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Zebrafish *kit* mutation reveals primary and secondary regulation of melanocyte development during fin stripe regeneration

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SUMMARY

Fin regeneration in adult zebrafish is accompanied by re-establishment of the pigment stripes. To understand the mechanisms underlying fin stripe regeneration and regulation of normal melanocyte stripe morphology, we investigated the origins of melanocytes in the regenerating fin and their requirement for the *kit* receptor tyrosine kinase. Using pre-existing melanin as a lineage tracer, we show that most fin regeneration melanocytes develop from undifferentiated precursors, rather than from differentiated melanocytes. Mutational analysis reveals two distinct classes of regeneration melanocytes. First, an early regeneration class develops dependent on *kit* function. In the absence of *kit* function and *kit*-dependent melanocytes, a second class of melanocytes develops at later stages of regeneration. This late *kit*-independent class of regeneration melanocytes has little or no role in wild-type fin stripe development, thus revealing a secondary mode for regulation of fin stripes. Expression of melanocyte markers in regenerating *kit* mutant fins suggests that *kit* normally acts after *mitf* and before *dct* to promote development of the primary *kit*-dependent melanocytes. *kit*-dependent and *kit*-independent melanocytes are also present during fin stripe ontogeny in patterns similar to those observed during regeneration.

Key words: Melanocyte, Fin, Regeneration, Regulation, *sparse*, *Danio rerio*, *kit*, *mitf*, *dct*

INTRODUCTION

Organisms maintain their form throughout life by replacing lost cells with new ones. In some cases, cells are replaced by proliferation of well-defined undifferentiated precursors. A variety of vertebrate tissues are maintained in this way, including the circulating blood (Morrison et al., 1995), the intestinal epithelium (Potten et al., 1997) and the hair follicle (Lavker et al., 1993). In other developmental scenarios such as the regeneration of the amphibian limb, cells are replaced by precursors that are not as well characterized (Brockes, 1997). The mechanisms that regulate the balance between precursor cells and their daughters, and the patterning of these cells to facilitate the maintenance of complex form are not well understood.

The zebrafish pigment pattern provides several opportunities to study the development of melanocytes from their undifferentiated precursors. Genetic dissection of the adult pigment pattern reveals multiple populations of melanocytes that develop during the transition from larval to adult form. The first of these populations begins to appear after 2 weeks of development and is absent in mutants for the zebrafish homologue of the *kit* receptor tyrosine kinase. The zebrafish *kit* locus was originally identified as *sparse* and subsequently renamed *kit,* to preserve a common nomenclature with amniotes (Parichy et al., 1999). A second population begins to appear during the fourth week of development (at 28°C) and is absent in *rose* (*ros*) or *leopard* (*leo*) mutants. Fish doubly mutant for *kit* and *ros,* or *kit* and *leo,* lack virtually all melanocytes in the body. Persistence of melanocyte stripes in the caudal and anal fins of *kit;ros* double mutants suggests the existence of yet a third distinct population of melanocytes, independent of the function of *kit* and *rose* (Johnson et al., 1995). The inference drawn from these studies is that precursors for adult pigment cells remain quiescent through embryonic and larval development and are recruited to re-enter the developmental pathway upon the onset of larval-adult metamorphosis. These precursors might also generate new cells during growth as new stripes are added or during regeneration of fin stripes following fin amputation.

Re-establishment of the pigment pattern in the regenerating zebrafish fin may provide insights into how quiescent melanocyte precursors are recruited to differentiate. Concomitant with regeneration of the fin, melanocytes (black pigment cells) and xanthophores (yellow pigment cells) re-establish stripes in the regenerated tissue. Goodrich and colleagues described two stages of melanocyte appearance during regeneration of the caudal fin, in which the melanocyte and xanthophore stripes are parallel to the fin rays, or parallel to the vector of growth (Goodrich and Nichols, 1931; Goodrich et al., 1954). During the first day following amputation, the wound epithelium forms and no melanocytes are present in the regenerate. Beginning approximately 3 days postamputation, a few melanocytes appear in regions immediately distal to the

preexisting melanocyte stripes (Fig. 1A). This phase is followed by the appearance of many melanocytes in a uniform band along the proximal regenerate parallel to the amputation plane (distal to both melanocyte and xanthophore stripes) beginning at approximately 4 days (Fig. 1B). The stripe pattern is then re-established by disappearance of melanocytes from regions distal to the original xanthophore stripes (Fig. 1D).

The origins of melanocytes in the regenerating zebrafish fin are not clear. Lacking methods to label melanocytes, Goodrich and his colleagues failed to distinguish between potential roles for pre-existing melanocytes migrating into the regenerating fin and melanocytes arising via de novo differentiation from unpigmented precursors (Goodrich and Nichols, 1931; Goodrich et al., 1954). The model that, in intact fins, unpigmented melanocyte precursors exist that could be recruited to re-establish the melanocyte stripe in regenerating fins was suggested by the de novo appearance of melanocytes in xanthophore regions following localized destruction of xanthophores (Goodrich et al., 1954). Unpigmented melanocyte precursors have also been shown to exist in adult mice, where they could contribute to the maintenance of the adult pigment pattern (Kunisada et al., 1998).

The processes by which melanocyte precursor cells are recruited to re-enter developmental pathways are poorly understood. Indeed, the existence of melanocyte precursors in zebrafish is only inferred, as molecular markers for these cells are not yet identified. To understand the role of melanocyte precursors in zebrafish fin stripe regeneration, we studied the regenerative development of wild-type caudal fins and identified mutants defective in those processes. We show that the fin stripe is re-established almost entirely by melanocytes that differentiate de novo from unpigmented precursors. Mutants for the zebrafish *kit* receptor are defective for this de novo melanocyte population, and analysis of early markers of melanocyte differentiation suggests *kit* is required for these primary regeneration melanocytes to progress beyond a *mitf*-positive stage (Lister et al., 1999). Study of *kit* mutants reveals a secondary class of de novo regeneration melanocytes that develops independently of *kit* function, and regulates its activity to reconstitute the stripes as fin regeneration passes to a late outgrowth stage (stage 8; see Johnson and Bennett, 1999). Furthermore, we demonstrate that these two de novo populations are similarly utilized during wild-type or *kit* mutant fin stripe ontogeny. This suggests that fin stripe growth and regeneration use common pathways to recruit melanocyte precursors and regulate stripe pattern.

MATERIALS AND METHODS

Stocks

Wild-type and mutant stocks were maintained on a 14L:10D light cycle at a constant temperature of 25°C. *lof*^{Dt2}, *nac*^{w2}, *jag*^{b230}, *leo*^{t1}, *ros*^{b140}, *kit*^{b5} and *kit*^{j1e1} have been previously described (Tresnake, 1991; Johnson and Weston, 1995; Lister et al., 1999; Johnson et al., 1995, 1996; Streisinger et al., 1986; Parichy et al., 1999). *kit*^{b134} is a spontaneous allele isolated and kindly provided by Charlene Walker. All references to *kit* mutants correspond to the *kit*^{b5} null allele, except where noted. *kit*^{b5} encodes a deletion at bp 846 of the coding sequence, resulting in a frame shift and premature stop codon (Parichy et al., 1999).

Fin regeneration and ontogeny experiments

For regeneration studies, caudal fins were amputated as described (Johnson and Weston, 1995). Briefly, mature fish (3-12 months of age) were anesthetized, the distal two-thirds of the caudal fin amputated with scissors or a scalpel, and fish returned to fresh water at 25°C on a regular feeding schedule for the duration of the experiment. Regenerating fins were staged by days postamputation according to Johnson and Weston (1995). Juveniles were staged according to body length and age. For phenylthiourea (PTU) treatment of fish, 0.1-0.2 mM PTU was added to the water and changed every 2 days (Milos and Dingle, 1978). Efficacy of PTU solutions was confirmed in parallel by ability to block pigmentation in 1 day embryos. Eight or more fish were analyzed at each time point and treatment.

Fin stripe melanocyte counts

To visualize individual melanocytes in adult fin stripes, fish were immersed in 1 mg/ml epinephrine for 5 minutes to contract melanosomes. Ontogenetic fin stripe melanocyte densities were assessed by observing intact caudal fins in wild-type and *kit*^{b5} adult siblings. Regenerated fin stripe melanocyte densities were determined by observing regenerated adult caudal fins in wild-type and *kit*^{b5} siblings 8 weeks following amputation. Melanocyte density was assessed in the three stripes of the caudal fin. Observation of late regeneration de novo melanocytes was accomplished by treating wild-type and *kit*^{b5} sibling regenerates with PTU during stages 7-13. Images were taken of each regenerate at stage 13 and several hours after PTU washout, and de novo melanocytes were identified as those unpigmented at stage 13 and present after PTU washout. Images of regenerating central fin stripes were then divided into thirds (proximal, medial and distal regenerate), and de novo melanocytes in each third of the central stripe were counted. Statistical analysis was performed with aid of JMP statistical package.

In situ hybridization

Whole-mount in situ hybridization of fins was performed according to Thisse et al. (1993), with the following modifications. Proteinase K digestion was carried out at 10 mg/ml for 30-45 minutes, and hybridization and stringency washes occurred at 68°C. Full-length antisense digoxigenin-labeled riboprobes for zebrafish *tyrosinase-related protein 2/dopachrome tautomerase (dct)* (Kelsh and Eisen, 2000), *microphthalmia-associated transcription factor (mitf)* (Lister et al., 1999), and *kit* (3545 bp; Parichy et al., 1999) were synthesized from linearized templates with T7 and T3 RNA polymerases.

RESULTS

Most regeneration melanocytes arise from unpigmented precursors

Melanocytes may appear in the regenerate through migration of pre-existing pigmented melanocytes from the old stripe, or through de novo differentiation from unpigmented precursors (de novo melanocytes). To distinguish between these models, we sought to identify previously differentiated melanocytes in the regenerating fin. We took advantage of the property of phenylthiourea (PTU) to inhibit the synthesis of new melanin, thereby preventing visualization of any newly differentiated melanocytes (Milos and Dingle, 1978). Thus, for regeneration in the presence of PTU, any pigmented melanocytes must have differentiated prior to amputation and drug treatment. At 3 days postamputation (stage 3), we observe that PTU-treated regenerates (not shown) are indistinguishable from untreated controls (Fig. 1A), each with a few melanocytes (approx. 2-10 per stripe) within 0.2 mm of the amputation plane and

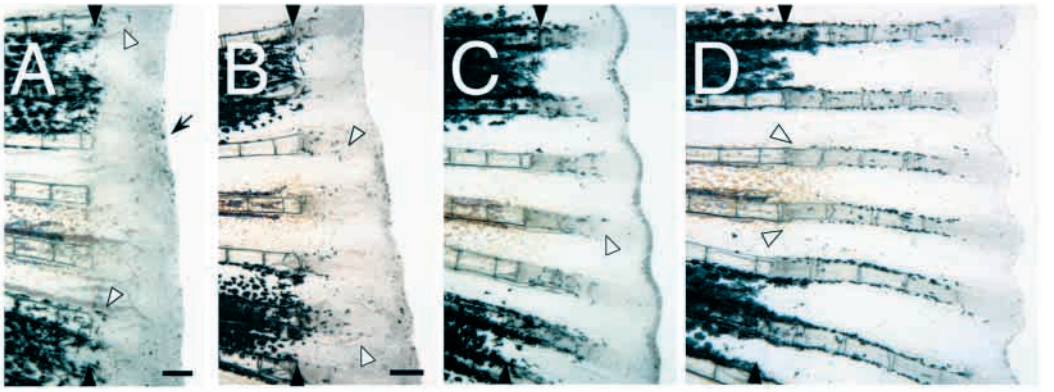

Fig. 1. Stages of melanocyte development during caudal fin regeneration. (A) Whole mount of stage 3 wild-type regenerate showing a few melanocytes (white arrowheads) just distal of the amputation plane (black arrowheads in all images) and old stripe stump, and melanocyte detritus in the distal regenerate (black arrow). (B) Stage 4 wild-type regenerate displaying melanocytes (white arrowheads) in the regenerate along the amputation plane, distal to both old melanocyte and xanthophore stripes. (C) Stage 5 wild-type regenerate showing increasing numbers of melanocytes in the regenerate (white arrowhead). (D) Stage 7 wild-type regenerate displaying more melanocytes in the regenerate, and the initial clearance of melanocytes from the presumptive xanthophore stripe (white arrowheads). Different fins are shown at each stage. Scale bars: A,B, 200 μm; C,D, same scale as B.

immediately distal of the old stripe. This pattern persists through stage 7 in PTU-treated fins (Fig. 2B), suggesting that previously differentiated melanocytes are a minor component of the total regeneration melanocyte pool.

In contrast, the vast majority of regeneration melanocytes develop by de novo differentiation from unpigmented precursors. When PTU-treated fish are returned to fresh water at stage 7 to remove the drug, lightly pigmented melanocytes become visible within a few hours in the normal pattern of untreated fish (Fig. 2C), indicating that these PTU-treated melanocytes had developed normally albeit without melanin. Since no melanin was apparent in these cells prior to drug washout, we conclude that the melanocytes that regulate fin stripe regeneration are derived from previously unpigmented precursors. Interestingly, wild-type fins are able to regenerate their stripes normally through at least ten rounds of amputation and regeneration (not shown), suggesting that these precursors have an apparently inexhaustible capacity for self-renewal. Therefore, although a few previously pigmented melanocytes migrate into the proximal regenerating stripe, fin stripes are reconstituted almost entirely by de novo melanocytes that differentiate beginning around stage 4.

Melanocyte precursors are uniformly distributed in the fin

We were interested to know whether melanocyte precursors are uniformly distributed throughout the fin, or instead reside in proximity to the differentiated melanocyte stripes in the fin. To begin to locate melanocyte precursors in the fin, we examined the expression of genes involved in early stages of melanocyte differentiation. In situ hybridization analysis reveals that *mitf*, the earliest known melanoblast marker in zebrafish (Lister et al., 1999), is expressed by mesenchymal cells in the stump beginning at stage 1 (Fig. 3A). The number of mesenchymal *mitf*-positive cells increases through stage 3, evenly distributed along a region approximately 1 mm proximal to the amputation plane (not shown; see Discussion). Later melanoblast markers, the *kit* receptor (Parichy et al., 1999) and the melanin synthesis enzyme *dct* (Steel et al., 1992; Kelsh and Eisen, 2000), are expressed in mesenchymal cells distributed throughout the

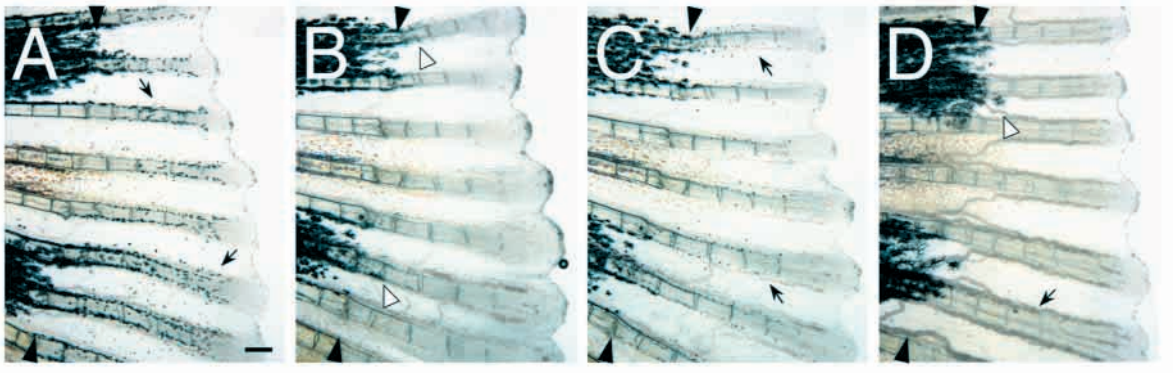

Fig. 2. Regeneration melanocytes arise by de novo differentiation from unpigmented precursors and are absent in *kit* mutants. (A) Stage 7 wild-type regenerate displays the normal pattern of regeneration melanocytes (black arrows). (B) Stage 7 wild-type after regeneration in the presence of PTU during stages 0-7 renders most regeneration melanocytes invisible. A few previously pigmented melanocytes migrate out from the stripe stump (white arrowheads). (C) The same PTU-treated fin several hours after PTU washout reveals many pigmenting melanocytes in the normal pattern (black arrows). (D) Stage 7 *kit* mutant regenerate lacks the normal pattern of regeneration melanocytes. Similar to wild type, a few pigmented cells migrate into the proximal regenerate (white arrowheads). A blood vessel is indicated by black arrow. Amputation planes are indicated by black arrowheads. Scale bars: A-D, 200 μm.

distal stump and the proximal regenerate beginning at stage 1.5 (Fig. 3C) and stage 2 (Fig. 3E) respectively. By stage 3, cells expressing *mitf* (Fig. 3G), *kit* (Fig. 3H) or *dct* (not shown) are also found to express melanin, verifying their melanoblast identity. Since cell division and gross morphological reorganization do not begin until around stage 1.5-2 of regeneration (Johnson and Bennett, 1999), we suggest that these observed patterns of differentiating *mitf/kit/dct*-positive cells early in regeneration reflect the position of de novo melanocyte precursors prior to amputation.

Although cell division and gross morphological reorganization do not begin until around stage 1.5-2 of regeneration (Johnson and Bennett, 1999), it remained possible that a few melanocyte precursors initially associated with the differentiated melanocyte stripes disperse laterally (approximately 1 mm) from positions in the stripe stump prior to stage 1 expression of *mitf*. To challenge this model, we examined fin melanocyte regeneration in *jaguar* (*jag*)*;long fin* (*lof*) double mutants, where the stripes are more widely spaced than in wild-type fins (Fig. 3I), but otherwise regenerate melanocyte pattern through stage 7 identical to wild type (not shown). This allowed us to resect the dorsal and ventral regions of *jag;lof* caudal fins, which contained melanocyte stripes, and then amputate the distal one-third of the remaining medial fin section. Following such compound amputation, fins have no differentiated melanocytes along the distal amputation plane. Moreover, the differentiated melanocytes closest to the distal amputation plane are at least 7 mm away in the proximal fin stumps. Melanocytes appeared dispersed throughout the distal regenerate as early as stage 3.5 after compound amputation (Fig. 3J), identical to the time when melanocytes regenerate in *jag;lof* mutants following simple distal amputation (not shown). Distal regeneration melanocytes in *jag;lof* compound amputees do not appear when regeneration occurs in the presence of PTU (not shown), consistent with our earlier finding (see above) that regeneration melanocytes do not arise from previously pigmented melanocytes migrating from the proximal fin stumps or the body stripes. Melanocyte regeneration along the proximal amputation planes occurs with similar timing (Fig. 3J), and the original fin pigment pattern is

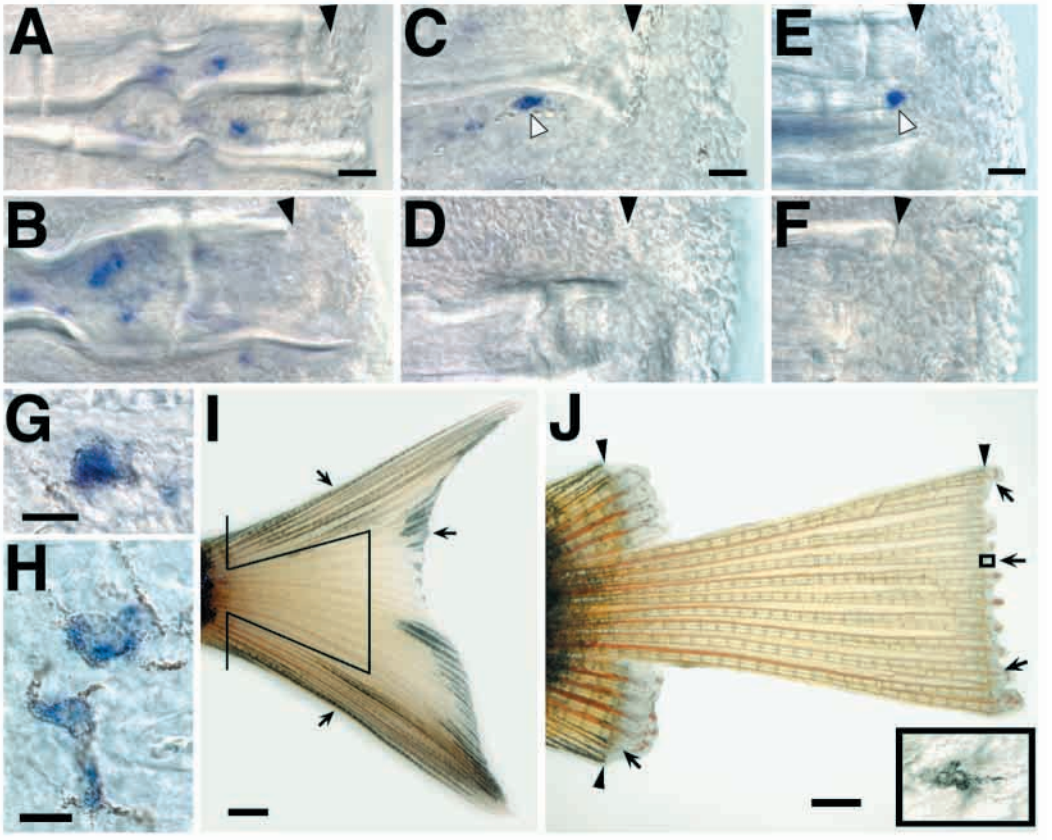

Fig. 3. Melanocyte precursors are distributed evenly throughout the fin and are unable to progress beyond a *mitf*-positive stage in regenerating *kit* mutants. Whole-mount in situs of wild-type (A) and *kit* mutant (B) regenerating fins reveals *mitf*-positive cells at stage 1 in the fin stump. *kit* transcript is found in cells proximal of the amputation plane as early as stage 1.5 in wild-type (C; white arrowhead), but is never observed in similarly staged *kit* mutants (D). Similarly, *dct* transcript is observed as early as stage 2 in wild-type regenerates (E; white arrowhead), but not in *kit* mutant regenerates (F). Cryosections along the proximodistal axis of stage 3 wild-type regenerates reveals *mitf*-positive (G) and *kit*-positive (H) mesenchymal cells along the amputation plane beginning to express melanin. In situ images were all taken from presumptive xanthophore stripes, which are devoid of previously pigmented melanocytes, although similar patterns are observed in presumptive melanocyte stripes (not shown). (I) Intact *jag;lof* caudal fins contain melanocytes only in the most dorsal, ventral and distal regions (black arrows). The portion of the fin to be resected is shown by a black line. (J) The same fin at stage 3.5 after amputation reveals several melanocytes in the distal and proximal regenerates (black arrows). The inset shows a melanocyte in a higher magnification of the area outlined in the black box. Distal is to the right in all images. Amputation planes are indicated by black arrowheads. Scale bars: A-F, 20 μm; G,H, 10 μm; I, 2 mm; J, 1 mm.

eventually reconstituted (not shown). Although we cannot formally exclude unusually rapid melanoblast migration from pre-existing stripes at the proximal amputation planes in this experiment, our finding of normal pattern and timing of melanocyte development in the distal region of these compound regenerates supports the notion suggested by in situ analysis of melanoblast markers (above, Fig. 3A,C,E) that melanocyte precursors are distributed evenly throughout the intact fin and are locally recruited by the amputation plane.

The normal development of new melanocytes is dependent on *kit*

To identify genes involved in fin stripe regeneration, we surveyed a panel of adult pigment pattern mutants (including *jag*^{b230}, *nac*^{w2}, *leo*^{t1}, *ros*^{b140} and *kit*^{b5}) for defects in fin stripe regeneration. After amputating and observing the regeneration of these different mutants, two mutations caused deficits in regeneration melanocytes at stage 7: *kit*^{b5}, a null-allele of the zebrafish *kit* gene (Fig. 2D; Parichy et al., 1999), and *nac*^{w2}, a loss-of-function allele of zebrafish *mitf* (not shown; Lister et al., 1999). Since *kit* mutants form melanocyte stripes during fin ontogeny (Johnson et al., 1995) yet show dramatic deficits in early stages of regeneration (see below), we further investigated the melanocyte regeneration defect in *kit* mutants.

kit mutant fin regeneration initially appears similar to wild type, with several melanocytes appearing distal of the old stripe stump by stage 3 (not shown). The migration of these cells into the fin regenerate is therefore not dependent on the *kit* gene. However, no additional melanocytes appear in the regenerate through stage 7, suggesting that *kit* mutant animals lack de novo melanocytes during fin stripe regeneration (Fig. 2D). To determine if this defect was due to mutations other than *kit*^{b5} fixed in the background, other *kit* mutant alleles were tested (*kit*^{j1e1}, *kit*^{b134}; not shown). These other *kit* alleles displayed regeneration defects identical to *kit*^{b5}, confirming that the observed de novo melanocyte deficit in *kit* mutants is a consequence of loss of *kit* function. Therefore *kit* is required for development of de novo regeneration melanocytes that pigment beginning at stage 4 in wild-type animals.

kit is required for melanoblast differentiation prior to *dct* expression

The *kit* mutant fin stripe regeneration phenotype suggests that *kit* promotes the development of melanocyte precursor cells. To help determine when *kit* might be required for melanocyte precursor development, we observed *kit* expression patterns using in situ hybridization. As previously described, *kit* is expressed during embryonic and larval development in the melanocyte lineage (Parichy et al., 1999), suggesting that the *kit* mutant regeneration defect could result from a requirement for *kit* in establishing melanocyte precursors early in ontogeny. Alternatively, *kit* may be required during regeneration to recruit melanocyte precursors to form melanocytes. As discussed above and shown in Fig. 3C, *kit* is expressed in melanoblasts as early as stage 1.5, consistent with a role for *kit* during regeneration. Almost all melanocytes in the regenerate express *kit* by stage 5, but this expression wanes and becomes undetectable in most melanocytes by stage 10 (not shown).

If *kit* is required during regeneration rather than for establishing precursor populations, then differentiating melanoblasts might develop up to the stage of *kit* requirement in *kit* mutant regenerates. To test this possibility, we observed melanoblast marker expression in *kit* mutant fins. Similar expression patterns of *mitf* in *kit* mutant and wild-type regenerates at stage 1 (Fig. 3A,B) and continuing through stage 3 (not shown) suggests that *kit* is not required for generating

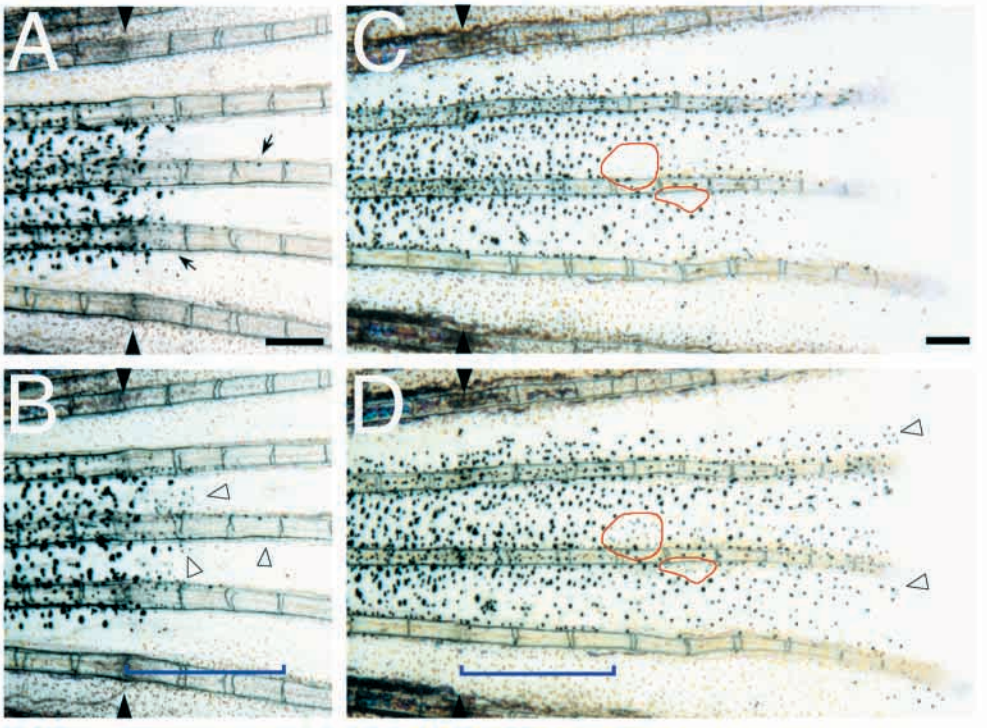

Fig. 4. Late regeneration melanocytes arise by de novo differentiation, and contribute differently to *kit* mutant and wild-type regeneration. (A) Stage 13 *kit* mutant regenerate after regenerating in the presence of PTU from stages 7-13 reveal few pigmented melanocytes in the regenerate (black arrows). (B) The same *kit* mutant fin several hours after PTU washout reveals many new melanocytes in the proximal regenerate (white arrowheads). (C) Stage 13 wild-type regenerate after regenerating in the presence of PTU during stages 7-13 of regeneration displays holes in the regenerated stripe (outlined in red). (D) The same wild-type fin several hours after PTU removal reveals new melanocytes filling the holes in the stripe, and in the distal regions of the fin (white arrowheads). The proximal one-third of the regenerated fins are indicated by blue brackets in B and D. Fish were treated with epinephrine prior to imaging to contract melanosomes. Amputation planes are labeled by black arrowheads. Scale bars: A-D, 200 μm.

mitf-positive presumptive melanoblasts (see Discussion). However, no mesenchymal *kit*-positive (Fig. 3D) or *dct*-positive (Fig. 3F) cells are detected through stage 7 in *kit* mutants, suggesting that *kit* is required for melanoblasts to progress to a *kit*/*dct*-positive stage during regeneration. In wild-type fins, low levels of *kit* transcript were detected in an unidentified population of cells scattered throughout the epidermis (not shown). The identity of these cells remains unclear. Interestingly, *kit* mutant fins retain the unidentified *kit*-positive epidermal cells (not shown), suggesting that their development is not dependent upon *kit* function.

kit-independent de novo melanocytes re-establish melanocyte stripes in *kit* mutant regenerates

Although regenerating *kit* mutant fins lack a wild-type pattern of de novo melanocytes, the fin stripes are eventually re-established. From stage 0-7, only a few melanocytes appear in the *kit* mutant regenerate immediately distal to the old stripe stump (Fig. 2D). These cells result from migration of pre-existing melanocytes into the regenerate (see below). Subsequent to stage 8, *kit* mutant melanocyte stripes are slowly re-established as melanocytes appear in the presumptive stripe until the regenerate stripe is filled to the distal edge of the fin (not shown). While a regenerating wild-type caudal fin stripe extends to the distal edge of the regenerating fin by stage 5, regenerating *kit* mutant fin stripes typically do not complete extension to the distal edge until around stage 20 (not shown). To distinguish whether *kit*-independent melanocytes arise from unpigmented precursors or from previously pigmented melanocytes, *kit* mutant caudal fins were amputated and challenged to regenerate in the presence of PTU. Since regenerating *kit* mutant fin stripes begin to extend distally around stage 8 and the efficacy of PTU treatment is temporally limited to about 7 days, *kit* mutant fish were treated with PTU from stage 7 to stage 13. Before PTU was removed at stage 13, melanocytes in the regenerating stripe were minimal in number and distal extension (Fig. 4A). At least some of these pigmented cells represent pre-existing melanocytes similar to those observed in wild-type and do not require *kit*, because *kit* mutant regenerates treated between stages 0 and 7 with PTU retain several melanocytes immediately distal of the stripe stump (data not shown). It is also possible that some of these pigmented cells represent *kit*-independent de novo melanocytes that pigment prior to stage 7. Following the removal of PTU at stage 13, melanocytes appeared in the presumptive stripe immediately distal to previously pigmented cells (Fig. 4B). Therefore, while regenerating *kit* mutant fins do not display a wild-type pattern of de novo melanocytes early in regeneration (primary regulation melanocytes), their stripes are eventually re-established by *kit*-independent de novo melanocytes beginning around stage 8 (secondary regulation melanocytes). Interestingly, wild-type regenerates also form de novo melanocytes during late regeneration; however, their pattern is distinct from that observed in *kit* mutants (Fig. 4C,D; see below).

Ontogeny of melanocyte fin stripes is similar to regeneration

Fin regeneration serves to investigate how cells are stimulated to replace missing tissue and regulate form, and may also provide an accelerated reflection of the processes employed during ontogenetic development. To explore the relationship of regeneration and ontogeny, we sought to determine whether ontogenetic development of caudal fin stripes displayed patterns of de novo melanocytes similar to those observed in regenerative development. Observation of wild-type and *kit* mutant juveniles suggested at least some similarity. Melanocytes initially appear in growing wild-type caudal fins around the 4.5 mm stage (approx. 21 days postfertilization at 25°C in these experiments, dpf) and cover the entire fin by the

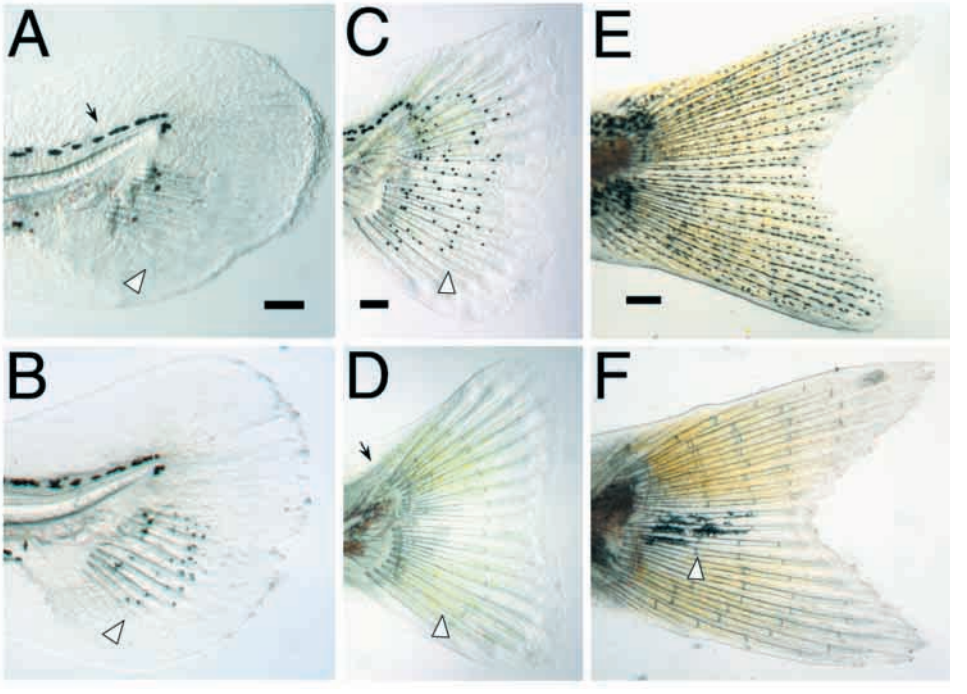

Fig. 5. *kit*-dependent and *kit*-independent de novo melanocyte contribution to fin stripe ontogeny in wild-type and *kit* mutants. (A) A 5 mm stage wild-type caudal fin after 6 days in the presence of PTU displays no melanocytes in the developing fin (white arrowhead), but does contain pre-existing melanocytes in the body (black arrow). (B) The same wild-type fin several hours after PTU removal reveals many new melanocytes (white arrowhead). (C) A 6 mm stage wild-type caudal fin displays melanocytes scattered through the fin (white arrowhead). (D) A 6 mm *kit* mutant larvae is devoid of any melanocytes in the caudal fin (white arrowhead) or the body (black arrow). (E) A wild-type fin at the 9 mm stage displays melanocytes throughout the entire fin. (F) A *kit* mutant at the 9 mm stage shows new melanocytes only in the proximal presumptive stripe (white arrowhead), similar to late stages of *kit* mutant regeneration. Scale bars: A-D, 100 μm; E,F, 200 μm.

6-7 mm stage (approx. 32 dpf; Fig. 5C). Reminiscent of the *kit*-dependent melanocytes at stage 4 of regeneration, 6 mm *kit* mutant siblings lack all melanocytes in their caudal fins (Fig. 5D). To determine if these *kit*-dependent melanocytes develop from unpigmented precursors, 4.5 mm wild-type juveniles were treated with PTU and allowed to continue development. After 6 days in PTU (approx. 5 mm stage), no new melanocytes were visible (Fig. 5A), while untreated siblings had developed more melanocytes in their fins (not shown). New fin melanocytes became visible after removal of PTU (Fig. 5B), confirming that fin stripe melanocytes develop from undifferentiated precursors rather than division of previously differentiated melanocytes in the body.

In contrast, melanocytes do not appear in developing *kit* mutant caudal fins until the 8-9 mm stage (approx. 40 dpf), when they fill in the presumptive stripe, progressing proximal to distal (Fig. 5F). Since *kit* mutant juveniles are devoid of both fin and body melanocytes at stages immediately prior to development of fin melanocytes (Fig. 5D; Parichy et al., 1999), these *kit*-independent melanocytes must also be formed by de novo differentiation. We conclude that both *kit*-dependent and *kit*-independent fin melanocytes develop with similar temporal and spatial relationships during ontogeny and regeneration of the fin.

kit-independent melanocytes regulate during *kit* mutant fin stripe development

Is there a role for *kit*-independent melanocytes during wild-type fin development? Previous studies of *kit* mutants showed that *kit*-dependent melanocytes form approximately 50% of the wild-type complement of body stripe melanocytes, and *kit*-independent melanocytes form the remaining 50% (Johnson et al., 1995). The inference drawn from these studies is that *kit*-independent melanocytes in the body stripes develop regardless of *kit*-dependent melanocytes. We were interested in whether *kit*-independent melanocytes contribute similarly to fin stripe development. Counts of wild-type and *kit* mutant fin stripe melanocytes revealed that these genotypes have similar melanocyte densities in both ontogenetic and regenerated fin stripes (Fig. 6A). This demonstrates that *kit*-independent melanocytes are able to regulate to wild-type melanocyte density during *kit* mutant fin development.

The above finding of similar melanocyte density in wild-type and *kit* mutant regenerated fins (Fig. 6A), together with the observation that regeneration melanocytes in *kit* mutants arise late at proximal positions (Fig. 4A,B), raises the possibility that the pattern of late proximally developing (*kit*-independent) melanocytes observed in *kit* mutants may be a specific response to lack of *kit* function. Alternatively, *kit*-independent melanocytes may develop during wild-type regeneration in a pattern similar to *kit* mutant regeneration. To distinguish between these two models, we compared the positions of late de novo melanocytes during wild-type and *kit* mutant regeneration. We amputated wild-type and *kit* mutant siblings and treated them with PTU during regeneration stages 7-13. We imaged the regenerating fins at stage 13 and after PTU washout, and assessed the number of de novo melanocytes in the proximal, medial and distal thirds of each regenerating central stripe. Comparison of wild-type and *kit* mutant fish revealed approximately five-fold greater numbers of de novo melanocytes in the proximal third of *kit* mutant

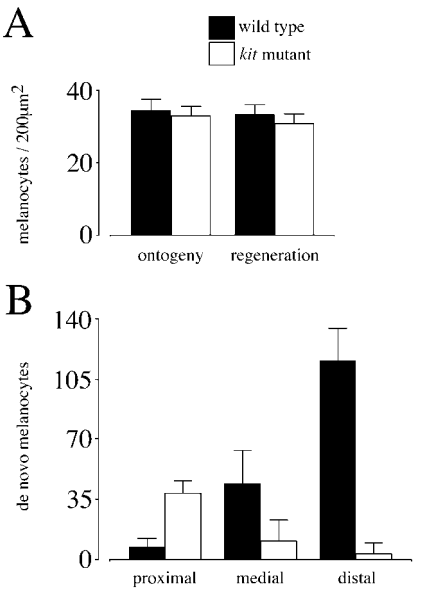

Fig. 6. *kit*-independent melanocytes regulate during *kit*-mutant fin stripe development. (A) Wild-type (black) and *kit* mutants (white) have similar densities of fin stripe melanocytes in both ontogenetic (t=0.736, df=20, P=0.47) and regenerated fins (t=1.884, df=24, P=0.0718). (B) *kit* mutants display significantly more late de novo regeneration melanocytes than wild-type in the proximal third of the regenerating central stripe (5.4-fold difference; t-test results: t=6.32, df=14, P<0.0001), while wild-type showed more de novo melanocytes than *kit* mutants in the medial (P<0.05) and distal thirds (P<0.0001). Wild-type and *kit* mutant regenerates were treated with PTU during stages 7-13. Regenerating central fin stripes were imaged at stage 13 and after PTU washout to visualize the number and position of de novo melanocytes.

regenerates than in wild type. In contrast, most late regeneration melanocytes in wild type appeared in the distal growth zone of the regenerate (Fig. 6B). This finding suggests that *kit*-independent de novo melanocytes do not develop in a similar pattern in wild-type regeneration compared to *kit* mutants. Rather, it suggests that development of these cells is a regulatory response to the lack of *kit* function or *kit*-dependent melanocytes during the late stages of regeneration.

DISCUSSION

Regeneration melanocytes arise from uniformly dispersed stem cells

PTU experiments described above show that most fin regeneration melanocytes arise from undifferentiated precursors. Although a few melanocytes migrate a short distance from the stump into the proximal regenerate and make no additional contribution to the regenerating stripe, the vast majority of regeneration melanocytes are PTU-sensitive (lack melanin during regeneration in PTU) and therefore arise from undifferentiated precursors. We can generally exclude the possibility that regeneration melanocytes arise from previously differentiated melanocytes that are partitioning their melanosomes to progeny, as we see no evidence of partitioned melanosomes at early stages of regeneration (S. L. J.,

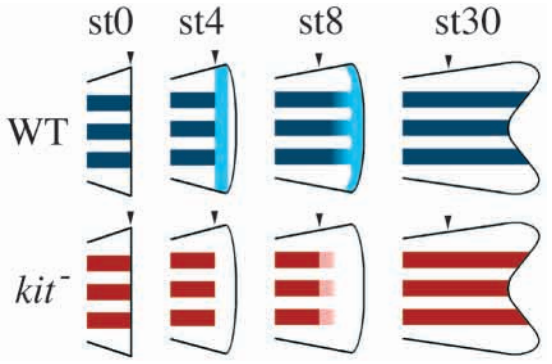

Fig. 7. Primary and secondary regulation of melanocyte development during fin stripe regeneration. De novo differentiation of primary *kit*-dependent melanocytes begins at stage 4 (st4) in wild-type (WT; *kit*-dependent melanocytes are represented in blue, with mature cells in dark blue and differentiating cells in light blue). These new cells appear in a band along the proximal regenerate. By stage 8 (st8), de novo differentiation of primary melanocytes occurs mainly in the distal regenerate. The secondary *kit*-independent class of melanocytes begin to differentiate around stage 8 in response to the absence of *kit* function or *kit*-dependent melanocytes (*kit*$^{-}$; *kit*-independent melanocytes are represented in red, with mature cells in dark red and differentiating cells in light red). These cells appear in the proximal presumptive melanocyte stripe. Both primary and secondary melanocyte classes re-establish the fin stripe by stage 30 (st30) in their respective genotypes.

unpublished data). Further, early melanoblast markers (*mitf, kit, dct*) identify cells that develop uniformly distributed in the distal stump at early stages (stages 1-2; Fig. 3A,C,E), or in the proximal regenerate at later stages (stages 3-4; Fig. 3G,H). This pattern of differentiating melanocytes persists in *jaguar;long fin* mutants where the amputation plane is selected at great distance from differentiated melanocytes (Fig. 3I,J), supporting our hypothesis that previously differentiated melanocytes do not give rise to undifferentiated precursors. We suggest in arguments below that these undifferentiated precursors are stem cells.

Several lines of evidence suggest that melanocyte stem cells exist in adult animals that provide new melanocytes in the regenerating fin. Here, we minimally define a precursor cell as one that can generate differentiated progeny, and a stem cell as a cell with the capacity for both producing differentiated progeny and for self-renewal. Goodrich first suggested the existence of such melanocyte stem cells by showing that new melanocytes could be recruited to develop in non-melanocyte regions in the intact fin (Goodrich et al., 1954). This model is also supported by the finding that unpigmented melanocyte stem cells are present in adult mice, where they might be responsible for maintaining the adult pigment pattern through successive hair follicle cycles (Kunisada et al., 1998). Our previous findings that melanocytes develop in larval *kit* mutants that lack all differentiated melanocytes shows that unpigmented melanocyte precursors are responsible for generating at least part of the adult zebrafish pigment pattern (Johnson et al., 1995). An alternative to the model that regeneration melanocyte stem cells exist in the intact fin is that stem cells are generated in the regeneration blastema. Our finding that fin stripe regeneration and ontogeny share developmental and genetic pathways tends to suggest that the stem cells contribute both to ontogenetic growth of the fin and to regeneration, rather than being a property solely of the regenerating fin (Fig. 5). Furthermore, their capacity for self-renewal appears to be significant, since wild-type fin stripes are able to form new melanocytes along a normal time course through multiple rounds of regeneration. Taken together, these data suggest that melanocyte stem cells scattered evenly throughout the intact fin are recruited following amputation to divide in a self-renewing manner to produce new melanocytes.

kit-dependent and *kit*-independent mechanisms of melanocyte regeneration in the fin

A surprising finding that prompted this study was that, while *kit* mutants have apparently normal fin stripes prior to amputation, these mutants show profound defects in melanocyte regeneration. Our finding that two different temporal classes of regeneration melanocytes contribute to the regeneration and development of the stripe tends to explain this result. The first class develops dependent on the *kit* gene early in regeneration (absent in *kit* mutants), and a second *kit*-independent class develops in late stage regeneration of *kit* mutants (see Fig. 7). Like *kit*-dependent melanocytes, we suggest that *kit*-independent regeneration melanocytes arise from unpigmented stem cells rather than by division of previously pigmented cells. This is supported by the findings that their pigmentation is inhibited by PTU (Fig. 4A,B), and that *kit* mutant juveniles develop fin stripe melanocytes prior to the formation of any other adult melanocytes (Fig. 5D,F). Although the regeneration stage when *kit*-independent melanocytes begin to appear is somewhat variable (Fig. 4A), these cells generally first appear around stage 8. Interestingly, stage 8 marks a transition from regenerative developmental anatomy to normal growth anatomy (Johnson and Weston, 1995; Johnson and Bennett, 1999), suggesting that *kit*-independent melanocyte differentiation may be a response to this transition. By analogy, the finding of two independent populations of melanocytes in the zebrafish body stripes, one dependent on *kit* and the other dependent on *rose* (Johnson et al., 1995), supports the model of two distinct populations of regeneration melanocytes. Furthermore, the *kit*-dependent melanocytes develop before the second melanocyte population in both the body stripe and regenerating fin. There are, however, important distinctions between body stripe development and fin stripe regeneration. For instance, fin stripe development and regeneration is largely independent of *rose*, as *kit;rose* double mutants develop or regenerate fin stripes indistinguishable from the *kit* single mutant (not shown). Additionally, *kit*-independent regeneration melanocytes are able to regulate their number to recover normal melanocyte density in the absence of *kit* function (see Fig. 6A). In contrast, *kit*-independent (*rose*-dependent) body stripe melanocytes fail to compensate for the missing *kit*-dependent melanocytes in *kit* mutants (Johnson et al., 1995).

The finding of distinct classes of differentiated melanocytes in the regenerating fin (as assessed by their different timing of development and different genetic control) does not distinguish between models for distinct or shared stem cells for the different types of melanocytes. Characterization of melanocyte stem cell potency and proliferative ability awaits the generation of molecular markers for these cells.

Several models could account for the defect in melanocyte development during *kit* mutant fin stripe regeneration. For example, *kit* could be required for proper establishment of *kit*-dependent melanocyte stem cells during the embryonic and larval stages. Alternatively, *kit* might be required for recruiting melanocyte stem cells to re-enter the developmental pathway during regeneration. Consistent with this second model, *kit* is expressed in developing melanocytes during fin regeneration (Fig. 3C,H). In situ hybridization in *kit* mutant regenerates reveals that normal *mitf* expression persists through stage 3 in the absence of *kit* function; however, *dct*-positive or *kit*-positive melanoblasts are not detected (Fig. 3A-F). Inability to detect *kit* transcript in *kit* mutant regenerates may be due to mutant transcript instability, rather than failure of *kit* mutant melanoblasts to proceed to a *kit*-positive stage of development. However, the normal development of *mitf*-positive cells and the absence of *dct*-positive cells in *kit* mutant regenerates suggests that *kit* acts downstream of *mitf* to promote progression to a *dct*-positive stage of melanoblast development in *kit*-dependent melanocytes. This inference is made cautiously, however, since *mitf* may be expressed in other cell types during regeneration that appear unaffected in *kit* mutants (Parichy et al., 2000). In the absence of molecular markers for melanocyte stem cells, these models could be further tested with a conditional allele of *kit*. Conditional mutations could be generated by screening for temperature-sensitive alleles (Johnson and Weston, 1995).

kit-independent melanocytes represent a secondary regulatory mode and are largely absent in wild-type regeneration

Is *kit*-independent de novo differentiation of fin melanocytes specific to *kit* mutant regeneration, or does this phenomenon also occur in wild-type regeneration? Considering the fact that new melanocytes develop in the proximal presumptive stripe after stage 8 in *kit* mutants (Fig. 4A,B), it was possible that the mutant revealed a role for these *kit*-independent melanocytes in the same pattern during late wild-type regeneration. This model tends to be refuted by the low number of late de novo melanocytes appearing in the proximal regenerating wild-type stripe compared to *kit* mutants (Fig. 6B). This suggests that *kit*-independent melanocytes or their precursors regulate their development in response to the absence of *kit* function and/or early *kit*-dependent melanocytes. The ability of the *kit*-independent melanocytes to regulate to fully populate the fin stripes is further demonstrated by the finding of similar melanocyte densities in wild-type and *kit* mutant fin stripes (Fig. 6A). This is in contrast to *kit*-independent (*rose*-dependent) melanocytes in the body stripe, which only form about half the wild-type complement of melanocytes (Johnson et al., 1995). We describe two redundant modes of regulation involved in reconstituting the melanocyte stripes of the zebrafish fin: a primary mode that develops melanocytes early in regeneration dependent on the *kit* gene, and a secondary mode that develops melanocytes late in regeneration in the absence of *kit* function (see Fig. 7).

Several models could account for the pattern of secondary regulation during *kit* mutant fin regeneration. By one model, there may be two distinct melanocyte stem cell populations, one for primary (*kit* dependent) regeneration melanocytes, the other for secondary (*kit*-independent) regeneration melanocytes. The absence of primary regeneration melanocytes (for instance, in *kit* mutants) may provide the opportunity for secondary stem cells to be recruited to develop after a lag of approximately one week. A second model posits a single stem cell population, which is first recruited to produce melanocytes in a *kit*-dependent fashion. Lacking *kit*, these differentiating melanoblasts presumably die, similar to *kit*-defective melanoblasts in fish or mouse embryos (Parichy et al., 1999; Wehrle-Haller and Weston, 1995). The subsequent absence of melanocytes in the regenerate may provide the conditions by which the same stem cell population is again recruited to produce melanocytes, this time by a *kit*-independent mechanism. A third model is that a single stem cell population is recruited only once to produce regeneration melanocytes. These differentiating melanoblasts may require *kit* to promote specific morphogenetic processes, such as the migration of developing melanoblasts into the regenerate, or their differentiation in the regenerate. Lacking *kit* function, these melanoblasts may arrest or delay their development at one of these steps. After a delay, secondary (*kit*-independent) mechanisms may be recruited to rescue the development of these cells, accounting for the apparent delay described for appearance of secondary regeneration melanocytes. We feel this last model is less likely, as other melanoblasts and melanocytes that utilize *kit* for their development die when *kit* function is ablated (Parichy et al., 1999; Wehrle-Haller and Weston, 1995), rather than simply delay development until other means can be found to promote their development. Rather, we support a model that *kit*-independent melanocytes are a distinct developmental lineage from *kit*-dependent melanocytes, arising either from a common stem cell population or alternatively from discrete stem cell populations.

These results show that *kit*-independent melanocytes arise through a secondary regulatory mode during *kit* mutant fin regeneration. Do they also play a minor role in wild-type fin development? One possibility is that *kit*-independent melanocytes function during wild-type development to help regulate the final form of the stripe. For instance, perhaps the mechanism of early *kit*-dependent stripe development leads to holes and gaps in the stripe that are later filled by *kit*-independent melanocytes (one possible example is shown in Fig. 4C,D). While regulation among melanocyte precursors and other neural crest derivatives during embryonic vertebrate development is well described (Huszar et al., 1991; Raible and Eisen, 1996; for review see Vaglia and Hall, 1999), our findings imply that at least partial regulatory potential is retained in adult neural crest derivatives. Although redundant secondary mechanisms of regulation are probably common in adult vertebrate development, they are only beginning to be appreciated (for instance, see Alison and Sarraf, 1998). The zebrafish genetic system raises the possibility of identifying mutants defective for the secondary *kit*-independent melanocyte class, allowing for understanding of the molecular mechanisms underlying the regulation of these cells.

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