Fifteen-year quest for microphthalmia-associated transcription factor target genes

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Summary

Microphthalmia-associated transcription factor (MITF) was initially shown to play a key role in melanocyte differentiation through the direct transcriptional control of *TYROSINASE, TYRP1* and *DCT* genes, encoding the three enzymes involved in melanin synthesis or melanogenesis. Sixteen years after the first description of MITF, more than 40 direct MITF target genes have been described. They play a key role in melanocyte, osteoclast and mast cell specific functions. Furthermore, several MITF target genes, e.g. *BCL2, CDK2, CDKN1A, CDKN2A, MET* and *HIF1A,* link MITF to general cellular processes such as growth or survival. In this review, we provide an overview of the MITF-regulated genes. We pay special attention to the MITF target genes in melanocytes and raise questions about target specificity.

Introduction

Melanocytes are highly differentiated cells responsible for pigment synthesis which derive from the neural crest cells. During embryogenesis, multipotent neural crest stem cells (NCSC) differentiate into neurons, glia and melanocytes (Bronner-Fraser and Fraser, 1988). These multipotent cells become committed to the melanocyte lineage, before or soon after the neural crest emerges from the neural tube, and next migrate along the dorsolateral migration pathway to join the ectoderm, where melanoblasts will be able to differentiate fully into mature melanocytes (Luo et al., 2003; Wilson et al., 2004). In human and mouse adult skin, a melanocyte regenerative pool, also called melanoblasts or melanocyte stem cells, is localized into a niche in the bulge of the hair follicle (Nishimura et al., 2005).

Melanocytes are found in various organs of the vertebrate body, such as hair follicle, basal layer of epidermis, inner ear, eye choroid Harderian gland and heart (Brito and Kos, 2008; Steingrimsson et al., 2004; Yajima and Larue, 2008). However, the main and most studied role of melanocytes is the production of melanin pigments to ensure hair and skin pigmentation. Skin melanin pigmentation is a key adaptive process playing a crucial role in protection against the noxious effects of solar UV radiation, which causes DNA damages and skin cancers. A well-characterized differentiation program allows melanocytes to produce and transfer melanin to surrounding keratinocytes. This program includes production of melanin within specialized organelles called melanosomes, which are transported to the dendrite tips of melanocytes and then to the surrounding keratinocytes, where melanin plays its photoprotective role.

This fascinating developmental and differentiation program has drawn the attention of researchers for a long time. In 1993 and 1994, the works of Arnheiter's and Read's groups gave a great impetus to the dissection of the molecular mechanism involved in melanocyte development and differentiation (Hodgkinson et al., 1993; Hughes et al., 1994). These groups identified a new transcription factor, the inactivation of which leads to microphthalmic and white mice. This transcription factor, called microphthalmia-associated transcription factor (MITF), was initially shown to play a key role in melanocyte differentiation through the direct transcriptional control of *Tyrosinase*, *Tyrp1* and *DCT* genes, encoding the three enzymes involved in melanin synthesis or

melanogenesis. However, the absence of melanocytes in Mitf-deficient mice also suggested a role of MITF in developmental processes such as growth or survival. More recently, several MITF targets have been identified (e.g. BCL2, CDK2, CDKN1A, CDKN2A) that provide the molecular links between MITF and growth or survival. Sixteen years after the first description of MITF, more than 40 MITF target genes have been reported, some endowed with antagonistic functions (e.g. *CDK2* and *CDKN1A*). Therefore, it is now clear that MITF, by controlling the transcription of numerous genes, is the source of multiple and complex molecular cascades that control melanocyte growth, survival and differentiation.

In this review, we aim to provide an update of the MITF target genes and to discuss the role of these genes in melanocyte biology.

MITF target genes involved in the melanocyte differentiation program

Melanin synthesis is an enzymatic process that converts tyrosine to melanin pigment. The first two steps in melanin synthesis, the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and the subsequent oxidation of DOPA to DOPA quinine, are catalysed by tyrosinase (TYR), the rate-limiting enzyme of this process. Two other enzymes, dopachrome tautomerase (DCT) and tyrosinase-related protein 1 (TYRP1), are involved in eumelanin synthesis. These enzymes, and specifically tyrosinase, are key players in the melanocyte differentiation program, as demonstrated by the dramatic consequences of their mutations on melanin pigment production. Therefore, numerous efforts have been made to elucidate the mechanisms by which the expression of these enzymes is regulated in melanocytes. Using a classical gene reporter approach, Bentley et al. (1994) showed that MITF transactivates the *TYR* promoter. Footprinting, electrophoretic mobility shift assay (EMSA) and mutational analysis identified two CATGTG motives in the proximal region of the promoter that mediate the effect of MITF. In the absence of antibody specific to MITF, it was not possible at that time to demonstrate that MITF indeed binds to this promoter region. Similar approaches with *Tyrp1* and *DCT* promoter activity reporter constructs and super shift assays with MITF antibodies demonstrated that MITF up-regulated TYRP1 and DCT promoter activity through binding to CATGTG motives (Bertolotto et al., 1998; Yavuzer et al., 1995). In *Tyrosinase*, *Tyrp1* and *DCT* promoters, a GTCATGTGCT motif, initially called M-box in 1992, is perfectly conserved and mediates most of the MITF effects (Lowings et al., 1992). Of note, MITF requires the ATP-dependent chromatin-remodelling enzymes SWI/SNF complexes to control the transcription of *TYR* and *TYRP1* (De La Serna et al., 2006). The regulation of the *DCT* promoter is more complex, as MITF cooperates with CREB (Bertolotto et al., 1998), Sox10 (Ludwig et al., 2004) or LEF-1 (Yasumoto et al., 2002) to stimulate DCT expression, whereas Pax3 antagonizes the effects of MITF (Lang et al., 2005) (Figure 1).

The unambiguous demonstration of the direct MITF binding to the *tyrosinase* promoter in intact cells was made by Goding and associates using the chromatin immunoprecipitation assay (ChIP) (Galibert et al., 2001). Later, the same binding to *Tyrp1* (De La Serna et al., 2006) and Dct promoters (Lang et al., 2005) was demonstrated.

As mentioned above, melanin synthesis takes place in melanosomes that function as an autonomous melanin factory. Melanosomes are lysosome-related organelles, whose biogenesis and transport are also key determinants of melanin synthesis and skin pigmentation (Wasmeier et al., 2008). In addition to Tyrosinase, Tyrp1 and Dct, numerous other proteins are present in melanosomes and play a pivotal role in melanogenesis, as their loss of function has also been associated with pigmentary defects. Among them, Silver homologue (Berson et al., 2001; Raposo et al., 2001) and Melan-a (MART1) (Hoashi et al., 2005), encoded in human by SILV and MLANA, respectively, are both involved in the formation of melanosomal matrix and melanosome maturation. Baxter and Pavan (2003) first predicted a role for MITF in the control of Silver homologue, as Pmel17 expression was undetectable in $Mitf^{mi}/Mitf^{mi}$ embryos. This hypothesis was confirmed using a large panel of approaches, including gene reporter assays, EMSA, ChIP and adenovirus forced expression of MITF by Du et al. (2003), which demonstrated that *SILV* and *MLANA* are direct transcriptional targets of MITF. This report links for the first time MITF to melanosome biogenesis. Two E-box sequences, CACATG and CACGTG, were suspected to mediate the effect of MITF on *MLANA* promoter transcription. It should be noted that these responsive elements are located rather far from the initiator (–1594 and –609, respectively), whereas in *Tyrosinase* (–110), *Tyrp1* (–36) and *DCT* (–130) they are located in the close vicinity of the transcription initiation start site. For *SILV*, MITF does not bind to the real promoter region, but to an enhancer element, CATGTG, within the first intron. Whether these E-boxes are required for regulation by MITF has not been determined in the context of a promoter construct.

Other proteins involved in melanosome biogenesis have also been identified as direct MITF targets. This is the case of ocular albinism type 1 *GPR143* (formerly *OA1*) gene. *OA1* encodes a G-protein-coupled receptor located on the melanosomal membrane that controls melanosome maturation and size (Cortese et al., 2005; Schiaffino and Tacchetti, 2005; Vetrini et al., 2004). ChIP assay, EMSA and mutated promoter construct identified an E-box, CACATG (–34), in the proximal promoter region that binds and mediates MITF transcriptional activity.

RAB27A, a small GTP-binding protein, forms a tripartite complex with melanophilin and Myosin Va, allowing

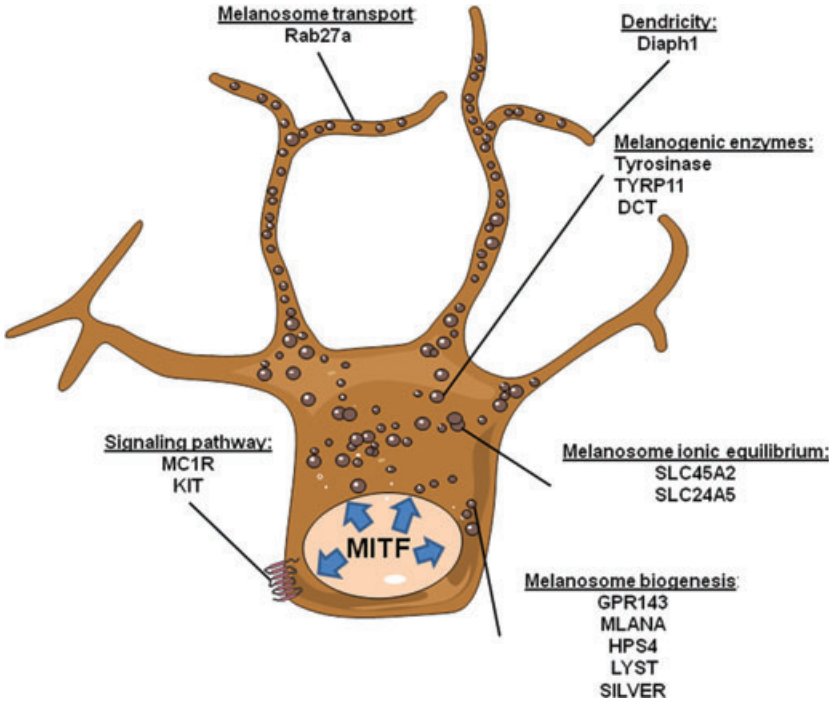

Figure 1. MITF actively controls the differentiation program. The scheme displays the different MITF target genes involved in the pigmentary signalling pathways, melanocyte morphology and melanosome biogenesis, structure, functioning and transport.

melanosome transport on the actin network (Bahadoran et al., 2001; Seabra and Coudrier, 2004). Mutation of one of the members of this complex results in the pigmentation dilution found in Griscelli syndrome (Menasche et al., 2003). Chiaverini et al. (2008), using loss (siRNA)/gain (adenovirus-mediated gene transfer) of function and reporter gene approaches, identified Rab27A as a target of MITF, thereby implicating MITF for the first time in the regulation of melanosome trafficking. Promoter mutagenesis, ChIP and EMSA with wild-type or mutated sequences revealed two proximal E-boxes, CATATG (–52) and CAGCTG (–40), responsible for *Rab27A* promoter regulation.

The genes mentioned above have been identified as direct MITF target genes, with the highest possible level of evidence and, generally, the MITF-responsive element is conserved at least in human and mouse. Several other genes are suspected to be MITF targets in melanocytes. However, no direct evidence is available to demonstrate that MITF directly regulates the transcription of these genes. For example, SLC45A2 (formerly AIM1 or MATP) is predicted to function in melanosome biogenesis, as mutations in this gene, found in OCA4, affect tyrosinase targeting to the organelles. A report identified *Slc45A2* as being downstream of MITF, but did not provide any evidence of direct MITF binding to the *Slc45A2* promoter or of a transactivation of this promoter by MITF (Du and Fisher, 2002).

MC1R, encoding a plasma membrane G-protein-coupled receptor that binds αMSH, plays a key role in the control of skin pigmentation (Busca and Ballotti, 2000). The human and mouse *MC1R* promoters contain a CATGTG (–454/–460) and are activated by MITF in gene reporter assays. However, it has been not determined whether this E-box binds MITF and is directly involved in the control of the promoter activity (Aoki and Moro, 2002).

A protein kinase C-dependent pathway has been shown to be involved in the regulation of melanogenesis. PKCβ phosphorylates the cytoplasmic domain of tyrosinase, leading to its activation and stimulation of melanogenesis (Park et al., 1999). A recent report shows that MITF regulates the expression of PKCβ through a transcriptional mechanism (Park et al., 2006). However, the binding of MITF to the *PKCβ* promoter has not been demonstrated.

Recently, a DNA microarray study using an SK-Mel28 melanoma cell line over-expressing MITF confirmed the regulation of the above genes and identified other possible MITF target genes (LYST, HPS4 and OSTM1) involved in melanosome biogenesis and functioning (Hoek et al., 2008). LYST mutations lead to the formation of giant melanosomes and have been detected in individuals with Chediak–Higashi syndrome, a rare autosomal recessive disease characterized by oculo-cutaneous albinism (OCA) and immunological deficiency (Spritz, 1998). HPS4 mutations have been implicated in Hermansky–Pudlak syndrome, a genetically heterogeneous group of related autosomal recessive diseases described in humans and mice, characterized by defects in the biogenesis and

function of organelles including melanosomes and platelets (Cutler, 2002; Dell'angelica et al., 2000). OSTM1 is located inside the grey-lethal (*gl*) allele and its mutation in mice induces severe osteopetrosis and grey coat colour as a consequence of melanin granule clumping in melanocytes (Gruneberg, 1935). In human, mutations in OSTM1 have been detected in a child suffering from autosomal recessive malignant infantile osteopetrosis (Ramirez et al., 2004). Compelling evidence from EMSA and promoter assay has been obtained in osteoclasts, demonstrating that MITF directly binds and transactivates the *OSTM1* promoter. Although four E-boxes (CAGGTG, CACCTG, CATGTG and CAGCTG) located between −50 and −480 have been detected in the human promoter, the MITF-responsive elements have not been clearly identified (Meadows et al., 2007). The function of OSTM1 in melanocyte is not known, but the pigmentation defects observed in *gl* mice indicate a role in pigment production.

Of note, Steingrimsson's lab described additional possible MITF targets (Hoek et al., 2008). These genes belong to vacuolar ATPases (ATP1A1, ATP6V1B2, ATP6V1C1) and Solute Carrier family (SLC1A4, SLC7A8, SLC11A1, SLC19A2, SLC24A5). Vacuolar ATPases have been involved in the control of melanosome pH and of melanin synthesis. Indeed, a V-ATPase inhibitor (bafilomycin) increases the pH of melanosome and stimulates melanin synthesis (Fuller et al., 2001). SLC proteins were implicated in ionic transport and some of them might play a role in the control of the ionic equilibrium in melanosomes. SLC11A1 (Gelineau-Van Waes et al., 2008), Slc7a11 (Chintala et al., 2005), SLC24A5 (Lamason et al., 2005) and SLC24A4 (Sulem et al., 2007) are already reported to be involved in pigmentation, or regulated by cAMP the main physiological stimulus of melanin synthesis (Cheli et al., 2009).

Taken together, the above studies demonstrate that, in melanocytes, MITF orchestrates several processes required for proper skin and hair pigmentation. MITF directly controls (i) the enzymes that synthesize melanin (Tyrosinase, Tyrp1, Dct), and the proteins required, (ii) for melanosome structure (SILVER, MART1), (iii) for melanosome biogenesis through OA1 and (iv) for melanosome transport through Rab27A (Figure 1). The observations mentioned above support the identification in the near future of new MITF targets involved in other processes essential for correct pigmentation, such as melanosome ionic equilibrium and pH.

MITF target genes involved in melanocyte proliferation and survival

A role for MITF in the development of melanocytes was first hypothesized following studies of mice with mutations at the *microphthalmia* (*mi*) locus. Mitf-deficient mice ($Mitf^{mi\text{-}vga9/mi\text{-}vga9}$ mice) show a loss of hair pigmentation, small eye (microphthalmic), and early onset of deafness (Hodgkinson et al., 1993). These phenotypes (except microphthalmia) are also observed, with some variations, in individuals suffering from Waardenburg type IIa and Tietz syndromes exhibiting mutations in the MITF gene (Amiel et al., 1998; Hughes et al., 1993; Opdecamp et al., 1998; Tachibana et al., 1994). Histological analysis of microphthalmic mice has linked the white coat colour to an absence of melanocytes. Therefore, it has been concluded that the devastating consequences on melanocyte development following MITF mutations might be due to reduced melanocyte precursor growth or survival (Figure 2).

Control of cell cycle

The observation that some melanoblasts, although in a reduced number, can be detected earlier during the embryonic development in $Mitf^{mi\text{-}ew}$ (eyeless white) mice but are undetectable at later stage could also suggest that the absence of melanocytes in the coat of these mutant mice could be ascribed to a secondary defect in the proliferation process (Nakayama et al., 1998) leading to growth arrest or cell senescence.

In this context, MITF was shown to control the transcription of *TBX2* (Bejar et al., 2003; Carreira et al., 2000), a transcription factor of the T-box family that blocks senescence through repression of $p21^{Cip1}$ and $p19^{ARF}$ (Jacobs et al., 2000; Prince et al., 2004; Vance et al., 2005). EMSA and mutated promoter analysis identified a CATGTG motif (−204) that seems to mediate the effect of MITF on the *TBX2* promoter. *TBX2* was the first described MITF target gene not directly involved in melanin synthesis but involved in the control of cell growth and invasion (Rodriguez et al., 2008). Later, David Fisher's group studied regulation of the cyclin-dependent kinase *2* (CDK2) by MITF. Human *CDK2* is located on chromosome 12 in the close vicinity of *SILVER* (but with the opposite orientation). Therefore, the *CDK2* promoter overlaps with the regulatory regions of *SILVER* that contain a CATGTG motif and binds MITF (Du et al., 2003). MITF, through the binding to a CATGTG motif (the same motif involved in the regulation of *SILVER* by MITF), located −1315 bp from the transcription start site of *CDK2*, regulates the transcriptional activity of the *CDK2* promoter, thereby controlling CDK2 expression and melanoma growth (Du et al., 2004).

More recently, another mechanism of melanoma growth arrest following MITF silencing has been described. Goding and associates showed that inhibition of MITF expression leads to the up-regulation of $p27^{Kip1}$ expression (Carreira et al., 2006). Accumulation of $p27^{Kip1}$ was the result of the down-regulation of diaphanous-related formin, DIAPH1. This in turn led to the inhibition of SKP2, which controls the level of $p27^{Kip1}$ through ubiquitination and degradation (Mammoto et al., 2004; Schulman et al., 2000). ChIP, EMSA and promoter activity assay demonstrate that MITF binds and activates the *DIAPH1* promoter. Three potential MITF-binding sites (CATGTG) were identified (E-box1: −509;

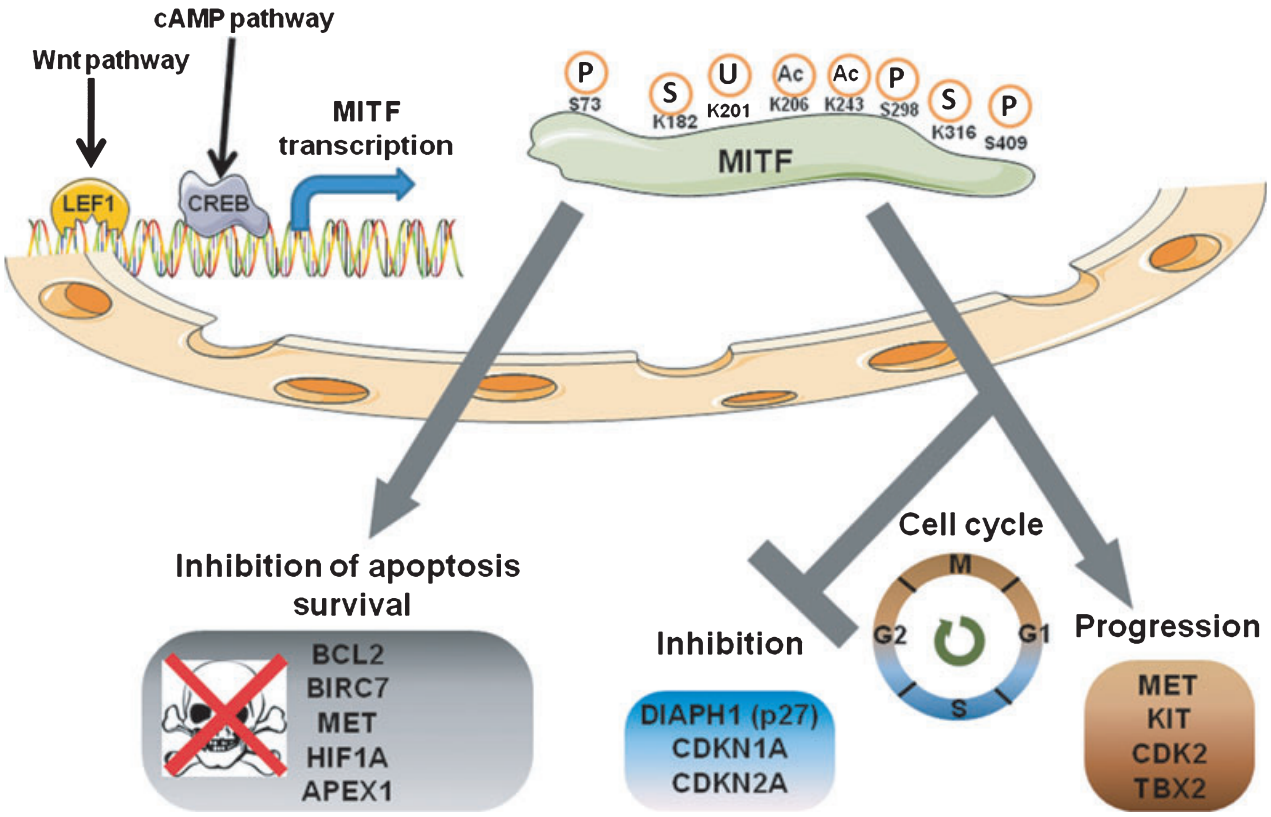

Figure 2. MITF controls melanocyte/melanoma cell survival and proliferation. Schematization of signalling pathways associated with MITF transcription and the different MITF post-translational modifications that will affect MITF activity. The left side displays the MITF targets that control cell survival and the right side shows the MITF targets that control either positively or negatively melanocyte or melanoma cell growth.

E-box2: –677; E-box3: –794) with E-box2 and E-box3 elements being the most critical for *DIAPH1* promoter activity. This report also showed that silencing of MITF blocks melanoma cell cycle progression in G0/G1. Further, MITF-depleted cells display exacerbated invasive properties linking for the first time MITF down-regulation to a metastatic phenotype.

Interestingly, forced expression of CDK2 restores the inhibition of melanoma colony formation caused by transfection of a dominant negative MITF (dn-MITF) construct (Du et al., 2004). This result indicates that CDK2 down-regulation is a key event in dn-MITF-induced melanoma growth arrest. However, one should expect that the inhibition of MITF function by dn-MITF would also lead to $p27^{Kip1}$ up-regulation. Therefore, CDK2 forced expression should not be sufficient to restore melanoma cell growth unless CDK2 regulates $p27^{Kip1}$ level or function. This is indeed the case, as cyclinE-CDK2 complex phosphorylates $p27^{Kip1}$ and increases SKP2 expression through proteins of the E2F family, both processes favouring $p27^{Kip1}$ degradation (Assoian and Yung, 2008). Consistently, the G0/G1 block elicited by siMITF is abrogated by $p27^{Kip1}$ inhibition, but in these conditions CDK2 expression does not seem to be affected (Carreira et al., 2006).

All these observations indicate that MITF favours growth, at least in melanoma. However, MITF plays a pivotal role in melanocyte differentiation, and the differentiation process is often linked to an arrest of the cell cycle. As MITF over-expression is able by itself to trigger the melanocytic differentiation program (Tachibana et al., 1996), it has been hypothesized that MITF could control the cell cycle in melanocytes to allow a coordinated response to the differentiation stimuli. In line with this, Mitf-heterozygous mice produced fewer melanoblasts from the neural crest, but they proliferated faster during the migratory phase in vivo, suggesting that Mitf could also inhibit cell proliferation (Hornyak et al., 2001). Consistently, in the same year using ChIP and gene reporter assays, it was reported that MITF controls *CDKN2A* and *CDKN1A* transcription and expression (Carreira et al., 2005; Loercher et al., 2005). An E-box (–635) has been identified by EMSA and mutagenesis as being the mediator of the MITF transactivating effect in the promoter of $p21^{Cip}$; however, the regulatory element in the $p16^{INK4A}$ promoter remains to be determined.

The regulation of $p21^{Cip}$ and $p16^{INK4A}$ by MITF suggests that MITF may favour *G1*-S arrest in melanocytes and melanoma cells. Indeed, MITF silencing decreases $p16^{INK4A}$ expression and stimulates proliferation of a

uveal melanocyte cell line, whereas forced expression of MITF increases $p21^{Cip}$ and $p16^{INK4A}$ expression and blocks growth in fibroblasts. In melanoma cells, the growth arrest evoked by MITF over-expression is mediated by $p21^{Cip}$ up-regulation rather than by an increased expression of $p16^{INK4A}$, which is frequently mutated or inactivated in melanoma (Bennett, 2008; Kido et al., 2009; Selzer et al., 2002).

Compelling evidence shows that *CDK2*, *DIAPH1*, *TBX2*, *$p16^{INK4A}$* and *$p21^{Cip1}$* are direct MITF targets. However, their contribution to the survival and differentiation of melanoblasts during development is less obvious and remains to be determined. Indeed, mice deficient for Cdk2, Diaph1, $p16^{INK4A}$ and $p21^{Cip1}$ (Berthet et al., 2003; Deng et al., 1995; Ortega et al., 2003; Sakata et al., 2007) show no noticeable coat colour phenotype, whereas Mitf-deficient mice exhibit severe melanocyte loss. Mice heterozygous for a *Tbx2* null mutation appear normal and *Tbx2* homozygous deletion is embryonically lethal (Harrelson et al., 2004). Therefore, the melanocyte-specific targeted disruption of *Tbx2* will help to investigate fully the role of Tbx2 in the melanocyte lineage in the developing embryo. Therefore, either these MITF targets only play a role in adult melanocytes or in melanoma proliferation, or their functions can be compensated by other factors during melanocyte development.

Control of cell survival

Less than 10 yr after the discovery of MITF, the molecular connection between MITF and melanocyte survival was disclosed. Microarray analysis of MITF-dependent gene expression identified the anti-apoptotic gene *BCL2* as a MITF-regulated gene. ChIP, promoter and gel super shift assays demonstrated that *BCL2* is a direct MITF target gene (Mcgill et al., 2002). An E-box motif CATGTG (–220) mediated the effect of MITF on the *BCL2* promoter. Further, enforced BCL2 expression rescued apoptosis induced by dominant negative MITF, suggesting that MITF controls melanoma cell survival via *BCL2*. This hypothesis was next confirmed by demonstrating the genetic interaction of *Mitf* and *Bcl2* in vivo. The role of Bcl2 was anticipated from the data obtained from Bcl2 knockout mice showing that these mice are born pigmented but then turn grey due to the loss of melanocyte stem cells (Mak et al., 2006; Veis et al., 1993).

In addition to BCL2, a recent report has identified another anti-apoptotic protein, BIRC7 (ML-IAP, LIVIN, or KIAP), as a direct MITF target (Dynek et al., 2008). MITF regulated the *BIRC7* promoter through binding to two E-boxes, CATGTG (–49 and –290), as shown by mutated promoter analysis, ChIP and band shift assays. On the other hand, MITF silencing by siRNA led to a reduced melanoma growth and decreased melanocyte cell viability, associated with an elevated caspase activity and a nuclear condensation, indicating induction cell of death. However, it should be noted that several groups did not report apoptosis in melanoma cells after MITF silencing by specific siRNA or shRNA (Busca et al., 2005; Carreira et al., 2006; Larribere et al., 2005; Liu et al., 2009).

Further, two reports showed that MITF regulates MET expression. The first report, using ChIP assay and EMSA, identified a CACGTG (–300) motif in the human *MET* promoter that binds MITF (Mcgill et al., 2006). However, no gene reporter activity confirmed the role of this E-box sequence. In the second report, using EMSA and gene reporter assay, a CACGTG (–109) motif was shown to mediate the transactivating effect of MITF on *Met* promoter (Beuret et al., 2007). Nevertheless, these two reports showed that activation of MET by HGF favours melanoma migration and protects melanocyte and melanoma cells from apoptosis.

The control of oxidative stress is particularly important in melanocytes that are physiologically exposed to environmental stresses such as solar UV radiation. In this context, oxidative DNA damages are managed by the base excision repair system to prevent DNA damage accumulation ending in cell growth arrest and apoptosis. Interestingly, a recent study indicated that the apurinic/apyrimidinic endonuclease1/redox factor-1 (*APEX1/Ref1*) is a MITF target gene, thereby associating MITF with the control of oxidative stress (Liu et al., 2009). ChIP and gene reporter experiments indicated that MITF binds to the *APEX1* promoter. MITF transactivates the *APEX1* promoter through three proximal E-boxes (E-box1: –10; E-box2: +52; E-box3: +73). E-box1 seems to be the most critical in this response and E-box2 also participates in this regulation. Although MITF knockdown per se does not promote cell death, MITF silencing leads to poor survival under oxidative stress. APEX1 forced expression partially rescues reactive oxygen species-induced cell death in MITF-depleted cells. Interestingly, APEX1 protein interacts with cAMP-response element binding protein (CREB) and activates hypoxia inducible factor 1 α (HIF1α), which has been also demonstrated to be a direct MITF target gene, as MITF binds to the *HIF1A* promoter and stimulates its transcriptional activity (Busca et al., 2005). Although the human *HIF1A* promoter contains 10 E-boxes, two of which match perfectly with the MITF consensus binding sequence (CACGTG), clear identification of the MITF-responsive elements still needs to be established. HIF1A also protected melanoma cells from apoptotic stimuli (Figure 2).

Kitamura's group identified *Kit* and *Ngfr* (p75 NGF receptor), two tyrosine kinase receptors, as direct MITF target genes (Jippo et al., 1997; Tsujimura et al., 1996). These receptors, upon the binding of their cognate ligands, activate ERK and PI3K signalling pathways, and both have been shown to prevent apoptosis and to favour cell survival. Using reporter gene assay and EMSA with very high amounts of recombinant MITF, these reports showed that MITF transactivates the *Kit*

promoter through the binding to a CACCTG (–351) motif and the *Ngfr* promoter through a CACTTG motif (–138). Even though the regulation of Kit and Ngfr by MITF was only demonstrated in mast cells, these receptors, in particular Kit, have been implicated in melanocyte development and functioning (Hou and Pavan, 2008; Yaar et al., 1994). Recently, MITF was shown to control the expression of endothelin receptor B (EDNRB), which plays a key role in melanocyte development (Sato-Jin et al., 2008). However, there was no direct evidence of a direct regulation of EDNRB by MITF. Therefore, the regulation of these receptors by MITF might have a physiological relevance in the control of melanocyte growth and survival.

Other MITF target genes

Several other MITF target genes have been identified. These genes do not function directly in differentiation, survival or growth processes. Among them, SLUG (*SNAI2*) was reported to be directly regulated by MITF. First, *Snai2*-deficient mice show white spotting and mid forehead depigmentation. MITF transactivates the *SNAI2* promoter through a CATGTG sequence located 1396 bp upstream of the transcriptional start (Sanchez-Martin et al., 2002). The *SNAI2* gene encodes a transcription factor of the zinc-finger type closely related to Snail (SNAI1). SNAI1 and SNAI2 play key roles in epithelial–mesenchymal transition (EMT) by repressing E-cadherin transcription and stimulating fibronectin expression. EMT is a crucial phenomenon during the normal developmental process, but also plays a role in invasion, metastasis and cancer stem cell production (Mani et al., 2008; Nieto, 2002). Therefore, SNAI2 links MITF to melanocyte precursor migration and melanoma metastasis.

Glycoprotein-nmb (*GPNMB/osteoactivin*) was shown to be a direct target of MITF, first in osteoclasts (Ripoll et al., 2008) and then in melanocytes (Hoek et al., 2008; Loftus et al., 2009). EMSA and reporter gene assay identified a CACATG sequence in the human promoter 38 bp upstream of the transcriptional start site that binds MITF and mediates the activation of the promoter by MITF. This sequence is perfectly conserved in mouse and chicken. The role of GPNMB in melanocytes is not known. GPNMB was identified in a mouse model of pigmentary glaucoma (Anderson et al., 2002). GPNMB shares amino acid homology with Silver and is also located in melanosomes (Tomihari et al., 2009). Therefore, GPNMB might be involved in the pigmentation process. Further, GPNMB, which promotes breast cancer metastasis to bones (Rose et al., 2007), is expressed in melanoma and might play a role in metastasis.

The melanoma marker melastatin/TRPM1 is a potential calcium channel family member whose expression in melanoma predicts non-metastatic behaviour and correlates with favourable outcome. ChIP, gel shift and promoter reporter assays showed that three E-boxes (–56, –392 and –479) in the human promoter bind MITF and allow MITF to activate the *TRPM1* promoter activity. Another channel, the bestrophin-1 (*BEST1/VMD2*), which was proposed to be a calcium-activated chloride channel, has also been identified as an MITF target gene. Compelling evidence (ChIP, EMSA, promoter assays) demonstrated that through an E-box sequence located –42 bp upstream of the initiation start site, MITF activates the *VMD2* promoter, thereby controlling VMD2 expression (Esumi et al., 2007). In eyes, VMD2 plays a key role in retina homoeostasis. Mutations of this gene are associated with macular dystrophies (Yu et al., 2007). In melanocytes, the role of VMD2 is not known. However, VMD2 is expressed in melanocytes of the skin (microarray database, NCBI), and is regulated by MITF in melanoma (Esumi et al., 2007).

Finally, MITF has been shown very recently to control ribonuclease DICER, which excises microRNAs from their precursors in cells and processes long double-stranded RNAs into short interfering RNAs, thereby demonstrating that MITF regulates miRNA production in melanocyte cells (Levy et al., 2009).

MITF targets in osteoclasts and mast cells

The observation that a few MITF alleles affect the development and function of other cell types, among which the most studied are mast cells and osteoclasts, suggests a role for MITF in these cells.

Mast cells are central effectors of normal physiologic processes such as innate immunity against parasitic and bacterial infections and are also mediators of allergic hypersensitivity reactions in pathologic conditions (Williams and Galli, 2000). Parasite infection leads to higher mortality of $MITF^{mi/mi}$ mice compared to their wild-type littermates (Kitamura et al., 2002), consistent with a mast cell defect in mice exhibiting MITF mutations. Several proteins such as proteases (mast cell proteases 2, 4, 8, 9, granzyme B, Tph1, Tpsb2), protease inhibitor (SerpinE1) (Ito et al., 1998; Jippo et al., 1999; Morii et al., 1996, 1997; Murakami et al., 2003, 2006), adhesion molecules (sgIGSF, integrin a4) (Ito et al., 2003; Kim et al., 1998), metabolic enzyme (hPGDs) (Morii and Oboki, 2004) and growth factor receptors (Kit, p75Ngfr) (Isozaki et al., 1994; Jippo et al., 1997; Kitamura et al., 2006) that play a key role in mast cell differentiation and functioning, have been identified as MITF target genes.

Like mast cells, osteoclasts are the progeny of multipotential hematopoietic stem cells. Osteoclasts play a critical role in normal bone remodelling, and alterations in their function have been involved in various mouse and human bone diseases such as osteopetrosis, a condition characterized by dense brittle bones and the lack of a marrow cavity (Everts et al., 2009). In mice, osteopetrosis is observed in some semi-dominant MITF

alleles. Consistently, MITF has been shown to be a regulator of osteoclast function by activating transcription of several genes – *TRAP*, *cathepsin K, OSCAR, E-cadherin* (Mansky et al., 2002), *OSTM1* and *Clcn7* (Meadows et al., 2007) – that are required for proper osteoclast functioning (Chalhoub et al., 2003; Kasper et al., 2005). Consistently, re-introduction of intact MITF into *mi/mi* mice-derived osteoclasts rescues their differentiation (Mansky et al., 2002).

Microarray analysis of MITF-regulated genes

More recently, high throughput gene expression profiling using DNA microarrays allowed a global characterization of the MITF-dependent transcriptome. Although this approach cannot formally identify direct MITF target genes, microarrays can be the first step towards the identification of new and unexpected MITF target genes. In addition to the microarray analysis briefly described above (Hoek et al., 2008), several other global analyses have been performed. The study of Mcgill et al. (2002) compared the transcriptomes of primary melanocytes infected with control or dominant negative MITF adenoviruses. That report identified *BCL2* as a direct MITF target gene (see above) and confirmed *TBX2* as an MITF-regulated gene. Among the numerous MITF-regulated genes identified in that study (which cannot be cited here), some of them, namely *RAB7* and *TAP1*, are of specific interest in the context of skin pigmentation. Indeed, RAB7 has been implicated into the trafficking of the early and intermediate stage melanosomes and interacts with RAB27A (Bahadoran et al., 2001; Jimbow et al., 2000; Jordens et al., 2006). TAP1 is a protein that has been genetically associated with vitiligo (Zhang et al., 2005).

Shahlaee et al. (2007) compared the pattern of MITF targets between the three isoforms that are present in mast cells, namely Mitf-mc, Mitf-e and Mitf-a. The authors used primary mouse mast cells isolated from vga9/vga9 ($Mitf^{-/-}$) mice that lack all Mitf transcripts and restored individual isoform expression by retroviral infection followed by antibiotic selection for stably transduced cells. A common set of 57 genes was up-regulated by the three isoforms. Most of these genes have been previously shown to be involved in mast cell biology.

Another study, designed to identify genes super-induced by Mitf over-expression in macrophage/osteoclast RAW cells, has been coupled to a bioinformatic analysis to find genes that display an E-box in their promoter sequence (Meadows et al., 2007). That study was able to identify Ostm1 and Clcn7 as a Mitf-A target in osteoclasts. In this screening, spleen tyrosine kinase (Syk) was also identified as a Mitf-A target. Syk is a non-receptor tyrosine kinase often lost during melanoma progression due to promoter methylation, and re-introduction of Syk decreases the metastatic behaviour of melanoma cells and acts as a tumour suppressant by inducing senescence-like growth arrest (Bailet et al., 2009; Hoeller et al., 2005).

A very recent report identified a set of 36 genes down-regulated by MITF siRNA in three melanoma cell lines (Kido et al., 2009). This report confirms *CDK2*, *MET*, *RAB27A* and *TRPM1* as MITF target genes. None of the other identified genes appeared to have a known implication in melanocyte biology.

Technical considerations

To be recognized as a MITF target gene, several conditions must be fulfilled. First, the target gene expression should parallel the expression of MITF in both MITF silencing or MITF over-expression experiments. However, a subtle difference should be noted between the two approaches. The MITF silencing approach will identify genes whose expression requires MITF, whereas the forced expression approach will identify genes for which MITF is sufficient. This second approach might not identify genes whose expression requires co-factors of chromatin modifiers. Indeed, it has been shown that forced expression of MITF was not sufficient to increase tyrosinase expression in B16 and 501mel cells (Gaggioli et al., 2003; Vachtenheim et al., 2001). This has been explained by the requirement of SWI/SNF chromatin modifier to make the chromosomal region of *tyrosinase* responsive to MITF (De La Serna et al., 2006). More recently, Hoek et al. (2008) have successfully shown that MITF forced expression is able to up-regulate tyrosinase in SK-Mel28 melanoma cells, suggesting the presence in these cells of sufficient levels of chromatin modifiers. Nevertheless, both approaches (gain and loss of MITF activity) identify MITF-dependent genes, but do not formally identify MITF target genes. This requires several additional experiments including promoter reporter and ChIP assays. Further, the identification of the regulatory region requires EMSA and mutated promoter experiments. However, it must be remarked that a promoter is properly identified if the initiation of transcription has been localized by an appropriate technique. Very few reports have indeed identified the promoter of the studied MITF target gene and therefore some approximations may exist in the localization of the MITF-responsive elements.

Finally, correlative analysis between MITF and the level of its target genes in different cell lines, human biopsies and/or mouse models will also give weight to the observations mentioned above to award a gene the 'MITF target' label. However, a lack of correlation cannot by itself disqualify a supposed MITF target gene. This is particularly true for MITF target genes involved in general cellular processes and ubiquitously expressed (*CDKN1A*, *HIF1A*), as they are obviously controlled by other transcription factors and regulatory processes.

MITF-binding consensus sequences

In 1998, Goding and colleagues, using a systematic mutational approach, defined the MITF-binding consensus sequences (Aksan and Goding, 1998). Their study showed that the binding of MITF to the canonical sequence CATGTG requires a T in 5′. At the time of the study, four genes, *tyrosinase*, *Tyrp1*, *DCT* and *Qnr-71*, were reported to be regulated by MITF. Without exception the promoter of these genes contains a TCATGTG sequence that is conserved from human to turtle, suggesting that this motif is a key determinant for the regulation of the genes involved in melanin synthesis by MITF. MITF can also bind to CACGTG with no specific requirement for the flanking sequence. However, it should be kept in mind that the binding to a specific sequence might also be regulated by post-translational modifications of MITF. In Table 1, we have compiled the sequences of all the MITF target genes described so far. This analysis, comprising more than 40 genes, reached the same conclusion as Goding's group, i.e. that the MITF-binding consensus sequence is T-C-A-T/C-G-T-G-A (Table 2). However, because binding specificity can change according to cellular context and MITF post-translational modifications (see below) this analysis cannot give very informative clues. No doubt, new approaches using the ChIP sequence will allow the identification of the genes interacting with MITF and will help to refine the characterization of MITF consensus binding sequences. However, it should be kept in mind that ChIP does not formally identify a direct interaction between a transcription factor and a regulatory sequence.

Specificity of MITF action

To date, 10 isoforms of MITF have been identified: MITF-A, B, C, D, E, H, J, Mc, CM and M. These

Table 1. Sequences of the MITF-binding regions in 47 MITF target genes

Symbol	5′	Core	3′	Function	Symbol	5′	Core	3′	Function
SNAI2	TT	CATGTG	AA	IN	ITGA4	GT	CACTTG	GT	IN, MC
ACP5	CT	CACATG	AT	O	KIT	AG	CACCTG	CC	D
APEX1	GT	CACGTG	GT	D	KIT	GC	CAGGTG	GC	D
APEX1	AT	CACGTG	AC	D	KIT	TG	CACTTG	GG	D
BCL2	GC	CATGTG	CC	D	***LYST***	***GG***	***CATATG***	***AC***	***DIF***
BEST1	GT	CAAGTG	AC	IN	***MC1R***	***GG***	***CATGTG***	***GC***	***DIF***
BEST1	CT	CACGTG	GG	IN	MCPT2	CC	CACATG	CT	MC
BIRC7	AG	CATGTG	AC	D	MCPT4	TC	CATGTG	CT	MC
BIRC7	CT	CACATG	TC	D	MCPT9	CC	CATATG	CT	MC
CADM1	CT	CATTTG	AT	MC	MET	GA	CACGTG	CT	D
CDH1	CT	CACCTG	GC	IN, MC	MET	CG	CAGGTG	AC	D
CDK2	TT	CATGTG	AT	CY	MLANA	TT	CACGTG	TG	DIF
CDKN1A	AG	CATGTG	AC	CY	MLANA	AC	CACATG	TC	DIF
CDKN1A	CT	CATGTG	TG	CY	NGFR	CT	CACTTG	AC	D
CDKN2A	AG	CACATG	AA	CY	OSCAR	CT	CACATG	GC	O
CDKN2A	TG	CATGTG	AA	CY	***OSTM1***	***TT***	***CAGCTG***	***CT***	***O, DIF***
CLCN7	AT	CACGTG	AG	O	***PKRCB1***	***CG***	***CAGCTG***	***GG***	***DIF***
CMA1	CA	CAGTTG	AG	MC	***PKRCB1***	***AG***	***CAGCTG***	***GG***	***DIF***
CTSK	GT	CACATG	TT	O	RAB27A	AA	CAGCTG	AC	DIF
CTSK	CT	CATGTG	AC	O	RAB27A	GC	CATATG	AC	DIF
CTSK	GT	CACATG	TG	O	SERPINE1	AT	CACGTG	GC	MC, IN
DCT	GT	CATGTG	CT	DIF	SILV	TT	CATGTG	AT	DIF
DIAPH1	AT	CACATG	GG	CY	***SLC11A1***		***ND***		
DIAPH1	GG	CACATG	TT	CY	***SLC24A5***	***CA***	***CAGTTG***	***CA***	***DIF***
DIAPH1	TT	CATGTG	TT	CY	***SLC45A2***	***CA***	***CAGCTG***	***CT***	***DIF***
GPNMB	AT	CACATG	AT	IN	TBX2	GA	CATGTG	AG	CY
GPR143	GG	CATGTG	AC	DIF	TPH1	AG	CAGGTG	TG	MC
GZMB	GT	CAGATG	TG	MC	TPSB2	GA	CACATG	TT	MC
GZMB	GC	CACGTG	GA	MC	TPSB2	GG	CATCTG	GG	MC
GZMB	AT	CACATG	AG	MC	TRPM1	GT	CATGTG	GG	IN
HIF1A	***AA***	***CACGTG***	***TG***	***D***	TRPM1	GC	CATGTG	CC	IN
HIF1A	***AG***	***CACGTG***	***AG***	***D***	TRPM1	CT	CACATG	CT	IN
HPGDS	AG	CACCTG	TG	MC	TYR	GT	CATGTG	CT	DIF
HPS4	***CG***	***CAGCTG***	***CG***	***DIF***	TYRP1	GT	CATGTG	CT	DIF

Bold and italics indicates those genes and sequences that have not been confirmed by experimental evidences. The 'function' column indicates the potential role of the gene in melanocytes. CY, cell cycle; D, cell death; IN, invasion; DIF, differentiation. O, osteoclast or MC, mast cell, indicates that the gene has a specific role in the mentioned cells.

Table 2. Statistical analysis of the MITF-binding sequences.

A	17	9	0	**100**	1	18	0	0	**24**	6
T	10	**32**	0	0	**24**	6	**100**	0	13	21
C	17	9	**100**	0	**31**	10	0	0	16	21
G	**24**	18	0	0	12	**34**	0	**100**	15	20
Consensus sequence	5′-G	**T**	**C**	**A**	**C/T**	**G**	**T**	**G**	**A**	N-3′

Bold values indicate the most significant base at the concerned position.

isoforms consist of common exons 2–9 and numerous alternative first exons, each with a unique promoter driving tissue-specific expression. The resulting proteins display unique amino termini but share transactivation, DNA binding and dimerization motifs. Some isoforms such as Mitf-A, Mitf-B, Mitf-D and Mitf-H can be found in many cell types but differences in their abundance have been reported. On the other hand, some isoforms show a tissue-restricted pattern of expression: for example, Mitf-M is specific for the melanocyte lineage, and Mitf-MC, Mitf-E and Mitf-A are expressed in mast cells. Mitf-A and Mitf-D are found in osteoclasts (Oboki et al., 2002; Steingrimsson et al., 1994; Takemoto et al., 2002) and MITF-CM, has been recently identified in human mast cells (Shiohara et al., 2009).

Each of these isoforms can potentially give rise to four transcripts as a consequence of alternative splicing of exon 2A/2B and 6A/6B (Hallsson et al., 2000; Steingrimsson et al., 1994). Each of these 40 transcripts may have some specificity and transactivate a distinct set of genes. Shahlaee et al. (2007) have shown that Mitf-mc-, Mitf-e- and Mitf-a-dependent transcriptomes are clearly different, but the three isoforms are expressed in mast cells (Shahlaee et al., 2007). It is also conceivable that MITF-M expressed in melanocytes and the three isoforms expressed in mast cells control different sets of genes. Indeed, mast cells do not express tyrosinase. This specificity could be borne by the MITF transcript itself, but could also be the consequence of the cellular context, which might carry specific co-factors dictating gene specificity.

Within melanocytes, MITF-M is able to control positive (*CDK2*) and negative regulators (*CDKN1A*, *CDKN2A*) of the cell cycle, but it seems unlikely that MITF regulates these genes simultaneously in physiological conditions. This apparent antagonistic function of MITF has been elegantly reconciled by Goding et al. They proposed that MITF might act as a rheostat of melanocyte cell cycle: depending on its level, MITF activates different sets of target genes, favouring either cell proliferation or cell cycle exit and differentiation (Carreira et al., 2006). Post-translational modifications, i.e. phosphorylation (Hemesath et al., 1998; Takeda et al., 2000; Wu et al., 2000), sumoylation (Miller et al., 2005; Murakami and Arnheiter, 2005), ubiquitination (Xu et al., 2000), and acetylation (Schepsky et al., 2009), might be critically involved in the specificity of MITF activity. Further, these modifications influence the interaction of MITF with co-factors that might also influence MITF specificity.

The specificity can be provided by the regulatory elements found in the target genes that allow the binding and the combined action of multiple transcription factors. This is the case for DCT, which is subjected to a synergistic regulation by both MITF and SOX10 (Ludwig et al., 2004). In osteoclasts, MITF collaborates with NFATc1 and PU.1 to regulate the gene expression required for their differentiation (Sharma et al., 2007). Finally, the chromatin context is also a crucial parameter, as MITF needs the chromatin-remodelling SWI/SNF enzymes to regulate the expression of tyrosinase and Tyrp1 (De La Serna et al., 2006).

Concluding remarks

Identification of MITF target genes and MITF-dependent transcriptome is of paramount importance to better understand the complex role of this transcription factor in melanocyte and melanoma biology. A more precise analysis, taking into account the cellular context and the post-translational modifications of MITF will further improve our understanding of MITF functions. It should be noted that all the MITF-regulated genes described so far are up-regulated by MITF. However, it would be surprising if MITF were unable to regulate negatively, directly or indirectly, a subset of genes whose functions might be crucial in melanocyte and melanoma cells.

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