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A requirement for *kit* in embryonic zebrafish melanocyte differentiation is revealed by melanoblast delay

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Abstract Exploring differences in gene requirements between species can allow us to delineate basic developmental mechanisms, provide insight into patterns of evolution, and explain heterochronic differences in developmental processes. One example of differences in gene requirements between zebrafish and mammals is the requirement of the *kit* receptor tyrosine kinase in melanocyte development. *kit* is required for migration, survival and differentiation of all neural crest-derived melanocytes in mammals. In contrast, zebrafish *kit* is not required for differentiation of embryonic melanocytes during normal development. When melanoblast development in zebrafish embryos is delayed by injecting morpholinos targeted to the *mitfa* gene, we show that these delayed melanoblasts fail to differentiate in *kit* mutants. Thus, we show that there is a *kit* requirement for melanocyte differentiation in zebrafish when melanoblast development is delayed. Furthermore, we show that *kit* is not involved in maintaining melanocyte precursors through the developmental delay, but instead is required for differentiation of melanocytes after the block on their development is removed. Finally, we suggest there is a heterochronic shift in the onset of melanocyte differentiation between fish and mouse, and developmental delay of melanoblast development in zebrafish removes this heterochronic difference.

Keywords Melanocyte · *kit* · *mitf* · Zebrafish · Heterochrony

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Introduction

An important lesson from the last few decades of research in developmental biology is that homologous developmental processes are often controlled by orthologous genes. While genes controlling developmental events in different organisms are shared, there may be differences in the specific events or tissues that require these genes. These differences are reflected in the extensive morphological diversity that exists between species. As differences in gene requirements across species could represent alternative developmental mechanisms, exploring the nature of these differences will help to define the basic requirements for normal development across species. In addition, it could aid in elucidating how mechanisms of development evolve, and provide molecular explanations for differences in development between organisms. One way differential development of species may have evolved is by changing the timing of gene expression, resulting in differential timing of developmental events, or heterochrony. Further examination of the difference in timing of gene requirements between organisms and how this is related to heterochronic shifts in development between species may allow us to understand how differences in timing of development have evolved.

Development of melanocytes from the neural crest and the genes required for their development have been extensively studied in mammals and teleost fish. As a result, many examples of the involvement of orthologous genes in pigment cell development in these two groups have been described. For instance, the G-protein-coupled receptor *ednrB* (*endothelin receptor B*), basic helix-loop-helix/leucine zipper transcription factor *mitf* (*microphthalmia-associated transcription factor*), and receptor tyrosine kinase (RTK) *kit* are each required for some aspects of melanocyte development in both zebrafish and mammals (reviewed in Rawls et al. 2001; Mellgren and Johnson2002; Quigley and Parichy 2002). However, there are differences in the requirements for these genes between mammalian and teleost pigment cell development.

One example of these differences comes from *EdnrB*, which is required for development of neural crest-derived melanocytes in the mouse (Baynash et al. 1994; Hosoda et al. 1994; Pavan and Tilghman 1994). In fish, however, mutants in *ednrB1 (rose)* lack only a subset of adult melanocytes, while embryonic melanocytes and other adult melanocytes are unaffected (Johnson et al. 1995; Parichy et al. 2000a). A second example is the *mitf* gene, which is required in mammals for neural crest-derived melanocytes and melanocytes of the retinal pigment epithelium (RPE; Hodgkinson et al. 1993; Hughes et al. 1993; Nakayama et al. 1998; Hallsson et al. 2000). In zebrafish, one of two *mitf* genes, *mitfa*, is only required for neural crest-derived melanocytes, although it is also expressed in RPE (Lister et al. 1999). The second *mitf* gene, *mitfb*, is also expressed in RPE, suggesting a possible role in RPE development (Lister et al. 2001). Thus, this difference in gene requirements between fish and mouse could be explained by the redundancy of the two *mitf* genes. A second possibility is that an alternative developmental mechanism for RPE development has evolved in fish. Thus, for both *ednrB* and *mitf* it remains unclear why the differences in gene requirements between mouse and fish exist.

Like the previous two examples, *kit* also has different requirements for melanocyte development between fish and mammals. Mouse mutants lacking either *steel* or *kit* fail to develop their melanocytes. Additionally, melanocyte precursors in these mutants fail to migrate along neural crest migratory pathways and are thought to subsequently die. Thus, *kit* and its ligand, *steel*, are required for melanoblast differentiation, migration and survival in mammals (Geissler et al. 1988; Morrison-Graham and Takahashi 1993; Cable et al. 1995; Wehrle-Haller and Weston 1995; Bernex et al. 1996; Ito et al. 1999). Embryonic zebrafish melanocytes share two of these requirements—they require *kit* for both migration and survival of melanocytes, but in contrast to mammals, zebrafish melanocytes differentiate in *kit* null mutants (Parichy et al. 1999). Therefore, *kit* is required for melanocytes to differentiate in mouse, but not in zebrafish.

In addition to the difference in *kit* requirements for differentiation between mouse and fish, there is also a difference in the timing of melanocyte differentiation. Zebrafish melanocytes differentiate as they migrate out from the neural crest. In mouse, melanocytes differentiate at a later stage of development, after they have finished most of their migration from the neural crest. Thus, melanocyte differentiation is one example of a heterochronic shift in the development of these two vertebrates.

Here, we examine the different requirements that zebrafish melanocytes have for *kit* when compared to mammals. We find that when melanocyte development is delayed in zebrafish embryos, *kit* is now required for melanocyte differentiation. We also find that *kit* is not required simply to sustain melanoblasts while their development is being blocked, but is required after the block on melanoblast development has been removed, to promote their differentiation. Together these findings reveal an underlying requirement for *kit* in embryonic melanocyte differentiation that has not previously been shown in zebrafish. We suggest that zebrafish embryonic melanocytes share the same requirements for *kit* tyrosine kinase activity as mammalian melanocytes, but the requirement for differentiation in zebrafish is obscured until melanocyte development is delayed. We also suggest that delaying zebrafish melanocyte development with *mitf*-MO injection phenocopies mouse development such that *kit* is now required for differentiation, and removes the heterochronic difference between mouse and zebrafish melanocyte differentiation.

Materials and methods

Fish stocks and embryo/larvae staging

Fish were reared by standard protocol on a 14 h light/10 h dark cycle at 28.5°C or 25°C unless otherwise described. Embryos used in the temperature-shift experiments were raised either at 25°C or 33°C as described. kit^{b5} (Parichy et al. 1999) and kit^{j1e99} (Rawls and Johnson 2001, 2003) have been described previously. Embryos were staged according to Kimmel et al. (1995). Thus, stages of embryos raised at 25°C or 33°C were converted to 28.5°C developmental stages by dividing time post-fertilization by 0.805 or 1.245, respectively (Kimmel et al. 1995; Rawls and Johnson 2003).

Morpholino injections and melanocyte counts

Morpholinos targeted to *mitf* (*mitf*-MO 5′-CATGTTCAAC-TATGTGTTAGCTTCA-3′; Nasevicius and Ekker 2000) as well as the control-MO (Genetools, LLC standard control-MO) were ordered from Genetools, LLC (Corvalis, Ore., USA) and diluted with sterile water to 2 mM and then to 3 ng/nl in 1× Danieau solution and 1% phenol red for injection. Injection amounts were determined by injecting into mineral oil and measuring the diameter of the injected sphere to calculate volume. Injection volumes were measured before and after sets of embryo injections to determine range of amounts injected into embryos. Embryos that lacked phenol red in blastomeres 2–4 h after injection were excluded from analysis.

When embryos are injected with less than 8 ng *mitf*-MO, melanocyte development is less delayed (not shown). Injection of higher doses of *mitf*-MO (21 ng) delays melanocyte development similarly to that observed for 8 ng injection. Because higher doses also result in some non-specific embryo lethality, we chose to use 8 ng (or a range of 8–16 ng) for our experiments. We used a slightly higher range (15–20 ng) for the *kit* temperature-sensitive reciprocal shift experiments, because 8 ng was not sufficient to completely abolish melanocyte development in embryos raised at 33°C.

After our initial experiments demonstrated the *mitf*-MO phenotype, the delay in melanocyte development in *mitf*-MO injected embryos, and the *kit* requirement for differentiation after *mitf*-MO injection compared to control-MO injected embryos was reproducible, we chose to use uninjected embryos to compare to the *mitf*-MO injected embryos.

To aid in melanocyte counts, 120 h post-fertilization (hpf) wild-type and kit^{b5} uninjected embryos and wild-type *mitf*-MO injected embryos were immersed in 10 mg/ml epinephrine for 10 min to contract melanosomes just prior to fixation in 4% paraformaldehyde.

RNA in situ hybridizations and temperature-shift experiments

RNA in situ hybridizations were performed as described by Jowett and Yan (1996) with washes performed by an Abimed in situ robot

(Abimed In Situ Pro, Intavis AG, Bergisch Gladbach, Germany). All embryos were color-developed manually. *mitf* (Lister et al. 1999) and *dopachrome tautamerase* (*dct*; Kelsh et al. 2000) RNA probes were synthesized from linearized template DNA. A *tyrosinase* probe was generated by amplification from 19.5 hpf embryo cDNA with primers designed using the published zebrafish tyrosinase sequence (Camp and Lardelli 2001). One primer sequence contained the T7 RNA polymerase-binding site for RNA probe synthesis from the PCR product template (protocol online at http://www.genetics.wustl.edu/fish_lab/frank/cgi-bin/fish/prot.html). The *tyrosinase* primers used were: forward primer 5′-ATGAACGGCTCCATGTCTTCAGTGC-3′, reverse primer 5′-TAATACGACTCACTATAGGGTGCTTGCATCCTTCTCTTGCTGAG-3′ (underlined sequence is the T7 RNA polymerase binding sequence).

For the temperature-shift experiments, following injection *kit*^{j1e99} *mitf*-MO embryos were raised at one temperature (25°C or 33°C) and then shifted to the other temperature to remove or restore *kit* function. At 72 hpf, they were fixed in 4% paraformaldehyde and melanocytes were counted.

Results

mitf-MO delays melanocyte development

Previous studies revealed that injection of antisense oligonucleotide morpholinos targeted to the *mitf* gene resulted in the *mitf* mutant phenotype of failure to develop embryonic melanocytes (Nasevicius and Ekker 2000). Embryonic melanocyte development was observed at later stages in these morphants, suggesting melanoblasts recover from the initial *mitf*-MO translational block. To examine this *mitf*-MO induced block on melanocyte development, we injected 8–16 ng *mitf*-MO into embryos and assessed how long the melanocyte deficit persisted in morphant embryos. In *mitf*-MO injected embryos, no melanocytes develop until 30 hpf, in contrast to control morpholino (control-MO) injected embryos where melanocytes first appear at 24 hpf (similar to uninjected embryos; Fig. 1a, b). At 30 hpf, delayed melanocyte differentiation begins, as *mitf*-MO injected embryos have a few melanocytes (average of 4.9 melanocytes, range 0–28 melanocytes). As delayed melanocyte development continues, the deficit in melanocyte numbers in *mitf*-MO embryos persists until approximately 120 hpf at which time the melanocyte number and pattern is indistinguishable from control-MO embryos (Fig. 1c–e). Therefore, injection of *mitf*-MO delays both the appearance of the first few melanocytes and significantly delays completion of larval melanocyte pigment pattern morphogenesis.

To investigate how *mitf*-MO blocks melanocyte development and how the subsequent recovery occurs, we next looked at expression of markers for stages of melanoblast development. We first examined expression of *kit* and *dct*, a specific marker for cells of the melanocyte lineage that requires *mitf* function for its expression (Kelsh et al. 2000; Lister et al. 1999). In wild-type embryos, expression of *kit* is seen as early as 18 hpf in neural crest cells (Parichy et al. 1999) and *dct* is seen by 19 hpf (Kelsh et al. 2000). In contrast, we detect few to no *kit*-expressing (not shown) or *dct*-expressing melanocyte precursors (melanoblasts) in *mitf*-MO embryos at 24 hpf (Fig. 2b, 3 out of 10 embryos have a few *dct*-positive cells) and only a few faintly expressing *kit*-positive (not shown) or *dct*-positive cells at 28 hpf (not shown, 16 out of 18 embryos have *dct*-positive cells). By 30 hpf, wild-type *mitf*-MO embryos have even more darkly expressing *kit*-positive (not shown) and *dct*-positive cells (Fig. 2d). This delay in *kit* and *dct* expression indicates that the translational block on *mitf* is relieved, beginning sometime after 24 hpf, but before 28 hpf, presumably immediately prior to the expression of *kit* and *dct*.

Since the *mitf*-MO blocks translation rather than transcription of the *mitf* gene, we can also examine melanoblasts containing the *mitf* transcript. At 24 hpf, *mitf*-MO embryos and uninjected embryos are similar, with *mitf*-positive cells in premigratory positions as well as a few dispersed to the periphery (not shown). Later, at 28 hpf in *mitf*-MO embryos, few cells are seen over the yolk (target location of migration) consistent with a defect in melanoblast migration (Fig. 2f). In contrast, in uninjected embryos, *mitf*-expressing cells are observed in migratory pathways or target locations such as over the yolk (Fig. 2e). In addition to melanoblasts, *mitf* is expressed in xanthophore precursors (Parichy et al. 2000b), which develop normally in *mitf* mutants, and may explain why we fail to see a complete deficit of migrating cells in *mitf*-MO embryos (not shown).

We conclude from our *mitf*, *kit* and *dct* expression studies that *mitf*-MO delays melanocyte lineage development by arresting melanoblasts at a *mitf*-positive/*kit*, *dct*-negative stage and that this delay or arrest is relieved beginning between 24 hpf, when we see few to no *kit*- and *dct*-expressing cells, and 28 hpf, when we observe the *kit*- and *dct*-expressing cells in most embryos. This *kit* and *dct* expression is followed by melanin synthesis and gradual accumulation of melanized melanocytes. The slow rate of melanocyte recovery after *mitf*-MO injections (Fig. 1e) raises the possibility that the mechanism or regulation of melanocyte differentiation following *mitf*-MO induced delay may differ from that used in normal embryonic melanocyte development.

Melanocyte recovery after delayed development is *kit*-dependent

To explore whether the mechanism regulating melanocyte differentiation during recovery was the same as that used for normal embryonic melanocyte development, we examined the role of *kit* in melanocyte recovery after *mitf*-MO injection of embryos. *kit* is not required for the differentiation of most melanocytes during normal development (see Discussion; Parichy et al. 1999). If the regulation of melanocyte differentiation is the same for *mitf*-MO delayed melanoblast differentiation, then we would expect similar melanocyte recovery in *kit* mutants injected with *mitf*-MO. To test this possibility, we first injected *mitf*-MO into *kit*^{b5} null mutants (*kit mitf*-MO embryos). We found that delayed melanocyte differentiation completely failed in *kit* mutant embryos injected with

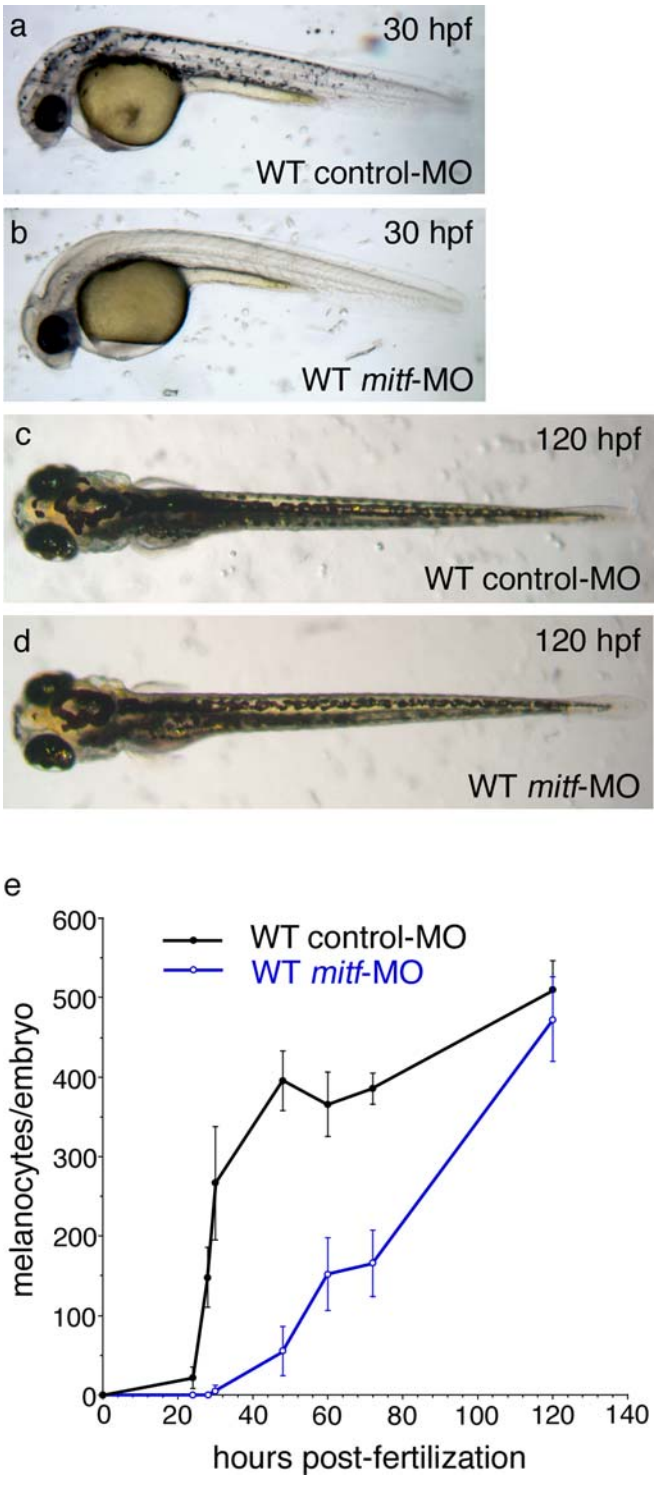

Fig. 1a–e The *microphthalmia-associated transcription factor*-morpholino (*mitf*-MO) induced block on melanocyte development is followed by melanocyte recovery. Wild-type (WT) embryos injected with a control-MO (**a**) have a normal number of melanocytes at 30 hpf, while embryos injected with *mitf*-MO (**b**) have few or no melanocytes at 30 hpf. Following this, melanocytes slowly recover and embryos eventually appear identical to wild-type by 120 hpf (**c**, **d**). Comparing numbers of differentiated melanocytes (melanin-containing cells) in wild-type control-MO embryos to wild-type *mitf*-MO injected embryos shows that initial melanocyte appearance is delayed by about 6 h (**e**). Furthermore, re-establishment of the larval melanocyte pigment pattern in *mitf*-MO embryos is significantly slower than wild-type control-MO injected embryos, as *mitf*-MO embryos have a slow recovery that is not complete until 120 hpf. The *error bars* on this graph represent standard deviation. Melanocytes from 4 to 16 embryos (average of 8.7±3.5 embryos/time point) were counted for each time point

mitf-MO, as melanocytes failed to differentiate in *kit mitf*-MO embryos through 5 days of development (Fig. 3a–d). Therefore, *kit* is required for melanocyte recovery after *mitf*-MO injection. This suggests that the mechanism regulating melanocyte differentiation after *mitf*-MO induced delay differs from normal melanocyte development, where *kit* is not required for melanocyte differentiation.

Melanoblasts persist through the stages when melanocyte development is delayed in *kit* mutants injected with *mitf*-MO

We next investigated how *kit* is required for melanocyte differentiation in *mitf*-MO delayed melanocyte development. One possibility is that *kit* could be required at early stages (before 24 hpf) to maintain melanocyte precursors through the *mitf*-MO delay. Alternatively, *kit* function may be required later (after 24 hpf), to recruit melanoblasts to differentiate after the block on their development is relieved. To distinguish between these possibilities we compared expression of melanoblast markers such as *dct* and *tyrosinase* (*tyr*), the rate-limiting enzyme in melanin synthesis, in wild-type and *kit mitf*-MO embryos. We found that *tyr* is expressed in uninjected *kit* mutant melanoblasts, indicating that in zebrafish *kit* is not required for *tyr* expression (not shown). We also find that *dct* and *tyr* are expressed in *kit* mutants injected with *mitf*-MO. The expression of these markers in *kit mitf*-MO embryos is delayed similarly to the delay observed in wild-type *mitf*-MO embryos in that they are not expressed at 24 hpf but are expressed at approximately 28 hpf (Fig. 3e, f). This indicates that delayed melanoblasts are able to progress to the *dct*/*tyr*-positive stage without *kit* function, but are unable to produce melanin and complete differentiation.

To further examine the fate of delayed melanoblasts in *kit mitf*-MO embryos, we next assessed the number of melanoblasts that persisted before and after the developmental delay in wild-type and *kit mitf*-MO embryos. As expression patterns of *dct* and *tyr* were similar in wild-type and *kit mitf*-MO embryos, we chose to focus on *tyr*-expressing melanoblasts. We counted similar numbers of *tyr*-expressing precursors in both wild-type and *kit* embryos injected with *mitf*-MO beginning at 24 hpf and increasing through 28 hpf. As melanization begins shortly thereafter in 30 hpf wild-type *mitf*-MO embryos (indicating the developmental delay is abating), this suggests that similar numbers of melanoblasts persist through the developmental delay in *kit* mutants compared to wild-type. By 32 hpf and later at 36 hpf, when the number of *tyr*-expressing cells continues to increase in wild-type *mitf*-MO embryos, the number of *tyr*-expressing cells in *kit mitf*-MO embryos does not increase (Fig. 3g–i).

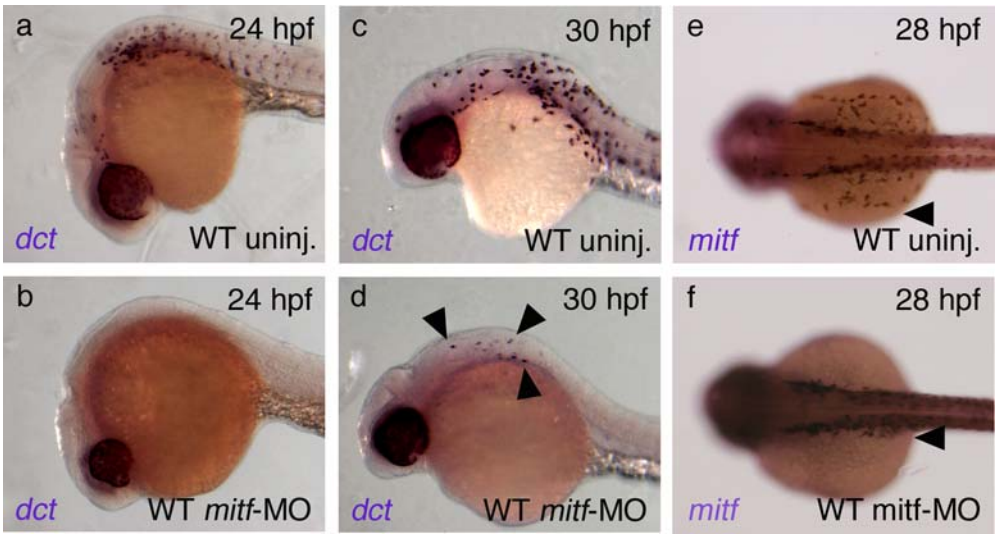

Fig. 2a–f Expression of melanoblast markers in wild-type *mitf*-MO embryos is delayed. In situ hybridizations with *dopachrome tautamerase* (*dct*) probe show that in wild-type uninjected embryos *dct* is expressed in migrating melanoblast precursors at 24 hpf (**a**), while in wild-type *mitf*-MO embryos *dct* is not yet expressed (**b**). While 30 hpf uninjected wild-type embryos have many *dct*-positive cells (**c**), in wild-type *mitf*-MO embryos few *dct*-expressing cells are observed (**d**). Expression analysis of *mitf* transcript reveals that *mitf*-positive cells have defects in migration. When compared to uninjected wild-type embryos at 28 hpf, *mitf*-expressing cells in wild-type *mitf*-MO embryos fail to migrate over the yolk, while melanocytes in wild-type uninjected embryos have migrated over the yolk (*arrowheads* show distance of melanocyte or melanoblast migration in **e** and **f**). Embryos in **a–d** were treated with 0.2 mM phenylthiourea (PTU) to block melanin synthesis so that melanoblasts were only visible by in situ hybridization with *dct* probe

Thus, in both wild-type and *kit mitf*-MO embryos, melanocyte precursors first show a delay in reaching the *dct*- and *tyr*-expressing stage. Next, in wild-type *mitf*-MO embryos, melanocyte precursors increase in number and begin differentiating. Likewise, in *kit mitf*-MO embryos, melanocyte precursors persist through the time when they would begin to differentiate in wild-type embryos injected with *mitf*-MO (28–30 hpf). However, unlike wild-type *mitf*-MO embryos, melanoblasts fail to increase in number or differentiate after the *mitf*-MO developmental block has been removed in *kit mitf*-MO embryos. Taken together these results show that *kit* is not required for maintenance of delayed *mitf*-positive; *dct*/*tyr*-negative melanoblasts through the developmental delay, but instead is required after the delay on melanoblast development is relieved, to promote the subsequent differentiation of *mitf*, *dct*, and *tyr*-positive melanoblasts into pigmented melanocytes. These results also indicate that *kit* is required for increasing the number of *tyr*-positive melanoblasts, although it is not clear whether *kit* is required for progression of additional *dct*/*tyr*-negative melanoblasts to *dct*/*tyr*-positive stages, or whether *kit* is required for division of the *dct*/*tyr*-positive melanoblasts observed at 28 hpf in wild-type and *kit mitf*-MO embryos (see Discussion).

The *kit* requirement for melanocyte recovery begins at 24 hpf and ends after 28 hpf

To further address the role of *kit* in promoting delayed melanocyte differentiation, we sought to determine when *kit* is required for melanocyte differentiation after *mitf*-MO injection. If *kit* is necessary for maintaining melanoblasts through the developmental delay, then *kit* function should be required early, during the delay (before 24 hpf). Alternatively, if *kit* is necessary for subsequent differentiation of melanoblasts after the developmental delay, we would expect *kit* function to be required after this delay on development is removed (after 24 hpf). To distinguish between these two possibilities, we took advantage of a temperature-sensitive *kit* mutation, kit^{j1e99} (Rawls and Johnson 2001). When kit^{j1e99} embryos are raised at the permissive temperature (25°C) they appear wild-type, and when raised at the restrictive temperature (33°C) they are indistinguishable from the *kit* null (kit^{b5}) phenotype. We injected kit^{j1e99} mutants with *mitf*-MO (kit^{j1e99}*mitf*-MO embryos) and performed a series of temperature-shifts on the developing embryos. Figure 4 shows one of the two series of temperature shift experiments performed. The number of melanocytes that developed following the various temperature shifts was compared to the number of melanocytes that developed in kit^{j1e99}*mitf*-MO embryos held continuously at either the permissive or restrictive temperatures.

While the number of melanocytes that developed in kit^{j1e99}*mitf*-MO embryos held at the restrictive temperature (1.8±1.8) closely resembled the few melanocytes that developed in *kit* null mutants injected with *mitf*-MO embryos, the number of melanocytes that developed in kit^{j1e99}*mitf*-MO embryos held at the permissive temperature (17.5±9.5) only reached approximately 11% of the number of melanocytes that developed in wild-type embryos injected with *mitf*-MO (165±41.7). We interpret this partial deficit to suggest that the conditional *kit* allele is somewhat compromised at the permissive temperature.

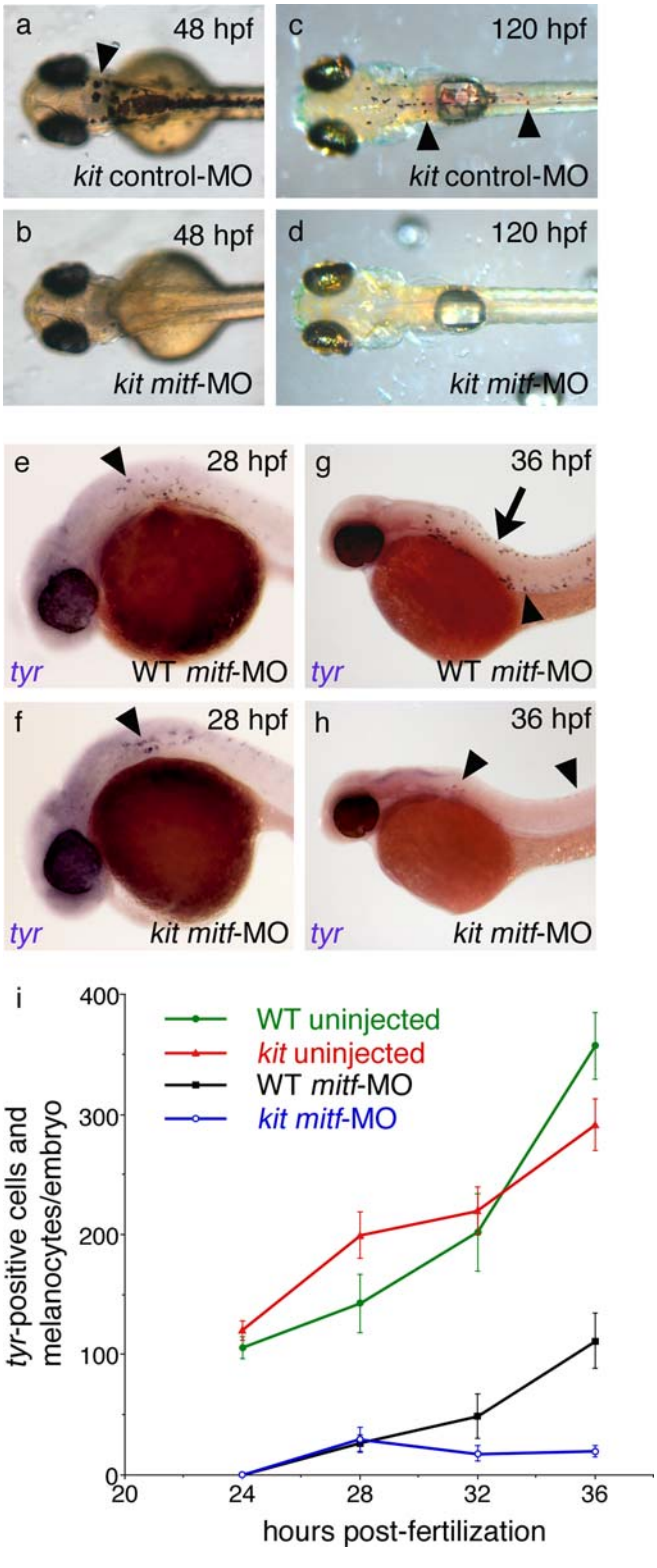

Fig. 3a–i *kit* is required for melanoblasts to differentiate after the block on melanoblast development is relieved. In 48-hpf control-MO injected *kit* embryos, normal-appearing melanocytes develop (**a**, *arrowheads*), but *kit mitf*-MO embryos lack melanocytes (**b**). *kit* mutants injected with *mitf*-MO fail to develop melanocytes through 120 hpf (**d**). In *kit* control-MO embryos at 120 hpf melanocytes are punctate (**c**, *arrowheads*), a sign of cell death which is a normal part of the *kit* mutant phenotype. In situ hybridizations with *tyrosinase* probe show that there are similar numbers of *tyr*-expressing cells (*arrowheads*) in wild-type (**e**) and *kit mitf*-MO embryos (**f**) at 28 hpf. At 36 hpf, *kit mitf*-MO embryos have few *tyr*-expressing cells (**h**, *arrowheads*), but there are many melanized melanocytes (*arrows*) and *tyr*-expressing cells (*arrowheads*) in wild-type *mitf*-MO embryos (**g**). **i** Quantitative analysis of *tyr*-positive melanoblasts or melanin-containing melanocytes between 24 and 36 hpf is compared in uninjected and *mitf*-MO injected *kit* and wild-type embryos. Note that the same numbers of *tyr*-expressing cells and melanocytes are present in wild-type *mitf*-MO and *kit mitf*-MO embryos through 28 hpf when melanoblasts have persisted through the block on their development and are just beginning to differentiate. After 28 hpf, the number of *tyr*-expressing cells increases in WT *mitf*-MO embryos but does not increase in *kit mitf*-MO embryos. *Error bars* represent standard deviation. Melanocytes and *tyr*-expressing cells from 4 to 15 embryos (average of 8.4±2.7 embryos/time point) were counted in each time point

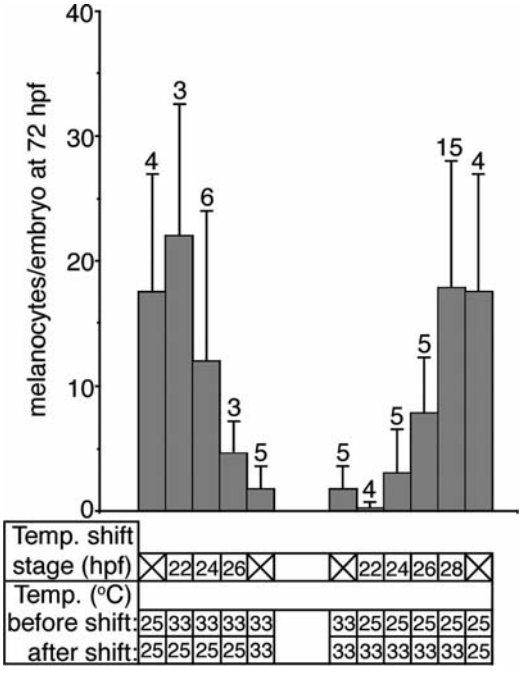

Fig. 4 Temperature-shift experiments show the temporal requirement for *kit* for delayed melanocyte differentiation. In this experiment, removing *kit* function by raising at 33°C for different amounts of time, then restoring *kit* function by shifting to 25°C, shows that *kit* is required after 24 hpf. Allowing *kit* to function (25°C) for different amounts of time then removing *kit* function (33°C) shows that *kit* is required through 28 hpf. *Error bars* represent standard deviation. *Numbers* at the top of each bar represent the number of embryos that were used for each time point

This decreased activity is not enough to impair melanocyte differentiation in otherwise normal embryos. That this decreased activity of the kit^{j1e99} allele is revealed in *mitf*-MO morphants suggests an increased requirement for *kit* function during this recovery stage. We took advantage of the residual activity at the permissive temperature promoting development of some melanocytes to explore the temporal role of *kit* in *mitf*-MO recovery.

Temperature-shift experiments reveal that *kit* is not required prior to 24 hpf for melanocyte recovery in *mitf*-MO injected embryos. When kit^{j1e99}*mitf*-MO embryos are raised at the restrictive temperature through 24 hpf, and

then shifted to the permissive temperature, they develop similar numbers of melanocytes as those held at the permissive temperature (Fig. 4). When *kit*^{j1e99}*mitf*-MO embryos are raised at the restrictive temperature for the first 26 hpf, the number of melanocytes that develop is fewer than the number that develops when held continuously at the permissive temperature, suggesting that the requirement of *kit* for delayed melanoblast differentiation begins before 26 hpf and after 24 hpf. A replicate series of temperature shifts (not shown) yielded similar results, except that they suggested a slightly later requirement for *kit* (after 26 hpf). Together these results indicate that *kit* begins to be required between 24 and 26 hpf.

In the reciprocal series of temperature shifts, when *kit*^{j1e99}*mitf*-MO embryos are raised at the permissive temperature for 24 hpf and then shifted to the restrictive temperature, a similar number of melanocytes develop as when embryos are held at the restrictive temperature, indicating *kit* function prior to 24 hpf is not sufficient for melanocyte recovery. When *kit*^{j1e99}*mitf*-MO embryos are raised at the permissive temperature for the first 28 hpf and then shifted to the restrictive temperature, the same number of melanocytes develop as those that develop in *kit*^{j1e99}*mitf*-MO embryos held continuously at the permissive temperature, indicating that *kit* function prior to 28 hpf is sufficient for melanocyte recovery. In our replicate of these temperature shifts, we observed that the *kit* requirement extended beyond 28 hpf. Together with the first trial, these data indicate that the critical period when *kit* is required for delayed melanocyte differentiation ends beyond 28 hpf. As the *kit*^{j1e99}*mitf*-MO embryos fail to develop the full compliment of melanocytes when raised at the permissive temperature, it may be that *kit* is required well beyond this time period for the complete larval pigment pattern to develop. Therefore our results suggest that the requirement for *kit* for differentiation starts to decline at 28 hpf.

Taken together, these results indicate that *kit* begins to be required between 24 and 26 hpf, and the requirement for *kit* begins to decline at 28 hpf for delayed melanocyte differentiation after *mitf*-MO injection. During this time period, delayed melanocyte precursors begin to express *dct* and *tyr*. Since this expression initiates normally in *kit* mutants injected with *mitf*-MO, we suggest that *kit* is required for progressing past the *dct*/*tyr*-positive stage, rather than progressing from a *dct*/*tyr*-negative to a *dct*/*tyr*-positive stage. Therefore, the temporal requirement for *kit* tends to exclude the hypothesis that *kit* is required for maintaining melanoblasts through the developmental delay, and supports the hypothesis that *kit* is required for progression of melanoblasts from a *dct*/*tyr*-positive stage to a pigmented melanocyte immediately after they become *dct*/*tyr*-positive.

Discussion

mitf-induced developmental delay of melanocytes reveals a requirement of *kit* for melanocyte differentiation

We show that *mitf*-MO causes a transient *mitf* phenocopy by delaying melanoblast development. This delay is in contrast to the permanent deficit of melanocytes in the *mitf* mutant. Moreover, the block on melanoblast development reliably wears off between 24 and 28 hpf, and although it is unclear how or why the morpholinos lose efficacy at this discrete stage, it allows us to explore the recovery of melanocytes after the *mitf*-MO induced delay. This demonstrates a previously unexplored feature of morpholino-based analysis—conditional abrogation of gene function. Although the conditional abrogation of gene function by morpholinos is limited to early developmental stages, we show they can be used effectively in the cases where conditional alleles of genes are not available.

Our results demonstrate that *mitf*-MO injection causes a delay in melanocyte development in wild-type embryos, and that differentiation of delayed melanoblasts is *kit*-dependent. While a requirement for *kit* to differentiate has been previously shown in mammalian melanocytes, these experiments now demonstrate that *kit* has a role for differentiation of neural-crest-derived melanocytes in zebrafish when their development is delayed. Thus, *kit* is required for differentiation in both organisms, but this requirement is not evident in zebrafish unless melanocyte differentiation is delayed. Moreover, our findings suggest that developmental requirements for *kit* in fish and mammals may be more conserved than previously appreciated, such that *kit* is required for migration, survival and differentiation in both mammals and zebrafish, even though the requirement for differentiation is only revealed in zebrafish by delaying melanocyte development.

Our results also show that *kit* is not required for maintenance of melanoblasts through the developmental delay, as melanoblasts in *kit* mutants injected with *mitf*-MO persist through the temporary arrest of melanocyte development to 28 hpf and become *dct*/*tyr*-positive. We also show the requirement for *kit* begins between 24 and 26 hpf, indicating that the *kit* requirement is for differentiation of melanoblasts following *mitf*-MO-induced melanoblast developmental delay (after 24 hpf) rather than the maintenance of melanoblasts through the delay (before 24 hpf). Together, the initial expression of *dct* and *tyr* between 24 and 28 hpf in delayed melanoblasts, the *kit*-independence of this initial *dct* and *tyr* expression, and the temporal requirement for *kit* after 24 hpf all suggest that *kit* is required for melanoblasts to differentiate immediately after they begin expressing *dct* and *tyr* for melanization. The specific differentiation mechanism for which *kit* is required remains unclear, but two possibilities are: (1) *kit* is required for expression of additional genes required for melanocyte differentiation, or (2) *kit* is necessary for

survival of melanoblasts once they become *dct/tyr*-positive.

In addition to the requirement for melanization, our results suggest that *kit* is also required for an increase in the number of *dct*/*tyr*-positive melanoblasts. This is consistent with what is observed in mouse *Kit* mutants, where there are fewer melanoblasts expressing a *lacZ* reporter gene under the control of the *dct promoter* than in wild-type siblings (Mackenzie et al. 1997). Although it is unclear how *kit* is required for expansion of melanoblasts, one possibility is that *kit* is required for proliferation of the *dct*/*tyr*-positive melanoblasts present in 28 hpf *mitf*-MO embryos. Alternatively, *kit* could be required for the differentiation of an additional melanoblast population to those observed at the *dct*/*tyr*-positive stage. Finally, it may be that some melanoblasts are dying prior to becoming *dct/tyr*-positive because *kit* is required for their survival. Future experiments where cell division in the melanocyte lineage is examined could reveal whether *kit* is required for division of melanoblasts or differentiation of additional melanoblasts from pluripotent precursors.

It is interesting to note that, although melanocytes differentiate in uninjected *kit* mutants, they fail to develop the same number of melanocytes as wild-type embryos during normal development. Zebrafish kit^{b5} (null) mutants have approximately 58% the number of melanocytes found in wild-type embryos (Parichy et al. 1999). Therefore, some melanoblasts may require *kit* to differentiate in normal zebrafish melanocyte development. Alternatively, it is also possible that *kit* is required for division of melanoblasts, or survival of melanoblasts prior to differentiation. It is possible that this *kit* role in generating the full compliment of melanocytes during normal embryonic development is related to the *kit* requirement for the correct number of melanoblasts in delayed melanoblast development.

Earlier we suggested two possible roles for *kit* that could explain the absence of melanocytes in *mitf*-MO injected embryos. The first possibility was that *kit* may be required before 24 hpf to sustain melanoblast precursors, and the second was that *kit* may be required after 24 hpf for differentiation of melanoblasts after the developmental delay. The normal expression of *kit* beginning at 18 hpf supports the notion that the *kit* requirement for delayed differentiation in *mitf*-MO injected embryos could also be prior to 24 hpf. Although we detect little or no *kit* expression in wild-type *mitf*-MO embryos at 24 hpf, it remained possible that *kit* was expressed at functional, albeit undetectable, levels. This compelled us to develop a functional assay for the role of *kit* prior to 24 hpf. Our temperature-shift experiments show that *kit* is required for melanoblast differentiation in *mitf*-MO embryos after 24 hpf. This indicates that *kit* is not required prior to 24 hpf to sustain melanoblasts during the developmental delay, but is required after the delay for their differentiation. The *kit* requirement for differentiation of melanocytes in *mitf*-MO embryos after 24 hpf correlates with the expression of melanoblast markers (*kit*, *dct*, *tyr*) in *mitf*-MO injected embryos after 24 hpf.

mitf-MO injections in zebrafish embryos cause a heterochronic shift in melanocyte development that mimics mouse embryonic melanocyte development

In mouse, melanoblasts migrate from the neural crest at early stages (E10–E12) and remain undifferentiated until later stages of development (E16–E18) when they enter hair follicles and begin producing melanin (Le Douarin 1982; Beermann et al. 1992). In contrast, in zebrafish, melanoblasts begin differentiating simultaneously with migration from the neural crest (Raible and Eisen 1994). Therefore, we suggest that there is heterochronic shift between mouse and fish melanocyte development with respect to when melanocytes differentiate and produce melanin (Fig. 5). It seems likely that this difference in timing of melanocyte pigmentation may reflect selective pressure for early pigmentation in teleost embryos and larvae, as they are subject to predation from the moment of fertilization, or alternatively, may reflect the lack of selection on mammalian embryos until after birth, a comparatively later event.

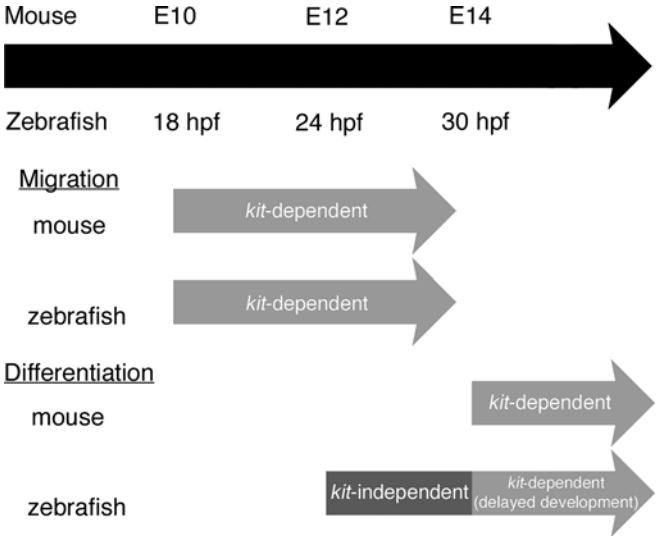

Fig. 5 A heterochronic shift in zebrafish melanocyte development caused by transient *mitf* depletion phenocopies some aspects of mouse melanocyte development. Our model shows *kit*-dependent migration (*upper light gray arrows*) occurs with the same relative timing in zebrafish and mouse. However, differentiation, here defined as the appearance of melanin-containing cells, occurs at different times in the two organisms (*lower light/dark gray* and *light gray arrows*). Mouse melanocytes melanize after they disperse from the neural crest (beginning at E10) and invade the epidermis (E12–14). Thus, they do not begin producing melanin until long after they leave the neural crest. In contrast, zebrafish melanocytes produce melanin at a comparatively earlier stage (beginning at 24 hpf), as they migrate out of the neural crest. Zebrafish embryos injected with *mitf*-MO phenocopy mouse embryos in that melanocytes differentiate at a later time and their differentiation is *kit*-dependent (*lower light gray arrows*). Whereas *kit*-dependent melanocyte development in both organisms (delayed melanocyte differentiation in zebrafish and normal differentiation in mouse) occurs within the same relative time, *kit*-independent differentiation occurs much earlier in zebrafish (*dark gray arrow*). We propose that *kit*-independent melanocyte differentiation in zebrafish is the result of an early functional redundancy with *kit* not yet identified (see text)

This heterochronic shift between melanocyte differentiation in mouse and fish correlates with heterochrony in expression of melanoblast markers. In mouse, *mitf*, *kit* and *dct* are expressed early in melanoblast development immediately before or just after melanoblasts leave the neural crest (E10–E12; Steel et al. 1992; Opdecamp et al. 1997; Nakayama et al. 1998; Hou et al. 2000). In contrast, *tyr* is not expressed until immediately before melanoblasts differentiate (E14.5–E16), after most melanoblasts have finished migrating out from the neural crest and invading the epidermis (Beerman et al. 1992; Steel et al. 1992; Hou et al. 2000). In zebrafish, all four of these melanoblast markers are first expressed at approximately the same time (18–19 hpf) coincident with melanoblast migration from the neural crest (Lister et al. 1999; Kelsh et al. 2000; Camp and Lardelli 2001). Thus, both the timing of melanocyte differentiation and *tyr* expression are delayed in mouse with respect to fish.

These differences in melanocyte differentiation between zebrafish and mouse are removed by delaying melanoblast differentiation in zebrafish embryonic melanocytes with *mitf*-MO. In these embryos, zebrafish melanocytes differentiate at a later embryonic stage comparable to the stage of embryonic melanocyte differentiation in mouse. *Tyrosinase* expression is also delayed in zebrafish *mitf*-MO embryos to the comparable stage in mouse. However, this *mitf*-MO induced heterochronic shift does not completely phenocopy mouse because expression of *kit* and *dct* is also delayed in zebrafish *mitf*-MO embryos. In addition to the differences in timing of melanoblast differentiation between fish and mouse, there is also a difference in the requirement for *kit* in differentiation. Mouse melanoblasts require *kit* for differentiation while in normal zebrafish melanocyte development melanoblasts develop without *kit* function. Thus, *mitf*-MO injection reveals a requirement for *kit* for differentiation of delayed zebrafish melanocytes similar to that observed in the mouse. Taken together, these results show that *mitf*-MO injection causes a heterochronic shift in zebrafish embryonic melanocyte differentiation that phenocopies mouse development in three ways—*tyrosinase* expression and melanocyte differentiation are delayed, and differentiation becomes completely dependent on *kit*.

Possible redundant gene function promoting *tyrosinase* activity early in development

Our results suggest that the role of *kit* in zebrafish and mouse melanoblasts is similar if development occurs with the same relative timing. One remaining question is how zebrafish melanoblasts that normally differentiate at earlier stages circumvent their requirement for *kit* for differentiation. Mammalian cell culture studies have shown that *kit* is required for the expression of *tyrosinase*, a crucial enzyme required for melanin synthesis, and melanin production (Hou et al. 2000). In contrast, we find that in zebrafish *kit* null mutants, *tyrosinase* is expressed (not shown), indicating that its expression is not controlled by *kit* alone. Although there are many possible explanations for how *tyrosinase* is expressed and melanocytes differentiate without *kit* function, here we consider the following possibilities: (1) an RTK product of a recent teleost-specific genome duplication event (a second *kit* ortholog) acts redundantly with *kit* to promote melanocyte differentiation; (2) a more distantly related RTK acts redundantly with *kit* to promote melanocyte differentiation; and (3) zebrafish use an alternative mechanism of melanocyte development relative to mammals.

Teleost fish are thought to have undergone a genome duplication event after the divergence of tetrapods from the fish lineage. There are many examples where there are duplicated genes in fish orthologous to a single gene in mammals (Barbazuk et al. 2000; Postlethwait et al. 1998). This suggests zebrafish may have an additional ortholog of *Kit* that may function in melanocyte development. In fact, zebrafish have a second *Kit* ortholog, which we refer to here as *kitb* (Ensembl predicted transcript ENSDART00000022982). However, unlike *kit* (now *kita*), *kitb* does not appear to promote melanocyte development in zebrafish. In situ expression analysis indicates that it is expressed in Rohon-Beard neurons rather than melanoblasts at the time we predict a redundant function of *kita* (E.M. Mellgren and S.L. Johnson, unpublished). This is inconsistent with the first possibility that a duplicate of *kita* gene is involved in melanocyte development.

As *kitb* does not appear to have a role in zebrafish melanocyte differentiation, a different RTK may have a role in melanocyte development in fish, or the function acting redundantly with *kit* is not another RTK but an alternative genetic pathway responsible for activating the MAPK pathway that promotes melanization. While we have reason to reject the first hypothesis of a recent paralog of *kit* promoting melanocyte differentiation in *kit* null mutants, these other two possibilities remain untested. No matter which of these is correct, identification of the gene or pathway functioning redundantly with *kit* should provide valuable insights into mechanisms of melanocyte differentiation and the evolution of heterochrony in pigment cell development.

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