loss, CDKN2A mutation tends to coincide with BRAF mutation [21].

The RB pathway regulates the G1/S cell cycle checkpoint. During normal cell cycle progression, the RB tumor suppressor is phosphorylated by mammalian G1 cyclin-CDK complexes. Hyperphosphorylation of RB triggers release of E2F family members, transcription factors that activate expression of genes important for entry into S phase and DNA synthesis. p16<sup>INK4a</sup> binds and inhibits cyclin-dependent kinases 4 and 6 (CDK4/6) from inappropriately phosphorylating the RB protein [68]. Thus, loss of p16<sup>INK4a</sup> facilitates RB phosphorylation and subsequent re-entry into the cell cycle. Point mutations or transcriptional silencing are responsible for loss of p16<sup>INK4a</sup> expression in 30–70% of melanomas, leading to increased cellular proliferation and escape from oncogene-induced senescence.

Activating mutations in CDK4 are found in a small number of melanomas. CDK4 germline mutations always occur at a conserved arginine residue, R24, that is necessary for regulatory inhibition of CDK4 by p16<sup>INK4a</sup> [8, 69]. 5% of melanomas



contain somatic CDK4 point mutation or amplification [70]. p16<sup>INK4a</sup> and CDK4 mutations are mutually exclusive [29, 71, 72].

While TP53 mutations are found in 5% of melanomas [73], the p53 apoptotic pathway is more often deficient due to loss of p14<sup>ARF</sup> function in melanomas [74]. p14<sup>ARF</sup> binds and inhibits the mouse double minute 2 homolog (MDM2). MDM2 encodes an E3 ubiquitin ligase that inhibits p53 transcriptional activity and targets p53 for proteasomal degradation. Inactivating p14<sup>ARF</sup> mutations permit the p53-antagonizing activity of MDM2 and subsequent genomic instability [75–77]. In rare cases, amplification of MDM2 without alterations in CDKN2A sequence or expression has been observed in melanoma [70].

## 2.5 MITF

MITF is the master lineage regulator of melanocyte development and survival. It serves as the transcription factor for differentiation and pigmentation genes such as TYR, tyrosinase-related protein 1 (TYRP1), dopachrome tautomerase (DCT), melanoma antigen recognized by T-cells 1 (MART1, also known as gp100), and premelanosome protein (PMEL17, also known as SILV). Although MITF is essential for melanocyte differentiation, it can alternatively promote malignant behavior in some melanomas. The most common genetic alteration of MITF is amplification, which occurs in 15–20% of melanomas with a higher prevalence among metastatic melanomas [78]. MITF amplification is thought to usually occur as a late event in melanoma progression and was associated with poorer 5 year survival in the pre-vemurafenib and ipilimumab era [79]. Many melanomas continue to depend on MITF expression for survival, and suppression of MITF *in vitro* is lethal to most melanoma cell lines [80, 81].

The transcriptional targets of MITF that mediate its oncogenic activity as distinct from its regulation of pigmentation and differentiation are not fully characterized. However, MITF is known to enhance expression of genes involved in cell cycle progression, cell proliferation, and cell survival. For example, MITF is a transcription factor for cell cycle kinase CDK2 [81], CDK inhibitors p16<sup>INK4a</sup> [82] and p21 [83], and anti-apoptotic mitochondrial membrane protein B-cell lymphoma 2 (BCL-2) [84] as well as its related family member BCL2A1 [85]. In melanomas with elevated MITF activity, increased expression of these MITF targets likely contributes to growth, invasion, and survival of melanoma cells.

MITF is known to cooperate with BRAF in melanoma transformation *in vitro* [78] and *in vivo* [86]. MAPK pathway activation, which is found in the majority of melanomas, results in MITF phosphorylation at Ser73 by ERK2 [87]. Phosphorylation at Ser73 affects MITF regulation in two ways: enhanced recruitment of p300, an MITF transcriptional coactivator and histone acetyltransferase, and increased ubiquitination of MITF [88, 89]. Because Ser73 phosphorylation ultimately accelerates proteasomal degradation of MITF, MAPK signaling in melanomas can reduce



expression of many MITF targets. BRAF inhibitors may enhance immunotherapy by stabilizing MITF and upregulating transcription of targets like MART1 and other antigens that are recognized by the immune response to melanoma [90].

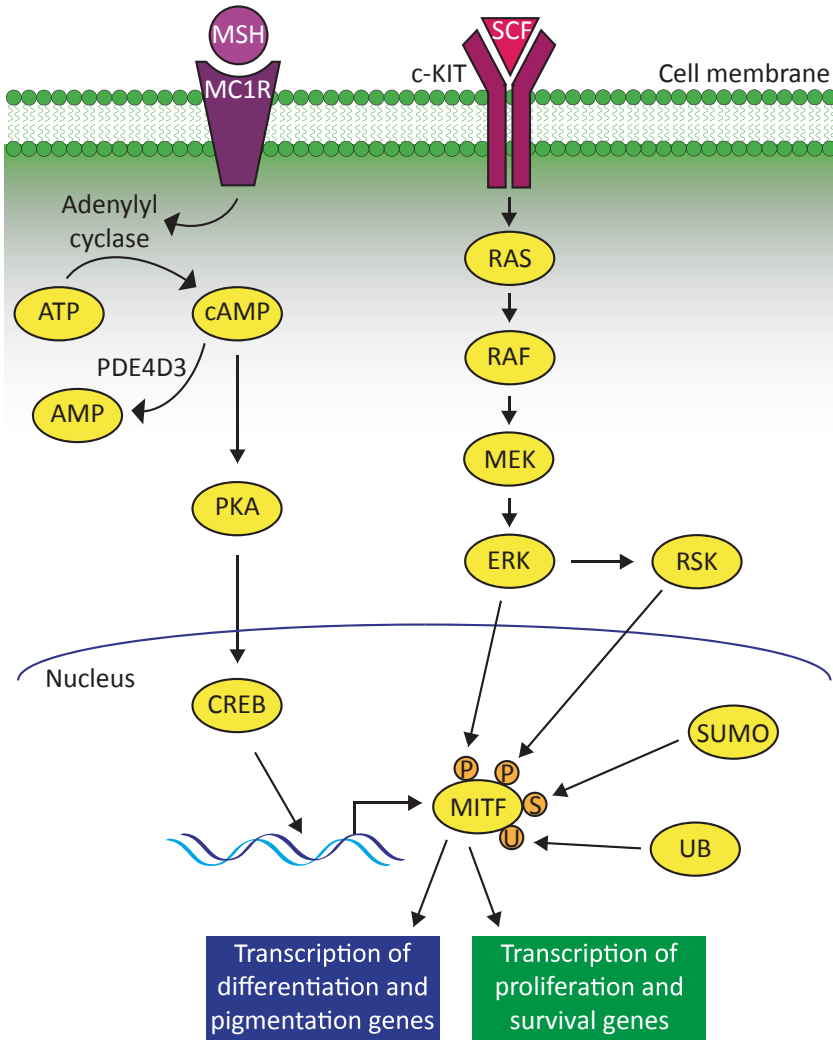
Other post-translational modifications of MITF include phosphorylation by ribosomal S6 kinase (RSK), glycogen synthase kinase-3 $\beta$  (GSK-3), and p38 and sumoylation by protein inhibitor of the activated STAT3 (PIAS3) [91–94]. MITF is also a substrate for proteolytic degradation by caspase 3 [95]. Protein kinase C interacting protein 1 and PIAS3, which preferentially binds Ser73-phosphorylated MITF, inhibit MITF binding to DNA [14, 96–98]. Sumoylation of MITF reduces transcription of a subset of MITF targets whose promoters contain multiple MITF binding sites [99, 100]. In light of this observation and the complexity of MITF regulation, it is tempting to speculate that post-translational modifications determine MITF target gene specificity in response to cell context. By such mechanisms, MITF may be able to switch between its two recognized functions of regulating melanocytic differentiation/pigmentation and modulating survival/proliferation effects capable of producing an oncogenic transcriptional program in melanoma.

Germline loss-of-function mutation of MITF in humans causes Waardenburg syndrome type IIA, an autosomal dominant inherited condition characterized by lack of melanocytes in the eye, forelock, and inner ear [101]. Melanocyte deficiencies in individuals with Waardenburg syndrome result in deafness, white forelock (unpigmented hair in the midline), and eye color variability [102]. In contrast, increased numbers of nevi and darker eye colors are associated with the gain-of-function mutation conferred by a germline missense mutation in codon 318 of MITF. As previously discussed, this mutation abrogates a sumoylation site, resulting in altered transcription of some MITF targets and elevated melanoma susceptibility [12, 13] (Fig. 2.2).

## 2.6 Acral and Mucosal Melanomas

KIT mutations and amplifications are the most common genetic alterations in melanomas arising in acral, mucosal, and chronically sun-damaged skin. Although KIT mutations are found in only 1 % of all melanomas, they are reported in 10 % of acral and 10 % of mucosal melanomas [103]. Less than 10 % of KIT mutant melanomas contain BRAF or NRAS mutations.

c-KIT encodes the receptor tyrosine kinase for stem cell factor. In response to ligand binding, KIT activates signaling of pathways such as RAS. The most commonly observed KIT variant in melanoma, found in a third of KIT-mutant melanomas, is L576P [55]. Activating mutations such as L576P promote melanocyte growth and survival by causing constitutive stimulation of MAPK and PI3K/AKT signaling. KIT mutations are associated with poorer clinical outcomes in acral and mucosal melanoma [119].



**Fig. 2.2 The MITFaxis.** In melanocytes, microphthalmia-associated transcription factor (*MITF*) is expressed in response to melanocortin 1 receptor (*MC1R*) signaling upon binding of melanocyte-stimulating hormone (*MSH*). Non-signaling variants of *MC1R* are associated with the red hair/fair skin phenotype and increased melanoma susceptibility. *MITF* activity is modulated by phosphorylation, sumoylation, and ubiquitination. *MITF* target genes include regulators of differentiation and pigmentation as well as proliferation and survival. *c-KIT* signaling is essential for melanocyte development. *c-KIT*, *NRAS*, *BRAF*, and *MITF* are known melanoma oncogenes in the *c-KIT* pathway. *SCF* stem cell factor, *cAMP* cyclic AMP, *PKA* protein kinase A; *CREB* cAMP-responsive element-binding protein

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