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mitfa is required at multiple stages of melanocyte differentiation but not to establish the melanocyte stem cell

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Abstract

The *mitfa* gene encodes a zebrafish ortholog of the microphthalmia-associated transcription factor (Mitf) which, like its counterparts in other species, is absolutely required for development of neural crest melanocytes. In order to evaluate mitfa's role in different stages of melanocyte development, we have identified hypomorphic alleles of *mitfa*, including two alleles that are temperature-sensitive for melanocyte development. Molecular analysis revealed that the *mitf*^{fh53}*ts* results from a single base pair change producing an asparagine to tyrosine amino acid substitution in the DNA-binding domain, and the *mitfa*^{vc7} *ts* allele is a mutation in a splice donor site that reduces the level of correctly-spliced transcripts. Splicing in the *mitfa*^{vc7} allele does not itself appear to be temperature-dependent. A third, hypomorphic allele, *mitfa*^{z25} results in an isoleucine to phenylalanine substitution in the first helix domain of the protein. Temperature upshift experiments with *mitfa*^{fh53}*ts* show that *mitfa* is required at several stages of melanocyte differentiation, including for expression of the early melanoblast marker *dct*, again for progression from *dct* expression to differentiation, and again for maintenance of dendritic form following differentiation. *mitfa*^{fh53}*ts* mutants recover melanocytes within 2–3 days when downshifted at all stages of larval development. However, when melanocyte stem cells (MSCs) are ablated by early treatment with the erbB3 inhibitor AG1478, melanocyte recovery is lost by 48 hours. This result indicates first that the MSC is established at the restrictive temperature, and that melanoblasts die or lose the ability to recover after being held at the restrictive temperature for approximately one day.

Keywords

melanocyte; MITF; zebrafish; neural crest

INTRODUCTION

The microphthalmia-associated transcription factor (MITF), a protein of the basic helix-loop-helix leucine zipper family, has been described as the master regulator of vertebrate melanocyte development (Widlund and Fisher 2003; Steingrimsson, et al. 2004; Levy, et al.

2006). Mutations in the *MITF* gene cause one type of the deafness/pigmentation disorder Waardenburg syndrome in humans, and over 20 alleles at the mouse *Mitf* locus comprise a complex series of coat color, eye development, osteoclast and mast cell phenotypes. *MITF* mutations have been described in several other vertebrates, including rat (Opdecamp, et al. 1998), hamster (Hodgkinson, et al. 1998), quail (Mochii, et al. 1998), and zebrafish (Lister, et al. 1999). In zebrafish, null alleles of the *MITF* ortholog *mitfa* result in a complete absence of neural crest-derived melanocytes as well as earlier markers of melanoblast specification including *dopachrome tautomerase* (*dct*) and the receptor tyrosine kinase *kit* (Lister, et al. 1999), but spare the retinal pigment epithelium. Transient knockdown of *mitfa* with morpholino oligonucleotides delays but does not eliminate differentiated melanocytes (Mellgren and Johnson 2004), but the complete role of *MITF/mitfa* over the course of differentiation of the individual melanocyte, or the full life history of the zebrafish, or any other vertebrate, is not clearly understood.

The many transcriptional targets of MITF suggest its involvement in multiple aspects of melanocyte biology (reviewed in Cheli, et al. 2010). Not surprisingly the first identified were those involved directly in melanin synthesis (*tyrosinase*, *dopachrome tautomerase*, *tyrp1*). More recently, MITF has been linked to control of expression in genes involved in survival (*bcl2*), cell cycle control (*cdk2*, *p16/INK4A*, *p21*), and cell morphology (*Dia1*). The potential of *MITF* to promote a melanocyte differentiation program has been further demonstrated by induction of melanocyte development following overexpression in medaka ES cells (Bejar, et al. 2003), or ectopic development following expression in zebrafish (Lister, et al. 1999) and xenopus (Kumasaka, et al. 2005) embryos. Dysregulation of *MITF* may contribute to the initiation and progression of melanoma in a variety of ways (reviewed in Levy, et al. 2006).

Although the existence of subpopulations of zebrafish melanocytes with distinct genetic requirements has been recognized for some time (Johnson, et al. 1995), recent progress in this area has been rapid, as a result of the development of techniques to ablate melanocytes (and thereby induce their regeneration; (Yang, et al. 2004; Yang and Johnson 2006)) and identification of mutations specific to one population (Budi, et al. 2008). In zebrafish, the embryonic pattern is generated primarily from a direct-developing population of melanocyte precursors (Hultman and Johnson 2010), while the melanocytes that will begin to create the adult pattern at the onset of metamorphosis, as well as those that can be recruited to replace embryonic melanocytes lost due to ablation, arise from a self-renewing, stem cell-like population established during the first two days of development (Budi, et al. 2008; Hultman, et al. 2009).

Conditional mutations, in particular temperature–sensitive alleles, have been exploited in genetic model organisms for decades. Temperature-sensitive alleles in zebrafish have proven to be particularly useful for understanding mechanisms of regeneration (Johnson and Weston 1995; Poss, et al. 2002; Nechiporuk, et al. 2003) and pigmentation (Rawls and Johnson 2001; Parichy and Turner 2003; Parichy, et al. 2003; Rawls and Johnson 2003). Previous studies in zebrafish showed that *mitfa* is required for development of all embryonic and adult melanocytes (Lister, et al. 1999). However, because of the complete absence of melanoblasts and melanocytes in fish homozygous for *mitfa* null alleles we were previously unable to address stage-specific roles for *mitfa* in melanocyte differentiation. Here we describe new alleles of *mitfa* which are temperature-sensitive, and use these new conditional alleles of zebrafish *mitfa* to test the requirement for *mitfa* activity at different stages of development and different points of melanocyte differentiation. We find that *mitfa* activity is required at multiple stages of melanocyte differentiation, but does not appear to be required for establishment of the melanocyte stem cell population.

RESULTS

Identification of temperature-sensitive alleles of mitfa

Three new ethlynitrosourea (ENU)-induced alleles of *mitfa*, *mitfa*^{fh53}, *mitfa*^{vc7}, and *mitfa*^{z25}, were identified through non-complementation of existing *mitfa* alleles (B. Draper, T. Linbo, M. Jurynek, unpublished results). In contrast to the two previously described alleles of *mitfa* (*mitfa*^{w2} and *mitfa*^{b692}) in which neural crest-derived melanocytes are completely absent through all stages of development (Lister, et al. 1999; Lister, et al. 2001), fish homozygous for *mitfa*^{fh53}, *mitfa*^{vc7}, and *mitfa*^{z25}, or in transheterozygous combination with the null alleles *mitfa*^{w2} and *mitfa*^{b692}, had variable numbers of melanocytes when reared under standard temperatures and conditions (28.5 C). (Figure 1, 2 and data not shown). Moreover, *mitfa*^{fh53} and *mitfa*^{vc7} developed no melanocytes at 32° C, but developed normal or nearly normal melanocyte patterns at 25° C (Table 1). Development at a variety of temperatures from 23–32 degrees suggest that the *mitfa*^{vc7} allele is still somewhat compromised for function at the lowest permissive temperature attempted (23° C), while the *mitfa*^{fh53} allele allows normal melanocyte development at 23° C, and nearly normal development at 24 and 25 degrees, significant deficits at 26° and 28.5° C, and complete ablation of melanocytes at 30° and 32°C. *mitfa*^{fh53} showed similar temperature sensitivity when challenged to grow at different temperatures through mature stages (Figures 1, 2 and Table 1). In contrast, *mitfa*^{z25} embryos and adults developed similar numbers of melanocytes at all temperatures (not shown). These results indicate that *mitfa*^{z25} is a temperature-independent hypomorphic mutation, while *mitfa*^{fh53} and to a lesser extent *mitfa*^{vc7} are temperature-sensitive alleles of *mitf*a.

Identification of distinct mutations

To further explore the hypomorphic or temperature-sensitive nature of the new *mitfa* alleles we sought to identify their molecular lesions. We first performed RT-PCR with gene specific primers to the 5′ and 3′ untranslated regions on mRNA from homozygous embryos (see Material and Methods). *mitfa*^{fh53} *and mitfa*^{z25} gave single PCR products (data not shown), which were sequenced directly to search for coding sequence changes. This analysis showed that *mitfa*^{fh53} is an A to T substitution that changes an asparagine to a tyrosine at amino acid 205 (N205Y) in the basic domain known to be the DNA binding portion of the molecule (Fig. 3A). This asparagine is absolutely conserved in all mitf/tfe proteins as well as in a large fraction of basic-helix-loop-helix proteins generally, including USF and all of the Myc/Mad/Max family proteins (Atchley and Fitch 1997). Although an MITF crystal structure has not been published, the structures of the Max and USF homodimers suggest that this asparagine makes contacts with the phosphate backbone (Ferre-D'Amare, et al. 1993;Ferre-D'Amare, et al. 1994;Hallsson, et al. 2007); changing this position to a tyrosine may therefore destabilize DNA binding by the mutant protein at higher temperatures. Mutation of this same codon in human *MITF* has been identified in a pedigree of a family with Tietz syndrome, although the substitution is for a lysine instead of a tyrosine residue (Smith, et al. 2000).

Our analysis of the *mitfa*^{z25} mutant sequence found that it results in an A-to-T substitution that changes an isoleucine to a phenylalanine (I219F)(Fig. 3B). Interestingly, this is the same amino acid change described for the *Mitf*$^{mi\text{-}enu122}$ allele in mouse (Steingrimsson, et al. 1998). Crystal structure analysis of homologous bHLH proteins show that this position is an an internal hydrophobic region of the protein that promotes dimerization (Ferre-D'Amare, et al. 1993;Ferre-D'Amare, et al. 1994;Hallsson, et al. 2007). Lack of temperature-dependence of this mutation suggests that the phenotype isn't simply a matter of thermodestabilization.

In contrast, RT-PCR from the *mitfa*^{vc7} mutant resulted in multiple PCR products. To understand this, we cloned each of the products, then sequenced them. The sequence from

the predominant RT-PCR product contained a T-to-A substitution in the intron 6 splice donor site (Figure 4), and retention of intron 6. The translation product of this mRNA is expected to extend through intron 6 and continue into exon 7 while preserving the correct reading frame, thus introducing 39 additional amino acids in the middle of the basic region. A minor RT-PCR product of the correct size showed proper splicing, suggesting that this splice donor mutation does not completely abrogate splicing, while a second minor product showed skipping of exon 6, which also preserves the reading frame but truncates the basic region. Semiquantitative RT-PCR shows that the proportion of intron-retaining message to properly-spliced message is identical in RNA harvested from animals reared at the permissive or restrictive temperatures (Fig. 4C), indicating that the temperature sensitivity of the *mitfa*^{vc7} mutation is not a result of temperature-sensitive splicing. Instead it raises the possibility that the threshold requirement for *mitfa* is less at lower temperatures. Thus, the small amount of normal protein produced from the minor, correctly spliced mRNA species may be sufficient at 23°C, but insufficient at 32°C. Alternatively, one or both of the translation products resulting from the misspliced messages may have temperature-dependent function.

mitfa is required at multiple steps in melanocyte development

We and others have previously shown that *Mitf/mitfa* is required for specification of melanocyte fate: neural crest cells in *mitfa* null mutants fail to express melanoblast markers such as *dopachrome tautomerase* (*dct*) (Opdecamp, et al. 1997; Lister, et al. 1999). This early and absolute requirement for *mitfa* for specification of the melanocyte lineage has prevented a more thorough understanding of subsequent roles for *mitfa* in melanocyte development and differentiation. We reasoned that we could use our ability to remove or add back *mitfa* function with temperature upshifts or downshifts at different stages to determine if *mitfa* is required once, to specify the lineage, or required at one or more successive stages in the differentiation and physiology of the melanocyte.

We first asked whether melanocyte lineage specification is similarly defective in the *mitf-ts* alleles as we had shown for the null mutations. We reared mutants for these alleles at the restrictive temperature for 70 hours at 31.5°C (equivalent of 82 hours standard development (Kimmel, et al. 1995)), fixed and probed them for expression of *mitfa* and *dct*. As a missense mutation, the *mitfa*^{fh53} transcript should be as stable as the wild-type message. At this stage, all of the direct-developing embryonic melanocytes have differentiated in wild-type larvae and strong expression of *mitfa* is restricted to a small number of cells in the head (Figure 5A). (Weak *mitfa* expression is detectable in differentiated melanocytes after 2 to 3 days of color development; data not shown.) In contrast, in *mitfa*^{fh53} homozygous larvae at this stage, strongly *mitfa*-expressing cells are still abundant, and although a large number around the ear appear not to have undergone extensive migration, many of these cells are found in positions characteristic of the dorsal, lateral, and ventral stripes. Despite abundant *mitfa+* cells in the temperature-sensitive mutants at the restrictive temperature (Fig. 5A), no *dct* expressing cells could be detected outside the pigmented retinal epithelium (Fig. 5B). Similarly, we find no expression of a variety of melanocyte lineage reporters such as a *kita* enhancer trap (Distel, et al. 2009), the *Gpnmb* enhancer reporter (Loftus, et al. 2009), the *fTyrp1* promoter reporter (Zou, et al. 2006), and *TYR*- and *DCT*- promoter and enhancer reporters (A. McCallion and S. Johnson, unpublished) (not shown). These results suggest that similar to the null mutants, the *mitfa-ts* mutants block melanocyte specification at early (pre-*kita* or pre-*dct* expressing) stages. The identity/fate of the *mitfa+* cells is not known (but see below).

In order to ask whether *mitfa* has subsequent requirements in differentiation we took embryos reared to the equivalent of stage 22 hpf at the permissive temperature and shifted them to the restrictive temperature. Embryos examined for *dct* expression at the time of the

upshift showed abundant *dct* expression, indicating that melanoblasts had been specified and were beginning to differentiate at the permissive temperatures (Fig. 6). However, no melanin was produced in *mitfa*^{fh53} embryos (n=58) that were upshifted but allowed to develop for an additional day or longer, with the exception of the RPE, which has no requirement for *mitfa*. These results show that *mitfa* is required both for initial specification of melanoblasts, but additionally after specification has been demonstrated, there remain additional roles for *mitfa* in melanocyte differentiation, including melanin production.

We were also interested in whether *mitfa* has roles in maintaining the normal physiology of the melanocyte after differentiation. When *mitfa*^{fh53} larvae carrying the *fTyrp>GFP*^{j900} transgene for differentiated melanocytes were shifted to the restrictive temperature following melanization and expression of *fTyrp>GFP*, GFP fluorescence was extinguished within 24 hours (data not shown). Possible explanations for this result include that *mitfa* is continuously required for expression of the *fTyrp>GFP* transgene, or that the melanocytes are dying, or both. One possibility is that *mitfa* is required for survival of melanocytes, which might be mediated through the requirement for *kit* (*or kita*) function. Use of a temperature-sensitive allele of *kit* has shown that *kit* is required at multiple stages for different melanocyte functions, including promoting survival after 48 hours (Rawls and Johnson 2003). Since *kit* expression is dependent on *mitfa* function, one prediction is that melanocytes shifted to the restrictive temperature at 48 hours would also die. To address this, we took *mitfa*^{fh53} embryos reared at the permissive temperature through 72 hours, at which stage they have the full complement of differentiated and melanized melanocytes, and shifted them to restrictive temperature. When larvae were examined 48 hours later, their melanocytes had lost dendricity and taken on a small, rounded appearance (Fig. 7). Although this is characteristic of dying melanocytes (Parichy, et al. 1999), we were unable to detect markers of cell death, and did not observe extrusion of cell carcasses from the epidermis characteristic of melanocyte death in *kit* mutants, even after several days. One possible explanation for the absence of overt melanocyte death and extrusion was that the *mitfa*^{fh53} mutation has residual activity at the restrictive temperature. This seems unlikely, since halving the dosage of *mitfa*^{fh53} by generating transheterozygotes with a null *mitfa* mutation (*mitfa*^{fh53}/*mitfa*^{w2}) also showed little or no extrusion. A second possible explanation, that lack of overt death and extrusion is due to a deficit in macrophages, seems unlikely since *mitfa* mutants stain normally for macrophages (data not shown) with the marker neutral red (Herbomel and Levraud, 2005). Whether there is residual *mitfa* activity or not, these results show that *mitfa* is still required for melanocyte physiology even after the transcript has become only weakly detectable.

mitfa is not required in the stem cell, but is required for maintenance of direct-developing precursors

Melanocytes in embryonic and larval zebrafish originate primarily from direct-developing embryonic (ontogenetic) precursors, however upon their ablation new melanocytes can be regenerated from a stem cell population which may be the same as that responsible for addition of melanocytes to the forming adult pigment pattern at metamorphosis (Hultman, et al. 2009). To determine if there are differential requirements for *mitfa* in these populations we performed temperature shift experiments. Melanocytes in *mitfa*^{fh53} homozygotes were observed to recover when animals were raised at restrictive temperature and downshifted after as many as 9 days, and indeed even in fish shifted to permissive temperature as adults (Figures 2, 8). These results suggest that embryonic melanoblasts may be arrested (albeit prior to becoming *dct*-positive) in the absence of *mitfa* activity, but capable of differentiating after *mitfa* is restored, and/or that *mitfa* activity is not required for the establishment or survival of the melanocyte stem cell.

Although the melanocyte stem cell has not yet been isolated in zebrafish, it is known that its establishment is dependent upon the activity of the *erbb3b* gene during a narrow window of embryonic development (Budi, et al. 2008; Hultman, et al. 2009). The stem cell can be eliminated by treatment with the drug AG1478 between 9 and 48 hours post fertilization (Budi, et al. 2008; Hultman, et al. 2009). To further test the relationship between the melanocyte stem cell and *mitfa* activity, we placed *mitfa fh53* homozygous embryos at restrictive temperature and treated them with AG1478, then downshifted to permissive temperature at various times to assess recovery of melanocytes (Figure 8). While AG1478-treated embryos downshifted at 24 hours showed comparable recovery to those treated with DMSO, embryos downshifted at later times showed progressively reduced capacity to recover (Figure 8). This sensitivity suggests that the majority of the melanocytes that develop in larvae downshifted after 48 h at the restrictive temperature do indeed derive from the *erbb3b*-dependent (stem cell) population. Moreover, because in untreated embryos these cells recover even when larvae are held at restrictive temperature during the critical period for MSC establishment, it argues that *mitfa* is not itself required for that establishment. We investigated the possibility that residual *mitfa* activity from the ts allele was responsible for promoting establishment of the MSC, by combining $mitfa^{fh53}$ (or $mitfa^{vc7}$) with the null *mitfa* allele, $mitfa^{w2}$. These animals should have one-half the residual *mitfa* activity as the homozygotes for the ts alleles. In both cases, the transheterozygotes recovered melanocytes similar to the homozygotes, tending to argue that there is little or no residual *mitfa* activity in the ts mutations at the restrictive temperature (not shown).

Finally, we examined the expression of *mitfa* by in situ hybridization under these conditions in wild-type and *fh53* homozygous embryos and larvae. Embryos were raised at restrictive temperature in the presence or absence of AG1478, and then fixed at the equivalent of 65 hours' development at standard temperature. As noted earlier, *mitfa* expression in wild-type larvae has greatly subsided by this stage; in AG1478-treated larvae, expression is reduced to an even greater degree (Figure 9). Likewise, the abundant expression of *mitfa* mRNA observed in *fh53* larvae at restrictive temperature is also greatly diminished by AG1478 treatment. Together with the loss of melanocyte recovery, these results raise the possibility that direct developing *mitfa*+ melanoblasts, in the absence of *mitfa* function, eventually die. Presumably, the MSC derived melanoblasts share a similar requirement on *mitfa* for their survival. Whether any of the *mitfa*-expressing population represents MSC's or rather committed progenitors recruited from the MSC awaits further investigation.

DISCUSSION

An understanding of the central role of Mitf in vertebrate pigmentation has been greatly aided by the large number of mouse *Mitf* alleles available for study (Hallsson, et al. 2000; Steingrimsson, et al. 2004). In this study we describe characterization of three new alleles of the zebrafish *Mitf* ortholog *mitfa*. We demonstrate that two of these alleles display temperature-dependent phenotypes, and exploit this conditionality to test the requirement for *mitfa* activity at distinct points of melanocyte differentiation and stages of ontogeny. Interestingly, although the *fh53* and *vc7* alleles display similar temperature dependence, the nature of the two mutations is quite distinct. $mitfa^{fh53}$ results from an amino acid substitution at a conserved position in the basic domain, which is responsible for DNA binding. In contrast, the $mitfa^{vc7}$ mutation is in a splice site, and leads to similar levels of mis-splicing both at permissive and restrictive temperatures. Whether the temperature sensitivity is a result of overall lower levels of Mitfa protein, or due to antimorphic effects from translation products of one or more of the mis-spliced transcripts, remains to be resolved.

Our results support previous studies of null alleles that *mitf* activity is required for specification of melanocytes (Lister, et al. 1999), as under restrictive conditions (high

temperature) we never observe expression of the specification marker *dct*. Additionally, we find that *mitfa* is required for later steps, as when the temperature is raised shortly after the onset of neural crest *dct* expression (i.e. specification) melanized cells are still not observed. Melanocytes that have undergone differentiation still require *mitf* function; although we did not observe melanocyte cell death at restrictive temperature, differentiated melanocytes lost dendricity and took on the appearance of dying cells (Parichy, et al. 1999). Similarly, melanoma cells depleted of Mitf by siRNA arrest in G1 and round up but do not die (Carreira, et al. 2006). It may be that even at restrictive temperature these conditional zebrafish alleles retain sufficient residual *mitfa* function to keep the melanocyte alive.

The fate of those cells which initiate *mitfa* expression but lack *mitfa* function is a key question. The finding that recovery of melanocytes following restoration of *mitfa* function is rapidly lost when MSCs were first ablated leads us to favor the model that these cells die. An alternative possibility is that these cells, failing in their efforts to specify along the melanocyte lineage, take on other pigment cell fates. Some support for this model is provided by previous reports of co-expression of *mitfa* with markers of other pigment lineages in wild-type embryos (Parichy, et al. 2000; Curran, et al. 2010) and of excess iridophores in *mitfa* null mutants (Lister et al, 1999). Similar increases in iridophores are also observed in the ts alleles at the restrictive temperature (data not shown). However, the number of excess iridophores (~ 20) can only partially account for the more than 400 direct developing melanocytes that fail to differentiate in *mitfa* mutants. Similarly, we fail to observe an excess of xanthophores in *mitfa* mutants.

In addition to addressing different stages in development of the melanocyte, we were able to use the conditional alleles to investigate the requirement of *mitfa* at different stages of the life history and in different populations of melanocyte precursors. Previous work has shown that the embryonic zebrafish melanocyte pattern is derived primarily from a population of direct-developing precursors, and that filling in (Hultman and Johnson 2010) or regenerating (Hultman, et al. 2009) the final pattern, as well as generating the metamorphic pattern (Budi, et al. 2008), is dependent on a stem cell population that is dependent on activity of the *erbb3b* gene between 9 and 48 hours post-fertilization. By combining temperature-shift experiments with drug treatments that block *erbb3b* activity, we find that *mitfa* activity is not required for establishment of the melanocyte stem cell: in mutant animals raised initially at restrictive temperature, shifts to permissive temperature even as late as adulthood result in recovery of melanocytes. This recovery arises largely from the *erbb3b*-dependent stem cell population as it is sensitive to early treatment with the antagonist AG1478. Similarly, the number of *mitfa*-expressing cells in *mitfa*^{fh53} embryos at restrictive temperature is greatly reduced by AG1478. This could argue that these mitfa expressing cells in *mitfa*^{fh53} embryos at restrictive temperature represent the MSC itself or their committed daughters at the first *mitfa*-requiring stage. That wild-type embryos have few cells expressing *mitfa* at this stage tends to argue that *mitfa* is not expressed in the stem cell, but rather in the committed daughter, at the time that *mitfa* is required to initiate melanocyte differentiation. We note that that in the absence of biochemical assays for Mitf activity, we have not completely excluded the unlikely possibility that there remains sufficient function at the restrictive temperature to rescue the MSC. Nevertheless, our finding is consistent with work in mouse, where *Mitf* does not appear to be a consistent marker of the MSC (Osawa, et al. 2005). Stable, long-term lineage tracing methods (such as via Cre/lox, or transposon marking) that have been available in other systems and are beginning to be established in zebrafish (Boniface, et al. 2009; Hans, et al. 2009; Hesselson, et al. 2009; Tu and Johnson 2010) are likely to aid in the resolution of this and other questions.

These results show that *mitfa* is required both for initial specification of melanoblasts, but additionally after specification has been demonstrated by *dct* expression, there remain

additional roles for *mitfa* in melanocyte differentiation. These roles may include expression of structural genes for melanosome biogenesis, such as *pmel17*, *mlana*, or *gpnmb*, or other enzymes that contribute to melanin synthesis, such as *tyr* or *tyrp1* (Cheli, et al. 2010). Expression of each of these genes is dependent on the transcription promoting activity of *Mitf* in mammalian cells. Whether these genes are expressed simultaneously with *dct* in the zebrafish melanoblast, or sufficiently late to account for the failure of $mitfa^{fh53}$ mutants to melanize after upshift to the restrictive temperature is not clear. These upshift experiments may provide an additional tool to understand the possible roles for temporal differences in control of gene expression by *mitfa*.

MATERIALS AND METHODS

Fishkeeping and husbandry

Adult fish of the wild-type AB strain, and *mitfa* alleles *w2* (ZDB-ALT-990423-22), *fh53*, and *vc7* were maintained on a 14 hour/10 hour light/dark cycle. Embryos were obtained from natural matings and staged according to Kimmel et al. (Kimmel, et al. 1995). In some cases, melanin synthesis was first suppressed by prior incubation with 0.2mM pheylthiourea (PTU, Sigma, P7629). All experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC) protocols. For all experiments, conditions, or datapoints, we analyzed at least 10 embryos, unless otherwise stated.

Identification of lesions

First strand cDNA was generated from total RNA isolated from homozygous $mitfa^{fh53}$ and $mitfa^{vc7}$ or transheterozygous ($mitfa^{fh53}/mitfa^{w2}$ or $mitfa^{vc7}/mitfa^{w2}$) embryos, and used as a template for PCR to amplify the *mitfa* open reading frame in two overlapping sections with the following primers: 5′forward: GGC CAA GAC GAC TGG TCA GTT CTT GCA C, 5′ reverse: TCT CTC TTT TGC CAG GGC TCT GAC TTC TGC, 5′ reverse2: ACG GAT CAT TTG ACT TGG GAA TTA AAG, 3′forward: GCA GAA GTC AGA GCC CTG GC, 3′reverse: GGT TCA TGA AAT TTA GTT GGC ATT GC. PCR products were sequenced directly, or subcloned by TOPO TA cloning (Invitrogen) and then sequenced. Sequence changes identified in cDNA were confirmed in genomic DNA.

Temperature shift experiments

For most experiments, $mitfa^{ts}$ embryos were generated from homozygous intercrosses. For the experiments shown in Table 1, $mitfa^{fh53}$ embryos were generated from heterozygous intercrosses. At 24–27 degrees, $mitfa^{fh53}$ mutant embryos were identified by ectopic iridophores, while at higher temperatures mutant embryos were clearly discerned by melanocyte phenotypes.

For adult experiments, fish from heterozygous intercrosses were were reared at restrictive temperatures for 3 days, mutant embryos selected and returned to 25 degrees for a further 10 days. They were then shifted to aquaria with individual heaters for 5 weeks, and scored for qualitative defects. Temperature in each tank was then recorded daily, and temperature range over the course of experiment is shown.

Drug treatments

AG1478/tyrphostin (LC Laboratories cat. no. T-7310) was prepared at a concentration of 6 millimolar in DMSO, and diluted further with DMSO before use to yield a final concentration of 3 micromolar in 0.5% DMSO in system water or embryo medium. Drug was added at 9 hours post fertilization and washed out at 48 hours post-fertilization unless otherwise noted. 10 fish were examined per timepoint for each treatment, and the experiment was replicated with similar results to those presented.

In situ hybridization

In situ hybridization was carried out as previously described (Thisse and Thisse 2008). Riboprobes for *mitfa* (ZDB-GENE-990910-11) and *dct* (ZDB-GENE-000508-1) have been described previously (Lister, et al. 1999; Kelsh, et al. 2000). For photography, samples were equilibrated in 50% glycerol/PBS and imaged with an Olympus SZ12 stereo dissecting scope and DP70 digital camera. Images were adjusted and color balanced in Photoshop (Adobe).

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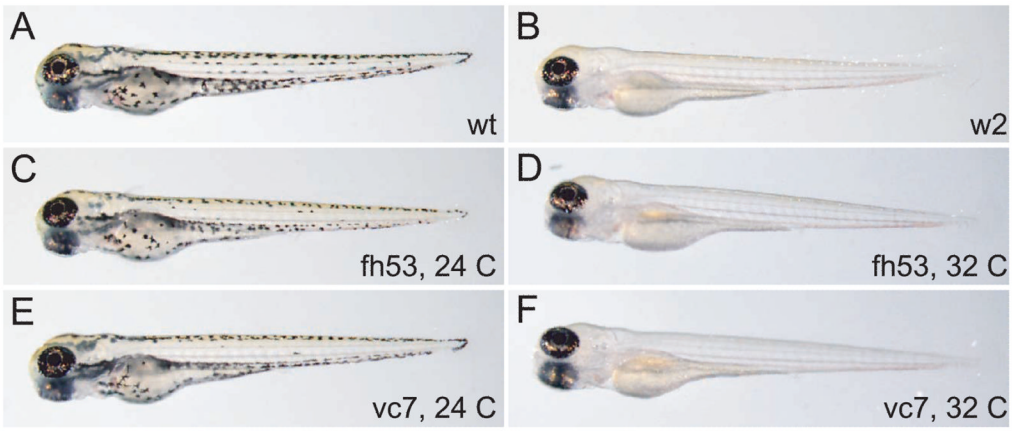

Figure 1.
Temperature sensitivity of two *mitfa* hypomorphic alleles: larval phenotypes. A) Wild-type, B) $mitfa^{w2}$, C) $mitfa^{fh53}$ raised at 24°C, D) $mitfa^{fh53}$ raised at 32°C, E) $mitfa^{vc7}$ raised at 24°C, F) $mitfa^{vc7}$ raised at 32°C.

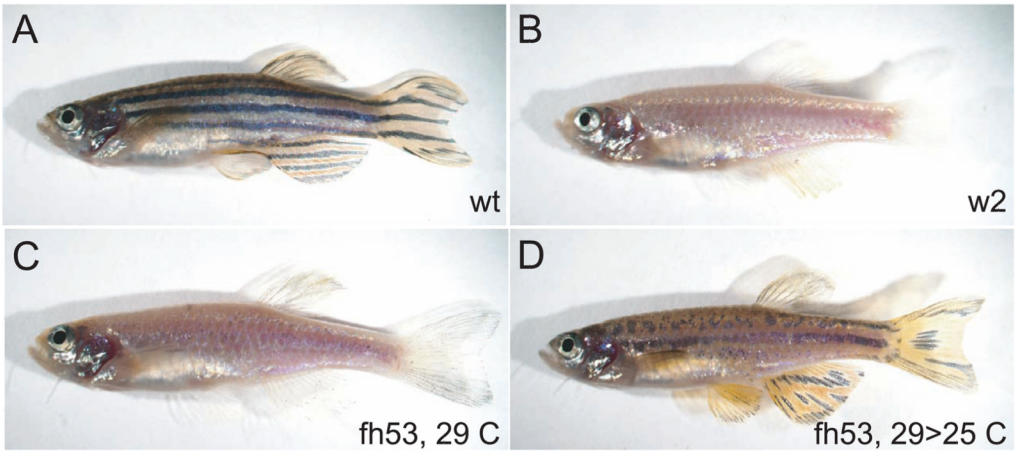

Figure 2.
Temperature sensitivity of the *mitfa fh53* allele: adult phenotypes. A) Wild-type, B) *mitfa*^{w2}, C) *mitfa*^{fh53} raised at 29°C, D) *mitfa*^{fh53} raised at 29°C to adulthood and then shifted to 25°C for one month, showing recovery of melanocytes.

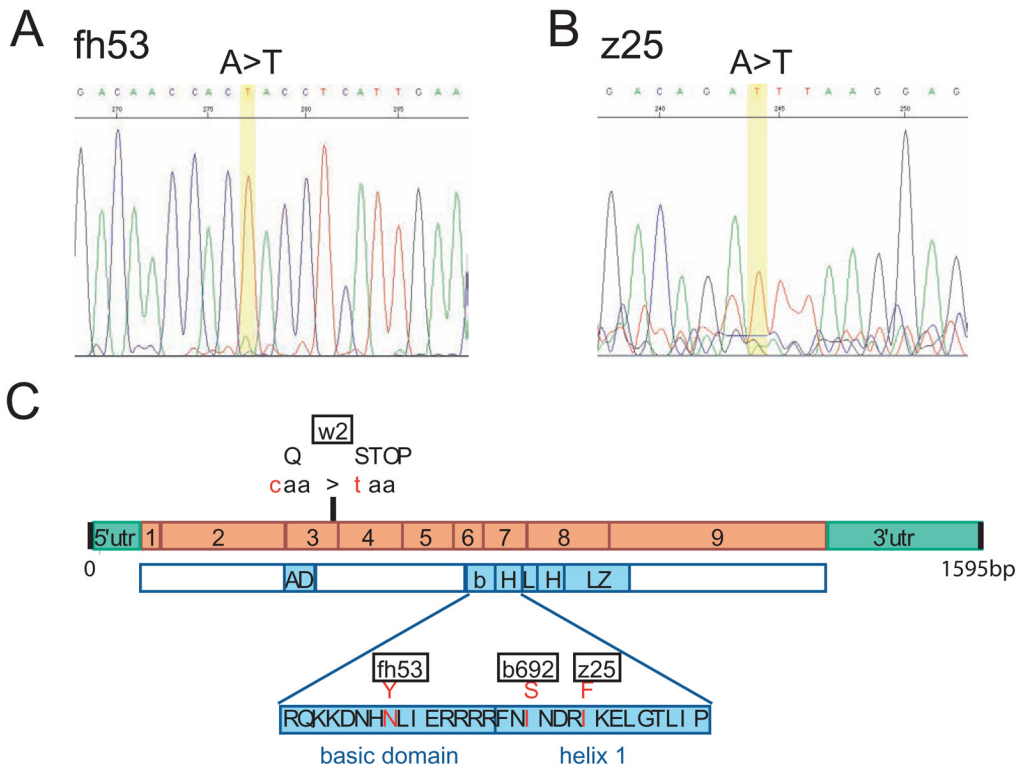

Figure 3.

Identification of mutations. A), B) Electropherogram of sequence obtained from *mitfa*^{fh53} (A) and *mitfa*^{z25} (B) alleles indicate single base substitutions that change amino acids. C) Location of *fh53* and *z25* mutations in the *mitfa* cDNA sequence. Previously characterized alleles *w2* and *b692* are also shown. Orange boxes indicate coding portions of exons.

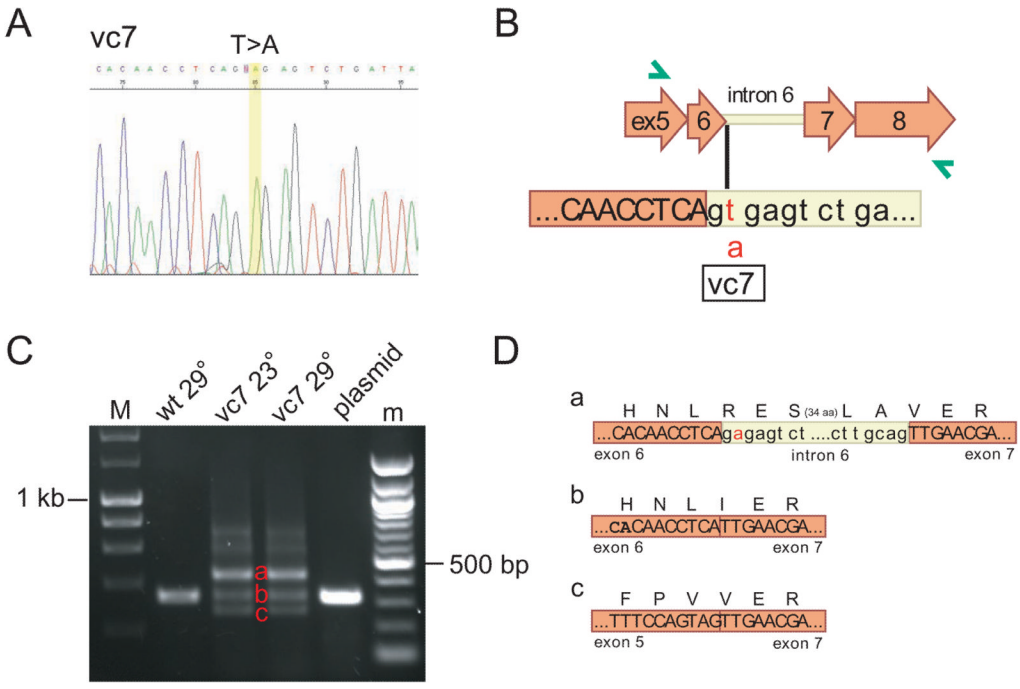

Figure 4.

The vc7 allele affects spicing of mitfa. A) Electropherogram of sequence obtained from *mitfa*^{vc7} allele. B) Location of *vc7* mutation in genomic sequence. C) Semi-quantitative RT/PCR shows that missplicing is not temperature-sensitive. *vc7* results in transcripts that (a) include intron 6 and (c) skip exon 6, in addition to correctly-spliced transcript (b). M, 200 basepair ladder; m, 100 basepair ladder. D) Reading frame is preserved in aberrantly-spliced transcripts a and c.

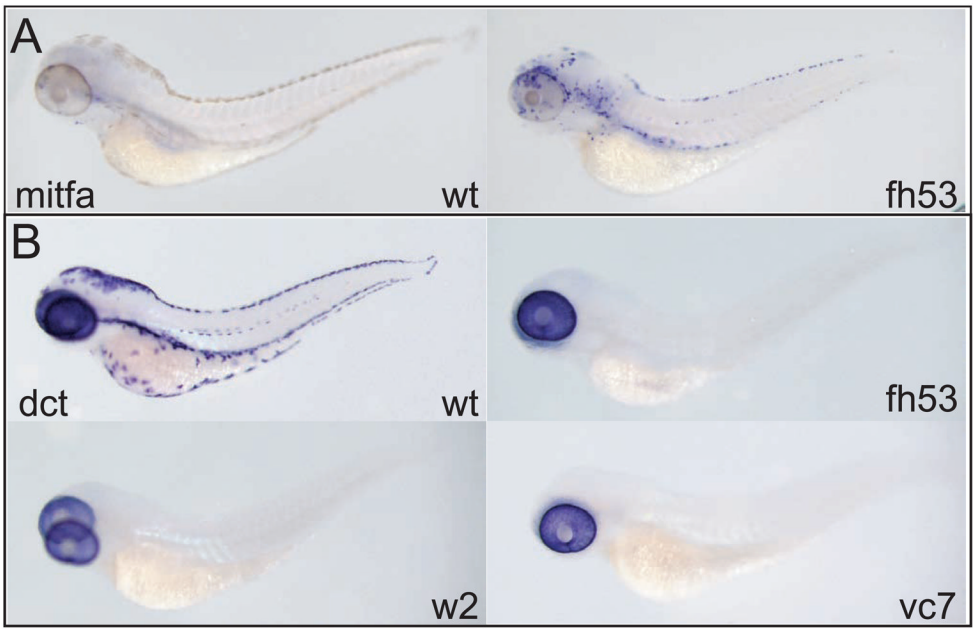

Figure 5.

mitfa is required at multiple steps in melanocyte development. A) *mitfa* expression persists at restrictive temperature. Wild-type and *mitfa*^{fh53} larvae are shown. B) No *dct* is expressed in neural crest cells when held at restrictive temperature, but retinal expression of *dct* is not affected. Wild-type, *mitfa*^{w2}, *mitfa*^{fh53}, and *mitfa*^{vc7} larvae are shown. Embryos were treated with 0.2 mM PTU to suppress melanin synthesis. All larvae are at the equivalent of 82 hours at standard temperature.

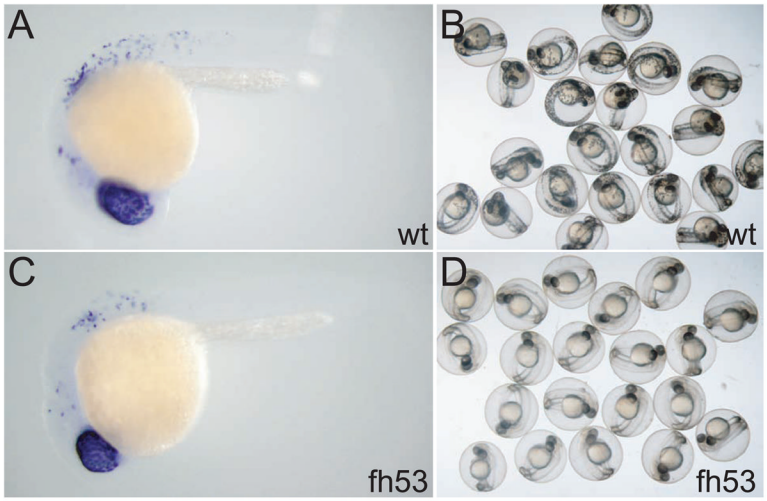

Figure 6.

Embryos raised at permissive temperature to *dct+* stage, when shifted show no melanized cells. A) Wild-type embryo showing dct expression in retinal pigment epithelium (RPE) and neural crest melanoblasts, B) wild-type embryos 20 hours after upshift (stage equivalent to 45 hours at standard temperature) showing differentiated melanocytes, C) *mitfa*^{fh53} embryo showing *dct* expression at time of temperature upshift, D) *mitfa*^{fh53} embryos 20 hours after upshift do not display any melanized cells save for RPE. In A and C, embryos were treated with 0.2 mM PTU to suppress melanin synthesis.

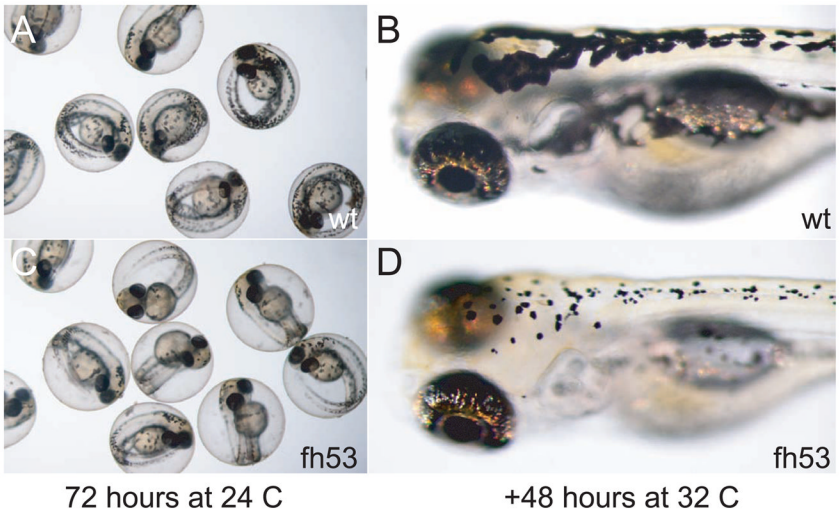

Figure 7.
Loss of dendricity in melanocytes at restrictive temperature. Wild-type and *mitfa*^{fh53} embryos were raised at permissive temperature for 72 hours, photographed (A,C) then shifted to 32°C for 48 hours (B,D).

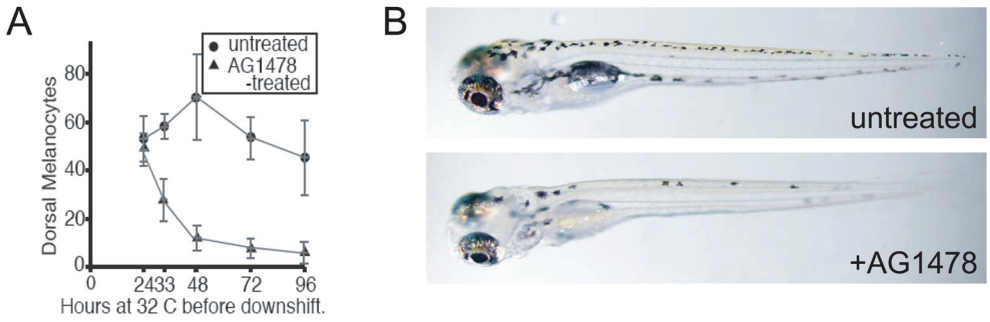

Figure 8.

mitfa is not required in the stem cell, but is required for survival of direct-developing melanocyte progenitors. A) graph showing dorsal melanocyte number in *mitfa*^{fh53} homozygous larvae held at 32°C for the indicated times and then shifted to permissive temperature, and melanocytes counted three days later. B) Homozygous *mitfa* ^{fh53} larvae held at 32°C in the presence of DMSO (top) or AG1478 (bottom) then downshifted to permissive temperature.

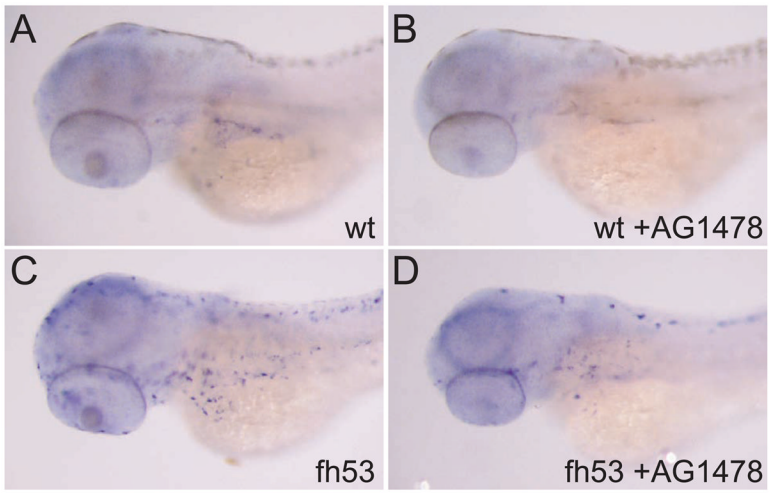

Figure 9.

in situ hybridization for *mitfa* in wild-type (A,B) and *mitfa*^{fh53} (C,D) larvae held at restrictive temperature with or without AG1478 treatment from 9–48 hours development. AG1478 treatment reduces the number of *mitfa*-expressing cells in both wild-type and mutant embryos. Larvae are at the equivalent of the 65 hour stage at standard temperature. Embryos were treated with 0.2 mM PTU to suppress melanin synthesis.

Table 1

Temperature dependence of *mitfa* alleles

	embryonic melanocytes(1)		adult melanocytes(2)	
temp.	*mitfa*^{vc7}	*mitfa*^{fh53}	temp. range	*mitfa*^{fh53}
23	+* (n=7)	+	25.0 – 26.9	regular stripes (n=5)
24	+* (n=10)	+* (n=5)	26.9 – 27.5	stripes have fewer melanocytes (n=6)
25	+* (n=6)	+* (n=9)		
27	+*	gaps (n=6)	28.6 – 29.5	very few melanocytes (< 50/side) (n=7)
28.5	1–10 mels/emb. (n=9)	10–50 mels/emb (n=6)	31.0 – 31.8	no melanocytes, solid xanthophore field (n=4)
30	none (n=8)	none (n=6)		
32	none (n=4)	none (n=4)		

(1) Embryos scored at ~ 80 hours post-fertilization, after shifting to indicated temperatures at ~ 10 hpf.

(2) Fish from heterozygous intercrosses were were reared at restrictive temperatures for 3 days, mutant embryos selected and returned to 25 degrees for a further 10 days. They were then shifted to aquaria with individual heaters for 5 weeks, and scored for qualitative defects. Temperature in each tank was then recorded daily, and temperature range over the course of experiment shown

* Full pattern development slower at these temperatures