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Zebrafish *sparse* corresponds to an orthologue of c-*kit* and is required for the morphogenesis of a subpopulation of melanocytes, but is not essential for hematopoiesis or primordial germ cell development

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SUMMARY

The relative roles of the Kit receptor in promoting the migration and survival of amniote melanocytes are unresolved. We show that, in the zebrafish, *Danio rerio*, the pigment pattern mutation *sparse* corresponds to an orthologue of c-*kit*. This finding allows us to further elucidate morphogenetic roles for this c-*kit*-related gene in melanocyte morphogenesis. Our analyses of zebrafish melanocyte development demonstrate that the c-*kit* orthologue identified in this study is required both for normal migration and for survival of embryonic melanocytes. We also find that, in contrast to mouse, the zebrafish c-*kit* gene that we have identified is not essential for hematopoiesis or primordial germ cell development. These unexpected differences may reflect evolutionary divergence in c-*kit* functions following gene duplication events in teleosts.

Key words: Neural crest, Melanocyte, c-*kit*, *Danio rerio*, Hematopoiesis, Primordial germ cell, Apoptosis, Cell migration

INTRODUCTION

Vertebrate melanocytes are derived from the neural crest (NC), a population of embryonic precursor cells that arises along the dorsal neural tube then disperses throughout the embryo. In addition to melanocytes, NC cells also contribute to the peripheral nervous system, craniofacial skeleton, heart, endocrine glands, and other tissues and organs (Le Douarin, 1982; Hall and Hörstadius, 1988; Erickson, 1993; Le Douarin et al., 1994; Groves and Bronner-Fraser, 1999). The morphogenesis and differentiation of melanocytes and other NC derivatives requires the transduction of extracellular signals through a variety of cell surface receptors (Weston, 1991; Barsh, 1996; Wehrle-Haller and Weston, 1997; Moellman and Halaban, 1998; Reedy et al., 1998). Among these is the product of the c-*kit* (*kit*) gene, a type III receptor tyrosine kinase expressed by melanocytes and melanocyte precursors, or melanoblasts (Qiu et al., 1988; Orr-Urtreger et al., 1990; Motro et al., 1991; Manova and Bachvarova, 1991; Giebel and Spritz, 1991; van der Geer et al., 1994; Wehrle-Haller and Weston, 1995; Bernex et al., 1996; Opdecamp et al., 1997). In mouse, mutants in *Kit* (formerly *W*) are dominant and long have been studied for their effects on the development of melanocytes, as well as hematopoietic precursors and primordial germ cells (PGCs; de Aberle, 1927; Little and Cloudman, 1937; Russell, 1949; Mintz and Russell, 1957; Mayer and Green, 1968; Silvers, 1979; Tan et al., 1990; Nocka et al., 1990; Tsujimura et al., 1991; Giebel and Spritz, 1991; Besmer et al., 1993; Spritz, 1998; Marklund et al., 1998). Mice homozygous for severe *Kit* alleles lack melanocytes, are deficient in PGCs, and die prenatally or perinatally of macrocytic anemia. Mice that are heterozygous, or homozygous for less severe and viable alleles, completely lack melanocytes or have melanocyte deficiencies, and also may exhibit sterility or impaired fertility, as well as anemia. Similar phenotypes are observed in murine mutants for *Steel* (*Mgf*), which encodes the Kit ligand, Steel Factor (SLF; also known as Mast Cell Growth Factor and Stem Cell Factor; Zsebo et al., 1990; Anderson et al., 1990; Huang et al., 1990; Besmer et al., 1993).

Despite the long-standing availability of mouse *Kit* and *Steel* mutants, questions remain about the roles of these genes in the development of the melanocyte lineage. During normal development in mouse, NC cells leave the dorsal neural tube and shortly thereafter a subpopulation of these cells begins to express Kit as well as early markers of melanocyte differentiation (e.g., the transcription factor Mitf and the melanin synthesis enzyme Dct/Trp2; Steel et al., 1992; Wehrle-Haller and Weston, 1995; Bernex et al., 1996; MacKenzie et al., 1997; Opdecamp et al., 1997). These melanoblasts then disperse along migratory pathways lined by cells expressing SLF, and ultimately enter the epidermis where they proliferate, colonize hair follicles, and begin to synthesize melanin (Serbedzija et al., 1990; Erickson and Perris, 1993; Wehrle-Haller and Weston,

1995; Yoshida et al., 1996; Reedy et al., 1998). In contrast, mutations in *Kit* or *Steel* lead to an early failure in melanocyte development: fewer melanoblasts arise from the NC, they fail to disperse into the periphery, and ultimately they disappear prior to overt differentiation (Besmer et al., 1993; Wehrle-Haller and Weston, 1995; Cable et al., 1995; Bernex et al., 1996; Spritz, 1998). A priori, these defects are consistent with several possible roles for Kit signaling in the morphogenesis or differentiation of cells in the NC-melanocyte lineage.

One potential morphogenetic role for *kit* is to promote the migration of melanocytes or their precursors as they disperse from the NC and home to various destinations throughout the skin. To date, however, various analyses of melanoblast and melanocyte distributions have provided conflicting evidence regarding such a role. For example, presumptive melanoblasts are restricted to the vicinity of the dorsal neural tube in mice mutant for null alleles of *Kit* or *Steel* (Wehrle-Haller and Weston, 1995; Bernex et al., 1996), and melanoblasts and melanocytes occur in regions not normally occupied by these cells in mice expressing a *Steel* transgene ectopically (Kunisada et al., 1998). These results support a model in which *kit* promotes both the initial dispersal and subsequent migration of melanoblasts. In contrast, studies of other alleles of *Kit* or *Steel* in mouse, and other anatomical regions, have failed to identify differences in the relative distributions of melanoblasts in comparison with wild type (Cable et al., 1995; MacKenzie et al., 1997). Moreover, avian melanoblasts do not express *kit* until well after their migration is underway, 2 days after leaving the NC (Lecoin et al., 1995). These observations raise the possibility that Kit signaling either is not essential for melanoblast dispersal and migration, or that any such roles are secondary to roles in other morphogenetic processes, such as melanoblast maintenance (see below). A function for *kit* in promoting the normal migration of cells in the melanocyte lineage thus remains largely unresolved.

A role for *kit* in promoting the survival of melanoblasts and melanocytes also remains somewhat ambiguous. Several studies of *Kit* or *Steel* mutant mice have shown that, although cells expressing markers of the melanocyte lineage are present at early stages, such cells are not found during later development (Cable et al., 1995; Wehrle-Haller and Weston, 1995; MacKenzie et al., 1997). Likewise, studies of cultured NC cells have demonstrated a decline or disappearance of melanocyte precursors when SLF is withheld or when Kit function is perturbed immunologically (Murphy et al., 1992; Morrison-Graham and Weston, 1993; Reid et al., 1995; Langtimm-Sedlak et al., 1996). These findings have been interpreted to reflect an essential role for *kit* in providing trophic support that maintains cells in the melanocyte lineage. Nevertheless, direct evidence of programmed cell death typically has not been provided, and thus the ultimate fate of melanocyte precursors in the absence of *kit* activity remains unclear. For instance, it remains possible that cells initially expressing markers of the melanocyte lineage – or their precursors – respond to the absence of Kit signaling by assuming alternative fates (e.g., as stem cells that either are unspecified or arrested at a stage of differentiation prior to the expression of melanoblast-specific markers; cf. Grichnik et al., 1996; Kunisada et al., 1998). Or, effects on melanoblast and melanocyte numbers could largely reflect *kit*-dependent changes in the proliferative activity of these cells (e.g., Lahav et al., 1994; Reid et al., 1995; MacKenzie et al., 1997).

The pigment pattern of the zebrafish *Danio rerio* provides an opportunity to extend studies of *kit* function: the zebrafish embryo is particularly accessible due to its transparency and oviparous development, and a large number of mutations affecting NC-derived pigment cells have been isolated (Kelsh et al., 1996; Odenthal et al., 1996). Intriguingly, several of these mutants exhibit melanocyte deficiencies that are reminiscent of *kit* mutants in mammals. For example, *sparse*, *rose* and *leopard* mutant adult zebrafish each exhibit only half the normal complement of stripe melanocytes (Johnson et al., 1995), raising the possibility that one of these mutants might correspond to a zebrafish orthologue of *kit*. Identification of such a mutant would, in turn, allow an independent test in zebrafish of roles proposed for *kit* activity based on studies of melanocyte development in mammals.

In the present investigation, we show that a zebrafish orthologue of *kit* maps to the same chromosomal region as *sparse*, and that lesions in *kit* cDNAs are present in multiple alleles of *sparse*, demonstrating the correspondence of *kit* and *sparse*. We then examine melanocyte development in *sparse* mutant embryos to test roles for *kit* in promoting the migration and survival of these cells. Finally, we test whether zebrafish *kit* is required for hematopoiesis or PGC development, as in mouse. Our findings provide new insights into roles for *kit* during the morphogenesis of NC-derived melanocytes, and also reveal evolutionary divergence in *kit* expression and function across phylogenetic lineages.

MATERIALS AND METHODS

Fish rearing and staging

Fish were maintained at a constant temperature of 25°C with a 14L:10D photoperiod. To facilitate comparison with other studies, we present all stages relative to development at the standard temperature of 28.5°C, using the conversion factor, hours at 28.5°C=0.81 × hours at 25°C (Kimmel et al., 1995).

Isolation and mapping of *kit* and identification of lesions in *kit* cDNAs

To map *kit* relative to *sparse*, we cloned a partial *kit* cDNA (644 bp) from wild-type *D. rerio*, using nested PCR across the 'kinase insert' domain with degenerate primers (all primer sequences available on request). We then used sequence from this fragment to design primers (forward: 5′-GCATCCTGCTGTGGGAGATC-3′; reverse: 5′-TCCCTGGATATGGACTACTC-3′) spanning the predicted location of intron 18 of the *kit* gene (Giebel and Spritz, 1991), and we tested for the presence of this sequence tagged site (KITin18) in genomic DNA isolated from wild-type embryos and embryos homozygous for γ-ray-induced chromosomal deficiencies. Upon mapping *kit* to the vicinity of *sparse* (see Results), we cloned a presumptive full-length wild-type *kit* cDNA by rapid amplification of cDNA ends (Marathon cDNA Amplification Kit; Clontech, Palo Alto, CA), using nested and overlapping primers in conjunction with Clontech RACE adapter primer AP1. Using sequences from these fragments, we designed primers that amplified a 3,545 bp *kit* cDNA that we subcloned into the TA-cloning vector pKRX (Schutte et al., 1997). To verify that the zebrafish gene that we isolated is orthologous to amniote *kit* genes, we reconstructed a phylogeny that included the gene identified in this study and other receptor tyrosine kinases (Rousset et al., 1995). Sequences were aligned with the PileUp program of the GCG software package (Genetics Computer Group, Madison WI), inspected by eye, then parsimony-based phylogenetic analyses were performed using the heuristic search option in PAUP 4.0.0d55 for Unix (Swofford, 1999) with 1000 bootstrap replicates, treating type V receptor tyrosine kinases as an outgroup. Analyses of amino acid

and nucleotide sequences yielded identical tree topologies and only the latter are presented here.

To search for *kit* lesions, we amplified multiple, overlapping *kit* fragments from first-strand cDNAs prepared from *sparse* mutant and wild-type strain SJD backgrounds. One allele of *sparse*, spa^{b5}, arose spontaneously in stocks at the University of Oregon and additional alleles were isolated in the SJD background by non-complementation of spa^{b5} using the point mutagen *N*-ethyl *N*-nitrosourea (Solnica-Krezel et al., 1994); all alleles are recessive and homozygous viable. We obtained multiple forward and reverse sequences by independent amplifications from 2-12 individuals of each genotype. Sequencing was performed using AmpliTaq FS or BigDye (Perkin Elmer, Norwalk, CT) dye-terminator sequencing chemistry and ABI373 and ABI377 automated sequencers.

In situ hybridization and imaging

In situ hybridization with antisense DIG- or fluorescein-labeled riboprobes was performed as described (Jowett and Yan, 1996), using 68°C hybridization and stringency washes, alkaline phosphatase-conjugated secondary antibodies and NBT/BCIP (Boehringer Mannheim, Indianapolis, IN) and FastRed (Sigma, St. Louis, MO) substrates. Sense and unrelated probes were used as controls. *kit* riboprobes were prepared using a full-length *kit* cDNA. A cDNA of the zebrafish melanoblast marker, the *Mitf*-related gene *nacre*, was generously provided by J. Lister and D. Raible, a cDNA for the PGC marker *vasa* (Yoon et al., 1997) was a gift of C. Yoon and N. Hopkins, and a cDNA for the hematopoietic lineage marker *GATA2* was provided by D. Stanier. For some rounds of staining, we treated embryos with 10 mM phenylthiourea (PTU) to partially inhibit melanin synthesis, thereby allowing easier visualization of blue NBT/BCIP and red FastRed precipitates in otherwise dark melanocytes. All images were captured using a Spot digital camera or Panasonic CCD camera on Zeiss compound epifluorescence microscopes or an Olympus stereomicroscope. Images were processed to correct brightness or color balance in Adobe Photoshop.

Cell transplantation

To test for cell-autonomous roles of *kit* in promoting melanocyte migration and survival, we constructed wild-type–*sparse* chimeras by transplanting (Ho and Kane, 1990) 20-100 cells between wild-type and spa^{b5} blastula stage embryos. To identify donor-derived cells in *sparse* mutant hosts, we used hosts doubly mutant for spa^{b5} and the golden (gol^{b1}) mutation, which results in lightly pigmented melanocytes that are easily distinguishable from wild type, allowing unambiguous identification of both donor and host melanocytes in chimeras. To identify *sparse* mutant cells in wild-type hosts, we used hosts homozygous for the *albino* (alb^{b4}) mutation. Like *golden*, *albino* acts cell autonomously to cause faintly pigmented melanocytes, with no gross changes in melanocyte numbers or distributions (e.g., Streisinger et al., 1986; Lin et al., 1992; Kelsh et al., 1996; S. L. J., unpublished data). Similarly, melanocyte distributions in *sparse; albino* and *sparse; golden* double mutants are similar to that of *sparse* single mutants (S. L. J., unpublished data), suggesting no genetic interaction that would interfere with the interpretation of cell distibutions in chimeric embryos. Accordingly, choice of *golden* or *albino* mutations was dictated largely by the availability of stocks as the experiments were performed.

Analyses of melanocyte distributions and death

To assess roles for *kit* in promoting melanocyte migration, we analyzed the distributions of melanocytes in embryos and early larvae. Melanocytes were examined after immersing fish in 1 mg/ml epinephrine, which rapidly induces the movement of melanosomes from peripheral processes towards the cell body in fish older than ~4 days, and thereby facilitates distinguishing adjacent cells. Melanocytes in the dorsal stripe posterior to the middle of the eye, or within ~125 μm anterior or posterior to the otocysts were classified as 'non-dispersed' (i.e., at the level of the premigratory NC, see below); all other melanocytes were classified as 'dispersed.'

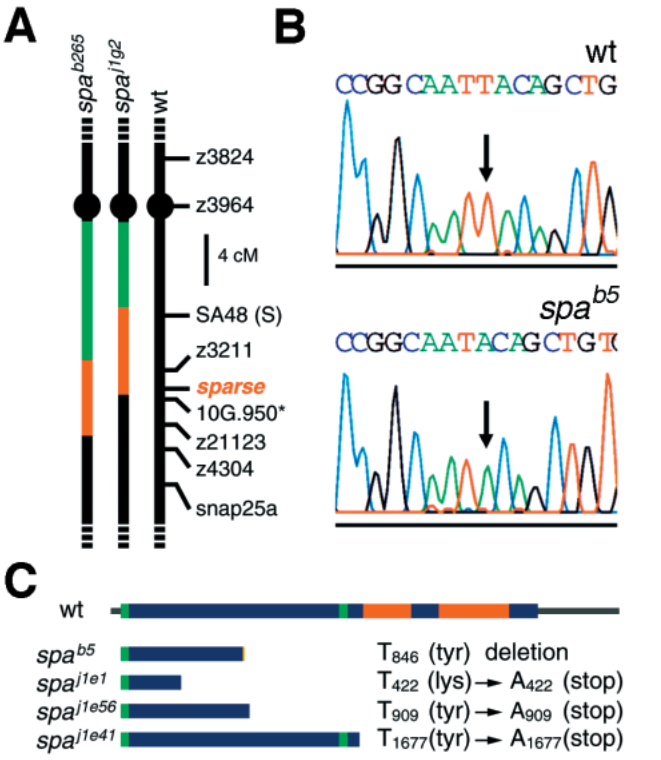

Fig. 1. Zebrafish *sparse* is allelic to *kit*. (A) Deletion mapping places *kit* in the vicinity of *sparse*. Shown are local genetic linkage maps of wild-type (wt) linkage group 20 (right chromosome; Johnson et al., 1996) and the γ-ray-induced chromosomal deficiencies, spa^{b265} and spa^{j1g2}; regions deleted are indicated in red and regions possibly deleted are indicated in green. KITin18 amplified from wild-type genomic DNA, but not homozygous spa^{b265} or spa^{j1g2} genomic DNA. (B,C) Cloning of wild-type *kit* cDNA and analysis of *kit* lesions in *sparse* mutant backgrounds. Using sequence from a *kit* fragment obtained by degenerate PCR, we cloned a presumptive full-length wild-type *kit* cDNA [total size: 3545 nt; open reading frame (ORF): 2928 nt], and also sequenced *kit* ORFs amplified from wild-type and homozygous *sparse* mutant backgrounds. (B) Partial electropherogram showing wild-type *kit* sequence (upper) and a 1 nt deletion in the spa^{b5} mutant background (lower). (C) Comparison of wild-type and *sparse* mutant *kit* cDNAs. Open reading frames are indicated in blue, signal and transmembrane domains are green, tyrosine kinase domains are red, and 5′ and 3′ untranslated regions in wild type are grey. Truncations in *sparse* ORFs are indicated, as is the location of each lesion relative to the initiator ATG for each of four alleles: spa^{b5}, spa^{j1e1}, spa^{j1e41} and spa^{j1e56}. For spa^{b5}, a short region encoding novel amino acids following the deletion and frameshift are shown in orange. A fifth allele, spa^{j1e15} did not exhibit a lesion within the ORF; the nature of this mutation remains unresolved.

To determine whether melanocytes undergo programmed cell death in the absence of *kit* activity, we used terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL; Zhang and Galileo, 1997; Smith and Cartwright, 1997; Wakamatsu et al., 1998). In brief, larvae were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) overnight at 4°C, then transferred to 100% methanol at –20°C for storage. Prior to staining, larvae were rehydrated through a graded methanol series, rinsed twice in PBS containing 0.1% Tween-20 (PBST), permeabilized for 20 minutes in PBS containing 0.1% Triton X-100 on a rocking platform

and rinsed twice in PBST. Larvae then were incubated for 1.5 hours at 37°C in a solution containing 0.2 U/μl TdT, TdT reaction buffer (Promega, Madison, WI), 100 μm dNTP and 1 mM fluorescein-labeled dUTP (Boehringer Mannheim), with gentle agitation every 15 minutes. After washing extensively in PBST, fluorescein-labeled dUTP was detected as described (Jowett and Yan, 1996) using anti-fluorescein, alkaline phosphatase-conjugated Fab fragments and FastRed substrate. After stopping the staining reaction and stripping away protruding yolk, larvae were mounted in glycerol between glass coverslips and TUNEL-stained melanocytes were identified by FastRed fluorescence under rhodamine epifluorescent illumination using a 63× oil immersion objective.

We tested whether melanocytes are extruded from the skin in the absence of *kit* activity by rearing wild-type or *sparse* mutant larvae over 'carpets' of white Hybond N+ nylon membrane (Amersham, Piscataway, NJ). Three replicates each of 100 wild-type or *spa*^{b5} mutant larvae were maintained above membranes in 35 mm plastic Petri dishes beginning at 3 days. A fine mesh was placed between larvae and membranes to prevent fish from contacting the membranes directly. At 24 hour intervals, larvae were transferred to new dishes with fresh membranes, exposed membranes were dried, and melanocyte carcasses were counted on each of three representative 4 mm^2 areas per exposed membrane. These counts were used to estimate the mean number of melanocyte carcasses recovered per larva per day (mean carcasses mm^{-2} × total membrane area × 100^{-1} larvae). These values then were summed across days to estimate the mean total number of melanocyte carcasses recovered per larva through the course of the experiment. Our counts underestimate the actual numbers of melanocytes extruded, however, as many melanocytes failed to stick to the membranes and were lost in the medium.

Classification of hematopoietic cell types

We assessed hematopoietic defects in *spa*^{b5} mutant embryos at 48 hours and 5 days by direct examination of peripheral blood flow ventral to the heart and in the tail, and also by o-dianisidine staining of erythropoietic cell populations (Iuchi and Yamamoto, 1983; also see: Detrich et al., 1995; Ransom et al., 1996). To determine whether *spa*^{b5} mutant adults exhibit defects in hematopoiesis, we assessed proportions of different hematopoietic cell types in smears of peripheral blood (obtained by cardiac puncture with heparanized capillary tubes), as well as touch preparations of dissected kidney (the major hematopoietic organ of the adult fish) and spleen in each of ten *spa*^{b5}/+ and *spa*^{b5} fish, and classified ~1000 cells per preparation.

Statistical methods

For analyses of proportions in assessing melanocyte distributions and hematopoietic lineages, we used *G*-tests for goodness of fit (Sokal and Rohlf, 1981) and adjusted significance levels to control for multiple comparisons using the sequential Bonferonni method (Rice, 1989). We used two-way analyses of variance (Sokal and Rohlf, 1981) to assess differences in melanocyte and PGC numbers across days in wild-type and *sparse* mutants. Statistical analyses were performed with JMP software for the Apple Macintosh (SAS Institute Inc., Cary NC).

RESULTS

Zebrafish *kit* corresponds to *sparse*

As a first step in assessing the correspondence of *kit* and previously isolated pigment pattern mutants, we cloned a partial *kit* cDNA using degenerate PCR and mapped *kit* with chromosomal deletions. Primers spanning the predicted location of intron 18 of the *kit* gene amplified a ~160 bp fragment from wild-type genomic DNA. In contrast, this fragment did not amplify from genomic DNA of embryos homozygous for either of two γ-ray-induced chromosomal deficiencies that include the *sparse* locus, *spa*^{b265} or *spa*^{j1g2} (Fig. 1A). These data place *kit* on linkage group 20 (Johnson et al., 1996) in the vicinity of *sparse*, and confirm *kit* as a candidate for *sparse*.

To test the correspondence of *kit* and *sparse*, we then cloned a presumptive full-length wild-type *kit* cDNA (GenBank accession number: AF153446) and sequenced open reading frames (ORFs) from wild-type and homozygous *sparse* mutant backgrounds. BLAST searches and phylogenetic analyses indicate the zebrafish gene that we have cloned is more similar to tetrapod *kit* genes than other genes encoding type III receptor tyrosine kinases (e.g., c-*fms*, *FLT3*, *PDGFRA*, Rousset et al., 1995; Fig. 2). In *kit* cDNAs amplified from *spa*^{b5} mutants, we found a 1 nt deletion resulting in a frameshift and premature stop codon (Fig. 1B,C). Consistent with the correspondence of *kit* and *sparse*, sequence analysis revealed no recombinants between this lesion and *spa*^{b5} in a 476 haploid embryo mapping panel and in situ hybridization showed greatly diminished levels of *kit* mRNA in *spa*^{b5} mutant embryos (not shown). In each of three additional ENU-induced *sparse* mutants, *kit* cDNAs exhibited different T→A

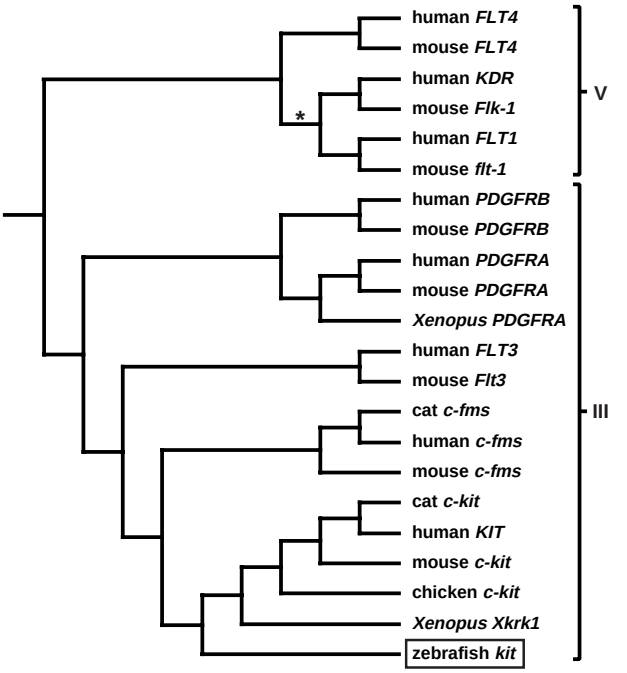

Fig. 2. Phylogenetic analysis demonstrates that zebrafish *kit* is orthologous to tetrapod *kit* genes. Shown is a maximum parsimony tree of selected type III and type V receptor tyrosine kinases, based on analyses of 2907 nucleotide positions of which 2890 were informative. Bootstrap support for all nodes was 100%, with the exception of the node leading to Flt-1 and KDR/Flk-1 genes (*), which was 94%. Analyses of nucleotide and amino acid sequences (not shown) both indicate that the zebrafish gene isolated here is more similar to *kit*-related genes of other taxa (including *Xenopus Xkrk-1*; Baker et al., 1995) than other type III receptor tyrosine kinases. Corresponding GenBank accession numbers from top to bottom in the figure are: 4503752; 293780; 3132832; 50976; 3132830; 2809068; 4505682; 53618; 189733; 200274; 214652; 406322; 50978; 163854; 29899; 50980; 913503; 4557694; 50423; 303532; 763033; 153446.

transversions resulting in premature stop codons (Fig. 1C). These data confirm that *kit* is allelic to *sparse.* Since all four mutant *kit* transcripts lack regions coding for transmembrane domains, tyrosine kinase domains, or both, all are likely to represent null alleles. Consistent with this interpretation, transheterozygotes of *spa^{b5}* over deficiencies of *sparse* are phenotypically identical to *spa^{b5}* homozygotes.

Zebrafish *kit* is expressed by the NC-melanocyte lineage

In contrast to mouse, homozygous *sparse* mutant zebrafish develop numerous melanocytes (Johnson et al., 1995; Kelsh et al., 1996; see below), demonstrating that *kit* is not essential for the differentiation of these cells. This observation, and the report that a *Xenopus kit*-like receptor is not expressed by melanocytes (Baker et al., 1995; see Discussion), raised the possibility that zebrafish *kit* might not be expressed by cells in the melanocyte lineage, in which case pigment pattern defects in *sparse* mutants would represent only an indirect effect of *kit* loss-of-function. To determine whether *kit* is expressed by melanocytes and their precursors in zebrafish, we examined the distribution of *kit* mRNA by in situ hybridization. These analyses revealed *kit* expression in dispersing neural crest cells, cells that co-express the *Mitf*-related gene *nacre*, and thus are likely to be melanoblasts (J. Lister and D. Raible, personal communication), as well as lightly pigmented and fully differentiated melanocytes (Fig. 3A-D). Thus, zebrafish *kit* is expressed by cells of the NC-melanocyte lineage, as in amniotes.

kit promotes the normal migration of cells in the melanocyte lineage

Because zebrafish *sparse* mutant melanocytes differentiate and are fully melanized, we can use this pigmentation as a marker to help address roles for *kit* in melanocyte morphogenesis. To investigate roles for zebrafish *kit* in the migration of cells in the NC-melanocyte lineage, we examined the distributions of melanocytes in zebrafish embryos. At 60 hours, *spa^{b5}* mutant embryos exhibit 58% as many melanocytes as wild-type and a greater proportion of these cells is found dorsally in the vicinity of the neural tube and otocysts ('non-dispersed' melanocytes in *spa^{b5}* and wild-type embryos: 72%, 52%, respectively; G=153.8, P<0.0001; n=4400 melanocytes, 12 embryos; Figs 4A,B, 5A). More striking differences in melanocyte distributions are found over the anterior head and behind the ear. In wild-type embryos, melanoblasts arise in large numbers from NC immediately posterior to the otocysts and at the lateral margin of the midbrain-hindbrain boundary; these cells then migrate dorsally and anteriorly (data not shown; D. Raible, personal communication) so that, by 60 hours, melanocytes are essentially absent anterior and posterior to the otocysts but are abundant over the anterior head (Fig. 4A; also see: Schilling and Kimmel, 1994; Bernex et al., 1996; MacKenzie et al., 1997). In *spa^{b5}* mutant embryos, however, melanocytes and melanoblasts are absent from the anterior head, and instead accumulate at or near their NC origin, posterior to the otocysts and at the lateral margins of the branchial arches (Fig. 4B). This accumulation of melanocytes indicates that the difference in relative melanocyte distributions between wild-type and *sparse* mutant embryos does not result from the selective death of a subpopulation of melanocyte precursors that normally migrates from the NC. Rather, the different melanocyte patterns between wild-type and *sparse* mutant embryos suggest that melanocytes fail to disperse properly from the NC in the absence of *kit* activity.

The relatively restricted distribution of melanocytes in *spa^{b5}* mutant embryos could reflect a direct, cell autonomous role for *kit* in promoting the migration of these cells or their precursors. For example, signal transduction through Kit might upregulate the expression of genes required for cell motility, or interactions between Kit and its ligand might serve adhesive or haptotactic functions (see Discussion). Alternatively, the different distribution of melanocytes in *spa^{b5}* could result from a less direct, cell non-autonomous role for *kit*. For example, NC cells of other species exhibit contact-stimulated migration and contact inhibition of movement, and these morphogenetic behaviors are thought to contribute to the normal dispersal of these cells from the neural tube (Erickson, 1986; Tucker and Erickson, 1986a; Thomas and Yamada, 1992). Thus, fewer cells in the NC-melanocyte lineage in *spa^{b5}* mutants might reduce 'population pressure' and the likelihood of melanoblasts dispersing to the periphery. To further test a role for *kit* in promoting the migration of melanocytes or their precursors, and to distinguish between cell autonomous and non-autonomous effects, we transplanted cells between wild-type and *sparse* mutant backgrounds. If *kit* promotes migration through a cell autonomous mechanism, donor cells should behave according to their genotype in host embryos. In contrast, if *kit* does not contribute to migration, or promotes migration through a cell non-autonomous mechanism, donor cells should behave according to the genotype of host embryos. At 60 hours, wild-type-derived melanocytes in *sparse* mutant hosts were distributed in a wild-type pattern: donor melanocytes were abundant over the anterior head but typically were not found lateral to the otocyst (Fig. 4C). In contrast, donor *sparse* mutant melanocytes in wild-type hosts were distributed in a *sparse* pattern: none were present over the anterior head, but a large proportion were found immediately posterior to the otocyst (Fig. 4D). These data are consistent with cell autonomous activity of zebrafish *kit* in promoting the normal migration of cells in the melanocyte lineage.

kit is essential for survival of embryonic melanocytes

Previous studies suggesting trophic roles for *kit* in maintaining amniote melanocyte precursors typically have relied on the disappearance of labeled cells, rather than direct evidence of programmed cell death. To assess roles for zebrafish *kit* in maintaining cells in the melanocyte lineage, we examined the fate of embryonic melanocytes during later larval development. In wild type, melanocyte numbers remain relatively constant (Figs 5A, 6A), and these cells are found spread beneath the epidermis (Fig. 6E). In contrast, *spa^{b5}* mutants, which initially have numerous melanocytes, lose virtually all of these cells by 11 days (Figs 4B, 5A, 6B); the melanocytes that are present during this time are rounded and found within the plane of the epidermis (Fig. 6F). The different melanocyte morphologies between wild-type and *spa^{b5}* mutants reflect a cell autonomous effect of *kit*: in chimeric larvae at 7 days, donor wild-type melanocytes exhibited a spread morphology in *spa^{b5}* mutant hosts, whereas *spa^{b5}* mutant melanocytes exhibited a

contracted, punctate morphology in wild-type hosts (data not shown).

To assess whether the difference between wild-type and *spa*^{b5} larvae in melanocyte number, tissue localization and morphology reflect programmed cell death in the absence of *kit* activity, we used TUNEL to identify apoptotic melanocytes. In wild-type larvae, melanocytes did not stain with TUNEL (Fig. 5B). In *spa*^{b5} mutant larvae, however, some rounded melanocytes within the epidermis were found to stain with TUNEL beginning ~4 days and continuing at least through 11 days (Figs 5B, 6G,H). These data provide definitive evidence for a role of *kit* in melanocyte maintenance.

Finally, the location of *spa*^{b5} melanocytes within the epidermis suggested that the gradual loss of these cells between 3 and 11 days might reflect their extrusion from the skin. To test this hypothesis, we reared free-swimming wild-type or *spa*^{b5} mutant larvae over 'carpets' of charged nylon membrane. Membranes beneath wild-type larvae remained unmarked (Figs 5C, 6I), whereas membranes beneath *spa*^{b5} mutant larvae collected spots of melanin of the same size as melanocytes remaining in *spa*^{b5} larvae, with a time course coinciding with the disappearance of melanocytes from the fish (Figs 5C, 6J). These data indicate that, in the absence of *kit* activity, embryonic zebrafish melanocytes die and are extruded from the skin, though we do not exclude the possibility that some melanocytes also may be consumed by macrophages or other cells (e.g., Dickman et al., 1988).

kit-independent cell lineages

Besides melanocytes derived from the NC, murine *Kit* mutants have defects in two additional stem cell populations: hematopoietic progenitors and PGCs. Mice homozygous for null alleles of *Kit* are severely anemic and also exhibit a loss of PGCs during their migration to the genital ridges; less severe and viable alleles also may be anemic and sterile, or can exhibit impaired fertility owing to defects in oocyte and spermatogonial development, or both (Russell and Fondal, 1951; Mintz and Russell, 1957; Geissler et al., 1981; Besmer et al., 1993; Broudy, 1997; Lyman and Jacobsen, 1998; Vincent et al., 1998). To assess roles for zebrafish *kit* in hematopoiesis or PGC development, we used in situ hybridization and quantitative analyses of hematopoietic and primordial germ cell lineages. At embryonic stages, zebrafish *kit* is expressed in mesodermal cells that give rise to hematopoietic precursors of the intermediate cell mass as evidenced by anatomical position (Fig. 3E-G) as well as co-expression of the hematopoietic lineage marker GATA2 by

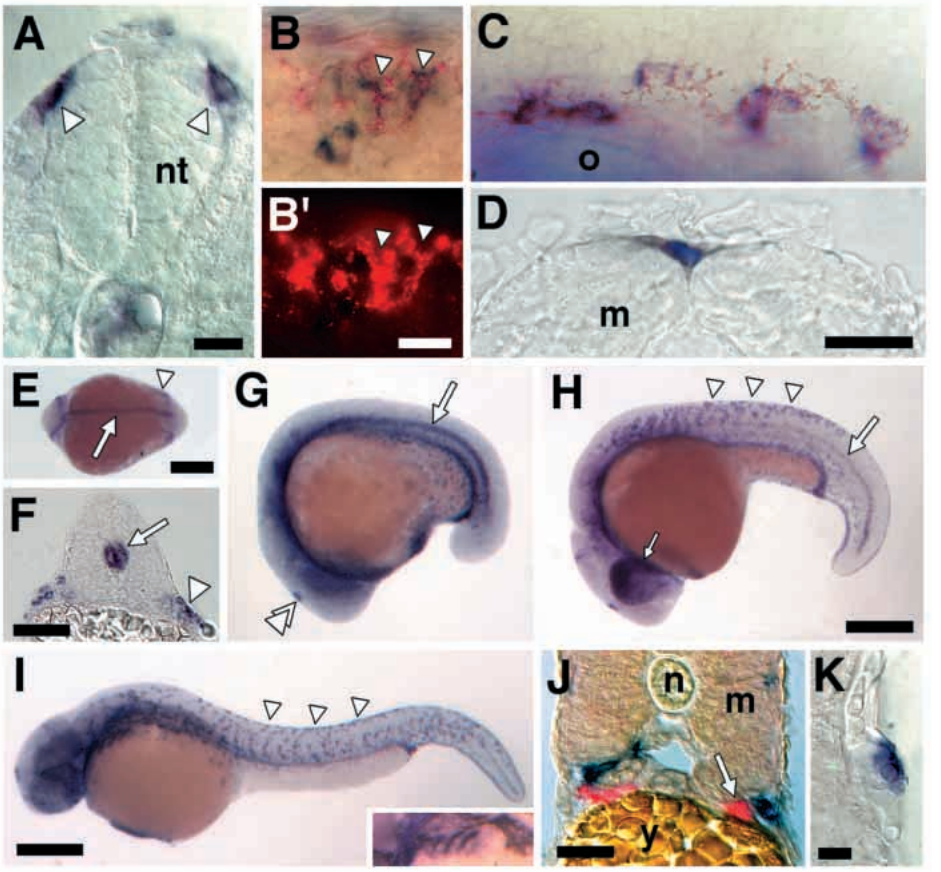

Fig. 3. *kit* is expressed by NC-derived melanoblasts and melanocytes during wild-type zebrafish development. (A) Transverse cryosection showing NC cells (arrowheads) expressing *kit* as they disperse from the neural tube (nt) (18 hours cryosection). (B) Two-color in situ hybridization reveals cells expressing both *kit* (blue, in B) and *nacre* (red, in B, B′) in the dorsolateral NC migratory pathway (24 hours whole mount). (C) Lightly pigmented melanocytes expressing *kit* dorsal to the otocyst (o) (30 hours whole mount). (D) Fully differentiated melanocyte in the dorsal stripe expressing *kit* (12 day larva, cryosection); m, myotome (treated with PTU to partially inhibit melanin synthesis and better visualize *kit* staining). (E-K) *kit* also is expressed by an array of other cell types. (E) *kit* expression in notochord (arrow) and lateral mesodermal cells that are hematopoietic precursors and will form the intermediate cell mass (arrowhead; Detrich et al., 1995) (14 hours, whole mount dorsal view, anterior to left). (F) Notochord (arrow) and lateral mesodermal staining (arrowhead; 18 hours, transverse cryosection). (G) Notochord staining and staining in the pineal gland (double arrowhead; 17 hours, whole mount). (H) Dispersing, *kit*-expressing NC cells in the trunk (arrowheads), retinal staining in the eye (small arrow) and posterior notochord staining (large arrow; 22 hours, whole mount). (I) *kit*-expressing NC cells in the trunk (arrowheads) and branchial arches (inset; whole mount, 30 hours). (J) Zebrafish PGCs were not observed to express *kit* between 14 hours and 6.5 days. Shown are merged fluorescence and brightfield views of a transverse cryosection with *vasa*-stained PGCs (red) that do not stain for *kit* (blue, in adjacent melanocytes; 42 hours). n, notochord; y, yolk. (K) *kit* also is expressed in the lateral line sensory system (transverse cryosection, 3 day larva). In contrast to a *Xenopus kit*-like receptor (Baker et al., 1995) expressed in migrating lateral line primordia, zebrafish *kit* is expressed in differentiated sensory cells of lateral line neuromasts. Staining with DASPEI (Parichy, 1996a) does not reveal differences in midbody lateral line neuromast number between *spa*^{b5} and wild type ($F_{1,15}$=0.77, P=0.4). Scale bars: (A) 10 μm; (B) 5 μm; (C, D) 30 μm; (E) 500 μm; (F) 40 μm; (G, H) 500 μm; (I) 250 μm; (J) 30 μm; (K) 10 μm.

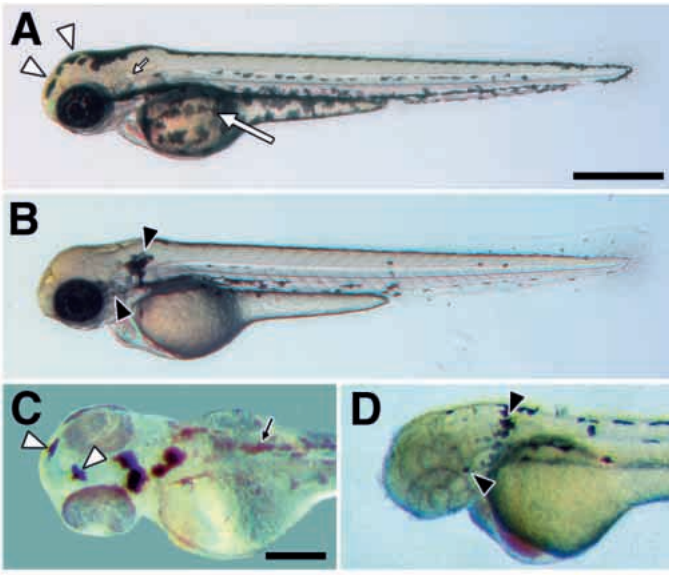

Fig. 4. Cell autonomy of *kit* in promoting melanocyte migration. (A) In wild type (60 hours), melanocytes occur in stripes dorsally at the apex of the myotomes, laterally near the horizontal myoseptum, ventrally at the ventral margin of the myotomes, and covering the yolk mass. (B) In *spa*^{b5} mutants (60 hours), however, a greater proportion of differentiated melanocytes is found dorsally as compared to wild type (see text); e.g., melanocytes covering the yolk in wild-type embryos (large arrow in A) are absent in *sparse* (B). Different melanocyte distributions also are evident on the head (Table 1). Melanocytes are abundant over the anterior head of wild-type embryos (12.9% of total; open arrowheads in A) but are virtually absent in *spa*^{b5} mutant embryos (0.6%); in contrast, melanocytes are abundant lateral to the otocyst (small arrow in A) in *spa*^{b5} mutant embryos (11.9%; closed arrowheads in B), but are essentially absent in wild-type embryos (0.3%). (C,D) Melanocyte distributions in wild-type–*sparse* chimeras reveal a cell autonomous role for *kit* in melanocyte migration. (C) Donor-derived wild-type melanocytes in *sparse* (*spa*^{b5}, *gol*^{b1}) hosts are abundant over the anterior head (12.8% of total transplanted melanocytes; open arrowheads) but not behind the ear (0.5%), as in wild-type embryos (*n*=20 chimeras, 415 melanocytes). Small arrow indicates lightly pigmented *spa*^{b5}, *gol*^{b1} host melanocytes. (D) *spa*^{b5} mutant melanocytes in wild-type (*spa*$^{+}$, *alb*^{b4}) hosts are absent from the anterior head (0%) and accumulate near the otocysts (20.7%; closed arrowhead), as in *sparse* (*n*=11 chimeras, 92 cells). Scale bars: (A,B) 500 μm; (C,D) 250 μm.

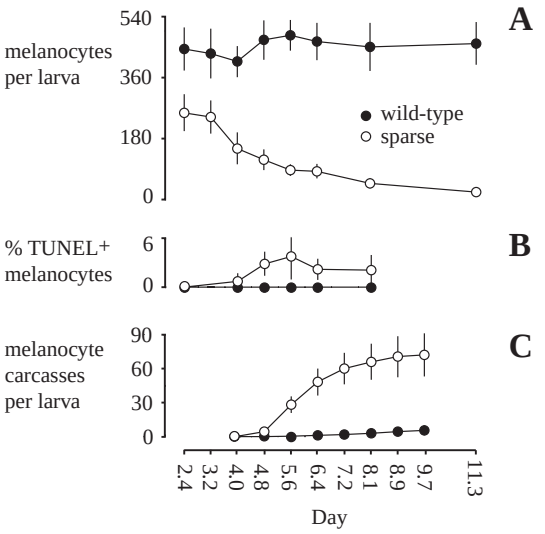

Fig. 5. Changes in melanocyte numbers (A), frequencies of apoptotic melanocytes (B) and collection of melanocyte carcasses on nylon membranes (C). (A) In wild type, melanocyte numbers do not differ among days during the first 11 days of development ($F_{7,35}$=1.20; P=0.3). In *spa*^{b5} mutants, fewer melanocytes than wild type are present at 2.4 days (60 hours; $F_{1,10}$=30.87; P<0.001) and these cells are lost over the next several days ($F_{7,33}$=45.8; P<0.0001). On each day, dorsal stripe melanocytes were counted in 5-7 larvae of each genotype. (B) TUNEL staining is evident in *spa*^{b5} mutant melanocytes by 4 days and persists at least through 8 days (*n*=43 larvae, 3903 melanocytes), but is not observed in wild-type melanocytes (*n*=15 larvae, 2025 melanocytes). TUNEL staining was assessed in dorsal stripe melanocytes in whole-mount, Fast-red-stained embryos. (C) The timing of melanocyte carcass recovery on nylon membranes corresponds to the time course of melanocyte loss from *spa*^{b5} mutant larvae. Error bars in A and B are 95% confidence intervals. Error bars in C are 95% confidence intervals, with intervals summed across days. Counts in A-C were gathered on different days using different groups of larvae and rearing densities.

some of these cells (not shown) (see Detrich et al., 1995). Nevertheless, visual examination of peripheral blood flow and o-dianisidine staining of erythropoietic cell populations in 48 hour embryos and 5 day larvae did not reveal any gross defects in *spa*^{b5} mutants as compared to wild type (data not shown). Likewise, examination of peripheral blood smears and classification of hematopoietic cell types in adults failed to demonstrate macrocytic anemia, as assessed by criteria used previously for identifying anemic mutants in zebrafish (Weinstein et al., 1996; Ransom et al., 1996); i.e., *spa*^{b5} mutant adults exhibited no gross deficits in hematopoietic cell numbers, no apparent alterations in blood cell morphology or blood coloration, and no overall increases in the proportions of immature cell types (Table 1). Small but statistically significant differences in proportions of some lineages in kidney and spleen may reflect minor effects attributable to *kit* loss of function, or differences in genetic background independent of *sparse*. Nevertheless, these data indicate that hematopoiesis proceeds essentially normally in zebrafish even in the absence of *kit* activity.

To test potential roles for zebrafish *kit* in PGC development, we examined the distribution of *vasa*-stained cells (Yoon et al., 1997) in wild-type and *spa*^{b5} mutant embryos and larvae. In contrast to mouse (Manova and Bachvarova, 1991; Bernex et al., 1996), two-color in situ hybridization failed to reveal *kit* expression in PGCs at any of 13 stages between 14 hours and 6.5 days (Fig. 3J), though we cannot rule out expression in this lineage at other stages, or at levels below the threshold of detection with this technique. To test for differences in PGC number between wild-type and *spa*^{b5} mutants, we counted *vasa*-stained cells on one side of embryos at 27 hours, 44 hours, 58 hours and 12 days. Two-way analysis of variance revealed no significant difference in PGC number between genotypes (pooled across days, wild type: mean=12.3, s.d.=4.66, *n*=66; *spa*^{b5}: mean=11.8, s.d.=5.82, *n*=95; $F_{1,153}$=0.794, P=0.4), and no significant genotype × day interaction ($F_{3,153}$=1.94, P=0.1), demonstrating that genotypes did not differ after controlling for overall changes in PGC number across days. Consistent with

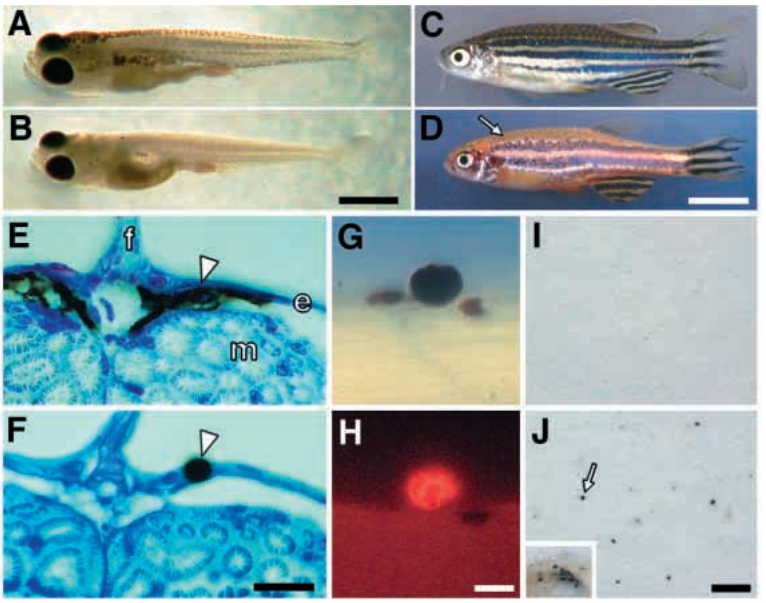

Fig. 6. Fate of melanocytes in *sparse* mutant larvae. (A,B) At 11 days, melanocytes persist in wild type (A), but are virtually absent from *spa*^{b5} mutants (B). (C,D) *sparse* mutant larvae recover melanocytes during the larval-to-adult transition. Thus, both wild-type (C) and *spa*^{b5} (D) adult zebrafish exhibit dark stripes of melanocytes and iridophores, alternating with light stripes of xanthophores and iridophores. Small arrow in D indicates the dorsal region where scale melanocytes are present in wild-type but absent in *spa*^{b5}. (E,F) In 7 day wild-type and *sparse* mutant larvae, cross sections (3 μm) of plastic-embedded specimens reveal a difference in melanocyte localization. In wild type (E), melanocytes (arrowhead) are spread beneath the epidermis; in *spa*^{b5} mutants (F), however, melanocytes are rounded within the plane of the epidermis (f, fin; e, epidermis; m, myotome). (G,H) *sparse* mutant melanocytes undergo apoptosis. TUNEL^{+} melanocytes are rounded and bulging outwards from the epidermis; this melanocyte morphology is not observed in wild-type larvae. Corresponding bright-field (G) and fluorescence (H) images of a *spa*^{b5} mutant, TUNEL^{+} melanocyte rounded within the epidermis near the base of the fin fold (5.5 days). (I,J) *sparse* mutant melanocytes are extruded from the epidermis as revealed by capture on nylon membranes. Membranes beneath free-swimming wild-type larvae is unmarked (I), whereas membranes beneath free-swimming *spa*^{b5} mutant larvae (J) collect spots of melanin (arrow) of the same size as melanocytes remaining in *sparse* larvae (inset: same magnification image showing melanocytes on *sparse* larva dried onto membrane). Scale bars: (A,B) 1 mm; (C,D) 8 mm; (E,F) 20 μm; (G,H) 12 μm; (I,J) 250 μm.

these data, *spa*^{b5} mutant adults exhibit no qualitative defects in fertility. Thus, zebrafish *kit* is not essential for PGC development.

Besides hematopoietic precursors, in situ hybridization reveals several additional cell types that express *kit* during embryogenesis yet exhibit no gross defects in *spa*^{b5} mutants, including notochord, NC-derived cells of the branchial arches, pineal gland, retina and mechanoreceptive sensory cells of lateral line neuromasts (Fig. 3E-I,K). In mouse, *Kit* is also expressed and required by intestinal pacemaker cells (Maeda et al., 1992; Bernex et al., 1996). We do not observe *kit* expression in the gut of zebrafish early larvae, nor are there apparent defects in digestive functions of *spa*^{b5} null mutant larvae or adults. Finally, in contrast to severe *Kit* mutants in mouse, examination of *spa*^{b5} mutant zebrafish reveals that not all melanocytes are *kit*-dependent: despite the absence of differentiated melanocytes in *spa*^{b5} larvae after 11 days (Fig. 6B), a new wave of melanocytes arises during the larval-to-adult transition, and these cells contribute to an adult pigment pattern (Fig. 6C,D; Johnson et al., 1995). Thus, although we cannot exclude the possibility of subtle non-lethal abnormalities in other lineages, our data suggest that zebrafish *kit* is required exclusively by a subset of pigment cells, in contrast to its requirement by multiple tissues and all NC-derived melanocytes in mouse.

DISCUSSION

We have shown that the zebrafish *sparse* mutant corresponds to an orthologue of *kit*, a gene long studied in amniotes for its roles in the development of melanocytes, hematopoietic stem cells and PGCs. Our analyses of *sparse* mutant embryos and larvae reveal that the zebrafish *kit* orthologue identified in this study plays essential roles in the morphogenesis of melanocytes that develop in embryos and give rise to the early larval pigment pattern. These findings are consistent with roles suggested for Kit signaling in the development of amniote melanocytes. In contrast to mouse, our analyses show that the zebrafish *kit* gene isolated here is not essential for hematopoiesis or the development of either PGCs or an additional class of melanocytes that contributes to the adult pigment pattern.

Zebrafish *kit* is essential for embryonic melanocyte morphogenesis

Melanocyte deficiencies in mouse *Kit* and *Steel* mutants are consistent with roles for Kit signaling in promoting the differentiation or morphogenesis of cells in the NC-melanocyte lineage. Although previous studies have suggested *kit* probably is not required for terminal stages of melanocyte differentiation, roles in earlier steps of specification (e.g., prior to the appearance of melanoblast markers) have not been excluded. Indeed, increased melanocyte numbers upon stimulation with SLF in vivo (Kunisada et al., 1998) and in vitro (Lahav et al., 1994; Guo et al., 1997; but see Langtimm-Sedlak et al., 1996) have been interpreted to reflect, in part, a role for *kit* in promoting the differentiation of cells in the melanocyte lineage. In zebrafish, the correspondence of *kit* and *sparse*, and the development of fully melanized melanocytes in *sparse* mutants, indicates that *kit* is not essential for melanocyte differentiation in this species. Nevertheless, as *sparse* mutant embryos and adults also exhibit deficiencies in the total numbers of melanocytes, we cannot exclude the possibility that Kit signaling might promote the differentiation of maximal numbers of melanocytes, either directly or through synergistic interactions with other factors (see below).

A potential role for *kit* in melanoblast migration remains controversial (see Introduction). If *kit* activity is required for

Table 1. Hematopoietic cell types in wild-type and sparse mutant adult zebrafish

	Peripheral blood		Kidney			Spleen		
Cell type	Wild type (mean % ± s.d.)	*sparse* (mean % ± s.d.)	Wild type (mean % ± s.d.)	*sparse* (mean % ± s.d.)	*G*, *P*	Wild type (mean % ± s.d.)	*sparse* (mean % ± s.d.)	*G*, *P*
band	0	0	12.3±1.27	13.8±0.55	10.88, 0.0010*	0	0	•
basophil	0	0	0.3±0.11	0.2±0.10	4.79, 0.0287	0.2±0.14	0.5±0.21	8.15, 0.0043*
eosinophil	0	0	<0.1±0.00	<0.1±0	1.38, 0.2401	0.1±0.05	0.2±0.04	0.11, 0.7370
erythrocyte	99.96±0.05	99.97±0.05	•	•	•	70.4±1.30	68.4±1.07	9.93, 0.0016*
macrophage	0	0	0	0	•	5.5±0.44	7.1±0.48	23.68, 0.0001*
monocyte	0	0	3.2±0.36	6.3±0.61	111.34, 0.0001*	9.1±0.68	8.4±0.57	3.17, 0.0750
neutrophil	0	0	13.1±0.99	9.9±0.55	52.70, 0.0001*	14.7±1.20	15.6±1.23	2.92, 0.0874
proerythroblast	0	0	25.9±1.89	25.1±1.36	2.10, 0.1470	0	0	•
proerythrocyte	0.04±0.05	0.03±0.05	26.9±1.62	28.6±1.03	6.81, 0.0091	0	0	•
promyelocyte	0	0	18.2±0.99	16.2±0.80	14.66, 0.0001*	0	0	•

Mean percentage±s.d. of cell types observed in peripheral blood, kidney, or spleen of adult zebrafish (n = 10 fish per genotype). Only erythrocytes and proerythrocytes were observed in peripheral blood and their proportions did not differ between wild type and *spa*^{b5} mutants (G = 0.143, d.f. = 1, P = 0.7). Since contingency table analysis revealed minor differences in the proportions of blood types observed between wild type and *spa*^{b5} mutant zebrafish in kidney (G = 187.077, d.f. = 7, P < 0.0001) and spleen (G = 38.840, d.f. = 7, P < 0.0001), comparisons of proportions between wild type and *spa*^{b5} mutants are provided for each cell type; (*) indicates differences significant after controlling for multiple comparisons (see Statistical methods). We did not count erythrocytes in kidney preparations because of the proximity of the kidney to the dorsal aorta, which normally is ruptured during dissection, thus making it impossible to confidently assign mature erythroid cells to a particular hematopoietic compartment. Although Kit is essential for the development of mammalian mast cells, these have not been identified in zebrafish, and we have not observed unmelanized *kit*-expressing cells in the skin of zebrafish larvae that might represent a previously cryptic mast cell population.

promoting the normal migration of these cells, a reasonable expectation is that the relative distributions of melanoblasts or melanocytes will differ between wild-type and *kit* mutants, independent of differences in total cell number. We demonstrate that, although *sparse* mutant zebrafish have fewer total melanocytes than wild-type overall, significantly greater proportions of *sparse* melanocytes are found in close proximity to their sites of origin, an effect especially apparent in the head of the embryo. These data support a role for Kit signaling in promoting the normal migration of zebrafish melanocytes. In this respect, our findings are similar to those of Wehrle-Haller and Weston (1995) and Bernex et al. (1996), who observed melanoblasts restricted to the vicinity of the NC in mouse *Kit* and *Steel* null mutants, as well as Kunisada et al. (1998), who observed an expansion of melanocyte distributions upon ectopic expression of a murine *Steel* transgene. Furthermore, our analyses of melanocyte distributions in wild-type–*sparse* mutant chimeras indicate that *kit* acts cell autonomously to promote the migration of cells in the melanocyte lineage: wild-type melanocytes were distributed in a wild-type pattern when transplanted to a *sparse* mutant background, whereas *sparse* mutant melanocytes were distributed in a *sparse* pattern when transplanted to a wild-type background. These findings may reflect a role for *kit* in upregulating genes required for melanocyte motility or adhesion, or direct adhesive interactions between Kit and its ligand. Consistent with these possibilities, SLF stimulation alters the expression of integrins by human melanocytes in vitro, and adhesive interactions between Kit and SLF – as well as chemotactic, haptotactic and chemokinetic effects of SLF – all have been demonstrated in cultured, non-NC-derived cell types (Adachi et al., 1992; Meininger et al., 1992; Hirata et al., 1993; Kinashi and Springer, 1994; Scott et al., 1994; Pesce et al., 1997; Kim and Broxmeyer, 1998). Finally, despite the apparent contribution of *kit* to the migration of cells in the melanocyte lineage, the extensive dispersal achieved by some zebrafish melanocytes even in a *kit* null mutant background differs from the nearly complete failure of melanoblast dispersal in *kit* null mutant mice. Thus, the relative importance of signaling through Kit for promoting melanoblast migration may vary among species, as further evidenced by the observation that avian NC cells do not begin to express *kit* until relatively late in their migration (Lecoin et al., 1995; but see below).

In addition to a role for *kit* in the migration of melanocytes or their precursors, our analyses demonstrate an essential role for *kit* in the maintenance of melanocytes that differentiate in embryos and contribute to the early larval pigment pattern: in the absence of *kit* activity, these melanocytes undergo programmed cell death and are extruded from the skin. These results confirm inferences from studies of amniotes that Kit signaling is required for maintaining cells in the melanocyte lineage (e.g., Wehrle-Haller and Weston, 1995; Langtimm-Sedlak et al., 1996), and are in accordance with the observation of ultrastructural features characteristic of apoptosis in murine melanocytes exposed to a Kit function-blocking antibody in vivo (Okura et al., 1995). In zebrafish, several mechanisms could be responsible for the death of melanocytes in *sparse* mutant larvae. For example, the absence of functional Kit could deprive cells of specific signals that normally prevent apoptosis. Or, melanocyte death could be a less direct effect of *kit* loss of function, perhaps resulting from a loss of cell-substratum adhesion. Further studies of the consequences of Kit signaling for downstream gene expression and morphogenetic behaviors (e.g., cell adhesion) should help to resolve these issues.

Finally, in addition to the death and disappearance of differentiated embryonic melanocytes, our analyses revealed an initial deficit in melanocyte numbers as early as 60 hours in *spa*^{b5} mutant embryos. This defect may indicate a role for *kit* in promoting the specification or maintenance of melanocyte precursors, or a failure of *kit*-dependent proliferation (e.g., Reid et al., 1995; MacKenzie et al., 1997). Consistent with the latter possibility, zebrafish melanocyte precursors divide on average only ~1.75 times between leaving the NC and establishing the early larval pattern (Raible and Eisen, 1994). Thus, a complete failure of these cells to proliferate would be

expected to result in only 57% of the normal complement of melanocytes, close to the 58% of normal that we observed in *spa*^{b5} mutant embryos. We are currently testing roles for zebrafish *kit* in promoting the maintenance and proliferation of melanocyte precursors.

Evolutionary divergence in *kit* function during development

Mouse *Kit* and *Steel* each have essential roles in hematopoiesis and PGC development (e.g., Mintz and Russell, 1957; Russell, 1979). Although our analyses reveal expression of zebrafish *kit* by hematopoietic precursors, *spa*^{b5} mutants were not grossly anemic and all hematopoietic lineages examined were both present and morphologically normal in adults. We did not detect expression of zebrafish *kit* in PGCs, and *spa*^{b5} mutants exhibited no apparent defects in PGC development or signs of impaired fertility. These results, coupled with other interspecific differences in the expression of *kit* orthologues suggest a marked evolutionary divergence in the roles for *kit*-related genes during development [cf. mouse: Bernex et al., 1996; chicken: Lecoin et al., 1995; *Xenopus kit*-like receptors *Xkrk1* and *Xkl-1*, which exhibit 95% amino acid identity to one another, and cluster together in phylogenetic reconstructions (not shown): Kao and Bernstein, 1995; Baker et al., 1995]. For example, all species examined thus far express *kit* orthologues in cells likely to be hematopoietic precursors, and all but *Xenopus* express *kit* in melanoblasts. In contrast, only zebrafish and *Xenopus* express *kit* in notochord whereas only chicken and *Xenopus* express *kit* in somitic mesoderm. Moreover, mouse and chicken (M. V. Reedy, personal communication) are the only species demonstrated to express *kit* in PGCs, and only chickens express *kit* in the limb apical ectodermal ridge. Although expression data alone do not adequately address functional requirements for *kit* activity, they point to a high degree of phylogenetic variation in presumed roles for *kit* orthologues during development, and may reflect evolutionary alterations in tissue-specific regulatory elements. Additional studies of *kit* and *steel* orthologues in other taxa (e.g., Mason et al., 1998; Parichy et al. 1999) should help to identify ancestral roles for these genes, as well as the molecular bases for evolutionary changes in their expression and function.

Finally, our demonstration that *sparse* corresponds to a zebrafish orthologue of *kit* also reveals that, in contrast to mouse, not all zebrafish pigment cells are *kit*-dependent. Although *spa*^{b5} mutants are virtually devoid of melanocytes by the middle of the larval period, a new population of melanocytes arises at metamorphosis, presumably from *kit*-independent precursors in the skin, and generates an adult pigment pattern that superficially resembles the wild-type pattern, though fewer melanocytes are found within stripes and dorsal scale melanocytes are absent (Johnson et al., 1995). Furthermore, NC cells of zebrafish and other ectothermic vertebrates give rise not only to melanocytes or melanophores, but also yellow xanthophores and silvery iridophores (Raible and Eisen, 1994; Parichy, 1996b), and neither of these populations exhibit gross defects in the absence of *kit* activity (though presently uncharacterized white cells in the tips of the fins are missing in *spa*^{b5} adults). The presence of multiple *kit*-independent blood, germ cell and pigment cell lineages in zebrafish may reflect a novel independence of these cells from the signal transduction mechanisms downstream of Kit. Alternatively, gene duplications (Rousset et al., 1995) or genome-wide duplications (Amores et al., 1998; Force et al., 1999) may have furnished paralogous genes that substitute for *kit* activity in these cell lineages. Indeed, the residual migratory capability and prolonged survival of melanocytes in *kit* null mutant zebrafish are consistent with partially redundant activity through an additional Kit-like receptor. Further examination of zebrafish mutations affecting pigment pattern or hematopoiesis (Weinstein et al., 1996; Ransom et al., 1996), and screening for additional *kit*-related genes, should illuminate the relative importance of changes in signal transduction mechanisms versus genomic architecture in shaping the cellular requirements for *kit* during vertebrate ontogeny.

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