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## MITF

### *A Matter of Life and Death for Developing Melanocytes*

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#### Summary

Since its discovery over a decade ago, the microphthalmia-associated transcription factor (MITF), has moved ever more to the center of pigment cell biology. Not only has MITF been found to regulate the expression of a number of genes involved in melanin biosynthesis, it is also essential in cell lineage determination, regulation of cell proliferation and cell survival, and replenishment of follicular melanocytes in the adult. To perform these multiple functions in a temporally and spatially appropriate manner, *Mitf* needs to be stringently regulated. Through the fruitful merging of genetics, biochemistry, and molecular and cell biology, it has become clear that *Mitf* is regulated both transcriptionally and posttranslationally in response to extracellular signaling and, hence, serves as a critical link between extracellular cues and gene expression. Intriguingly, many of the molecular pathways important for pigment cell development are also implicated in the formation of melanoma; therefore, the mechanisms controlling the development of pigment cells may provide invaluable insights into the cells' malignant transformation.

**Key Words:** Neural crest; melanoblast; retinal pigment epithelium; Kit; Kitl; transcription regulation; posttranslational regulation; serine phosphorylation; cell proliferation; cell differentiation.

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## INTRODUCTION

Melanoma cells and developing melanocytes share many intriguing similarities. Both cells, for instance, engage in comparable complex behaviors that include the dissociation from an epithelial environment, invasion of the surrounding tissue, and migration to distant locations. Both cells respond to microenvironmental signals that regulate their growth, and both express common molecular markers, including microphthalmia-associated transcription factor (MITF), which links extracellular-signaling pathways with gene expression and seems crucial for the cells' survival. Hence, similar laws may govern melanocyte biology during development and malignant transformation, and understanding melanocyte development may help in understanding the formation and progression of melanoma.

### *Development of Mammalian Pigment Cells*

Mammalian melanocytes are derived from multipotent precursors in the embryonic neural crest, which is formed from specific cells residing at the junction between the surface ectoderm and the neural plate (for a comprehensive review of the neural crest, *see ref. 1*). Neural crest cells undergo an epithelial-to-mesenchymal transition, dissociate from each other, proliferate, and start to express distinct molecular markers. The expression of particular sets of markers is associated with biased cell fate choices that ultimately lead to the generation of a number of different cell types. These include, besides melanocytes, all cells of the peripheral nervous system, smooth muscle cells, and cartilage cells. The precursors to melanocytes are called "melanoblasts" and can be defined operationally as cells expressing the high mobility group transcription factor SOX10; MITF; the tyrosine kinase receptor, KIT; the G-coupled receptor, EDNRB; and the melanogenic enzyme, DCT (formerly called tyrosinase-related protein-2 or Tyrp-2); expression of these markers does not, however, preclude potential cell fate changes later in development. Melanoblasts migrate over considerable distances from the sites of their initial generation to their final destinations and start their journey approx 1 d later than other neural crest-derived cells. In areas where somites are present, melanoblasts migrate on a dorso-lateral path rather than the ventro-medial path along the side of the neural tube that is taken by other crest cells. Although the mechanisms responsible for these characteristic temporal and spatial migration patterns are not understood in detail, it appears that cadherins, integrins, and extracellular-signaling pathways operating through EDNRB are involved (reviewed in refs. 2 and 3).

While traveling to their final destinations, melanoblasts sequentially express additional melanogenic genes, many of them regulated by MITF (for an overview, *see ref. 4* and references therein). In the mouse, the sequence of expression of these genes culminates with the appearance of tyrosinase, the rate-limiting enzyme in melanin synthesis, approx 4–5 d after the first expression of MITF (for review, *see refs. 4–6*). The emergence of tyrosinase marks the beginning of differentiation into mature, melanin-positive melanocytes that finally take up residence in skin and hair follicles, the oral mucosa, the choroid in the back of the eye, the iris, and several internal sites, such as the poorly understood periorbital Harderian gland, the leptomeninges (which form the connective tissue around the brain), and the inner ear (Fig. 1A). In the inner ear, melanocytes are located in a specialized part of the lateral wall of the cochlear duct, the stria vascularis, in which they participate by still unknown mechanisms in the regulation of

the ionic homeostasis of the potassium-rich endolymph. In the absence of stria melanocytes, regardless of the genetic cause, the electrical potential between endolymph and perilymph is close to 0 mV, instead of the normal 100 mV, and auditory hair cells no longer transduce mechano-sensory signals. In fact, in viable mice with *Kit* mutations, there often are asymmetries between left and right ears, and ears containing pigment cells in their stria display an endocochlear potential, whereas ears lacking stria pigmentation do not (7,8). In addition, in human Waardenburg syndrome (of which there are several subtypes including one, Waardenburg IIa, linked to mutations in *MITF*; see ref. 9), congenital deafness is associated with pigment disturbances in hair, skin, and iris that may serve as outward signs of melanocyte deficiencies in the inner ear. Importantly, however, it is melanocytes and not their melanin that is required for normal hearing; the lack of melanin *per se*, such as in albino organisms that retain unpigmented melanocytes in their stria, causes mild, if any, hearing problems (10), although the sensitivity to ototoxic drugs may be increased (11).

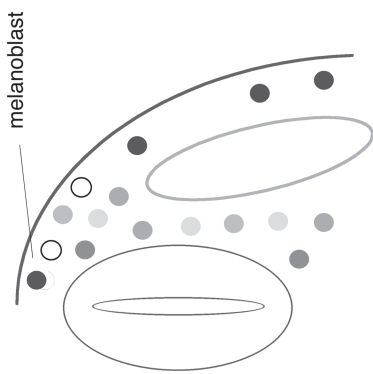
In the eye, neural crest-derived melanocytes populate the choroid and the anterior layer of the iris and act as light screens. In addition, as shown in Fig. 1B, the eye contains a specialized layer of pigment cells, the retinal pigment epithelium, or RPE, that is derived locally from the neuroepithelium of the optic vesicle. This neuroepithelium is developmentally bipotential and can give rise to either neuroretina or RPE, and disturbances in cell fate decisions between the future retina and the RPE can lead to developmental abnormalities that ultimately result in coloboma, retinal malformations, and microphthalmia (for a recent review, see ref. 12). Postnatally, disturbances in RPE cells can lead to retinal degeneration because of the critical functions that RPE cells play in photoreceptor cell physiology and maintenance.

Thus, it follows, that the biology of pigment cells reaches far beyond creating the variety and beauty of an animal's or person's pigmentation and touches on many other disciplines, including sensory organ physiology and oncology.

### ***Melanocyte Development Is Controlled by a Genetic Network***

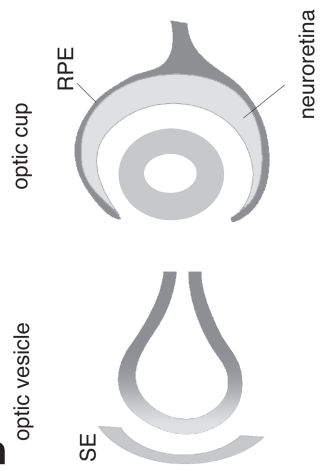
Much of our knowledge about the network of the molecules controlling birth, proliferation, migration, differentiation, death, or malignant transformation of pigment cells comes from the successful integration of biochemistry, molecular biology, and genetics. Fig. 2 shows classical examples from mouse genetics depicting phenotypes associated with heterozygosity for certain mutant alleles of five distinct genes. They include *Sox10*, *Pax3*, *Kit ligand (Kitl)*, *Kit*, and *Mitf*. Each of the mutant alleles produces similar white belly spots where neural crest-derived melanocytes are missing. Overlapping phenotypes often suggest that the products of the genes in question participate in common molecular pathways, and it is a gratifying finding that the five white spotting genes depicted in Fig. 2 indeed seem functionally linked in a common pathway. *Sox10* and *Pax3* encode transcription factors that, although more widely expressed than *Mitf*, activate at least one of the many *Mitf* promoters in vitro (13–15). *Kitl* and *Kit* encode a ligand/receptor pair (for a recent review, see ref. 16), whose activation leads to multiple post-translational modifications of MITF protein that affect MITF's transcriptional activities on target genes (17–19). It thus appears that a network of extracellular and intracellular regulatory proteins all converge on the single transcription factor, MITF, which, in turn, serves as the nexus to a set of downstream target genes that execute the requisite program of melanogenesis (for a review, see ref. 20). Here, we highlight these recent findings and

**A**



melanoblast

**B**

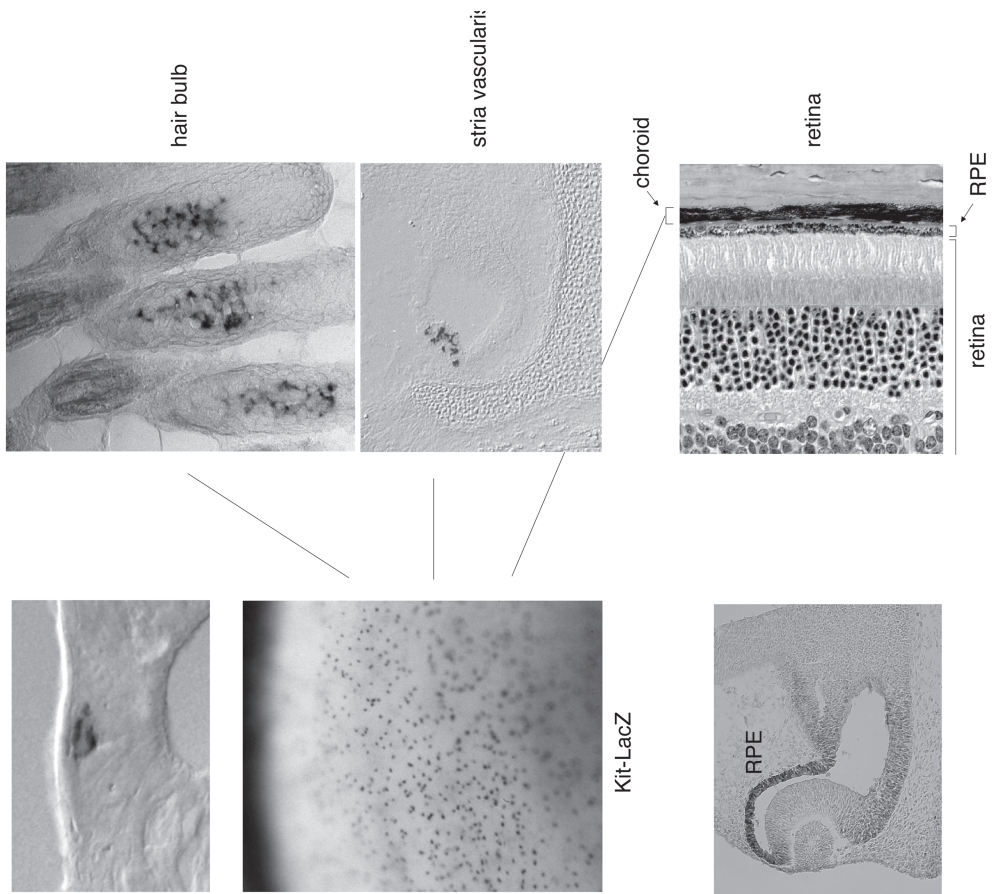


optic vesicle

optic cup

RPE

neuroretina



hair bulb

stria vascularis

choroid

retina

RPE

retina

Kit-LacZ

RPE

focus in particular on mouse *Mitf*, because *Mitf* research started in mice, and mice provide a rich resource of *Mitf* mutations and will continue to yield important insights into the function of *Mitf*. For more comprehensive reviews on the transcriptional regulation of *Mitf* and its target genes, however, we refer the reader to Chapters 3 and 4 in this book, and to other recent reviews (21,22).

## **MITF: EXPRESSION, ALLELES, AND DEVELOPMENTAL PHENOTYPES**

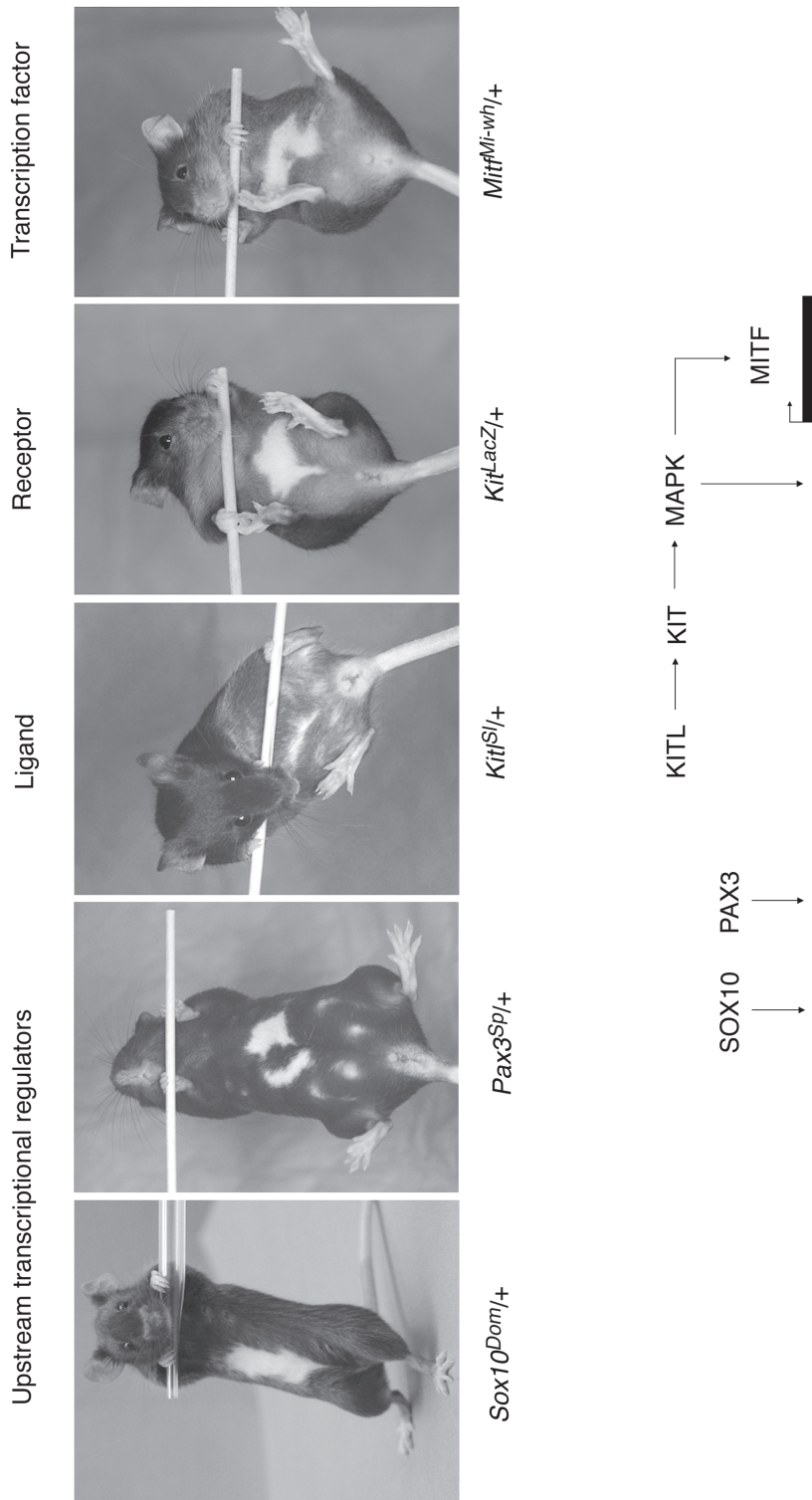
### ***The Mitf Gene and Its Protein Products***

The *Mitf* gene was first isolated in 1993 from lines of mice with transgenic insertional mutations at the *microphthalmia* (*mi*) locus (23,24). This locus was originally described more than 50 yrs earlier with a single mutant allele, *mi* (25). Meanwhile, more than 30 additional alleles—many of them spontaneous, and some induced chemically, by irradiation or by targeted mutagenesis—have been isolated (refs. 26 and 27, and unpublished results). Similar to mice with phenotypically severe *mi* alleles, mice homozygous for the transgenic insertion *Mitfvga-9*<sup>1</sup> lack neural crest-derived pigment cells in coat, eye, and inner ear; have an abnormal RPE; small, degenerating eyes; and are profoundly deaf (23,28–30). In these transgenic mice, extraneous sequences are by chance inserted into the promoter region of a gene encoding a novel member of the basic helix-loop-helix–leucine zipper (bHLH-LZ) class of transcription factors (23) that we later termed *Mitf* (31) (Fig. 3). Such factors are known to participate in a variety of biological processes, including the regulation of cell fate specification, proliferation, and differentiation. The bHLH-LZ domain is the critical domain that allows these proteins to form obligatory homodimers and heterodimers and to bind specific DNA elements, Ephrussi (E)-boxes, 5'CANNTG3', in target gene promoters. Because all known *mi* alleles in mice turned out to have mutations in this gene, it is now firmly established that *Mitf* is indeed the single *mi* gene in this species (32,33).

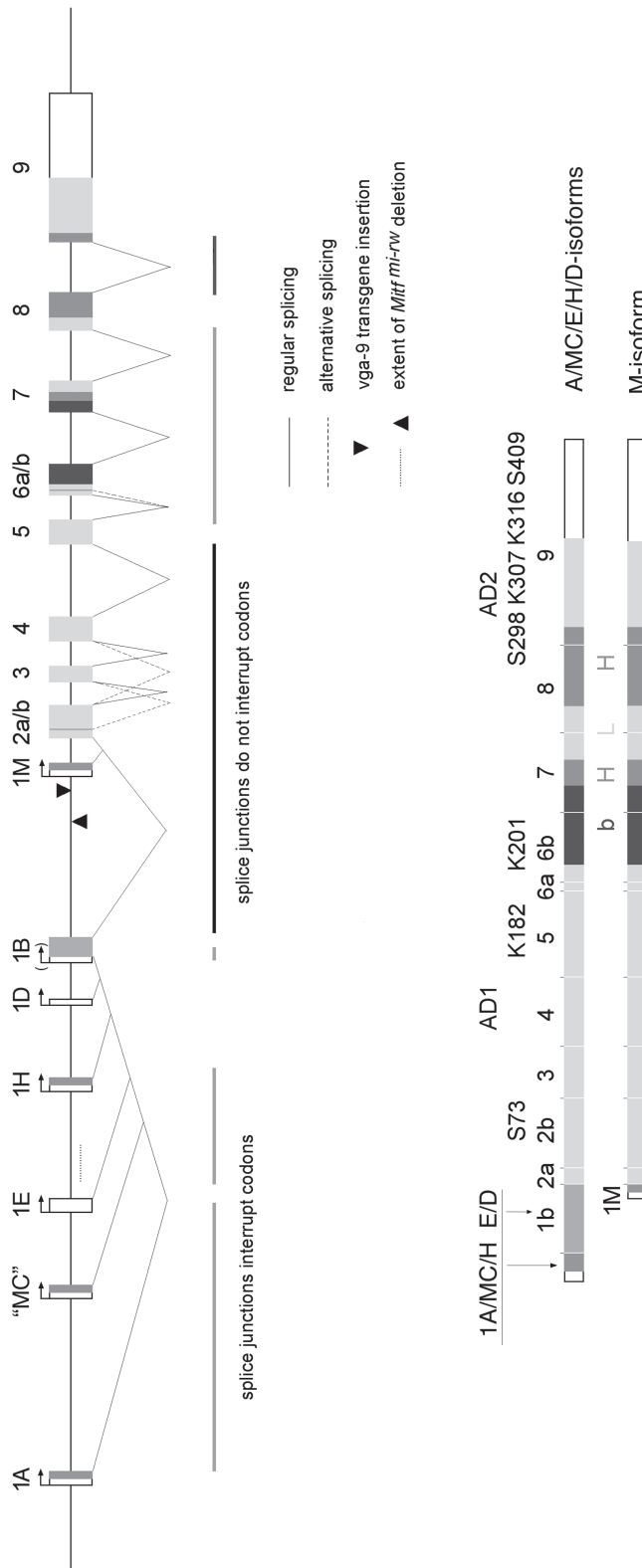
**Fig. 1.** (*opposite page*) Development of vertebrate pigment cells. (A) One source of pigment cells is the neural crest. In the trunk area of a wild-type mouse embryo, *Mitf*-positive melanoblasts (●) originate at the rooftop of the neural tube and then migrate underneath the surface ectoderm on a dorsolateral pathway. Other neural crest derivatives (gray circles) take the ventro-medial route. Some cells (○) may not yet express any cell type-specific marker and represent uncommitted stem cells. A *Kit*-LacZ transgene allows migrating *Kit*-positive melanoblasts to be labeled en face underneath the surface ectoderm. These melanoblasts migrate and differentiate and finally take up residence at various sites, such as in hair bulbs, the stria vascularis of the inner ear, or in the choroid behind the retina. (B) Another source of vertebrate pigment cells is the optic neuroepithelium which evaginates as an optic vesicle from the telencephalon. After invagination to form the optic cup, the part of the optic neuroepithelium exposed to growth factors emanating from the closely juxtaposed surface ectoderm (SE) finally gives rise to a domain that goes on to form the retina. The part closer to the brain will give rise to a domain that goes on to form the retinal pigment epithelium (RPE). Each part of the optic neuroepithelium is initially bipotential, that is, capable of giving rise to either retina or RPE, and addition or removal of growth factors or genetic manipulations can change the normal fate determinations (119).

<sup>1</sup>“vga-9” was the ninth transgenic line made with a transgene comprised of a mouse vasopressin promoter,  $\beta$ -Gal reporter, and human vasopressin polyA signal.





**Fig. 2.** Five genes controlling melanocyte development are linked in a common genetic pathway. Heterozygosity for particular alleles of these five genes causes similar belly spots in the mouse. They include the genes for the transcription factors SOX10 and PAX3, the ligand/receptor pair KITL/KIT, and the transcription factor MITF. KIT signaling activates the mitogen-activated protein kinase (MAPK) pathway that can affect the transcription of the *Mitf* gene as well as modulate MITF activity through serine phosphorylation.



**Fig. 3.** *Mitf* gene structure and protein isoforms. The mouse *Mitf* gene on chromosome 6 spans approx 200 kbp and contains multiple exons. Most mRNA isoforms contain exons 2–9 and either the 1m exon, or exon 1b or part of exon 1b along with one of the upstream exons (1a, 1c, 1mc, 1h). The site of the vga-9 transgene insertion and the 5' and 3' boundaries of the *Mitf**mi-rw* deletion are indicated, as are standard and alternative splice forms. The basic (DNA-binding) domain is encoded by parts of exons 6 and 7, and the HLH-Zip domain by the remainder of exon 7, exon 8, and the beginning of exon 9. The encoded MITF proteins contain two activation domains (AD1 and AD2) and several residues that are subject to posttranslational modifications. They include the serines S73, S298, S307, and S409 as sites of phosphorylation, the lysines K182 and K316 as sites of sumoylation (91,92), and K201, reported as a site for ubiquitination (19).

*Mitf* is approx 100 megabasepairs (Mbp) from the centromere on mouse chromosome 6, and its human homolog, approx 70 Mbp from the telomere on chromosome 3p (31). *Mitf* is most closely related to three other bHLH-LZ genes, *Tfeb*, *Tfe3*, and *Tfec*. The proteins encoded by these related genes and *MITF* can form stable dimeric combinations among each other but not with other bHLH-LZ or bHLH proteins (34,35), and, together, constitute the MITF-TFE subfamily of bHLH-LZ proteins (35). In both mouse and humans, *Mitf* transcription is initiated from at least nine distinct promoters, giving rise to mRNAs encoding proteins that differ at their amino-termini but usually share the sequences of eight exons (Fig. 3). Reverse transcription-polymerase chain reaction (RT-PCR) approaches, along with the analyses of *Mitf* mutations, have revealed a variety of alternatively spliced mRNAs lacking particular exons or parts of exons. Intriguingly, as shown in Fig. 3, the 3' splice junction of exon 1b; the 3' splice junction of exon 1m; all junctions of exons 2, 3, and 4; and the 5' junction of exon 5 correspond to in-frame codons. This means that exons 2, 3, and 4 can be spliced out safely without disrupting the remaining open reading frame, and their inclusion or exclusion could therefore be regulated depending on the developmental or proliferative state of a given cell. By contrast, selective elimination of the entire exons 5, 7, or 8, the latter two including sequences encoding the bHLH-LZ domain, would create mRNAs with premature stop codons, expected to be subject to nonsense-mediated degradation. Elimination of exon 6, however, does not lead to an interruption of the open reading frame, but, nevertheless, creates a nonfunctional protein, as seen in mice with the *Mitf<sup>mi-ew</sup>* mutation (32). Also, the 3' splice junction of the exons 1a–1h interrupt the codons corresponding to the predicted amino-terminal reading frames, precluding the maintenance of open reading frames for a number of alternative potential splice arrangements such as splicing directly into exon 2. In fact, the elimination of exon 1b, as seen in *Mitf<sup>mi-rw</sup>* mice, in which the upstream exon A is spliced directly into exon 2, creates mRNAs with premature stop codons (32,33).

Based on the observed gene structure, the single *Mitf* gene of mammals could theoretically give rise to at least 48 distinct, functional mRNAs and protein isoforms that retain the full bHLH-LZ domain. For some of these, such as splice forms lacking exon 6a, a genetic role has been established, but for many others, a specific role still needs to be explored by further mutational analyses. Intriguingly, the genes encoding the related proteins TFEB, TFE3, and TFEC are organized in a similarly complex manner (36). Their capacity to dimerize with MITF when coexpressed may thus lead to a vast set of distinct dimers, each potentially with discrete stabilities and activities.

### ***The Developmental Role of Mitf: Expression Patterns and Genetics***

The cell-autonomous role of *Mitf* in melanocyte and RPE development is evident from its expression patterns and its genetics. Evolutionarily ancient—homologs are found in *Caenorhabditis elegans* and *Drosophila* (37)—*Mitf* is clearly expressed in developing pigment cells starting with urochordates, which have a notochord like vertebrates, but lack a neural crest (38). Interestingly, tadpoles of the urochordate *Halocynthia roretzi* sport exactly two melanin-containing pigment cells, an anterior one called the otolith that serves as a balance organ akin to that in the inner ear of vertebrates, and a juxtaposed posterior one that is part of the ocellus, a primitive structure involved in light perception.

In vertebrates, *Mitf* is expressed in melanoblasts and melanin-containing melanocytes (called melanophores in fish, amphibia, and reptiles) and RPE cells (30,39–41). This is not to say that *Mitf* is only found in pigment cells. For instance, in mammals, some



isoforms are expressed in unrelated cell types such as osteoclasts (42), NK cells (43), macrophages (44), B-cells (45), and mast cells (46), all of which are affected, if not by null alleles, at least by dominant-negative alleles of *Mitf*. *Mitf* is also expressed in heart (23,30), in which its function has yet to be established. In fact, by RT-PCR techniques, *Mitf*, at least its exon 1b, is expressed widely, if not ubiquitously, as shown in the gene expression database GXD (47). It would appear, then, that, evolutionarily, the *Mitf* gene must be under considerable constraints to maintain proper expression patterns and regulation to serve the needs of so many different cell types. It also implies that *Mitf* expression does not inevitably lead to the activation of the melanogenic program. Rather, it is the developmental history of cells, or the presence of specific *Mitf* isoforms, or a combination of history and isoforms, that is associated with the development of the respective lineages. Hence, despite the demonstration that *Mitf* or at least some isoforms are capable of recruiting cells other than melanocytes to become pigmented (48), the broad term “melanocyte master regulator” should be applied with caution.

Whereas *C. elegans*, *D. melanogaster*, and *H. roretzi* each seem to have only one *Mitf* gene, teleost fish (40,49) and *Xenopus* (50) have two separate genes. One, *Mitf-a* (nomenclature according to ref. 40), is expressed in the neural crest and the RPE, and the other, *Mitf-b*, in the RPE, epiphysis (zebrafish and frog), and olfactory bulb (zebrafish) but not, or less abundantly, in melanoblasts. In birds and mammals, there is no evidence for two separate genes, and tissue-specific roles may be fulfilled by the distinct isoforms. M-Mitf, the homolog of fish *Mitf-a*, for instance, is prominently expressed in the neural crest and not the RPE of mice, and A-Mitf (the homolog of fish *Mitf-b*) and D-Mitf are expressed in the RPE (ref. 51, and unpublished results). Their respective roles can be assessed in organisms with distinct *Mitf* mutations, which are quite abundant in vertebrates.

Mutations in *Mitf-a* in the *nacre* zebrafish are associated with a selective loss of melanophores, while other types of pigment cells (xanthophores, iridophores, and RPE cells) are not affected (39). Although no isolated mutation in zebrafish *Mitf-b* has yet been found, it is clear that *Mitf-b* retains the potential to generate melanophores, because it can rescue *Mitf-a* mutations, whereas the related zebrafish *Tfe3*, for instance, cannot (40). *Mitf* mutations have also been found in quail (52), hamster (41), rat (53,54), mice (23,32,33), and humans (9). In mice, even the mildest alleles affect neural crest-derived melanocytes, but only more severe mutations affect the RPE as well. The phenotypes associated with each of these alleles are far from being uniform or merely gradations in severity, however. Indeed, a trained eye can distinguish the 30 different mouse alleles and many of their heteroallelic combinations alone by visual inspection of their carriers.

### ***A Variety of Alleles and Phenotypes: The Bane and Beauty of Mitf***

One of the many reasons why distinct alleles cause such a variety of phenotypes lies in the fact that MITF is a protein with multiple, functionally distinct domains, some of which are subject to further posttranslational modifications. Mutations that eliminate or distort the protein's DNA binding basic domain, the dimerization domain, or one of its activation domains usually result in an early abrogation of the melanocyte lineage and a hyperproliferation of the RPE followed by subsequent derailment of eye development (32,55). Hence, mice homozygous for such mutations are white, deaf, and microphthalmic, similar to mice lacking MITF protein altogether. Nevertheless, the complete lack of MITF protein is milder in its effects than the presence of an MITF protein that, although unable to bind DNA, still can dimerize or interact with other proteins and exert

dominant-negative activities. Milder still are alleles that leave the bHLH-LZ domains intact but affect other protein domains. No doubt the mildest among all published alleles is *Mitfmi-sp* (*mi-spotted*), which, when homozygous, has no obvious pigmentation phenotype at all, although a reduction in tyrosinase levels in the skin has been observed. Only in combination with other *Mitf* alleles do carriers of *Mitfmi-sp* become conspicuous by the presence of white spots, and it is this fact that originally led to the discovery of *Mitfmi-sp* when it appeared *de novo* in a colony segregating *MitfMi-wh* (*Mi-white*) (56). *Mitfmi-sp* is caused by the insertion of an extra base pair in exon 6a, leading to the exclusive expression of mRNAs lacking the exon 6a-associated six codons (encoding ACIFPT) normally present in at least half of the *Mitf* mRNAs (32). Lack of these residues slightly lowers the protein's avidity for DNA (35) and its capacity to stimulate transcription. Another mild allele is *mi-vitiligo* (*Mitfmi-vit*), which leads to a combination of white spots and large pigmented areas that prematurely become gray as the animal goes through its molting cycles. Premature graying is likely caused by premature loss of melanocyte stem cells in the hair bulb niche (57,58). A similar, though less extensive, premature graying is also seen with other alleles, but with certain alleles, such as *mi-red eyed-white* (*Mitfmi-rw*), pigmented spots do not seem to prematurely gray. Further, there are alleles such as *mi-brownish* (*Mitfmi-b*) or heteroallelic combinations between *MitfMi-wh* and several other alleles that lead to changes in the hue of pigmentation (55,59). Such color changes show that *Mitf* does not only regulate melanoblast development but also the quality of melanin in the differentiated melanocyte, consistent with *Mitf*'s direct transcriptional stimulation of pigmentation genes or its effects on melanocyte dendricity or other aspects of melanocyte biology.

The different domains appended to the amino-termini of MITF may also contribute to allele-specific phenotypes. Available genetic evidence suggests, for instance, that selective deficiencies in M-MITF result in lack of pigmentation in the coat, choroid, and anterior layer of the iris, but not the RPE (60), hinting at the possibility that selective deficiencies in other amino-terminal isoforms might likewise lead to cell type-specific phenotypes. It is not known, however, whether such cell type specificities are simply owing to differences in expression patterns or to functional differences between the different polypeptides. In other words, it is not known to what degree one isoform might substitute *in vivo* for another. Nevertheless, functional differences between isoforms have been suggested by a recent study showing that melanoma cells that lack M-Mitf expression (although expressing other isoforms), when reconstituted with an M-Mitf expression plasmid change their morphology and growth characteristics after *in vivo* transplantation (61).

A further reason for allele-dependent phenotypic distinctions is heterodimerization of MITF with TFE proteins. The lack of either TFE3 or TFEC is without obvious phenotypic consequences in the mouse and the lack of TFEB is associated with disturbances in placental vascularization (62). Combinations with *Mitf* mutations do not result in novel phenotypes except when both *Tfe3* and *Mitf* are missing. Mice lacking both TFE3 and MITF suffer from severe osteopetrosis that interferes with normal tooth eruption and is fatal at the time of weaning (62). Indeed, mutations in *Mitf* alone, when homozygous, can lead to fatal osteopetrosis if the mutant MITF protein is still capable of heterodimerization with TFE3 but cannot bind DNA, thus mimicking the combined lack of TFE3 and MITF (53,62). Importantly, however, the simple lack of MITF in osteoclasts does not result in osteopetrosis, and the lack of TFEB, TFE3, or TFEC does not seem to affect melanogenesis (62). This suggests that although heterodimers between MITF and

TFE proteins are found *in vivo*, such as in osteoclasts (53), they are not essential to the function of each protein. MITF-TFE proteins, therefore, do not seem to follow the rationale of regulation by heterodimerization that is seen with the MYC/MAD/MAX group of bHLH-LZ proteins or the myogenic MYOD group of bHLH proteins. This is surprising, because MITF, like these other proteins, also links cell proliferation with cell differentiation (*see* page 41).

From the genetic evidence it follows, then, that MITF must have multiple functions in melanogenesis: it recruits cells to the pigment lineage, regulates their proliferation and survival, induces their differentiation into mature melanocytes, regulates their differentiated state, and is responsible for their maintenance throughout adulthood.

## REGULATION OF *Mitf*

### *Transcriptional Regulation*

Not surprisingly, a factor as potent as MITF must be under stringent regulation to safeguard against two dangerous derailments: premature cell differentiation, potentially leading to deficiencies in cell numbers, or prolonged cell proliferation, potentially leading to an overproduction of immature cells. Ultimately, what needs to be regulated is *Mitf*'s function in the broadest sense, which depends not only on *Mitf* mRNA and protein levels, but also on posttranslational modifications of MITF and the availability and activity of cofactors.

A first level of control is exerted, positively and negatively, by a combinatorial set of transcription factors that each binds specific motifs in the promoter elements of the *Mitf* gene and regulates *Mitf* mRNA expression. One of these, PAX3, is a paired homeodomain protein required for the proper generation of the neural crest and other lineages including, for instance, limb muscle precursors. For melanoblasts, PAX3 is limiting, because *Pax3* heterozygous mice have belly spots (Fig. 2), and *PAX3* heterozygous humans have the typical combination of pigment disturbances and deafness of Waardenburg I syndrome (63). Another transcription factor is SOX10, associated with belly spots when mutated in mice (Fig. 2) and with Waardenburg-Shah syndrome when mutated in humans (64). In fact, several groups have shown cooperativity of PAX3 and SOX10 on the M-*Mitf* promoter (13,14). Additional positive regulators for M-*Mitf* include members of the LEF1/TCF family of proteins, which interact with  $\beta$ -catenin and link *Mitf* expression to Wnt-signaling (65,66); this link provides the rationale for the observation that Wnt signaling increases the generation of melanocytes in zebrafish (67), quail (68), and mouse neural crest cell cultures (69), and that Wnt1/Wnt3a double mutant mouse embryos have a substantial reduction in *Dct*-positive melanoblasts (70). Intriguingly, LEF1 can interact with the bHLH-LZ domain of MITF and cooperate in the activation of the M-*Mitf* promoter, and this cooperation is even seen with a mutant MITF protein incapable of efficient DNA binding (71,72). This observation is consistent with the facts that the M-*Mitf* promoter does not itself contain MITF binding sequences, and that *Mitf* mutations affecting DNA binding do not hamper early developmental *Mitf* expression. Consequently, by interacting with LEF1, MITF may potentially regulate its own expression.

There are a number of additional factors that have been implicated in the regulation of M-*Mitf* expression, mostly based on *in vitro* results. The bLZ protein cAMP responsive element binding protein (CREB), for instance, binds a cAMP-responsive element (CRE) in the M-*Mitf* promoter and links *Mitf* expression to cAMP responsiveness and,

through CREB phosphorylation, to the mitogen-activated protein kinase (MAPK) pathway (73; for review *see ref. 74*). Although CREB would come across as an all-purpose activator that is stimulated by many signal-transduction cascades and activates many target genes (75), the CRE of M-Mitf is quite tissue-specific, likely because its activation codepends on SOX10 (76). Additional factors involved in M-Mitf regulation include the transcription factor Onecut2, which belongs to a family of homeodomain proteins involved in lineage determination (77), and the POU domain protein BRN2, which has been proposed to serve as a negative regulator (21). Interestingly, *Brn2* expression is itself upregulated by Wnt signaling *in vitro*, as is M-Mitf expression, but *Brn2* is not seen in *Dct*-positive melanoblasts, only later in hair follicle melanocytes (78). Hence, *Brn-2* may not exert its potential negative activity on M-Mitf early in development. When prematurely activated in tyrosinase promoter/ $\beta$ -catenin transgenic mice, however, it interferes with normal melanogenesis (78).

Although the M-Mitf promoter has been studied in some detail, little is known about the regulation of the other promoters. A recent report shows that for RPE precursors, the paired domain proteins PAX2 and PAX6 act as critical, though redundant, positive regulators of *Mitf* (79), as do the homeodomain proteins OTX1/OTX2 (80). Furthermore, the paired-like homeodomain protein CHX10 represses MITF in the part of the neuroepithelium destined to become retina. In fact, the lack of MITF repression in the future neuroretina is part of the ocular phenotype in mice with *Chx10* mutations (refs. 81 and 82, and *see page 42*).

### ***Posttranslational Regulation***

Besides regulation at the transcriptional level, MITF is also controlled post-translationally by several modifications, the best characterized of which is serine phosphorylation by MAPK signaling. As mentioned above, it is well established that MAPK signaling initiated by KITL-activated KIT is of critical importance to melanocyte development. In fact, potential links between KIT signaling and *mi* have been reported even before *Mitf* was cloned. For instance, a suggestion that the two genes might interact came from the observation that the extent of white spotting in mice heterozygous for both *MitfMi-wh* and *KitW-36H* vastly exceeded that of their single heterozygous parents (83), a finding that we confirmed using the null allele *KitlacZ* (4). Importantly, however, combinations of *KitLacZ* with other *Mitf* alleles, including the null allele *Mitfvga-9*, do not show such phenotypic enhancements, suggesting that *Kit* heterozygosity does not lead to further loss of melanocytes in combination with *Mitf* alleles unless acting through mutant MITF protein, in particular dominant-negative MITF (unpublished results; for further discussion of this point, *see page 40*). Another study showed that the tyrosine kinase receptor c-FMS, although capable of rescuing *Kit* mutant mast cells, was incapable of rescuing *mi* mutant mast cells (84). This suggested that either *Mitf* was downstream of the *Kit/c-fms* signal-transduction pathway or played entirely independent roles. In yet another study, however, *Kit* expression was found to be low in *mi* mutant mast cells, suggesting that *Mitf* was upstream of *Kit*. These latter two results are not necessarily conflicting, because they may simply reflect a feedback loop between *Kit* and *Mitf*. In fact, it was later shown that in primary melanoblast cultures, *Mitf* is required for upregulation and maintenance of *Kit* expression (4,29), and that in melanocyte or melanoma cell lines, KIT signaling regulates MITF protein through phosphorylation (17–19).

Following KIT signaling, phosphorylation occurs at two serine residues, serine-73 (S73), which is phosphorylated by the MAPK–stimulated extracellular signaling-regulated kinase ERK1/2 (17–19), and serine-409 (S409), which is phosphorylated by p90<sup>RSK</sup>, itself activated by ERK1/2 and other kinases (18). Although S73-phosphorylated MITF can be conveniently detected because of its reduced electrophoretic mobility (17), S409 phosphorylation does not change the protein’s electrophoretic mobility, which makes its detection less straightforward. Importantly, however, phosphorylation of neither one of these two serines is necessary for phosphorylation of the other, and either one can be phosphorylated regardless of the presence or absence of the genetically important exon 6a-encoded residues.

What is the biological consequence of MITF phosphorylation? It appears that unlike what is observed with other transcription factors, such as the SMAD proteins, phosphorylation does not regulate nuclear accumulation of MITF because both wild-type MITF, whether S73 phosphorylated or not, and MITF whose S73 and S409 have been substituted for nonphosphorylatable alanines, accumulate efficiently in the nucleus (unpublished results). Phosphorylation at S73 does, however, increase MITF’s transcriptional activity on the tyrosinase promoter. This is inferred from the fact that a S73LA substitution yields a protein with a two- to threefold lower activity compared with wild type, as originally tested in the presence of constitutively active RAF and wild-type MEK (17), or later without these activators (18). The increased activity following S73 phosphorylation is thought to result from an increased association with the transcriptional cofactors CBP/p300 (85,86). CBP/p300 are histone acetyl transferases best known for their ability to modify chromatin architecture. It is not clear, however, whether the molecular target of these proteins is chromatin of *Mitf* target genes or MITF protein itself, which also has potential acetylation sites; acetylation has been demonstrated to modify the activity, for instance, of NF- $\kappa$ B (87). Alternatively, CBP/p300 might also act as bridging proteins between MITF and basal transcription factors, as scaffold proteins required for setting up the transcriptional machinery or as ubiquitin ligases.

Phosphorylation at S73 alone (19) or only in conjunction with phosphorylation at S409 (18) also renders MITF less stable. This loss of stability is thought to result from an increased polyubiquitination at lysine 201 (19) followed by proteasome-mediated degradation. For instance, 5 h after addition of KITL to human melanoma cells, there was a marked reduction of MITF, which could be prevented by addition of MAPK or proteasome inhibitors, or combined alanine substitutions at S73 and S409 (18). Despite an increase in stability, the double alanine substitutions rendered the protein transcriptionally inactive, yet still capable of binding DNA (18). In contrast to S73/409 unphosphorylated wild type or alanine-mono-substituted MITF, however, the doubly substituted MITF also displayed a surprising increase in electrophoretic mobility (ref. 18, unpublished results), suggesting either a change in conformation or in other modifications of MITF.

S409 phosphorylation has also been implicated in regulating the interaction of MITF with the zinc finger protein, PIAS3, which was found to repress the transcriptional activity of wild-type MITF but not of S409→D MITF, a substitution made to mimic the charge of phospho-S409 (88). Consistent with this finding, PIAS3 bound and inhibited wild-type MITF and S409→A mutated MITF but not S409→D mutated MITF. PIAS3, similar to other PIAS proteins, serves as E3 ligase for SUMO-1 (small ubiquitin-like modifier, a 101-residue-polypeptide), whose attachment to  $\epsilon$ -amino groups of lysines



embedded in an (I/L/V)KXE motif results in a branched polypeptide (for a recent review, *see ref. 89*). Sumoylation has been shown to attenuate the activity of transcription factors, such as LEF1, by sequestering the sumoylated protein into nuclear bodies (90). It would be intriguing if S409 phosphorylation modified MITF activity and/or stability by interfering with PIAS3-mediated sumoylation. It has recently been found that MITF is indeed sumoylated at two lysines, K182 and K316, and that sumoylation decreases MITF's transcriptional activity. This sumoylation depends on SUMO E1 activating enzyme (SAE I/SAE II) and E2 conjugating enzyme (Ubc9). Nevertheless, coexpression of PIAS members reduced the transcriptional activity of wild-type MITF and K182/K316→R mutated MITF to the same extent, suggesting that PIAS does not suppress MITF by modulating sumoylation (91,92).

Besides phosphorylation at S73 and S409, phosphorylation at two additional serines has been implicated in the regulation of MITF. Signaling by the stress kinase p38 leads to phosphorylation at S307 and, concomitantly, to an increase in MITF's transcriptional activity (93). This study was limited to osteoclasts, but because p38 is activated by UV (as are melanocytes), S307 phosphorylation might quite possibly play a role in melanocytes as well. It has also been proposed that S298 is phosphorylated by GSK-3 $\beta$ , which is inhibited in the canonical Wnt/ $\beta$ -catenin pathway but activated by cAMP, known to stimulate melanogenesis in an *Mitf*-dependent manner (73,94,95). This serine is substituted for a proline in an individual with Waardenburg syndrome (96). In vitro, substitutions for either proline or alanine impair MITF's activity (96), but so do substitutions for aspartate or glutamate, despite the fact that these two residues mimic the charge of p-S298 (94). In fact, to date, S298 fulfills only one of several criteria proposed for standard GSK-3 $\beta$  targets (97), and until it is unequivocally proven that GSK-3 $\beta$  phosphorylates S298, one might explain the results also by assuming that substitutions at this residue might interfere with phosphorylation at other sites.

Regardless of the precise residue that becomes phosphorylated and the kinases that are involved, a consensus has emerged that phosphorylation increases MITF's transcriptional activity. Conversely, as seen, at least with S73 and S409 phosphorylation, it also decreases MITF stability. That stability can regulate the relative abundance of a transcription factor, and that relative abundance can determine the extent of target gene stimulation, has been amply demonstrated, for instance, for  $\beta$ -catenin or p53 (98). At first sight, then, MITF's phosphorylation-dependent loss of stability and its gain of activity are two opposing principles, whereby one may win over the other, or they may cancel each other out, depending on when precisely and how efficiently phosphorylations and dephosphorylations occur during development (for a discussion of the importance of the kinetics of phosphorylation/dephosphorylation events in vitro, *see ref. 99*). Interesting situations may arise with dominant-negative MITF proteins, whose stability can be regulated by phosphorylation but whose activity cannot be regulated because of their intrinsic inability to bind DNA. Such proteins should manifest their dominant-negativity more prominently if they are underphosphorylated. Intriguingly, as mentioned on page 38, white spotting is increased in *Kit* heterozygotes if they carry dominant-negative *Mitf* alleles but not if they carry *Mitf* null alleles (refs. 4 and 83, and unpublished results). However, based on the tight coupling between instability and activity observed in a number of other transcription factors, including the bHLH-PAS factor AhR (100), SMAD2 (101), or STATs (102), a different model of transcription regulation has emerged, in which ubiquitination and proteasome-mediated degradation of enhancer-bound factor are *prerequisite* for transcription initiation and elongation (103). Accord-



ing to this model, a new molecule from the pool of unbound factor would have to be recruited to bind DNA for each round of transcription initiation, a mechanism that would allow for a stringent regulation of gene expression, provided the pool of unbound factor is limiting (which may again depend on the protein's stability). Future experiments will show whether MITF might operate along these lines or not. We find it to be of critical importance, however, that any in vitro results, however compelling, be confirmed in the intact organism, be it by testing targeted mutations or by rescue strategies using appropriate transgene constructs.

### THE BUSINESS END OF *Mitf*: THE REGULATION OF CELL PROLIFERATION AND DIFFERENTIATION

In 1993, when *Mitf* was cloned, it was already known that the expression of melanocyte pigmentation genes, such as tyrosinase, depended on crucial *cis* elements called M-boxes (104). In their core, M-boxes contain a CATGTG E-box, which, in a way, was puzzling because there is hardly anything melanocyte-specific about an E-box and no melanocyte-specific E-box-binding bHLH or bHLH-LZ proteins were known. The identification of *Mitf* as a bHLH-LZ gene was met with some relief because it went a long way toward explaining pigment gene regulation via E-boxes, not only in melanocytes but also in the RPE. However, although much has since been learned about target gene regulation and the multiple levels of the regulation of *Mitf* itself, many fundamental questions remain unanswered. For instance, E-boxes are found in many promoters, and even the refinement of the optimal MITF binding sequence (105) would not seem to provide the required element of specificity. Furthermore, *Mitf* is not specific to pigment cells, although during development it is most prominently expressed in the pigment lineage (30). Hence, are pigment gene E-boxes characterized by a particularly high threshold of activation, and only MITF can meet this threshold because of its high level of expression in these cells? If so, which other factors, if experimentally expressed to similar levels, would substitute for *Mitf*? Or does *Mitf* cooperate with a distinct set of other factors to provide the necessary specificity? How does *Mitf* achieve the correct temporal sequence with which these target genes are expressed during development? What factors are responsible for the maintenance of target gene expression once *Mitf* is downregulated in differentiated cells, as is the case in the RPE? And finally, what precisely is the set of genes through which *Mitf* regulates the specification of precursor cells, the behavior of melanocyte stem cells, their survival, proliferation, migration, homing, and, ultimately, differentiation? Interestingly, an ever-growing list of genes including *Matp* (also known as *Aim-1*, underlying the *underwhite* mutation), *Mart1*, *Silver*, *OAI*, *Ink4a*, *Cdk2*, *Mif1*, and *Slug*, turn out to be targets of *Mitf* (106–112), and their identification will undoubtedly shed light on some of these questions.

As pointed out on page 29, the lack of *Kit* or *Kitl*, similar to the lack of *Mitf*, leads to the demise of the melanocyte lineage. A lack of maintenance of KIT protein expression would therefore seem sufficient reason to explain the early loss of *Mitf* mutant melanoblasts (29). However, things are rarely that simple. In fact, mutations in other genes, such as endothelin-3 (*Edn3*) and its receptor, Endothelin receptor B (*Ednrb*) may also lead to an early loss of melanoblasts (113,114), but a direct link between *Mitf* and *Edn3/Ednrb* has not yet been established. We have recently found, however, that KITL generated by EDN3-responsive “nonmelanoblasts” can rescue *Ednrb*-deficient melanoblasts (115), suggesting that there is perhaps an indirect link to nonmelanoblastic *Mitf* because of its response to KIT signaling. Furthermore, *Mitf* may regulate cell survival via yet another

factor, the anti-apoptotic gene *Bcl2*. *Bcl2*-deficient mice are born pigmented but rapidly lose their pigmentation with the first hair cycle, earlier than *Mitf<sup>mi-vit/mi-vit</sup>* mice (116). In vitro evidence suggests that *Bcl2* and *Mitf* might act in the same pathway. For instance, chromatin immunoprecipitation assays indicate that MITF associates with the *Bcl2* promoter in melanoma cells, binds to an E-box in this promoter, and mutation of this E-box abolishes the stimulation of a reporter after MITF overexpression (117). It has yet to be demonstrated, however, whether *Mitf* and *Bcl2* act in vivo in the same pathway. In fact, a recent study on hair graying suggests that they may not act in the same pathway (58).

RPE cells react differently to *Mitf* mutations than melanocytes (30). Rather than dying, RPE precursors survive and, in fact, hyperproliferate and transdifferentiate into a second neuroretina (118,119). It is tempting to speculate, therefore, that if *Mitf* mutant melanoblasts could somehow be coerced to survive as well, they would hyperproliferate like RPE cells, suggesting that *Mitf* may serve as an inhibitor of cell proliferation. As listed in Table 1, although there is much indirect evidence suggesting that *Mitf* is antiproliferative, there are other studies arguing that it promotes proliferation.

To illustrate how *Mitf* might regulate cell proliferation negatively in vivo, let us turn away from pigment cells and consider retinal precursors. Early in mouse eye development (at the optic vesicle stage, *see* Fig. 1), *Mitf* is expressed in both the future retina and the future RPE. Experiments with explant cultures of such early eyes have shown that under the influence of the surface ectoderm, the juxtaposed presumptive neuroretina begins to express, or maintains expression of, the paired-like homeodomain transcription factor, CHX10 (119). This factor is instrumental in the rapid downregulation of *Mitf* in the future neuroretina, because, in *Chx10* null mutant embryos, *Mitf* is no longer downregulated or is downregulated very slowly. This has the consequence that what should normally develop into a proliferating multilayered retina, now develops into a hypoplastic retina or, worse, a pigmented RPE-like monolayer in addition to the normal RPE (81,82). If, however, a *Chx10*-mutant embryo also carries an *Mitf* mutation, the retina develops nearly normally, suggesting that an aberrant maintenance of wild-type *Mitf* expression slows down neuroretinal cell proliferation. Interestingly, like *Mitf* mutations, null mutations in p27Kip1 also correct the phenotype of a *Chx10* mutation (114,115). p27Kip1, along with p21Cip1, belongs to a class of proteins that inhibit cdk2 and hence inhibit cyclinE/cdk2-mediated G1/S progression. One might postulate, therefore, that *Mitf* inhibits cell cycle progression in the *Chx10*-mutant retina by stimulating p27Kip1 expression. This is in line with the observation that p27Kip1 is normally expressed in the RPE during mid to late embryonic stages, when it might control the timing of cell cycle withdrawal (122). A similar mechanism may operate in melanocytes, in which *Mitf* stimulates p21 expression (123). Nevertheless, other studies show that *Mitf* activates the T box gene, *Tbx2*, which in turn represses the p21 promoter through a GTGGTA motif (124). Moreover, Cdk2, whose product promotes G1→S progression, is stimulated by *Mitf* in some cell lines (111,125). The decision on whether MITF acts in a pro- or antiproliferative way may well depend on posttranslational modifications and on the relative abundance of each splice isoform. We recently found, for instance, that the isoform lacking exon 6a has little, if any, antiproliferative activity, in contrast to the isoform containing exon 6a (126). It would therefore be of interest to analyze which isoforms are expressed in melanomas with *Mitf* gene amplifications (127). In any event, it appears that there are multiple pathways with opposite activities, the perfect

Table 1  
Evidence for a Role of *Mitf* in Regulating Cell Proliferation

Observation	Reference
1. Evidence consistent with inhibition of cell proliferation	
Hyperproliferation of RPE cells in <i>Mitf</i> mutants	(30,118)
Misexpression of <i>Mitf</i> in presumptive neuroretina reduces cell proliferation	(81)
Increased rate of melanoblast proliferation in the head region of <i>Mitfmi</i> heterozygous embryos	(131)
Activation of MITF by p38, itself associated with reduced cell proliferation	(93)
Mitogenic stimuli activate MAP kinase pathway and stimulate MITF degradation	(18,19)
Activated form of the E1A oncogene represses <i>Mitf</i>	(132,133)
<i>Mitf</i> stimulates p21 expression, an inhibitor of CyclinE/CDK2, which normally promotes G1→S progression	(123)
<i>Mitf</i> stimulates <i>Ink4a</i> , whose product promotes cell cycle exit	(110)
Overexpression of MITF inhibits DNA synthesis	(123,126)
2. Evidence consistent with promotion of cell proliferation	
In B16 melanoma cells, overexpression of wild-type β-catenin stimulates <i>Mitf</i> expression and increases the number of cells in S phase. This effect can be reversed by adding dominant-negative TCF or dominant-negative MITF.	(130)
<i>Cdk2</i> , which promotes G1→S progression, is positively regulated <i>Mitf</i> (111). <i>Mitf</i> activates <i>Tbx2</i> which in turn suppresses p21, a negative regulator of cell cycle progression (124).	

situation to allow fine-tuning of a system that is critical for achieving the appropriate balance between cell proliferation and differentiation.

All of this, of course, follows the assumption that MITF integrates the regulation of cell proliferation and differentiation solely in the capacity of what transcription factors are best known for, namely, binding enhancer motifs in target genes to stimulate or repress transcription. This, however, need not necessarily be so as MITF proteins with DNA binding mutations can retain their antiproliferative capacity upon transfection into heterologous cells (126). That transcription factors can regulate cell proliferation without directly regulating transcription has been seen with other proteins as well. For instance, the homeodomain protein Six3, can promote cell proliferation by sequestering a negative regulator of cell cycle progression, geminin (128). Another example is MYOD, which in proliferating, mitogen-stimulated myoblasts, is sequestered by the cyclin D1-responsive cyclin-dependent kinase, CDK4, and hence, prevented from activating muscle differentiation genes. This interaction goes both ways: MYOD also inhibits CDK4 activities, such as its inhibition of the retinoblastoma protein. Once mitogenic signals are reduced and cyclinD1 levels decrease, CDK4 is translocated to the cytoplasm, thereby allowing MYOD to form active complexes with E proteins and to activate muscle gene expression (129). Taken together, the results suggest that cell proliferation and differentiation in this system are regulated by the relative levels of MYOD and CDK4:

overexpression of MYOD will override mitogenic stimulation, and overexpression of Cyclin D1 will override MYOD-mediated differentiation. If MITF regulation followed similar principles, conclusions based on cell lines in which MITF is expressed above its physiological levels would have to be taken with a grain of salt.

## CONCLUSIONS AND PERSPECTIVES

Thus, it follows that similar pathways may regulate cell survival in melanoblasts and melanoma. In melanoma, the Wnt/ $\beta$ -catenin pathway is constitutively active, and when suppressed, for instance, by dominant-negative TCF, interferes with growth (130). As mentioned, Wnt signaling is likewise critical for melanoblast development. Activating mutations in *BRAF*, which occur in a majority of melanoma, activate the MAPK-signaling pathway, and this pathway is likewise crucial for melanoblasts. For both signaling pathways, *Mitf* is a critical target. Moreover, *Brn2*, greatly overexpressed in melanoma, may suppress *Mitf* expression to some degree, and, hence, suppress an antiproliferative action of *Mitf*. *Brn2* is not expressed during early development, however, only later when melanoblasts are in the epidermis and in hair follicles, where it may function as a stimulator of cell proliferation.

These observations all suggest that a number of shared pathways operate during development, postnatal replenishment of melanocytes, and melanoma formation. No doubt, however, by homing in on a few pathways, often chosen because of the chance availability of striking genetic models or of convenient assays, we vastly simplify the intricate molecular complexities that characterize the generation of melanocytes and RPE cells and that are altered when these cells derail during malignant transformation. As time progresses, we expect that other pathways may gain favor and shine in the limelight of scientific interest, for shorter or longer periods. We hope that when all is said and done, a picture will emerge with each actor standing in his proper place on the common stage. This, we hope, will finally provide the required knowledge to design therapeutic strategies to lastingly control the growth and spread of melanoma.

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