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REVIEW

Transcriptional and signaling regulation in neural crest stem cell-derived melanocyte development: do all roads lead to Mitf?

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Human neurocristopathies include a number of syndromes, tumors, and dysmorphologies of neural crest (NC) stem cell derivatives. In recent years, many white spotting genes have been associated with hypopigmentary disorders and deafness in neurocristopathies resulting from NC stem cell-derived melanocyte deficiency during development. These include *PAX3*, *SOX10*, *MITF*, *SNAI2*, *EDNRB*, *EDN3*, *KIT*, and *KITL*. Recent studies have revealed surprising new insights into a central role of *MITF* in the complex network of interacting genes in melanocyte development. In this perspective, we provide an overview of some of the current findings and explore complex functional roles of these genes during NC stem cell-derived melanocyte development.

***Keywords*:** neurocristopathy, Waardenburg syndrome, white spotting mice, pigment cells, differentiation
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Neural crest stem cell development

The neural crest (NC) is a unique embryonic structure and contains a remarkable multipotent stem cell population that arises during vertebrate embryogenesis [1, 2]. NC has been referred to as the fourth germ layer because of its great importance during development [3]. NC stem cells arise from the dorsal neural tube during neurolation in early development, then migrate out from the neural tube and along defined pathways throughout the body, where they contribute to numerous cell types and tissues, including melanocytes, ocular and periocular structures, bone and cartilage cells of the cranial skeleton, odontoblasts, autonomic neurons, sensory neurons, enteric neurons, smooth muscle, endocrine cells, chromaffin cells, and glial cells [1]. Although it has long been thought that the fates of NC-derived lineages are controlled by transcription and growth factors, the physiological functions of these factors are not fully known.

Understanding NC development is medically important because defective derivatives of aberrant NC cell development give rise to numerous human diseases known as neurocristopathies [4]. These diseases include ocular diseases (such as iris hypoplasia and optic nerve head melanocytoma), cardiocutaneous syndromes, craniofacial malformations of mesoectodermal origin, DiGeorge syndrome, Ewing's tumors, Hirschsprung disease, lentigo, medullary carcinoma of the thyroid, melanotic nevi, melanoma, multiple endocrine neoplasia (types 2A and 2B), neuroblastoma, neurocutaneous syndromes, neurofibromatosis type 1, PCWH (Peripheral demyelinating neuropathy, Central dysmyelinating leukodystrophy, Waardenburg syndrome (WS), and Hirschsprung disease), PHACES syndrome (Posterior fossa abnormalities and other structural brain abnormalities, Hemangioma(s) of the cervical facial region, Arterial cerebrovascular anomalies, Cardiac defects, aortic coarctation and other aortic abnormalities, Eye Anomalies, Sternal defects, and/or Supraumbilical raphe), pheochromocytoma, piebaldism, WS, Tietz syndrome, and more [4]. Among these WS is an autosomal-dominant subtype of complex NC diseases and is named after the Dutch ophthalmologist who, in 1947, first described a patient with heterochromia iridis (different eye colors), congenital deafness, and dystopia canthorum (lateral displacement of the inner canthi of the eyes leading to a wide nasal bridge).

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WS patients also show additional defects, including white forelock, pigmentary disturbance of the skin, upper limb abnormalities, and megacolon [5]. To date there are at least four types of WS that are due to mutations in separate transcription factors, including *SOX10*, *MITF*, *PAX3*, and *SNAI2*, and in signaling molecules, including *EDNRB* and *EDN3*. The four WS types are categorized based on presentation of various subsets of the phenotypic characteristics of the syndrome. For example, WS type 1 patients have craniofacial defects, WS type 3 patients have craniofacial and limb defects, and WS type 4 patients have megacolon. Intriguingly, distinct subtypes of WS and piebaldism, which is associated with mutation of *KIT*, often have the common phenotype of hypopigmentation, which is due to melanocyte defects in the skin. The comparable hypopigmentation defect in these diseases reflects a possible functional relationship among the disease-associated genes in melanocyte development. In this review, we discuss the known functional roles of these genes during NC stem cell-derived melanocyte development and propose alternative models of functional roles of these genes, with a focus on the central role of *MITF*.

White spotting mouse disease models and melanocyte development

Gene expression programs that direct the development of distinct cell lineages from unspecified precursor cells are the result of complex interactions between cell-extrinsic signals and transcription factors. An excellent system to study such interactions is provided by the development of melanocytes. Their precursor cells, the melanoblasts, originate from multipotent NC stem cells and migrate along characteristic pathways to various destinations such as the iris and the choroid of the eye, the inner ear, the dermis, and the epidermis. In the skin, these precursors differentiate into melanin-producing cells that determine skin color and protect the organism from UV radiation, one of the risk factors for skin cancers such as melanoma [6]. In addition, the precursors distribute into the bulged region of developing hair follicles, where they persist as self-renewing stem cells in the niche [7]. For their development, melanoblasts depend on numerous transcription factors and signaling systems. These include the transcription factors PAX3 [8, 9], SOX10 [9-11], and MITF (Microphthalmia-associated Transcription Factor) [12], the WNT signaling pathway [13, 14], G protein-coupled endothelin receptor B (EDNRB) and its ligand, endothelin 3 (EDN3) [15, 16], and receptor tyrosine kinase KIT and KIT-ligand (KITL) [17, 18]. Among the genes encoding these factors, *Mitf*, *Sox10*, *Pax3*, *Kit*, and *Kitl* comprise a particularly intriguing set, since heterozygosity for certain mutations in each of these genes leads to the strikingly similar phenotype of belly spotting in mice (Figure 1). Since many *in vivo* and *in vitro* observations suggest that there are mutual interactions between these genes [9, 19], a possible functional regulatory relationship may exist among these genes in melanocyte development.

Mouse mutations have long served as human disease models for many aspects of developmental studies [20, 21]. In addition, mouse coat color mutants serve as an excellent model for the study of melanocyte development and pigmentation [22]. Owing to the shared embryonic origin of various tissues or pleiotropic effects, these mutants also serve as models of disorders in vision, hearing, craniofacial development, enteric nervous system development, and neural tube closure. White spotting mutations produce white hair and skin in regions where melanocytes normally appear. This phenotype can result from a defect of survival, migration, proliferation, or differentiation at a particular time of melanocyte development when the specific gene product is required. The best-characterized models used in the studies of melanocyte development are *Microphthalmia* (*Mi*), *Dominant megacolon* (*Dom*) , *Splotch* (*Sp*) , *Dominant white spotting* (*W*), *Steel* (*Sl*), *Piebald-lethal* (*sl*) , and *Lethal spotting* (*ls*) (Figure 1). All of the mutated genes associated with these models are cloned, and they belong to two categories: transcription factors and receptor/ligand systems. These mutations provide a rich genetic resource for investigation of the mechanisms of melanocyte development at the molecular, cellular, and physiological levels.

Microphthalmia

This locus encodes the basic-helix-loop-helix-leucine-zipper transcription factor *Mitf* [12]. At least 25 different murine mutant alleles of *Mitf* have been identified, providing a useful genetic resource for studies of development and disease [23, 24]. *Mitf* homozygous mutant mice, such as $Mitf^{Mi}$ (*microphthalmia*), $Mitf^{mi\text{-}ew}$ (*mi-eyeless-white*), $Mitf^{Mi\text{-}wh}$ (*Mi-white*), or $Mitf^{mi\text{-}vga\text{-}9}$ (a transgenic insertional allele), typically survive but are microphthalmic, deaf, and completely white, reflecting the complete abolishment of melanocytes (Figure 1) [12, 25]. Additionally, a few mutations result in osteoclast defects.

Mutations of human *MITF* are associated with 10-15% of WS type 2, and patients show skin hypopigmentation, ocular pigmentation defects, and deafness caused by defects of melanocytes of the inner ear [26]. Tietz syndrome shows more obvious hypopigmentation and deafness that is also associated with mutations in *MITF* [27].

Dominant megacolon (Dom)

This mouse mutant exhibits white spotting and mega-

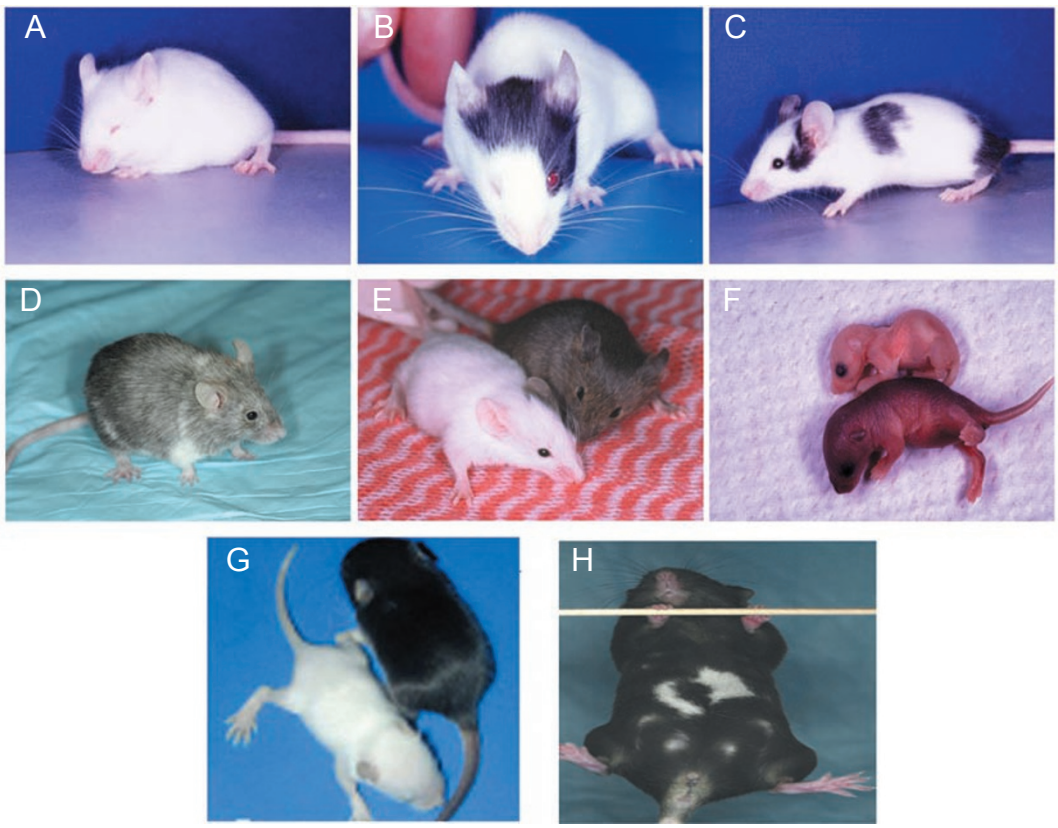

Figure 1 Mutations in the genes encoding key transcriptional factors or signaling molecules result in characteristic white coat color phenotypes, reflecting the ability of these genes to regulate NC stem cell-derived melanocyte generation. Among these genes *Mitf* affects development of both melanocytes and RPE, whereas others affect only melanocyte development. **(A)** A *Mitf*$^{mi\text{-}vga\text{-}9}$ homozygote (a null allele). *Mitf*$^{mi\text{-}vga\text{-}9}$ homozygotes are white, with small eyes, whereas heterozygotes have normal pigmentation. **(B)** A *Mitf*$^{mi\text{-}rw}$ (microphthalmia-red eyed white) homozygote, which contains a genomic deletion starting downstream of exon 1E and ending upstream of exon 1M and encompassing the exons 1H, 1D, and 1B1a/1B1b and their flanking sequences [147]. *Mitf*$^{mi\text{-}rw}$ homozygotes show abnormal RPE development and primarily white fur, but always display a black head spot, reflecting normal melanocyte generation in that region. **(C)** A *Mitf*$^{mi\text{-}bws}$ (microphthalmia–black and white spotting) homozygote. These mice harbor a point mutation that results in altered splicing so that the *Mitf* produces not only a wild-type transcript that contains exon 2b, but also a transcript that lacks exon 2b, which contains a KIT signaling-dependent phosphorylation site, serine-73. This causes deficiency of many skin melanocytes without affecting RPE development, resulting in widespread white spotting and black eyes. **(D)** A 10- month- old *Mitf*$^{mi\text{-}vit}$ (microphthalmia-vitiligo) homozygote. This allele contains a G to A transition that leads to an aspartate to asparagine substitution at amino acid 222 in the helix 1 region of MITF protein. *Mitf*$^{mi\text{-}vit}$ homozygous mice are born normally pigmented, but gradually lose their melanocyte stem cells with aging, resulting in a gray coat phenotype [146]. **(E)** *Ednrb*^{tm1Myks} homozygote (left) and heterozygote littermate (right). This homozygote contains a transgenic insertion of a *LacZ* reporter gene at the *Ednrb* locus, resulting in absence of *Ednrb* expression. Homozygotes are almost completely white, with pigmented regions remaining in the head and rump, and die from megacolon as juveniles [129]. **(F)** *Kit*^{tm1Alf} (also known as *Kit*$^{W\text{-}lacZ}$) homozygote (top) and normal littermate (bottom), age P3. This mouse is homozygous for the transgenic insertion of a *LacZ* reporter gene at the *Kit* locus [148]. Absence of normal Kit causes complete lack of skin melanocytes, resulting in white fur. **(G)** A *Sox10*^{Hry} homozygote (left) and normal littermate (right). This mouse contains a 15.9- kb deletion of non-coding sequence located 47.3 kb upstream of the transcription start site in the gene *Sox10*, leading to loss of skin melanocytes and megacolon [30]. **(H)** A *Pax3*^{Sp} heterozygote, which displays a characteristic white belly patch. Mice harboring heterozygous mutations of certain alleles of *Sox10*, *Mitf*, *Kit*, *and Kitl* show a similar belly spotting phenotype.

colon in heterozygotes. Homozygous *Dom* mutations are embryonic lethal (at E13.5) and exhibit absence of melanocytes and enteric neurons, size reductions in the dorsal trigeminal and facial ganglia, and defects in dorsal root ganglia, sympathetic ganglia, and terminal oligodendrocyte differentiation in spinal cord. This locus encodes *Sox10* (Sry-like HMB box 10), a member of the high mobility group (HMG) family of transcription factors, showing

HMG domain homology to the testis determining factor SRY [28, 29]. The *Sox10*^{Dom} allele results from a point mutation that introduces a frameshift and early truncation that generates a truncated SOX10 protein lacking the transcription activation domain [11]. A transgene-insertion mutant mouse line (*Hry*) was shown to be the result of a 15.9- kb deletion of a non-coding sequence located 47.3 kb upstream of the transcription start site in *Sox10* [30].

Mutations in human *SOX10* are associated with WS type 4, also known as Waardenburg-Shah syndrome. The patients show WS characteristics of the white forelock and eyelashes, abnormal iris pigmentation, and deafness, along with enteric aganglionosis, which is seen in patients with Hirschsprung disease [10, 11]. Recently, a complex neurocristopathy, PCWH, which shows WS phenotypes along with additional neurological defects, has been shown to result from mutated *SOX10* mRNA escaping the nonsense-mediated decay pathway [31]. Recent evidence suggests that some WS2 patients harbor *SOX10* deletions, and some of these patients also show the neurological phenotypes of PCWH [32].

Splotch

This locus encodes a paired-box homeodomain transcription factor, *Pax3*, and the *Splotch* mouse mutant was due to a *Pax3* loss- of- function mutation [33]. Mice harboring heterozygous *Pax3* mutations show ventral spotting, whereas homozygous mutations are embryonic lethal. *Pax3* belongs to the *Pax* gene family, which is highly conserved across species and whose members contain a paired DNA-binding domain [34].

Mutations in human *PAX3* are associated with WS type 1 and type 3 or Klein- WS [35]. WS type 1 patients show dystopia canthorum, hypopigmentation most often manifested as a white blaze of hair at the forehead or leukoderma, heterochromia iridis, and deafness. WS type 3 patients show additional skeletal abnormalities and cardiopulmonary defects.

Piebald (s) and lethal spotting (ls)

Mutations in the recessive mutants *s* and *ls* also disrupt normal melanocyte development. The *s* locus encodes G protein-coupled *Ednrb* [16] and the *ls* locus encodes *Edn3*, a 21-residue peptide ligand with high affinity for EDNRB [15]. The related ligands, EDN1 and EDN2, can also bind EDNRB. Activating mutations in the Ga subunits *Gnaq* and *Gna11* can promote expansion of the early melanoblast population, suggesting that the G protein-coupled receptor plays an important role in regulating melanocyte development [36].

Mutations at the human *EDNRB* and *EDN3* loci are also associated with WS type 4 or Waardenburg-Shah syndrome, which is inherited as an autosomal recessive trait. As described above, the patients show pigmentary defects and enteric aganglionosis [37, 38]. Additionally, ABCD syndrome, named for the patients' phenotypic presentation of albinism, black lock, cell migration disorder of the neurocytes of the gut, and deafness, has been identified as a homozygous nonsense mutation in the *EDNRB* gene [39]. *EDNRB* is also associated with melanoma risk and is required for the expansion of malignant melanoma [40, 41].

Dominant white spotting (W) and Steel (Sl)

Similar to mice with defects in EDN3/EDNRB signaling, mutations in *W* and *Sl* also disrupt normal melanocyte development. *W* encodes the receptor tyrosine kinase *Kit* (also known as *c-Kit*) [17]. *Sl* encodes *Kitl*, also known as stem cell factor (SCF) and mast cell growth factor (MGF) [18]. Most alleles of *W* and *Sl* in heterozygotes show head and belly spots and the homozygotes are often embryonic lethal; those homozygotes that survive are black-eyed white, sterile, and anemic [42]. *Kitl* produces two KITL proteins, a transmembrane form and a soluble form. The membrane-bound form is required for melanocyte precursor survival in the dermis, whereas the soluble form is needed for melanocyte precursor dispersal on the lateral pathway and/or for their initial survival in the migration staging area [43]. In addition, *Kit* and *Kitl* mutants have defects in the intestinal pacemaker system, T-cell precursors, and hippocampal learning and hearing [44-46].

Mutations in human *KIT* are associated with piebaldism, a rare autosomal- dominant disorder in which patients show patches of white skin and white hair [47]. *KIT* mutations are also associated with human gastrointestinal stromal tumors, urticaria pigmentosa, and aggressive mastocytosis in which KIT proteins are constitutively activated [48, 49]. To date *KITL* mutations have not been found in human patients.

Snai2 knockout mice

Recent evidence suggests that mutations in human *SNAI2* are associated with WS2 and piebaldism [50, 51]. The initial description of *Snai2* knockout mice reported no NC defects and described normal melanocyte generation, migration, and development [52]. However, another report showed a strain-dependent phenotype of a small amount of white spotting in the homozygous *Snai2* knockout mice [50]. The functional role of *Snai2* in melanocyte development is not known and requires further investigation.

Microphthalmia-associated transcription factor

The first identification of the *microphthalmia* gene, now termed *Mitf*, was provided by cloning the gene from

a microphthalmic and hypopigmented transgene-insertion mutant mouse line [12]. *Mitf* encodes a transcription factor of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) class, which, together with TFE3, TFEB, and TFEC, belongs to the MITF-TFE subfamily of bHLH proteins. The four mammalian members of this subfamily share very similar bHLH and leucine zipper domains and *in vitro* form all possible combinations of homo- and heterodimers with each other, but do not interact with other bHLH and bHLH-Zip proteins [53]. Intriguingly, it has been shown that knockouts of *Tfe3, Tfeb*, and *Tfec* did not affect melanocyte development, suggesting that heterodimeric interactions are not essential for MITF-TFE function in melanocyte development [54, L Hou and H Arnheiter, unpublished results). The *Mitf* gene is quite complex, with at least nine promoters producing multiple isoforms, here termed A-MITF, J-MITF, C-MITF, MC-MITF, E-MITF, H-MITF, D-MITF, B-MITF, and M-MITF (Figure 2). These isoforms differ in their amino termini but share exons 2-9, which include all bHLH-Zip domains. MITF is broadly expressed though the protein levels and isoforms differ among cell types (for detailed structures of MITF, see Steingrimsson *et al*. [24] and Arnheiter *et al.* [23]). M-MITF is a major isoform in NC stem cell-derived melanocytes. All of the isoforms also produce alternative splice forms modifying exon 6 that lead to inclusion (+) or exclusion (–) of the sequence ACIFPT upstream of the basic domain. The function of MITF (+) or MITF (–) forms are not fully understood in melanocyte development, but they may be related to cell proliferation and different transcriptional activities [55. 56]. Distinct extracellular signaling pathways, such as those of WNT, KIT, EDNRB, and α-melanocyte-stimulating hormone (MSH), also regulate Mitf [57-60]. MITF proteins are modified by phosphorylation, ubiquitination, sumoylation, and acetylation [58, 61-63], and the protein inhibitor of activated STAT3 (PIAS3) inhibits MITF transcriptional activity [64].

Mitf is expressed in developing NC-derived melanocyte precursors before the initial expression of *Dopachrome tautomerase* (*Dct*) and in the neuroepithelium-derived retinal pigmented epithelium (RPE) of the eye beginning at E10. On the basis of the coexpression of markers such as *Kit* and *Dct*, these NC-derived *Mitf*-positive cells are defined as melanocyte precursors [19, 65]. *Mitf* is one of the key transcription factors regulating many aspects of melanocyte development and has been referred to as the melanocyte master regulator [66, 67]. MITF is required for melanocyte cell survival by directly regulating Bcl2 and MET, the receptor for hepatocyte growth factor [67, 68], and is involved in melanocyte proliferation and cell cycle progression by its regulation of *Tbx2*, *INK4A/p16*, *p21,* and *CDK2* [69-73]. MITF can also control melanocyte differentiation by directly activating transcription through E-box (CATGTG) binding sites in the melanocyte-specific genes, *Dct*, *Tyrosinase* (*Tyr*), *Tyrosinase related protein 1* (*Tyrp1*), and *Silver/Pmel17, Aim-1, Mart1,* and *MC1R* [62, 74-76]. Interestingly, recent work suggests that MITF is not the sole regulator of *Dct* and *Tyr* in melanocyte development. SOX10 also regulates *Dct* expression by directly binding to the promoter of *Dct* [77] and melanocyte-specific expression of *Dct* is dependent on its synergistic activation by SOX10 and MITF [78, 79]. In addition, MITF is not

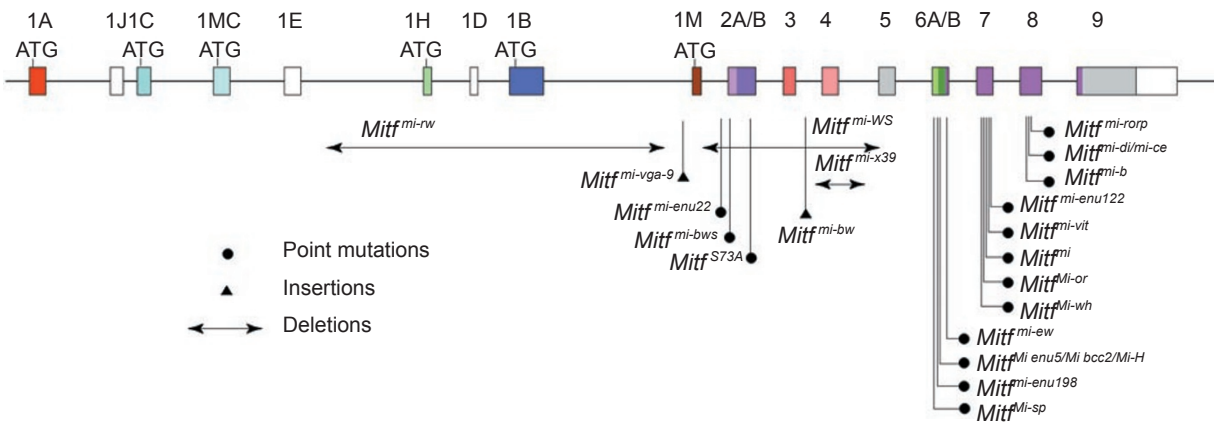

Figure 2 Schematic diagram of the mouse *Mitf* gene and its mutations. The upper part of the figure shows the genomic organization of the gene. The boxes represent exons, with the numbers written on top indicating the corresponding nine distinct exons: 1A, 1J, 1C, 1MC, 1E, 1H, 1D, 1B, and 1M, each associated with a distinct mRNA isoform, and the common exons 2-9. The bHLH-Zip domain (colored pink) is contributed by part of exon 6B, all of exon 7 and 8, and part of exon 9. The lower part of the figure shows 20 of the currently known alleles that have been described in the literature [24, 25, 125, 147]. Filled circles represent point mutations, filled triangles represent insertion mutations, and lines represent deletions (Courtesy: Heinz Arnheiter).

sufficient to induce *Tyr* expression and full melanocyte differentiation in the absence of functional SOX10, suggesting that *Sox10* also may control expression of other melanocyte-specific gene(s) [80].

SOX10 and PAX3 are both broadly expressed in NC stem cells [81, 82]. Supporting this similarity in expression patterns, ectopic expression of a *Sox10* transgene under the control of regulatory regions from the *Pax3* gene in *Sox10*-deficient NC cells rescues melanocyte differentiation [83]. Intriguingly, *Sox10* and *Pax3* have more expansive expression patterns than M-*Mitf* and are required for several lineages of NC cells; yet, M-*Mitf* is activated in a small subset of NC cells and is only required for melanocyte development. These observations suggest that additional extrinsic signaling control must be involved in *Mitf* regulation. In support of this idea, we have found that MITF is not sufficient to induce the expression of *Tyr* without functional KIT signaling [19], suggesting that KIT signaling modulates the activity of MITF either directly or indirectly in melanocyte development. Further studies are needed to understand the roles of MITF in melanoblast survival, proliferation, differentiation, and disease.

Transcriptional regulation of *Mitf*

MITF plays an essential role in survival, migration, proliferation, and differentiation of melanocytes during development. Therefore, understanding the transcriptional regulation of *Mitf* will help us identify the transcriptional hierarchy that directs the development of melanocytes from NC stem cells. Here we discuss two transcription factors, SOX10 and PAX3.

SOX10 is expressed in NC stem cells [81] and in NC-derived structures during embryonic development [11], and is required for proper development and survival of NC-derived melanocyte, glial, and enteric neuron lineages [84-86]. Sox10 function is regulated by sumoylation in Xenopus NC development [87]. SOX10 has been shown to strongly activate *Mitf* expression in cultured cell lines [9, 88] and to regulate *Dct* expression [77]. In addition, SOX10 is required not only for inducing *Mitf* expression in NC cells, but also for Mitf-dependent *Tyr* expression [80]. These results suggest that SOX10 regulates the expression of other melanocyte-specific gene(s) in addition to *Mitf* in melanocyte development. In contrast, in zebrafish melanocyte development Sox10 is only required for directly activating *mitf*, which, independent of the further actions of Sox10, rapidly stimulates downstream target genes and hence pigmentation [89]. These results clearly show that distinct species differ in usage of homologous regulators and their targets for melanocyte development. In zebrafish, *sox10*, *mitf*, and downstream pigment genes are linked in a linear, seemingly simple, regulatory chain in which *sox10* controls the expression of *mitf*, which in turn is sufficient to regulate melanocyte-specific gene expression and pigmentation. In mice, the situation is apparently more complex in that the generation of melanocytes requires both *Sox10* and *Mitf*, and neither gene alone can overcome the lack of the other to generate *tyrosinase*-expressing, mature melanocytes (schematically illustrated in Figure 3). This regulatory model was confirmed in mouse melanocytes, in which it was shown that SOX10 cooperates with MITF to regulate *Tyr* gene expression by direct activation of the *Tyr* distal regulatory element [90]. SOX10's interacting factors and its downstream targets are yet to be fully elucidated in NC stem cell and melanocyte development.

PAX3 is expressed in NC cells and is required for early NC and melanocyte development [33, 82, 91]. PAX3 controls neural tube closure through inhibition of p53-mediated apoptosis [92]. PAX3 also up-regulates *Tyrp1* promoter activity [93], and overexpression of *Pax3* induces tyrosinase activity in ascidian embryos [94]. It has been shown that PAX3 weakly transactivates the *Mitf* promoter [8] and that PAX3 synergistically transactivates the promoter of *Mitf* with SOX10 [8, 9, 88]. However, contradictory data showing that PAX3 does not synergistically act with SOX10 to activate *Mitf* transcription have also been reported [95]. *In vitro* studies have shown that the phorbol ester, 12-tetradecanoylphorbol 13-acetate (TPA), induces melanocyte differentiation from NC cells through *Mitf* up-regulation, but that *Pax3* expression level is not altered by the treatment [96]. It is currently unknown whether PAX3 directly regulates *Mitf* in melanocyte development *in vivo*. Recently it has been shown that PAX3 represses *Dct* expression in the absence of activated β-catenin, and such repression is relieved by activated β-catenin in melanocyte stem cells [97]. The precise function of PAX3 in NC stem cell and melanocyte development, however, is poorly understood and requires further investigation.

Signaling regulation of *Mitf*

During melanocyte development, the three major signaling pathways involving WNT, KIT, and EDNRB play essential roles, whereas the roles of other signaling pathways are not readily apparent from analysis of mouse models. For example, despite expression of *Met* and *Erbb3* in melanoblasts, there were no melanocyte defects in *Met* and *Erbb3* knockout mice [98, K Buac and WJ Pavan, unpublished results]. Although α-MSH utilizes cAMP to trigger melanin synthesis and pigmentation of melanocytes through activation of *Mitf* and *Sox10* [59, 99], it does not affect melanocyte differentiation in mouse. However, it does function in this capacity in other vertebrates, such as

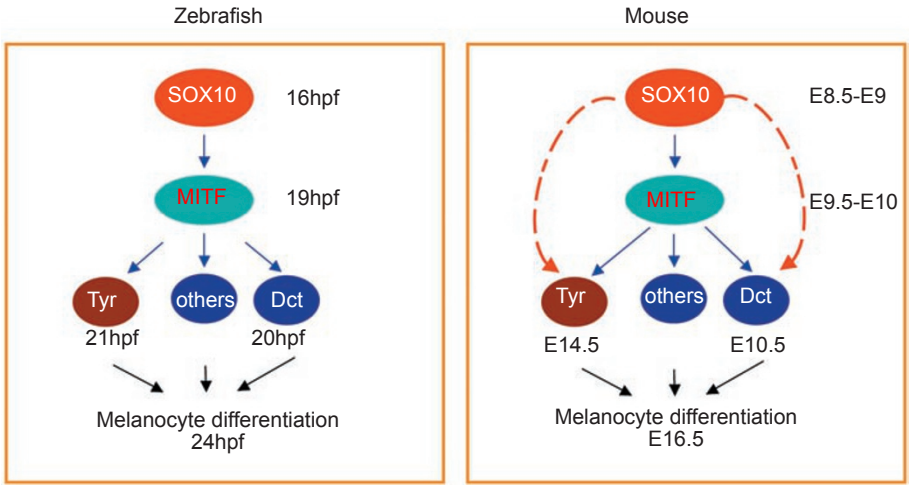

Figure 3 The transcriptional regulatory hierarchy of *Sox10* and *Mitf* in melanocyte development and differentiation is distinct in zebrafish and mice. Examples of the transcriptional regulatory network models are based on reference [149]. In zebrafish, melanocyte development exhibits a simple regulatory chain model. Here, Sox10 directly activates *mitf* and Mitf, independent of the further actions by Sox10, and rapidly stimulates downstream target genes and hence pigmentation. Mouse melanocyte development exhibits a more complex feed-forward loop network model. Here, SOX10 directly regulates *Mitf* and subsequently cooperates with MITF and/or additional SOX10-dependent regulators to activate downstream target genes, including *Tyr*. This model allows for temporal control of melanogenic gene expression in mouse.

reptiles [100, 101]. Below we discuss the roles of WNT, KIT, and EDNRB signaling in melanocyte development (Figure 4).

WNT/β-catenin signaling

WNT signaling is essential for NC induction and melanocyte development. Activation of Frizzled receptors by WNT leads to activation of downstream signal transduction molecules, such as β-catenin, PKC, CAMKII, PKA, and Rho GTPase, resulting in WNT-mediated complex cellular actions. In the best understood canonical WNT/β-catenin signaling pathway, when extracellular WNT ligand binds to its receptor (Frizzled), β-catenin accumulates, enters the nucleus, and subsequently interacts with members of the lymphoid enhancer binding factor 1/T-cell specific factor (Lef1/Tcf) family of transcription factors, which then modulate transcription of target genes [102]. *Wnt1* and *Wnt3* are expressed in the dorsal part of the neural tube in spatiotemporal patterns consistent with the timing of NC induction [57, 103], and *Dct*-positive cells are markedly reduced in *Wnt1*/*Wnt3* double knockout mouse E11.5 embryos [13].

In vivo and *in vitro* studies also indicate that the WNT/β-catenin signaling pathway is required for induction of melanocyte and other cell fates. Overexpression of β-catenin in zebrafish promotes melanoblast formation and reduces formation of neurons and glia [57]. Similarly, WNT3a or WNT1 promotes the differentiation and expansion of melanocytes in cultured chick NC cells and in cultured mouse NC cells [104, 105]. Furthermore, both melanoblasts and sensory neurons are absent in β-catenin conditional knockout mice during embryonic development [106]. Interestingly, there is a highly conserved binding site for LEF-1 in the *Mitf* promoter [14, 107], and the interaction between MITF and LEF-1, but not TCF-1, results in synergistic transactivation of the *Dct* gene promoter [108]. In addition, MITF can interact directly with β-catenin and can redirect its transcriptional activity away from canonical WNT signaling-regulated genes toward MITF-specific target promoters to activate transcription [109]. Together these studies suggest that WNT/β-catenin signaling promotes melanoblast development by regulating MITF.

KIT signaling

KIT signaling is required for normal development of three migratory cell populations: blood cells, melanocytes, and primordial germ cells [17, 18, 110]. Activation of KIT by KITL leads to receptor dimerization and autophosphorylation of specific tyrosine residues in the kinase domain. This activates downstream signal transduction molecules, such as MAPK, phosphatidylinositol 3′-kinase (PI3K), JAK/STAT, and Src family members. Although KIT signaling-induced activation of PI3K is required for male fertility, this activation is not essential for melanocyte development [111].

KIT is expressed in developing NC cells, hematopoietic stem cells, and primordial germ cells, whereas KITL is expressed in tissues associated with KIT-expressing

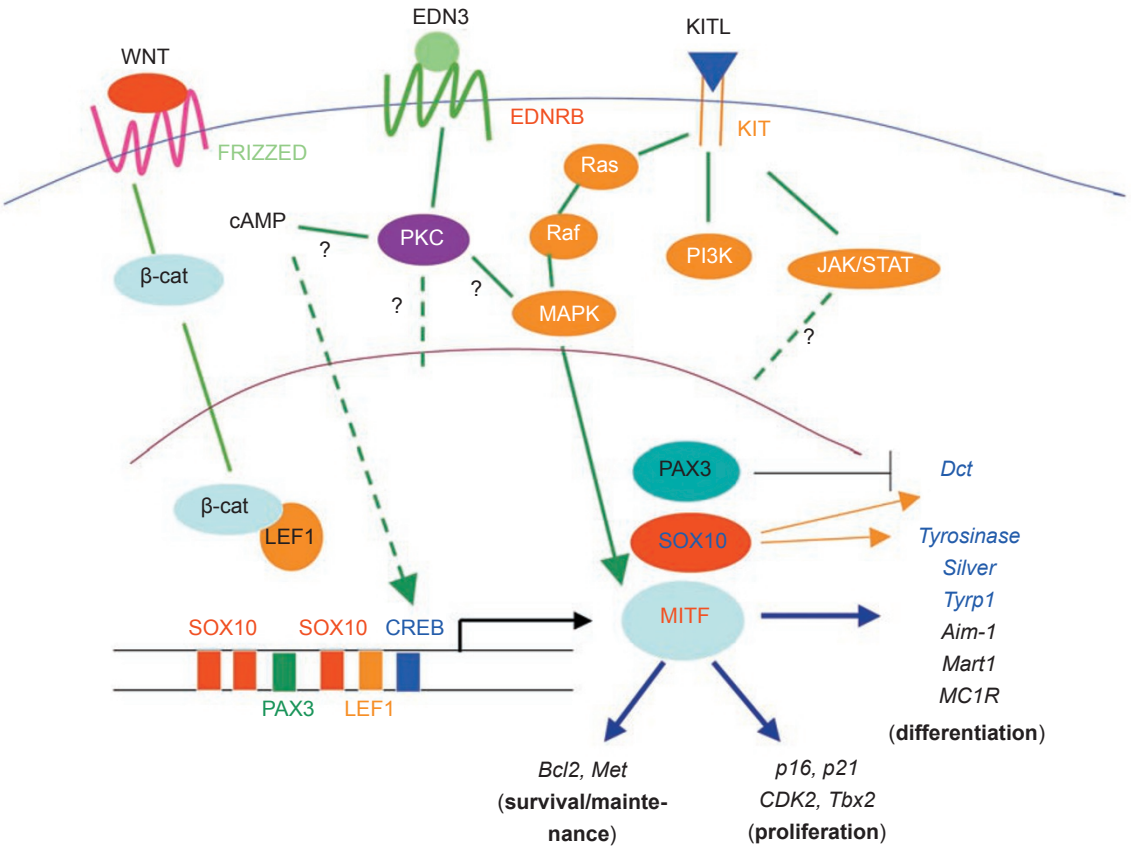

Figure 4 A simplified schematic showing the features of key signaling pathways in melanocyte development. Green lines represent three major signaling pathways, WNT, KIT, and EDNRB, which are all connected to *Mitf*. WNT/β-catenin signaling promotes melanoblast development by regulating MITF transcription. KIT and EDNRB signal pathways are not required for the initial expression of Mitf in melanocyte development, but both pathways induce the phosphorylation of MITF in mature melanocytes. However, the KIT signaling-dependent phosphorylation site at serine-73 is not essential for melanocyte development. It is unknown whether melanocytic KIT and EDNRB signaling pathways act through regulation of MITF and whether EDNRB signaling-dependent MITF phosphorylation plays any role in melanocyte development *in vivo*. MITF is involved in melanocyte survival, proliferation, and differentiation by regulating downstream genes. Blue lines represent MITF target genes, which include genes involved in cell survival (*Bcl2* and *Met*), cell proliferation (*p21*, *p16*, *CDK2* , and *Tbx2*), and differentiation (*Tyr*, *Tyrp1*, *Dct*, *Silver/Pmel17*, *Mart1*, *Aim-1*, and *MC1R*). During melanocyte lineage development, the transcription factors SOX10, PAX3, and LEF1 regulate expression of the melanocyte-specific *Mitf* isoform. The relative positions of the binding sites for these factors within the proximal *Mitf* promoter are shown. MITF is insufficient to induce *Tyr* expression and full melanocyte differentiation in the absence of SOX10. SOX10 is required for *Dct* and *Tyrosinase* expression in addition to the control of Mitf. Although PAX3 is known to repress *Dct* expression, the precise function of PAX3 in melanocyte development is poorly understood. In addition, MSH can elevate cAMP levels that subsequently activate both the cAMP and the MAP kinase pathways, resulting in elevated *Mitf* promoter activity in melanogenesis. However, how *CREB* activation is involved in melanocyte development is unknown to date. KIT signaling-induced activation of PI3 K is not essential for melanocyte development [111]. Question marks indicate insufficient data to describe the involvement of these signaling pathways in melanocyte development.

cells and in the neural tube [43, 112-115]. KIT signaling is necessary for the survival and/or migration of melanoblasts [43, 116, 117]. Injection of KIT antibody into early mouse embryos blocks proper melanocyte development [118, 119], and ectopic expression of KITL promotes migration, proliferation, and differentiation of melanocyte precursors [120]. Tyrosine residues 567 and 569 of KIT are crucial for its function in melanocyte development, as specific mutation of both residues results in complete loss of melanocytes [121].

Understanding of the complex relationship between KIT signaling and MITF function also concerns their temporal expression patterns in melanocyte development. One possible model is that MITF can activate *Kit* transcription [122]

and thus upregulate KIT expression [65]. This model is supported by observations that zebrafish *mitf* mutants fail to express *kit*, suggesting that the initial expression of KIT is dependent on MITF [123]. Another possible model is that KIT signaling induces the transcriptional activity of MITF. This model is supported by studies on cultured melanocytes and melanoma cells in which KIT signaling leads to an increase in MITF phosphorylation, which is associated with an enhanced recruitment of the transcriptional coactivator p300/CBP and a concomitant stimulation of MITF transcriptional activity [58, 124]. This increase is transient and followed by rapid ubiquitination and proteasome-mediated degradation of MITF [61]. However, we have shown that the initial expression of *Kit* is not dependent on MITF and that the initial expression of *Mitf* is not dependent on KIT. In addition, we have shown that the presence of MITF alone is not sufficient for *Tyr* expression in melanoblasts in the absence of functional KIT signaling, and that KIT signaling influences gene expression through MITF in a gene-selective manner during melanocyte development [19]. Taken together, these results suggest that MITF and KIT are not related in a simple linear regulatory chain, and that both cooperatively regulate the expansion of melanocyte precursors in development. To date, it is unknown when and where KIT signaling-dependent MITF phosphorylation occurs and whether this post-translational modification plays any role in melanocyte development *in vivo*. Interestingly, one mutant *Mitf* allele that results in reduced skin melanocytes, $Mitf^{mi\text{-}bws}$ (*mi-black and white spotting*), produces not only a wild-type transcript that contains exon 2b, but also a transcript that lacks exon 2b, which contains a KIT signaling-dependent phosphorylation site, serine-73. This suggests that exon 2b may play a role in melanocyte development, potentially through KIT signaling-dependent phosphorylation. However, targeted mutation of serine-73 to alanine leads to normally pigmented mice [125]. This indicates that mutation of this phosphorylation site is not deleterious to melanocyte development. The developmental mechanism of $Mitf^{mi\text{-}bws}$ mutant mice and the precise function of Kit signaling in melanocyte development require further investigation.

EDNRB signaling

EDNRB exerts pleiotropic effects on mouse development, and its function is required for the normal development of NC-derived melanocytes and enteric ganglia [15, 16]. Binding of EDNRB by EDN3 leads to the activation of downstream signal transduction pathways, including PKC, CamKII, and MAPK [126]. EDNRB is expressed in developing NC stem cells, melanoblasts, and enteric ganglia in mouse embryos [16, 127-130], whereas EDN3 is expressed in tissues associated with Ednrb-expressing cells [127]. Studies on mice harboring mutant *Ednrb* alleles showed that EDNRB signaling functions are necessary for the development of melanoblasts and enteric neural precursors [15, 16, 128, 131]. Likewise, transgenic expression of *Edn3* prevents aganglionosis and piebaldism in lethal spotted mice [132]. Experiments in *Ednrb* mutant mice have shown that EDNRB is not needed for melanoblast formation, but is needed for migration of melanoblasts and enteric neuroblast precursors prior to cell differentiation, between E10.5 and E12.5 [133]. In addition, it is also required for melanoblast development in the epidermis beyond E12.5 [119]. Avian NC cells express an additional *Ednrb* gene, *Ednrb2*, which is involved in melanoblast differentiation and migration [134, 135].

Although EDNRB is expressed in unspecified NC cells and melanocyte precursors, it is not clear whether it acts solely in a cell-autonomous manner [16, 130, 136]. By cross-explantation of embryonic tissues and NC cells, it has been found that the melanoblasts of the hypomorphic $Ednrb^{s}$ (*piebald*) allele show increased survival on *in vitro* cultured wild-type skin compared with mutant skin [137]. To address the question of whether melanocyte development depends entirely on the cell-autonomous action of EDNRB, we have performed a series of tissue recombination experiments *in vitro* using NC cell cultures from $Ednrb^{lacZ}$ embryos, which contain a functionally null allele of *Ednrb*. These studies showed that EDNRB plays a significant role during melanocyte differentiation by sequential cell-autonomous and non-autonomous actions [138]. Recently it has been shown that endothelin signaling leads to an increase in MITF phosphorylation and CREB phosphorylation in cultured human melanocytes [60]. However, it is unknown whether melanocytic EDNRB signaling acts through regulation of MITF, when and where the MITF post-translational modifications occur, and whether EDNRB signaling-dependent MITF phosphorylation plays any role in melanocyte development *in vivo*.

In addition, how EDNRB itself is regulated in melanocyte development is unknown. Interestingly, it has been shown that SOX10 directly activates *Ednrb* transcription in NC stem cell-derived enteric neurons [139], but genetic evidence suggests that SOX10 does not directly activate *Ednrb* transcription in the melanocyte lineage [140]. However, contradictory data showed that SOX10 transactivates the *Ednrb* promoter in human cultured melanocytes [141]. These results suggest that SOX10 may regulate differentiation-related downstream target gene(s) based on the cellular context in development.

Notch signaling

Recent work showed that Notch signaling is involved in the maintenance of melanoblasts and melanocyte stem cells

[142, 143]. The precise function of Notch signaling in the maintenance of melanocyte stem cells, however, requires further investigation. For detailed information on the Notch signaling pathway and its general role in melanocytes, we refer the readers to other recent reviews [144, 145].

Conclusions

White spotting genes play essential roles in NC stem cell-derived melanocyte development and related diseases. Nevertheless, much work is required to complete our functional understanding of these genes in melanocyte development. One of the important questions is when and how this complex network of genes interacts with other genes to regulate proper melanocyte development. Current evidence suggests that MITF is extensively involved in melanocyte development, providing a central link between transcription factors and signaling pathways (Figure 4), and is also involved in melanocyte stem cell maintenance [146]. Do all transcription factors and signaling pathways use MITF to regulate melanocyte development? More research is needed to answer this question. For example, it is unknown if and/or how PAX3 or SNAI2 regulates melanocyte development via *Mitf*. In addition, it will be very interesting to determine how WNT, KIT, and EDNRB signaling pathways regulate MITF and whether Notch signaling influences the maintenance of melanocyte stem cells through regulation of MITF. It is unknown whether KIT- and EDNRB-dependent MITF phosphorylation plays any role in melanocyte development *in vivo*. Increasing evidence suggests that signaling proteins tend to form networks of interactions rather than simple linear pathways. Therefore, most importantly, we need to further understand how distinct signaling pathways form interacting networks to regulate specification, survival, migration, proliferation, and differentiation of melanocyte precursors during development and how these pathways influence the dynamic balance between stem cell maintenance and differentiation in tissues of mature melanocytes. The field of melanocyte research has grown to include developmental cell biology and cancer biology, and will continue to provide a fruitful ground for basic and translational research in the future.

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