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Requirements for the *kit* receptor tyrosine kinase during regeneration of zebrafish fin melanocytes

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SUMMARY

Embryonic neural crest-derived melanocytes and their precursors express the *kit* receptor tyrosine kinase and require its function for their migration and survival. However, mutations in *kit* also cause deficits in melanocytes that make up adult pigment patterns, including melanocytes that re-establish the zebrafish fin stripes during regeneration. As adult melanocytes in mice and zebrafish are generated and maintained by stem cell populations that are presumably established during embryonic development, it has been proposed that adult phenotypes in *kit* mutants result from embryonic requirements for *kit*. We have used a temperature-sensitive zebrafish *kit* mutation to show that *kit* is required during adult fin regeneration to promote melanocyte differentiation, rather than during embryonic stages to establish their stem cell precursors. We also demonstrate a transient role for *kit* in promoting the survival of newly differentiated regeneration melanocytes.

Key words: Melanocyte, Stem cell, Regeneration, *kit*, *sparse*, Conditional, Temperature sensitive, Zebrafish, *Danio rerio*

INTRODUCTION

Stem cells are found in a variety of vertebrate tissues where they replace differentiated cells lost through cell turnover and tissue injury. A complete understanding of stem cell development requires the identification of mechanisms that underlie the initial establishment of stem cell populations, and the recruitment of stem cells later in development to produce differentiated progeny. The genetic system of the zebrafish, *Danio rerio*, provides opportunities for investigating the mechanisms that promote these different stages in the development of stem cells, such as those cells responsible for re-establishing the melanocyte stripes during fin regeneration (Rawls and Johnson, 2000).

Following partial amputation, zebrafish fins rapidly regenerate to replace the missing tissue. Wound healing occurs in the first stage after amputation (1 stage is the equivalent of 1 day of regeneration at 25°C; Johnson and Weston, 1995), followed by recruitment of cells into the cell cycle between stages 1.5 and 2 (Johnson and Bennett, 1999), formation of visible blastemata by stage 3 and outgrowth of the regenerate through completion of regeneration (approximately stage 25-30). Precursors of regeneration melanocytes are observed by in situ hybridization proximal to the amputation plane as early as stage 1 to 1.5, and then distal to the amputation plane by stage 2 to 3. Pigmented de novo melanocytes are first observed throughout the regenerate at stage 4, and continue to differentiate in the distal-most, or developmentally youngest, part of the regenerate, until completion of regeneration. The observation that fish can regenerate their fins and re-establish their melanocyte stripes from undifferentiated cells in the stump through numerous cycles of amputation and regeneration, suggests the existence of melanocyte or pigment cell stem cells (Rawls and Johnson, 2000).

We have previously shown that zebrafish mutant for the *kit* receptor tyrosine kinase (formerly called *sparse*; Parichy et al., 1999) fail to develop regeneration melanocytes between stage 4 and stage 7. In the absence of *kit* function and absence of early regeneration melanocytes, a secondary regulatory class of melanocytes differentiates (starting around stage 8) and eventually re-establishes the fin stripes. Secondary regulatory melanocytes have little or no role in normal stripe regeneration (Rawls and Johnson, 2000), therefore the melanocytes that re-establish the fin stripes during normal regeneration are entirely or almost entirely *kit* dependent. However, to avoid complications arising from the possible minor contribution of secondary regulatory melanocytes to normal regeneration after stage 8, we now focus on melanocytes that develop prior to stage 7 in investigating the role of *kit* in regeneration melanocyte development.

In mammals, adult melanocyte pigment pattern is also maintained by stem cells (Kunisada et al., 1998), and loss of *kit* function similarly causes deficits in adult melanocytes (Silvers, 1979; Geissler et al., 1988; Nocka et al., 1990; Tan et al., 1990; Giebel and Spritz, 1991; Tsujimura et al., 1991; Besmer et al., 1993; Marklund et al., 1998). As *kit* function is also required for the migration and survival of embryonic neural crest-derived melanocytes and their precursors (Motro

et al., 1991; Cable et al., 1995; Wehrle-Haller and Weston, 1995; MacKenzie et al., 1997; Parichy et al., 1999), it has been proposed that adult pigment pattern phenotypes in *kit* mutants might be due to requirements for *kit* during early stages of ontogeny to promote the development of adult melanocyte stem cells (Huszar et al., 1991; Wehrle-Haller and Weston, 1995; Yoshida et al., 1996; MacKenzie et al., 1997; Rawls and Johnson, 2000). For example, *kit* may be required during embryogenesis to establish melanocyte stem cells, or during larval development to maintain melanocyte stem cells. Adult roles for Kit in melanocyte development have been previously suggested by studies in mice, using conditional abrogation of gene function using Kit-directed antibodies (Nishikawa et al., 1991; Kunisada et al., 1998). In the absence of molecular markers for melanocyte stem cells, we sought to distinguish between possible roles for *kit* during early developmental stages, in establishing or maintaining adult melanocyte stem cells, or during regeneration, in promoting melanocyte differentiation, using a temperature-sensitive mutation of *kit*.

The utility of temperature-sensitive mutations to remove or restore gene function has been useful in dissecting a variety of biological processes in yeast (Hartwell et al., 1974), worms (Vowels and Thomas, 1992) and flies (Suzuki et al., 1976). As poikilothermic vertebrates such as zebrafish can grow in a wide range of temperatures (Schirone and Gross, 1968), temperature-sensitive mutations in fish can also be identified (Abdelilah et al., 1994; Johnson and Weston, 1995; Winkler et al., 2000). We therefore generated a temperature-sensitive allele of zebrafish *kit* in order to assess the temporal requirements of *kit*. We show that *kit* is required after fin amputation to promote the population of the regenerate by melanoblasts, rather than during earlier developmental stages to establish melanocyte stem cells. As early regeneration melanocytes do not form in the absence of *kit* function, we also used the temperature-sensitive *kit* mutant to determine the role that *kit* plays in these cells following differentiation. These studies revealed a transient role for *kit* in promoting the survival of differentiated regeneration melanocytes.

MATERIALS AND METHODS

Fish husbandry and regeneration staging

Fish were maintained at either 25°C or 33°C on a 14L:10D cycle. For fin amputation, fish were anesthetized and the distal two-thirds of the caudal fin resected with a scalpel. One stage of regeneration corresponds to 24 hours at 25°C or 12 hours at 33°C (Johnson and Weston, 1995). All references to kit^{j1e99} and kit^{b5} mutants refer to homozygous animals. In situ hybridization using DIG-labeled full-length zebrafish *kit* riboprobes (Parichy et al., 1999) was carried out as previously described (Thisse et al., 1993), with the exceptions that proteinase K digestion was performed at 10 μg/ml for 40 minutes, and hybridization and stringency washes occurred at 68°C.

Non-complementation screen for temperature-sensitive *kit* alleles

Pre-meiotic ENU-mutagenesis (Solnica-Krezel et al., 1994) was performed on wild-type SJD males. Mutant clones produced after such treatment typically account for less than 0.1% of sperm from individual males (S. L. J., unpublished). Pooled sperm from subsets of 45 mutagenized males was used to fertilize eggs from homozygous kit^{b5} females in a C32 background, and progeny were initially reared at 33°C. Non-complementing embryos displaying the *kit* mutant embryonic melanocyte phenotype (Parichy et al., 1999) were shifted to 25°C and reared to maturity for further analysis. Over 83,000 embryonic progeny were screened in this manner, yielding 247 non-complementing mutants. Temperature sensitivity of new mutants was assessed by backcrossing founders to homozygous kit^{b5} testers, splitting the clutch in half between the permissive temperature (25°C) and the restrictive temperature (33°C). Clutches with approximately 50% *kit* mutant phenotype embryos and 50% wild-type embryos at 25°C, and 100% *kit* mutant phenotype at 33°C were considered to be temperature sensitive. Sixty different founders, most of them presumably identifying independent alleles, were screened in this manner. From these, six temperature-sensitive *kit* alleles were identified and five of these alleles were recovered. Although it remains possible that these temperature-sensitive *kit* alleles are not independent, the lack of duplicated lesions in the 10 *kit* alleles so far sequenced from this screen (Parichy et al., 1999; this study; J. F. R. and S. L. J., unpublished) suggests that this possibility is remote.

Quantitative assessment of regeneration melanocyte survival

Fins regenerated at 25°C until upshift to 33°C at stage 7, 11, 15 or 20. At the time of upshift, animals were treated with 1 mg/ml epinephrine for 5 minutes to contract melanosomes and facilitate cell counting (Johnson et al., 1995; Rawls and Johnson, 2000; Sugimoto et al., 2000) and photographed. Following upshift to 33°C, fish were maintained in 0.2 mM phenylthiourea (PTU) to block melanin synthesis and therefore inhibit pigmentation of any new melanocytes (Milos and Dingle, 1978; Rawls and Johnson, 2000). After six stages at 33°C, fish were again treated with epinephrine and photographed. The rate of melanocyte survival during these treatments was assessed by dividing the number of melanocytes in the presumptive central stripe in an individual regenerate after six stages at 33°C, by the number of melanocytes in the presumptive central stripe at the time of upshift (7-10 individuals per timepoint). We define the presumptive central stripe as the region of the regenerate distal of the central stripe in the stump. As the pigmentation of further de novo melanocytes is inhibited with PTU and migration of differentiated regeneration melanocytes is minimal (C. Beckett and S. L. J., unpublished), no further melanocytes become located in the presumptive central stripe following upshift for the duration of the experiment.

RESULTS

Temperature-sensitive *kit* mutation

We conducted a screen for ENU-induced temperature-sensitive alleles of *kit* (see Materials and Methods). The most robust of these, kit^{j1e99}, encodes a T to C transition at nucleotide 2325, resulting in a leucine to proline substitution at residue 754 in the second kinase domain (not shown). This conserved leucine residue has not been previously implicated as important for *kit* function (for a review, see Linnekin, 1999). Animals homozygous for kit^{j1e99} that were reared and challenged to regenerate their fins at the permissive temperature (25°C; Fig. 1B) developed and regenerated their fin melanocyte pattern similar to wild-type fish (Fig. 1A). kit^{j1e99} animals reared at the restrictive temperature (33°C) developed embryonic and adult pigment pattern phenotypes identical to kit^{b5} null mutants (not shown). When then challenged to regenerate their fins at the restrictive temperature, these mutants failed to form regeneration melanocytes by stage 7 (Fig. 1E), a phenotype identical to that of kit^{b5} null mutants (Fig. 1D). Thus, the kit^{j1e99} allele encodes a temperature-sensitive mutation that

allows for removal or restoration of *kit* gene function at different stages of development.

kit is required following amputation to promote regeneration melanocyte development

To test whether *kit* is required prior to amputation (for example, during embryonic or larval stages) to establish or maintain adult melanocyte stem cells, we reared kit^{j1e99} animals to maturity at the restrictive temperature and shifted them to the permissive temperature after fin amputation. Following downshift (Fig. 1C), kit^{j1e99} regenerates developed de novo melanocytes identically to kit^{j1e99} fish that were held constantly at the permissive temperature (Fig. 1B). We conclude that stem cell precursors of adult regeneration melanocytes develop and persist through adult stages in the absence of *kit* function.

To determine if *kit* is required during regeneration to recruit melanocyte precursors to form differentiated melanocytes, we amputated fins from kit^{j1e99} animals reared at the permissive temperature, and then shifted them to the restrictive temperature. These regenerates failed to develop de novo melanocytes by stage 7 (Fig. 1F), indicating that *kit* is required after amputation to promote development of regeneration melanocytes.

kit is required after stage 2 to promote population of the regenerate by melanoblasts

To determine the role of *kit* during regeneration in promoting the development of de novo melanocytes at stage 4, we first observed the location of melanoblasts in wild-type and mutant regenerating fins using in situ hybridization. During wild-type fin regeneration, *kit*-expressing presumptive melanoblasts are located in the stump and at the amputation plane as early as stage 1.5 (Rawls and Johnson, 2000). By stage 4 in wild-type regenerates, *kit*-expressing melanoblasts have typically migrated into the regenerate and have begun expressing melanin (Fig. 2A). In contrast, we detected *kit*-expressing melanoblasts in the stump and at the amputation plane but not in the regenerate in stage 4 kit^{j1e99} mutants held at the restrictive temperature (Fig. 2B). This shows that *kit* is required to promote population of the regenerate by *kit*-expressing melanoblasts, and is consistent with possible roles for *kit* in migration of melanoblasts into the regenerate as well as the subsequent survival of melanoblasts in the regenerate prior to differentiation. As previously described (Rawls and Johnson, 2000), *kit* transcript was undetectable in kit^{b5} homozygotes (Fig. 2C). Presumably this failure to detect kit^{b5} transcript was due to degradation of the mutant transcript via nonsense-mediated mRNA decay (Culbertson, 1999) caused by the premature stop codon encoded in the kit^{b5} lesion (Parichy et al., 1999).

Further temperature shift experiments revealed that the initial requirement for *kit* in melanocyte differentiation is between stages 2 and 3 of regeneration. Shifting regenerates from the restrictive to the permissive temperature as late as stage 2 had no effect on the time of appearance or number of pigmented melanocytes in the regenerate (Fig. 2D). However, kit^{j1e99} fish held at the restrictive temperature until stage 3 failed to form melanocytes by stage 4 (Fig. 2D). Instead the appearance of regeneration melanocytes in this experiment was delayed until stage 5 (not shown). This suggests that *kit* is initially required between stage 2 and stage 3 of regeneration to promote melanocyte development.

Requirement for *kit* extends until late stages of melanocyte differentiation

In a reciprocal series of temperature shifts, kit^{j1e99} regenerates shifted from the permissive temperature to the restrictive temperature as late as stage 3.5 failed to develop regeneration melanocytes (Fig. 2E). In shifts to the restrictive temperature at stage 4, when some regeneration melanocytes had already developed at the permissive temperature prior to the upshift, few or no additional melanocytes developed following the shift

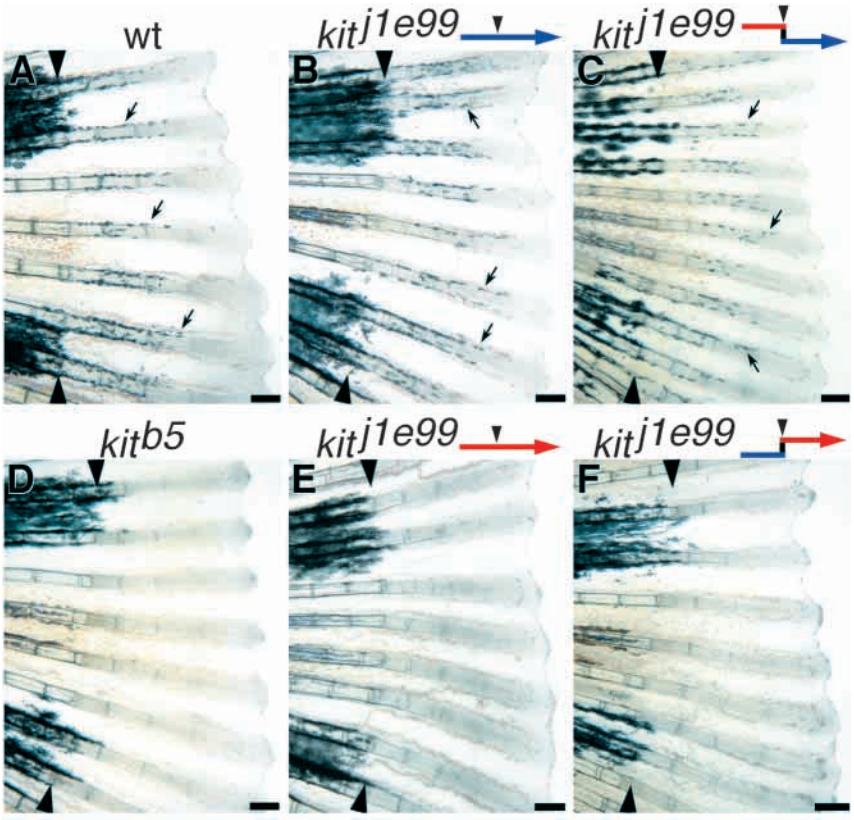

Fig. 1. Requirement for *kit* function in regeneration melanocyte development in regenerating fins. Shown are stage 7 regenerates from (A) wild type, (B) temperature-sensitive *kit* mutant (kit^{j1e99}) reared and regenerated constitutively at 25°C, (C) kit^{j1e99} fish reared at 33°C and shifted to 25°C upon amputation, (D) kit^{b5}-null mutant, (E) kit^{j1e99} mutant reared and regenerated constitutively at 33°C and (F) kit^{j1e99} mutant reared at 25°C and shifted to 33°C upon amputation. Regeneration melanocytes are indicated by black arrows. Amputation planes are indicated by black arrowheads, with distal to the right of figures. In Figures 1-3, red lines and arrows represent growth at 33°C, blue lines and arrows represent growth at 25°C, and associated small black arrowheads represent time of amputation. Scale bars: 200 μm.

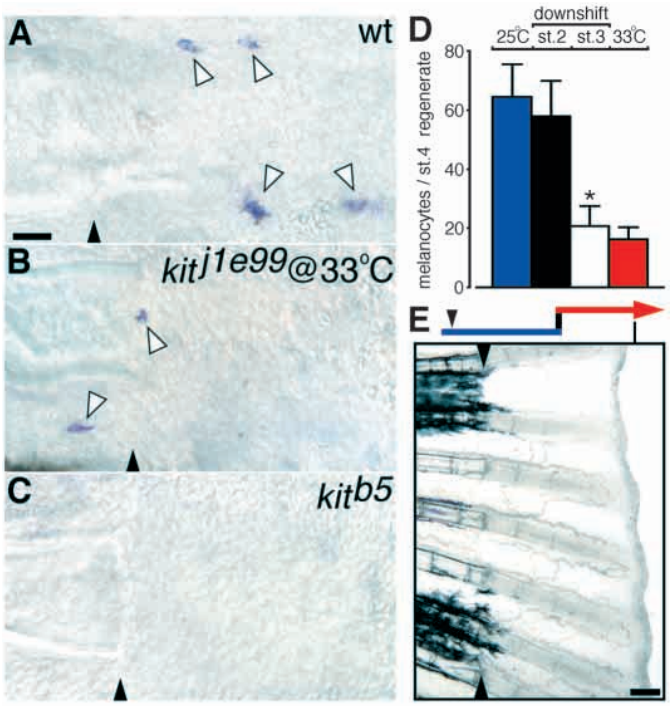

Fig. 2. Temporal requirement for *kit* function in regeneration melanocyte development. In situ hybridization detects *kit*-expressing melanoblasts (white arrowheads) in stage 4 regenerates in (A) wild type and (B) *kit*^{j1e99} mutants regenerated at 33°C, but not in (C) *kit*^{b5}-null mutants (Parichy et al., 1999). (D) Histogram shows average number of pigmented melanocytes in stage 4 regenerates of *kit*^{j1e99} mutants held constitutively at 25°C (blue), at 33°C for stages 0-2 and then shifted to 25°C for remainder of experiment (black), at 33°C for stages 0-3 and then shifted to 25°C (white), and held constitutively at 33°C (red). Melanocytes indicated by white and red bars consist largely of cells that were pigmented prior to amputation and subsequently migrated into the proximal regenerate (see Rawls and Johnson, 2000). Error bars show 95% confidence intervals. (E) Stage 6 *kit*^{j1e99} regenerate shifted from 25°C to 33°C at stage 3.5. Amputation planes are indicated by black arrowheads, with distal to the right of figures. Scale bars: 20μm (A,B,C), 200μm (E).

to the restrictive temperature (through stage 7; not shown). These results suggest that a temporal requirement for *kit* extends until late stages of melanoblast differentiation, perhaps as late as onset of pigmentation. That this requirement persists beyond stage 4 in fin regeneration presumably reflects the continuous differentiation of new melanocytes as the fin regenerate grows.

Consistent with a role for *kit* in the continuous formation of new melanocytes through later stages of regeneration, we find that regeneration melanocyte precursors in *kit*^{j1e99} regenerates at the restrictive temperature remain competent to respond to restoration of *kit* function. In *kit*^{j1e99} animals reared at the restrictive temperature and then shifted to the permissive temperature during later stages of regeneration (stages 7-11), de novo melanocytes subsequently appeared in distal positions in the regenerate (not shown). Notably, these new melanocytes consistently appeared within two stages of downshift to the permissive temperature, but never earlier than stage 4, which is when regeneration melanocytes normally form. This shows that *kit* is required continuously for new melanocyte differentiation, and suggests that melanoblasts or their precursors remain competent to respond to *kit* function beyond stage 4, which is when they would normally complete pigmentation.

kit is required for survival of differentiated regeneration melanocytes

Because regeneration melanocytes fail to form in the absence of *kit* function, the role of *kit* in regeneration melanocytes following differentiation was unknown. As larval melanocytes in *kit* mutants undergo programmed cell death and extrusion from the animal within ~6 days of differentiation (Parichy et al., 1999), we hypothesized that differentiated regeneration melanocytes would also require *kit* function for survival. To test this model, we permitted *kit*^{j1e99} fish to develop regeneration melanocytes normally at the permissive temperature until specific stages of regeneration, when they were shifted to the restrictive temperature to remove *kit* function. To monitor regeneration melanocyte survival, fish were maintained in the presence of the melanin synthesis inhibitor, phenylthiourea (PTU; Milos and Dingle, 1978; Rawls and Johnson, 2000), after the shift to the restrictive temperature. This enabled us to follow selectively those melanocytes that were differentiated prior to PTU treatment and upshift. *kit*^{j1e99} fins regenerating at the permissive temperature formed typical dendritic melanocytes throughout the dermis of the regenerate by stage 7 (Fig. 3A). However, shifting those fish to the restrictive temperature caused regeneration melanocytes to undergo typical teleost melanocyte cell death (Parichy et al., 1999; Sugimoto et al., 2000), as evidenced by contraction of their dendritic processes and displacement from the dermis into the epidermis by stage 10 (Fig. 3C), and their disappearance from the fin by stage 13 (Fig. 3B). This shows that *kit* is required for the survival of differentiated regeneration melanocytes, in addition to the aforementioned requirement for their differentiation.

Regeneration melanocytes acquire *kit* independence as they mature

Quantitative assessment of melanocyte survival revealed that the dependence on *kit* function for survival is transient. Although only 38% of melanocytes present in the *kit*^{j1e99} regenerate at the time of shift to the restrictive temperature at stage 7 persisted through six subsequent stages in the presence of PTU, survival increased to 68% when the upshift was delayed until stage 11 and increased to 92% survival after upshift at stage 20 (Fig. 4). Interestingly, the first melanocytes to acquire *kit* independence were located in the proximal portion of the presumptive stripe, while the cells that disappeared after shifting to the restrictive temperature were located in the distal portion (Fig. 3A-E). As the developmentally youngest regeneration melanocytes are typically located in the distal portion of the regenerate and the oldest in the proximal regenerate (Rawls and Johnson, 2000), we interpret these results to suggest that regeneration melanocytes transiently require *kit* after differentiation and subsequently acquire *kit* independence as they mature. An alternative possibility was that *kit*-dependent melanocytes died and were replaced by a second population of melanocytes that differentiates in the absence of *kit* function after stage 7 (secondary regulatory melanocytes; Rawls and Johnson,

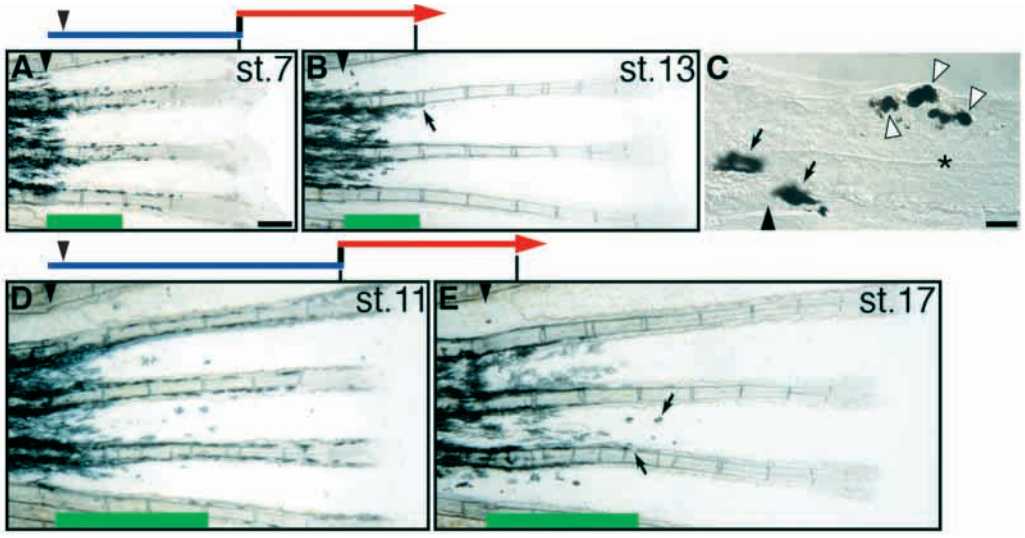

Fig. 3. Transient requirement on *kit* for differentiated melanocyte survival. *kit*^{j1e99} mutant regenerating fin photographed at stage 7 following regeneration at 25°C (A), and immediately shifted to 33°C and later photographed at stage 13 (B). Note most regeneration melanocytes are lost by stage 13, with persistent melanocytes (black arrows in (B) and (E)) located only in the most proximal region of the regenerate (indicated by green bars in A,B,D,E). (C) Longitudinal section of a stage 10 *kit*^{j1e99} regenerate shifted to 33°C at stage 7 shows punctate distal melanocytes in the epidermis (white arrowheads), and proximal dermal dendritic melanocytes (black arrows). Basement membrane is indicated by an asterisk. (D,E) *kit*^{j1e99} mutant regenerating fin photographed at stage 11 following regeneration at 25°C (D), immediately shifted to 33°C at stage 11 and later photographed at stage 17 (E). Note that region of persistent melanocytes (green bars) has expanded distally. Amputation planes are indicated by black arrowheads, with distal to the right of figures. Scale bars: 200 μm (A,B,D,E), 20 μm (C).

2000). However, this seems unlikely, as we observed no melanocyte turnover (death and replacement) after stage 7 in *kit*^{j1e99} mutants held at the permissive temperature (Fig. 4 and not shown).

DISCUSSION

We have used a temperature-sensitive mutation of the zebrafish *kit* gene to determine the temporal requirements of *kit* in promoting development of melanocytes from their stem cell precursors during adult zebrafish fin regeneration. While deficits in adult melanocytes resulting from mutations in *kit* have been well described (Silvers, 1979; Geissler et al., 1988; Nocka et al., 1990; Tan et al., 1990; Giebel and Spritz, 1991; Tsujimura et al., 1991; Johnson et al., 1995; Marklund et al., 1998; Parichy et al., 1999; Rawls and Johnson, 2000), it remained unclear when during development *kit* was required to promote adult melanocyte development. For example, the role of *kit* in migration and survival of embryonic melanocytes in fish (Parichy et al., 1999) or their precursors in mouse (Motro et al., 1991; Cable et al., 1995; Wehrle-Haller and Weston, 1995; MacKenzie et al., 1997) have led to the model that adult phenotypes in *kit* mutants result from loss of melanocyte precursors or stem cells during embryonic development (Huszar et al., 1991; Wehrle-Haller and Weston, 1995; MacKenzie et al., 1997; Rawls and Johnson, 2000). In contrast, adult roles for the *kit* receptor in promoting the formation of new melanocytes from stem cells have been previously suggested by studies in mice using conditional abrogation of gene function with Kit-directed antibodies (Nishikawa et al., 1991; Kunisada et al., 1998). Using a conditional zebrafish *kit* mutant, we found that *kit* is not required during embryonic stages to promote the formation of adult melanocytes, such as those that re-establish the melanocyte stripe during fin regeneration. Thus, the model

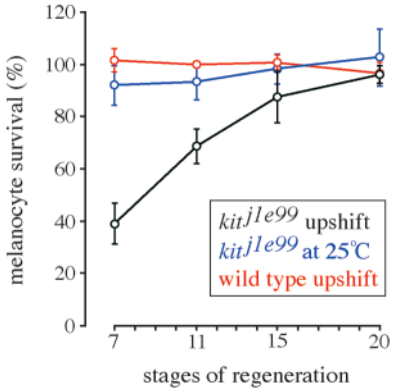

Fig. 4. Regeneration melanocytes acquire *kit*-independence as they mature during late stages of regeneration. Melanocyte survival rates (*y*-axis) from *kit*^{j1e99} (black) and wild-type regenerates (red) shifted from 25°C to 33°C at the stages of regeneration indicated (*x*-axis), or *kit*^{j1e99} held constitutively at 25°C (blue) are shown. To document the acquisition of *kit*-independence by regeneration melanocytes, melanocytes in the presumptive central stripe of caudal fins regenerating at 25°C were counted at the stages of regeneration indicated. The fish were then moved into PTU to inhibit the appearance of new melanocytes, and either shifted to 33°C or maintained at 25°C for six subsequent stages. The final number of melanocytes in the presumptive stripe from an individual regenerate after six stages was assessed and then divided by the initial number of melanocytes to determine the percent survival rate. The persistent melanocytes in *kit*^{j1e99} regenerates following upshift at stage 7 (black; 38%) consist largely of cells which were pigmented prior to amputation and subsequently migrated into the proximal regenerate (not shown). Error bars show 95% confidence intervals. (See Materials and Methods for further details.)

that adult *kit* mutant phenotypes in other vertebrates result from roles for *kit* early in development in establishing their precursors may need to be re-evaluated. Our studies suggest

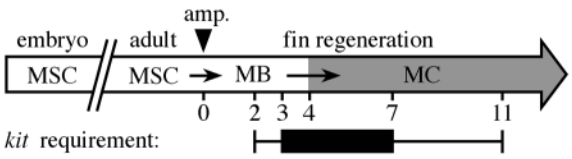

Fig. 5. Temporal requirement for *kit* in regeneration melanocyte development. Requirement for *kit* in development of melanocytes that develop first at stage 4 in regenerating wild-type fins. Melanocytes stem cells (MSC) are presumably established during embryonic development and persist through adult stages. After fin amputation (amp.), melanocyte stem cells are recruited to form melanoblasts (MB) by regeneration stage 2. Melanoblasts differentiate into melanocytes (MC) by stage 4, as evidenced by their expression of melanin (indicated by shaded area). Minimal stages of *kit* requirement are spanned by a black bar, with maximal possible stages of *kit* requirement spanned by error bars. Requirement for *kit* in the regeneration melanocyte lineage begins between regeneration stages 2 and 3 and extends beyond the onset of melanin synthesis at stage 4. *kit* is required for survival of stage 4 regeneration melanocytes until they acquire *kit*-independence between stages 7 and 11. Regeneration melanocytes which pigment later than stage 4 require *kit* for a similar duration of time before and after their differentiation (not shown). Note that *kit* is not required during embryonic or adult stages to promote the establishment or maintenance of melanocyte stem cells.

that the *kit* receptor tyrosine kinase functions, instead, to promote development during post-stem cell stages of the adult melanocyte lineage. Our model showing temporal requirements for *kit* in regeneration melanocyte development is shown in Fig. 5.

Temperature-shift experiments revealed that *kit* is first required between regeneration stages 2 and 3 to promote the differentiation of melanocytes in the regenerate by stage 4. This temporal requirement corresponds to the stage when cells in the stump are being mobilized to enter the regeneration blastema (Johnson and Bennett, 1999). We find that this requirement for *kit* persists until late stages of melanoblast differentiation, possibly as late as melanin synthesis (Fig. 5). Observation of *kit*-expressing melanoblasts in kit^{j1e99} regenerates at the restrictive temperature suggests that this temporal requirement corresponds to a morphological requirement for *kit* in promoting population of the regenerate by melanoblasts. Furthermore, our findings suggest that *kit* continues to be required for development of de novo melanocytes that pigment later at more distal locations (developmentally equivalent to the first melanocytes that differentiate at stage 4). The finding that *kit* is required for survival of differentiating regeneration melanocytes (Fig. 3; see below) leads us to suggest that *kit* may only play a role in the survival of melanoblasts during their population of the fin regenerate. However, we cannot exclude possible additional roles for *kit* in melanoblast differentiation or in melanoblast migration into the regenerate.

Conditional mutations facilitate the dissection of known mutant phenotypes, as well as uncover later roles for genes in cell types that are missing in constitutively mutant animals. Since *kit*-dependent regeneration melanocytes fail to develop in *kit* null mutants, we were able to assess late roles for *kit* in the temperature-sensitive mutant by allowing regeneration melanocytes to develop first at the permissive temperature before removing *kit* function by shifting to the restrictive temperature. Temperature shift experiments revealed a transient requirement for *kit* in promoting the survival of differentiated regeneration melanocytes. Because de novo melanocytes first appear at stage 4, and the first regeneration melanocytes to acquire *kit* independence between stages 7 and 11 are the developmentally oldest, we infer that the first regeneration melanocytes to pigment at stage 4 subsequently acquire *kit* independence between stages 7 and 11 (Fig. 5). While acquisition of both growth factor and growth factor receptor independence in neuronal development has been well documented in vivo by disrupting gene function with neutralizing antibodies (Johnson et al., 1980; Schwartz et al., 1982), the mechanisms that underlie this phenomenon remain largely unknown. Although dependence on *kit* and its growth factor ligand, *steel*, for melanocyte precursor survival and subsequent acquisition of independence has been described (Nishikawa et al., 1991; Morrison-Graham and Weston, 1993; Yoshida et al., 1996), our results now demonstrate transition to *kit*-independence in differentiated melanocytes, as revealed by genetic manipulation.

Our finding that *kit* is required only during post-stem cell stages in the zebrafish melanocyte lineage is in contrast to the temporal roles for murine *Kit* during germ cell development. In the germ cell lineage, *Kit* and its ligand have been shown to be required early in ontogeny to establish primordial germ cells in the gonad, as well as during later stages of germ cell maintenance and differentiation (see Sette et al., 2000). Interestingly, the temporal requirements for zebrafish *kit* in the melanocyte lineage are similar to the requirements for *Kit* during murine hematopoietic development. In the hematopoietic lineage, *kit* and its ligand are not required to establish totipotent hematopoietic stem cells, but are required later in development to promote their maintenance and recruitment into specific developmental pathways (see Broudy, 1997). As zebrafish *kit* is not required in the germ cell or hematopoietic lineages and *kit*-null mutants are therefore fertile and viable (Parichy et al., 1999), genetic analysis of *kit*-dependent pathways in the zebrafish melanocyte lineage may inform future studies of *Kit*-dependent processes in mammals.

Transgenic techniques for generating conditional mutations have been effective for analysis of temporal gene requirements in mice and flies (for example, see Shin et al., 1999), yet such tools have not yet been developed for the zebrafish. However, the ability of this poikilothermic vertebrate to live at a fairly wide range of temperatures (Schirone and Gross, 1968) permits the conditional disruption of gene function using temperature-sensitive mutations (Abdelilah et al., 1994; Johnson and Weston, 1995). The frequency of temperature-sensitive alleles found at the *kit* locus described in this study (6/60 or 10%) is consistent with frequencies of temperature-sensitive mutations in other model systems (Suzuki et al., 1967; Bartel and Varshavsky, 1988). Therefore, the frequency of temperature-sensitive mutations in zebrafish is sufficiently large to prompt screening for temperature-sensitive mutations in forward genetic screens (Johnson and Weston, 1995; J. F. R. and M. R. F., unpublished).

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