

# Temporal and cellular requirements for Fms signaling during zebrafish adult pigment pattern development

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## SUMMARY

Ectothermic vertebrates exhibit a diverse array of adult pigment patterns. A common element of these patterns is alternating dark and light stripes each comprising different classes of neural crest-derived pigment cells. In the zebrafish, *Danio rerio*, alternating horizontal stripes of black melanophores and yellow xanthophores are a prominent feature of the adult pigment pattern. In *fms* mutant zebrafish, however, xanthophores fail to develop and melanophore stripes are severely disrupted. *fms* encodes a type III receptor tyrosine kinase expressed by xanthophores and their precursors and is the closest known homologue of *kit*, which has long been studied for roles in pigment pattern development in amniotes. In this study we assess the cellular and temporal requirements for Fms activity in promoting adult pigment pattern development. By transplanting cells between *fms* mutants and either wild-type or *nacre* mutant zebrafish, we show that *fms* acts autonomously to the xanthophore lineage in promoting the striped arrangement of adult melanophores. To identify critical periods for *fms* activity, we isolated temperature

sensitive alleles of *fms* and performed reciprocal temperature shift experiments at a range of stages from embryo to adult. These analyses demonstrate that Fms is essential for maintaining cells of the xanthophore lineage as well as maintaining the organization of melanophore stripes throughout development. Finally, we show that restoring Fms activity even at late larval stages allows essentially complete recovery of xanthophores and the development of a normal melanophore stripe pattern. Our findings suggest that *fms* is not required for establishing a population of precursor cells during embryogenesis but is required for recruiting pigment cell precursors to xanthophore fates, with concomitant effects on melanophore organization.

Movies and supplemental figures available on-line

Key words: Melanophore, Xanthophore, Pigment pattern, Zebrafish, *fms*, *Csf1r*, Neural crest

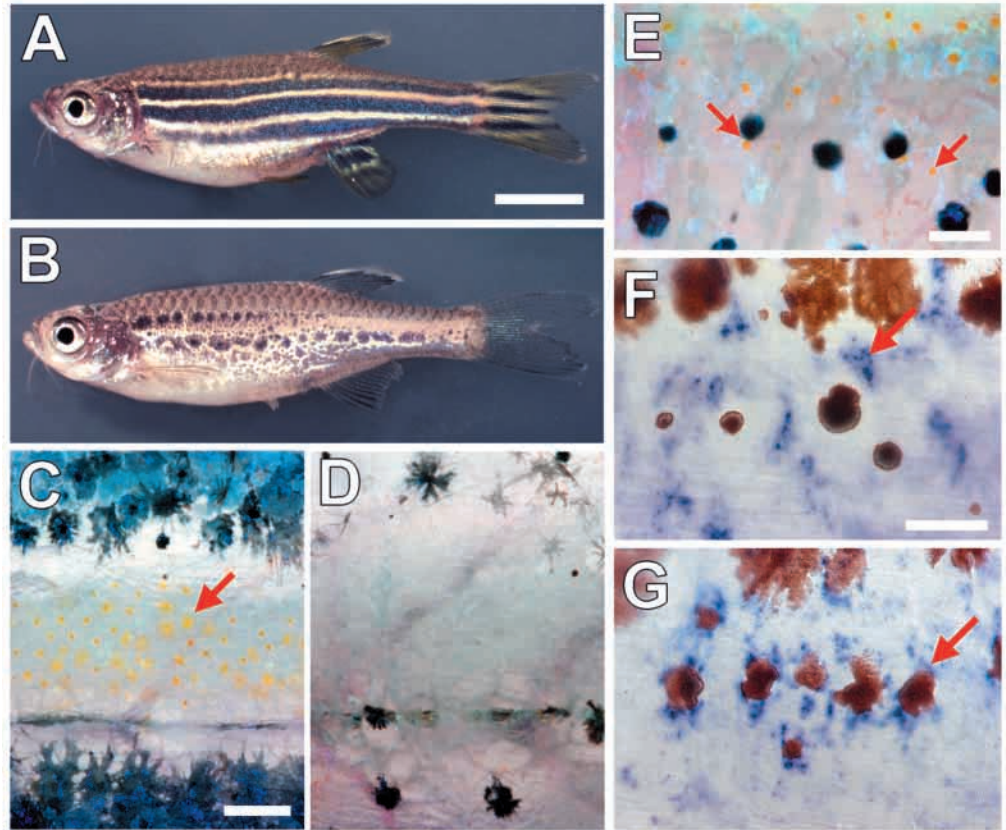
## INTRODUCTION

Pigment patterns have long been used as models for understanding the cellular and genetic bases of pattern formation in vertebrates (Goodrich and Nichols, 1931; Twitty, 1936; Jackson, 1994). In amniotes, coat and plumage patterns result largely from the spatial and temporal pattern of melanocyte differentiation and the transfer of melanin to developing hair or feathers, respectively (Nordlund et al., 1998). By contrast, pigment patterns of ectothermic vertebrates such as fishes and amphibians largely reflect the spatial arrangements of several classes of pigment cells, or chromatophores, that retain their pigments intracellularly (Bagnara, 1998). These include black melanophores, yellow or orange xanthophores and iridescent iridophores. The combinations of these and other chromatophore classes generate an extraordinary diversity of pigment patterns that serve a variety of roles across species, from crypsis and predator avoidance to schooling and mate recognition (e.g.,

Keenleyside, 1955; Endler, 1987; Houde, 1997; Couldrige and Alexander, 2002).

Fishes of the genus *Danio* exhibit a range of pigment patterns including horizontal stripes and vertical bars, as well as mottled and uniform patterns (Fang, 1997; Fang, 1998; Fang, 2000; Quigley and Parichy, 2002). Some insights into the mechanisms underlying these patterns can be gained by analyzing wild-type and mutant zebrafish, *D. rerio* (Parichy and Johnson, 2001). During normal development, zebrafish develop an embryonic and early larval pigment pattern comprising several stripes of melanophores with widely scattered xanthophores. This pattern persists for about 2 weeks, at which time an adult pattern begins to form. Over the following 2-3 weeks, a juvenile/early adult pattern develops consisting initially of two dark 'primary' melanophore stripes with an intervening light stripe. Subsequently, additional 'secondary' melanophore stripes are added as the fish grows (Fig. 1A) (Goodrich and Nichols, 1931; Kirschbaum, 1975; Johnson et al., 1995; Parichy et al., 2000a; Parichy and Johnson, 2001). Dark stripes comprise principally

**Fig. 1.** *fms* is essential for development of xanthophores and adult melanophore stripes, but is not expressed by melanophores. (A) Wild-type (strain AB<sup>UT</sup>) zebrafish exhibits several well-organized dark stripes that include melanophores with intervening light stripes that include xanthophores. (B) *fms*<sup>blue</sup> mutant adult, as a representative of *fms* mutants, lacks xanthophores and exhibits a disorganized pattern of melanophores. (C,D) Details of wild-type and *fms* mutant adult pigment patterns. (C) In wild type, melanophores are abundant in dorsal and ventral melanophore stripes, and a lighter interstripe region contains numerous yellow-orange xanthophores (arrow). Horizontal line is the horizontal myoseptum. (D) In a *fms*<sup>blue</sup> mutant, melanophores are reduced in number and fail to form normal stripes, and xanthophores are not present. The fish in C and D are illuminated so as to avoid reflections from iridophores throughout this region. (E) Detail of wild-type stripe margin in which melanosomes are contracted within melanophores, allowing a few xanthophores (arrows) to be discerned within the boundary of the dark melanophore stripe. Iridescent iridophores appear bluish in this image. (F,G) mRNA in situ hybridizations of zebrafish larvae during late stages of pigment pattern metamorphosis. (F) *fms* expression is not apparent in melanophores, but staining is observed in adjacent presumptive xanthophores (arrow). (G) In contrast, expression of the *fms* homologue, *kit*, is readily detected in melanophores (arrow). Scale bars: (A,B) 4 mm, (C,D) 60  $\mu$ m, (E) 80  $\mu$ m, (F,G) 120  $\mu$ m.



melanophores and iridophores, though occasional xanthophores can be found within these stripes as well (Fig. 1C,E). Light stripes lack melanophores and include only xanthophores and iridophores.

Of particular interest for understanding pattern-forming mechanisms and their evolution are mutants that alter the normal adult striped pattern, either by perturbing the development of particular classes of chromatophores or by affecting the extracellular environment in which these cells reside. Molecular analyses identified one such mutant (previously, *panther*) as an orthologue of the *fms* (*Csf1r*) locus (Parichy et al., 2000a). *fms* encodes a type III receptor tyrosine kinase known previously in mammals for roles in reproduction as well as the development of macrophages and osteoclasts (e.g., Marks and Lane, 1976; Motoyoshi, 1998; Dai et al., 2002; Tagoh et al., 2002). In zebrafish, *fms*<sup>-</sup> mutants exhibit a normal pattern of embryonic and early larval melanophores. In contrast, the pattern of adult melanophore stripes is severely disrupted (Fig. 1B). This defect is associated with fewer melanophores, disorganized melanophore movements, and both increased and disorganized melanophore death [which occurs normally in developing interstripe regions (Parichy et al., 2000a)].

Previous analyses suggest that *fms* and its homologue, *kit*, promote the development of temporally distinct populations of adult stripe melanophores in zebrafish. *kit* is essential for

the migration and maintenance of melanophores and their precursors, and *kit* mutants lack early metamorphic melanophores that normally arise in a dispersed pattern over the flank and ultimately migrate into stripes (Johnson et al., 1995; Parichy et al., 1999) (see also Rawls and Johnson, 2001; Quigley and Parichy, 2002). In contrast, *fms*<sup>-</sup> mutants retain these cells and instead exhibit an increasingly severe deficit of stripe melanophores beginning during middle metamorphic stages and extending into late metamorphosis and adult life (Parichy et al., 2000a). These observations suggested that *fms* promotes the development of a late-appearing metamorphic melanophore population, distinct from an early-appearing metamorphic melanophore population that requires *kit*. Consistent with this model, fish doubly mutant for both *fms* and *kit* exhibit additive effects of the two mutations and lack nearly all melanophores. A direct role for *fms* in establishing or maintaining melanophore precursors was also suggested by the observation that many cells migrating from the embryonic neural crest express both *fms* and either *mitfa* or *endothelin receptor b1* (*ednrb1*), two genes associated with melanophore development [though all of these loci are also co-expressed at early stages with xanthophore lineage markers, suggesting these cells may not yet be specified for one or another cell lineage (for details, see Quigley and Parichy, 2002)].

Despite genetic evidence for a *fms*-dependent population of adult melanophores, *fms* is not detectably expressed in

melanophores at the time when the migration and survival of these cells differs between wild-type and *fms*<sup>-</sup> mutants (though both melanophores and melanoblasts express *kit*; Fig. 1F,G) (Parichy et al., 1999; Parichy et al., 2000a). Thus, it remains unclear how, or when, *fms* promotes melanophore development and adult stripe formation. A possible explanation comes from the finding that, in contrast to the situation in melanophores or late stage melanoblasts, *fms* is expressed in yellow xanthophores and their precursors, xanthoblasts. Moreover, *fms*<sup>-</sup> mutants lack xanthophores and xanthoblasts in both embryos and adults, indicating an essential role for *fms* in the development of the xanthophore lineage (Fig. 1D) (Parichy et al., 2000a). In turn, these and other observations (e.g. Goodrich et al., 1954; Goodrich and Greene, 1959) suggested a model in which *fms* (i) acts directly to promote the establishment or maintenance of a subpopulation of stripe melanophore early precursors (above); and (ii) also acts indirectly through the xanthophore lineage to promote melanophore morphogenesis. Thus, interactions between melanophores and *fms*-dependent xanthophores were hypothesized to contribute to adult stripe formation. Such interactions could have broad phylogenetic significance, as alternating patterns of melanophores and xanthophores are found in a diverse array of ectothermic vertebrates, including fishes, frogs, salamanders and reptiles (e.g. Brodie, 1992; Seehausen et al., 1999; Parichy, 2001a; Parichy, 2001b).

In this paper, we investigate how and when *fms* activity is essential for the generation of an adult pigment pattern. Using cell transplantations, we show that *fms* acts through the xanthophore lineage to promote the arrangement of melanophores into stripes. To assess when *fms* is required for xanthophore development and melanophore stripe formation, we isolated temperature sensitive *fms* alleles and used reciprocal temperature shift experiments to enhance or curtail Fms activity at a range of stages from embryo to adult. These analyses show that *fms* is essential throughout development for maintaining cells of the xanthophore lineage and also for maintaining the integrity of melanophore stripes. Our results further suggest that *fms* is not required for establishing a population of precursor stem cells during embryogenesis (though it may serve to maintain precursors once established); rather, Fms activity is essential for recruiting precursor cells to the xanthophore lineage during later post-embryonic and adult development.

## MATERIALS AND METHODS

### Fish stocks and rearing conditions

Zebrafish were reared under standard conditions (28.5°C, 14 hours light:10 hours dark), except for temperature shift experiments in which individuals were typically reared at 24°C or 33°C (see below). Embryos were reared on rotifers until they were able to eat dry food, on which larvae and adults were maintained exclusively. Mutant alleles of *fms*, *fms*<sup>4e1</sup> (*fms*<sup>1</sup>) and *fms*<sup>4blue</sup> (*fms*<sup>blue</sup>), as well as *nacre* (*nacre*<sup>w2</sup>) have been described previously (Parichy et al., 2000a; Lister et al., 1999). *fms*<sup>1</sup> and *fms*<sup>blue</sup> are recessive homozygous viable, and are each predicted to encode proteins with substitutions in the functionally important and phylogenetically conserved kinase domains; these are likely to be null or severe loss of function alleles (Parichy et al., 2000a). *nacre*<sup>w2</sup> is recessive homozygous viable and acts as a null allele (Lister et al., 1999). Fish transgenic for green

fluorescent protein (GFP) under the control of the  $\beta$ -actin promoter were generously provided by K. Poss and have been maintained in the AB<sup>UT</sup> genetic background.

### Cell transplantation

Cell transplantations were performed on 3.3- to 3.8-hour embryos using a Narishige IM-9B micrometer-driven microinjection apparatus mounted on a Narishige micromanipulator. For operations, embryos were placed in wells formed in agar-lined dishes containing 10% Hanks solution (Westerfield, 1993) plus 1% penicillin/streptomycin. Typically 50-100 blastomeres were transplanted per embryo. To identify donor cells in host backgrounds, we used donors that were homozygous transgenic for GFP under the control of the  $\beta$ -actin promoter for wild-type  $\rightarrow$  *fms*<sup>-</sup>, *fms*<sup>-</sup>  $\rightarrow$  wild-type, and *nacre*<sup>-</sup>  $\rightarrow$  *fms*<sup>-</sup> chimeras. To identify donor melanophores in wild-type  $\rightarrow$  *nacre*<sup>-</sup> and *fms*<sup>-</sup>  $\rightarrow$  *nacre*<sup>-</sup> chimeras, we used the endogenous melanin of donor melanophores as an autonomous marker of cell lineage, as *nacre*<sup>-</sup> hosts are unable to produce melanophores owing to a mutation that acts autonomously to the melanophore lineage (Lister et al., 1999) see below); donor cell types other than melanophores were not assessed in these chimeras. For transplants involving  $\beta$ -actin-GFP transgenic donors, we identified GFP<sup>+</sup> donor cells under epifluorescent illumination using an EGFP filter set on a Zeiss Axioplan 2i microscope. Although xanthophores autofluoresce green as well (Raible and Eisen, 1994; Parichy et al., 2000a), GFP fluorescence is markedly brighter and color-shifted relative to endogenous xanthophore autofluorescence. To prevent melanin from quenching GFP fluorescence in melanophores, we treated fish with 2-3 drops of 10 mg/ml epinephrine prior to viewing, thereby causing melanin-containing melanosomes to be contracted towards cell centers; GFP fluorescence could then be clearly observed in cell peripheries. Individuals completely lacking GFP<sup>+</sup> cells, or comprising greater than ~75% GFP<sup>+</sup> cells were discarded and are not included in the analyses below; typically, however, chimeras exhibited relatively low percentages of donor cells (<25%) that were often distributed widely in the adult fish with patches of donor cells consisting of only one or a few cell lineages (see below). Cell transplants employing both *fms*<sup>1</sup> and *fms*<sup>blue</sup> yielded equivalent results; thus both genotypes are referred to below as *fms*<sup>-</sup>.

### Mutagenesis and non-complementation screening

To isolate temperature-sensitive alleles of *fms*, we crossed homozygous *fms*<sup>1</sup> or *fms*<sup>blue</sup> females to AB<sup>UT</sup> males that had been mutagenized three times over the course of three weeks with 3 mM N-ethyl-N-nitrosourea (Sigma) (Solnica-Krezel et al., 1994). Embryos were incubated at 33°C until hatching at which time individuals lacking xanthophores were transferred to 28.5°C and reared through sexual maturity. To test whether newly isolated mutants were allelic to *fms*, and to test for temperature sensitivity, mutant fish were backcrossed to *fms*<sup>1</sup> or *fms*<sup>blue</sup> and sibships were split between 24°C and 33°C. Mutants were considered alleles of *fms* if at 33°C none of the offspring developed wild-type phenotypes; mutants were identified as temperature-sensitive if at 24°C approximately half of the offspring developed *fms* null phenotypes (presumptive *fms*<sup>-</sup>/*fms*<sup>-</sup>) and half of the offspring developed less severe or wild-type phenotypes (presumptive *fms*<sup>TS</sup>/*fms*<sup>-</sup>). At least 100 offspring were examined at each temperature.

### Sequencing and genotyping

Sequencing of mutant alleles was performed following RT-PCR of *fms* cDNA from haploid embryos. Sequencing reactions were performed with BigDye dye terminator sequencing chemistry and resolved on an ABI-377 automated sequencer. Resulting sequences were compared to those of unmutagenized AB<sup>UT</sup> harboring the ancestral unmutagenized chromosome and wild-type *fms* sequence. For primer extension genotyping of *fms*<sup>ut.r4e174A</sup>, forward and reverse primers (*fms*174a-F: TCGAGTTCCTTTGTTTCTCCGAG; *fms*174a-R: CTCCGATTCT-

AGCGCAGCAAATG) flanking the mutant lesion were used to amplify genomic DNA and excess primers were digested using shrimp alkaline phosphatase and exonuclease (Amersham). Primer extension reactions were performed in 20  $\mu$ l volumes with 10  $\mu$ l PCR, 0.5 U Thermosequenase (Amersham), 50  $\mu$ M ddGTP, 50  $\mu$ M dATP, 50  $\mu$ M dTTP, 12.5 pmol *fms*<sup>174a-R</sup> and supplied reaction buffer. After denaturing (94°C, 2 minutes) reactions were run for 50 cycles at: 94°C, 5 seconds; 43°C, 15 seconds; 60°C, 1 minute (Hoogendoorn et al., 1999). After denaturing (94°C, 1 minute), primer extension products were resolved by HPLC on a Transgenomic WAVE DNA Fragment Analysis System at 80°C. Wild-type, *fms*<sup>l</sup>, or *fms*<sup>blue</sup> alleles result in addition of 3 nucleotides (ddGAT) whereas the *fms*<sup>ut.r4e174A</sup> allele results in addition of 2 nucleotides (ddGT) to the extension primer.

### Histology

mRNA in situ hybridization employed riboprobes for *fms* as well as *GTP-cyclohydrolase* (*gch*) and *xanthine dehydrogenase* (*xdh*), which have been described previously (Parichy et al., 2000a). mRNA in situ hybridization of embryos followed the method of Jowett and Yan (Jowett and Yan, 1996).

To test for apoptosis, TUNEL assays were performed as described (Zhang and Galileo, 1997; Parichy et al., 1999). After TUNEL staining, embryos were examined in whole mounts and numbers of TUNEL<sup>+</sup> cells were counted along the dorsal neural tube and in neural crest migratory pathways from just anterior to the midbrain-hindbrain junction to a point one-third of the distance along the tail (extensive genotype and temperature-independent cell death in posterior tail tips precluded accurate counts in this region). Stained cells ventral to the ventral margins of the myotomes (and likely to include macrophages rather than neural crest-derived cells) (Herbomel et al., 2001) were not included in counts. Embryos were fixed and examined in whole mount. To identify dead chromatophores in adults, fish were fixed in 4% paraformaldehyde in PBS. After washing in PBS, fins were removed and mounted on glass slides and trunks were embedded in OCT and cryosectioned.

### Morphometrics, image analyses and statistical methods

Fish sizes were quantified by measuring the length from the tip of the snout to the caudal peduncle (standard length, SL), as well as the height of the flank at the anterior and posterior margins of the anal fin. To quantify patterns, fish were imaged using a combination of incident and transmitted illumination with a Zeiss Axiocam digital camera mounted on either an Olympus SZX-12 epifluorescence stereomicroscope, or a Zeiss Axioplan 2i equipped with differential interference contrast optics. Both xanthophores and melanophores could be readily identified under these conditions. To allow accurate cell counts in temperature shift experiments, fish were treated with epinephrine (above) prior to imaging, and images were acquired using the square root transformation and color enhancement feature in Zeiss Axiovision 3.0. Xanthophore and melanophore densities were calculated for a representative region of the flank bordered by: anteriorly, the anterior margin of the dorsal fin; posteriorly, the posterior margin of the anal fin; ventrally, the ventral margin of the flank; and dorsally, a position just ventral to the dorsal margin of the flank (this position was determined by estimating the total height of the dorsal flank at the posterior margin of the anal fin, then selecting a point 10% of this distance from the dorsal edge; this restriction reduced variation among individuals owing to curvature of the fish in this region). Pigment cells in Adobe Photoshop images were identified by eye and marked digitally; the IPTK 4.0 software package (Reindeer Graphics) was then used to automatically count marks and to calculate total measurement areas. For imaging of some whole-mount embryos following in situ hybridizations, the Extended Focus module of Zeiss Axiovision 3.0 software was employed to flatten multiple focal planes into a single plane (which results in characteristic fringing at the edges of some features, such as melanophores).

All statistical analyses were performed using standard methods

(Sokal and Rohlf, 1981) in the JMP Statistical Analysis Package for Macintosh (SAS Institute, Cary, NC). Analyses of xanthophore and melanophore numbers and distributions were performed by treating size at temperature shift as both a continuous and ordinal factor and after controlling for variation in individual size at the time of imaging. Analyses of stripe breaks were done using maximum likelihood estimation and significance of effects were estimated using likelihood ratio tests. Nearest neighbor distances among melanophores were assessed initially as hierarchical mixed model analyses of covariance, with individual melanophore measurements nested within individuals (a random effect), and individuals nested within temperature treatments (a fixed effect); this approach avoids pseudoreplication from analyzing individual melanophores as independent data points. Methodological details and complete statistical analyses are available on request.

## RESULTS

### Autonomous and non-autonomous roles for *fms* in adult pigment pattern development identified by cell transplants between wild-type and *fms* mutants

A summary of the phenotypes and results of cell transplantation experiments described in this section and the next are provided in Tables 1 and 2. Overall, these analyses strongly suggest that *fms* acts, at least in part, through the xanthophore lineage to promote adult melanophore stripe development.

As a first step in testing how *fms* promotes adult pigment pattern development, we asked whether *fms* acts autonomously to pigment cell lineages during xanthophore development and melanophore stripe formation. In principle, xanthophore development and melanophore stripe patterning could depend on several potential sources of *fms* activity: early (possibly unspecified) precursor cells that coexpress *fms* and genes associated with melanophore development (e.g., *mitfa*, *ednrb1*); cells of the xanthophore lineage that express and require *fms* and in turn influence melanophore behavior; or melanophores themselves, if these cells in fact express *fms* at functionally significant but undetectable levels. To address these issues, we constructed chimeras between wild-type and *fms*<sup>-</sup> mutant embryos [for a general discussion of this approach in the context of analyzing functions of identified genes see Rossant and Spence (Rossant and Spence, 1998)]. We predicted that if *fms* acts autonomously to pigment cell lineages in promoting xanthophore development and melanophore stripe formation, then donor wild-type *fms*<sup>+</sup> melanophores and xanthophores should develop in *fms*<sup>-</sup> mutant hosts, and these cells should be capable of forming a wild-type stripe pattern (that might or might not include host *fms*<sup>-</sup> mutant

**Table 1. Summary of genotypes**

Genotype	Phenotypes		
	Mel	Xanth	Melanophore stripes
Wild type	+++	+++	+++
<i>fms</i> <sup>-</sup>	++	-	-*
<i>nacre</i> <sup>-</sup>	-	++†	-

\*Disrupted melanophore stripes and dispersed melanophores posteriorly; weak and irregular stripes anteriorly (Fig. 1B).

†Fewer, less heavily pigmented xanthophores than wild type (Lister et al., 1999) (D. M. P., unpublished data).

**Table 2. Cell transplantation experiments**

		Chimera cell types and pigment patterns						
Donor	Host	Donor derivatives*		Host chromatophores		Melanophore stripe development		
		Mel	Xanth	Mel	Xanth	Morphology <sup>†</sup>	Donor <sup>‡</sup>	Host <sup>§</sup>
Wild type	→ <i>fms</i> <sup>-</sup>	+	+	+	(-) <sup>¶</sup>	+++	+	+
<i>fms</i> <sup>-</sup>	→ wild-type	+	-	+	+	+++	+	+
<i>nacre</i> <sup>-</sup>	→ <i>fms</i> <sup>-</sup>	-	+	+	(-) <sup>¶</sup>	++	-	+
<i>fms</i> <sup>-</sup>	→ <i>nacre</i> <sup>-</sup>	+	nd**	-	+	++	+	-

\*Donor derivatives other than melanophores or xanthophores included iridophores, epidermis, muscle, nerves, lateral line, endoskeletal bone, fin rays and gut. Classes of derivatives produced did not differ noticeably among genotype combinations.  
<sup>†</sup>Scoring represents quality of stripes relative to wild-type adults: +++, melanophore stripes could be nearly indistinguishable from wild-type when donor pigment cells present; ++, melanophore stripes present when xanthophores present, but stripes less distinctive than wild type.  
<sup>‡</sup>Presence of donor melanophores within melanophore stripes.  
<sup>§</sup>Presence of host melanophores within melanophore stripes.  
<sup>¶</sup>Host xanthophores not observed, though presence of some unobserved cells cannot be excluded.  
\*\*Not determined.

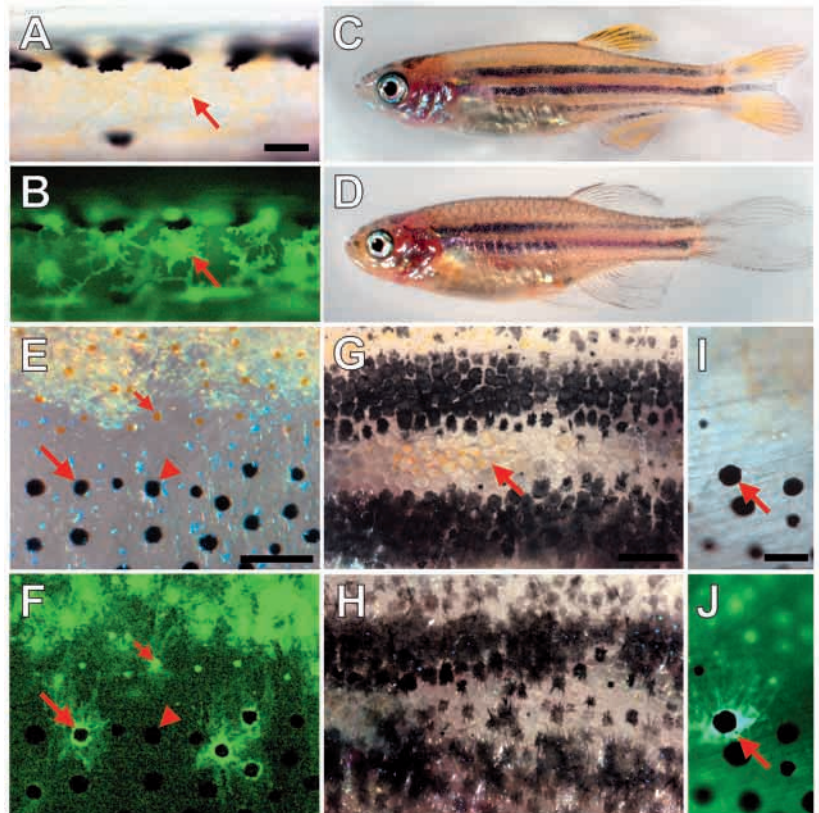
melanophores; see below). If *fms* acts non-autonomously to pigment cell lineages, then donor wild-type *fms*<sup>+</sup> melanophores and xanthophores might not develop in *fms*<sup>-</sup> mutant hosts, stripes might not develop, or both. To test these predictions, we reared wild-type → *fms*<sup>-</sup> (as well as *fms*<sup>-</sup> → wild-type) chimeric embryos 8-12 weeks through metamorphosis and adult pigment pattern formation. To distinguish donor from host cells, we used donors that expressed GFP ubiquitously under the control of the β-actin promoter.

For all genotypes examined (Table 1, and below), chimeras reared to adult stages exhibited donor cells in a variety of derivatives, including muscle, gut, lateral line, epidermis, dermal bone and endoskeleton, similar to chimeras typically examined at embryonic stages (e.g. Ho and Kimmel, 1993;

Halpern et al., 1993). A subset of chimeras exhibited donor-derived pigment cells, and these frequently occurred in the absence of other donor-derived cell types (see below).

Results from wild-type ↔ *fms*<sup>-</sup> chimeras suggested that *fms* acts autonomously to promote xanthophore development. When wild-type cells were transplanted to *fms*<sup>-</sup> hosts, donor-derived (*fms*<sup>+</sup> GFP<sup>+</sup>) melanophores and xanthophores could be observed on the flank in both embryos and adults (Fig. 2A,B,E,F; Table 2). Of the chimeras examined at adult stages, ~40% exhibited donor-derived pigment cells, including donor xanthophores. Frequently, these cells were distributed widely over the flank and apparently independently of other donor-derived cell types (Fig. 2F and data not shown). The wide distribution of donor-derived melanophores and xanthophores

**Fig. 2.** Chimeras reveal cell autonomous and non-autonomous roles for *fms* during adult stripe development. (A,B) Bright-field (A) and fluorescence (B) micrographs of early larva (72 h) showing donor wild-type (*fms*<sup>+</sup> GFP<sup>+</sup>) xanthophores over the dorsal myotomes of a *fms*<sup>-</sup> mutant host. (C,D) Wild-type → *fms*<sup>-</sup> chimeras reared to adult stages (n=20) develop well-formed (C) or partial (D) melanophore stripes when donor melanophores and xanthophores are present. (E,F) Detail of wild-type → *fms*<sup>-</sup> chimera showing organized stripes that include donor (*fms*<sup>+</sup> GFP<sup>+</sup>) melanophores (large arrow) and xanthophores (small arrow), as well as host (*fms*<sup>-</sup> GFP<sup>-</sup>) melanophores (arrowhead). This is the same individual as in C; note the absence of GFP donor cells in other tissues, such as myotomes or epidermis. (G,H) Melanophore stripe morphology depends on the presence of donor wild-type pigment cells. Opposite sides of a single wild-type → *fms*<sup>-</sup> chimera are shown in which well-defined melanophore stripes are present on the side exhibiting donor melanophores and xanthophores (arrow, G) but not on the side lacking donor pigment cells (H). (I,J) *fms*<sup>-</sup> → wild-type chimeras reared to adult stages (n=15) developed wild-type stripes. Although donor *fms*<sup>-</sup> cells contributed to epidermis, nerves, bone and other derivatives, only one chimera exhibited donor (*fms*<sup>-</sup> GFP<sup>+</sup>) melanophores (arrow) and these were present within host melanophore stripes; donor xanthophores were not observed. Scale bars, (A,B) 30 μm, (E,F) 200 μm, (G,H) 250 μm, (I,J) 60 μm.



is not unexpected given the migratory nature, invasiveness, and proliferative capabilities of these cells [particularly xanthophores (e.g. Tucker and Erickson, 1986a; Parichy, 1996a; Parichy, 1996b; Wilkie et al., 2002) (D. M. P., unpublished data)]. Conversely, when *fms*<sup>-</sup> cells were transplanted into wild-type hosts, donor *fms*<sup>-</sup> cells contributed to a range of non-pigment cell derivatives, but only a single individual (of 15 surviving chimeras) had a few donor (*fms*<sup>-</sup> GFP<sup>+</sup>) melanophores (Fig. 2I,J); donor *fms*<sup>-</sup> xanthophores were not observed. Although the paucity of donor melanophores in *fms*<sup>-</sup> → wild-type chimeras raises the possibility of an autonomous role for *fms* in melanophore development (consistent with genetic analyses; see Introduction), we cannot yet exclude the formal possibility that differences in genetic background unrelated to *fms*<sup>-</sup> might have biased the differentiation of donor cells away from melanophore fates.

Pigment patterns of wild-type ↔ *fms*<sup>-</sup> chimeras further suggested that adult melanophore stripes result from *fms* acting autonomously to pigment cell lineages, but non-autonomously relative to the melanophore lineage. In wild-type → *fms*<sup>-</sup> chimeras with donor (*fms*<sup>+</sup> GFP<sup>+</sup>) melanophores and xanthophores, stripes developed that frequently resembled wild-type stripes and were considerably more organized than patterns of *fms*<sup>-</sup> mutants (Fig. 2C; compare with Fig. 1A,B). The degree of melanophore stripe organization appeared to depend on the distribution of donor wild-type (*fms*<sup>+</sup>) cells, as individuals with more donor melanophores and xanthophores had more distinctive stripes than individuals with few donor melanophores and xanthophores (Fig. 2D). Similar variation in stripe morphology could be observed in individuals with donor melanophores and xanthophores only in discrete regions (Fig. 2G,H). (These qualitative interpretations are supported by quantitative analyses of temperature shift experiments below.) Importantly, melanophore stripes that developed in wild-type → *fms*<sup>-</sup> chimeras always included host *fms*<sup>-</sup> melanophores interspersed with donor *fms*<sup>+</sup> melanophores. Moreover, in regions with donor pigment cells and stripes, host *fms*<sup>-</sup> melanophores were not found in xanthophore-rich interstripe regions (Fig. 2E,F), suggesting that wild-type *fms*<sup>+</sup> donor cells were able to organize *fms*<sup>-</sup> mutant host melanophores into stripes. Owing to the difficulty of examining GFP expression in single cells of adult fish, it was not possible to quantitatively assess whether wild-type *fms*<sup>+</sup> melanophores or xanthophores that affected the arrangement of *fms*<sup>-</sup> mutant melanophores also affected the numbers of *fms*<sup>-</sup> mutant melanophores that differentiated. In reciprocal *fms*<sup>-</sup> → wild-type chimeras, the only individual that developed donor *fms*<sup>-</sup> melanophores exhibited these cells only within the melanophore stripes (Fig. 2I,J). Taken together, these results suggest that: (i) *fms* acts autonomously to pigment cell lineages to promote xanthophore development (and possibly melanophore development); and (ii) *fms* acts non-autonomously on the melanophore lineage to promote the arrangement of both *fms*<sup>+</sup> and *fms*<sup>-</sup> melanophores into stripes.

#### **Xanthophore-autonomous role for *fms* in promoting melanophore stripe development revealed by transplants between *nacre*<sup>-</sup> and *fms*<sup>-</sup> mutants**

Pigment cell distributions in wild-type ↔ *fms*<sup>-</sup> chimeras suggested that melanophore stripe development depends in part on *fms* acting non-autonomously relative to individual

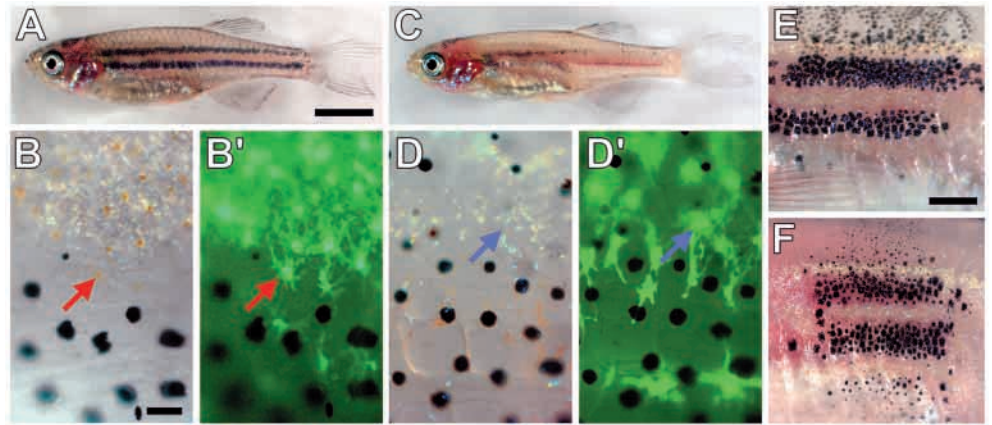
melanophores, since even *fms*<sup>-</sup> mutant melanophores were arranged in stripes in the presence of *fms*<sup>+</sup> pigment cells. We reasoned that at least two explanations could account for such non-autonomous effects on melanophore distributions. First, *fms* could be expressed at undetectable but functionally important levels by a subset of melanophores that, in turn, organize other melanophores into stripes. This model would be consistent with previous genetic analyses that identified distinct *fms*-dependent and *kit*-dependent melanophores constituting adult stripes (Parichy et al., 2000a). Second, *fms*-dependent cells of the xanthophore lineage could be essential for promoting the organization of melanophores into stripes. To distinguish between these models, we constructed chimeras between *fms*<sup>-</sup> mutants and *nacre*<sup>-</sup> mutants. *nacre*<sup>-</sup> mutants lack melanophores because of a mutation in *mitfa*, which encodes a microphthalmia-like transcription factor that acts cell autonomously during melanophore specification (Lister et al., 1999). Since *nacre*<sup>-</sup> mutants retain xanthophores, however, comparisons of cell distributions between wild-type ↔ *fms*<sup>-</sup> and *nacre*<sup>-</sup> ↔ *fms*<sup>-</sup> chimeras allows the isolation of a potential role for *fms* in promoting the organization of melanophore stripes via interactions among melanophores themselves. Thus, we predicted that if *fms* acts through the melanophore lineage to promote the organization of melanophores into stripes, then *nacre*<sup>-</sup> ↔ *fms*<sup>-</sup> chimeras should not form melanophore stripes, since *fms*<sup>+</sup> melanophores would not be present. Conversely, if *fms* acts through the xanthophore lineage to promote the organization of melanophores into stripes, then *nacre*<sup>-</sup> ↔ *fms*<sup>-</sup> chimeras should be capable of developing melanophore stripes, since *fms*<sup>+</sup> xanthophores would be contributed by the *nacre*<sup>-</sup> mutant background.

As previously, *nacre*<sup>-</sup> cells transplanted into *fms*<sup>-</sup> hosts contributed to a range of tissues including muscle, epidermis, nerves and others. As expected, and consistent with previous analyses of the *nacre*<sup>-</sup> mutation (Lister et al., 1999), none of the *nacre*<sup>-</sup> → *fms*<sup>-</sup> chimeras exhibited donor (*fms*<sup>+</sup> *nacre*<sup>-</sup> GFP<sup>+</sup>) melanophores. However, ~24% of chimeras reared to adult stages exhibited donor (*fms*<sup>+</sup> *nacre*<sup>-</sup> GFP<sup>+</sup>) xanthophores and these fish were invariably striped (Fig. 3A,B). By contrast, *nacre*<sup>-</sup> → *fms*<sup>-</sup> chimeras that lacked xanthophores failed to develop distinctive, well-organized melanophore stripes and instead exhibited melanophore patterns indistinguishable from *fms*<sup>-</sup> mutant controls; this was true of chimeras in which *nacre*<sup>-</sup> donor cells developed as non-pigment cell lineages, as well as chimeras in which *nacre*<sup>-</sup> donor cells developed as the third major class of pigment cells, iridescent iridophores (Fig. 3C,D). In reciprocal experiments, *fms*<sup>-</sup> → *nacre*<sup>-</sup> (as well as wild-type → *nacre*<sup>-</sup>) chimeras also developed regions of well-formed melanophore stripes (Fig. 3E,F). Together, these results demonstrate that non-autonomous roles for *fms* in promoting melanophore stripe development act largely or entirely via the xanthophore lineage.

#### **Isolation of temperature-sensitive *fms* alleles**

As mRNA in situ hybridization reveals *fms* expression in cells of the xanthophore lineage throughout embryonic and larval stages, as well as in embryonic cells expressing markers of multiple pigment cell lineages (see Introduction), we asked whether critical periods exist for Fms activity in promoting xanthophore development and adult stripe formation. To this end, we screened for temperature-sensitive alleles by non-

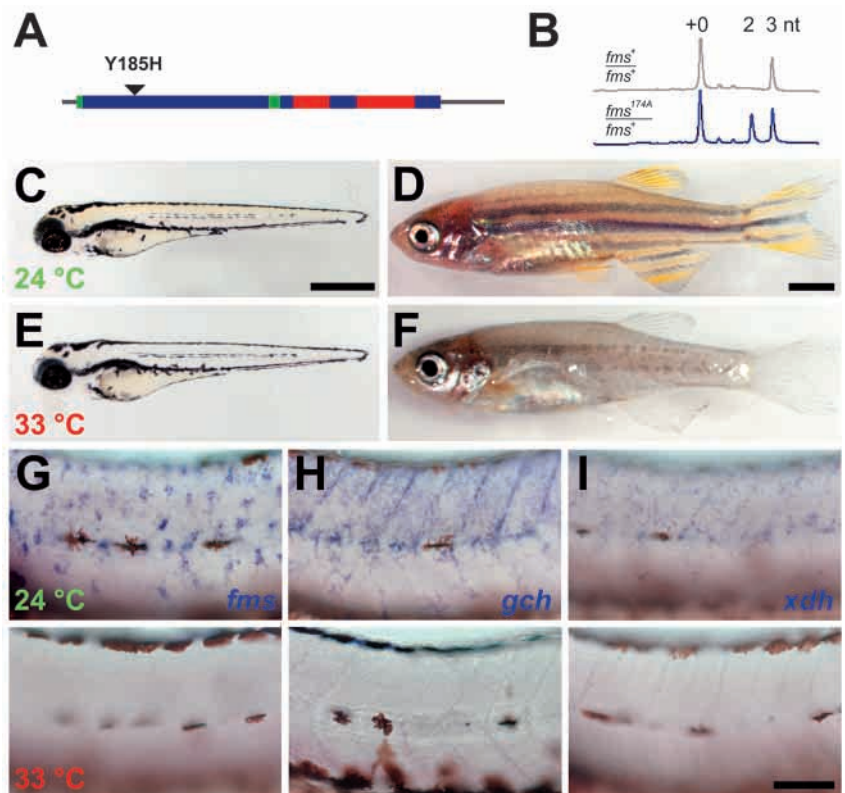
**Fig. 3.** *nacre* mutant reveals that *fms* acts through the xanthophore lineage to promote melanophore stripe formation. (A,B) *nacre*<sup>-</sup> → *fms*<sup>-</sup> chimeras reared to adult stages (*n*=29) that developed donor (*nacre*<sup>-</sup> *fms*<sup>+</sup> GFP<sup>+</sup>) xanthophores also developed organized melanophore stripes (*n*=7). (B,B') Corresponding bright-field and fluorescence micrographs of the individual in A showing donor xanthophores (e.g., red arrow) adjacent to the melanophore stripe. (C) *nacre*<sup>-</sup> → *fms*<sup>-</sup> chimeras, lacking xanthophores, failed to develop organized melanophore stripes (*n*=22). An individual exhibiting donor (*nacre*<sup>-</sup> *fms*<sup>+</sup> GFP<sup>+</sup>) iridophores (*n*=6) is shown. (D,D') Corresponding bright-field and fluorescence views of the individual in C showing donor iridophores (e.g., blue arrow). Note that the orange color in some regions is due to reflections from iridophores rather than differentiated xanthophores. In contrast to melanophore arrangements, however, average melanophore densities did not differ dramatically between *nacre*<sup>-</sup> → *fms*<sup>-</sup> chimeras that either developed or failed to develop donor xanthophores (means=373, 316 melanophores/mm<sup>2</sup>, s.d.=111, 69, *n*=4, 11, respectively; *t*<sub>13</sub>=1.0, *P*=0.3). (E,F) Development of melanophore stripes in *nacre*<sup>-</sup> hosts. (E) Wild-type cells transplanted to *nacre*<sup>-</sup> hosts develop regions of well-formed stripes. (F) *fms*<sup>-</sup> cells transplanted to *nacre*<sup>-</sup> hosts contribute to stripes resembling those formed by wild-type cells. Scale bars, (A,C) 4 mm, (B,D) 100 μm, (E,F) 800 μm.



complementation of *fms*<sup>blue</sup> or *fms*<sup>l</sup>. We isolated ~75 new alleles and tested 42 for temperature sensitivity. Three alleles (*fms*<sup>ut.r4e174A</sup>, *fms*<sup>ut.r4e536</sup>, *fms*<sup>ut.r4e564</sup>) resulted in presumptive *fms* null phenotypes (Parichy et al., 2000a) at a restrictive temperature (33°C) and wild-type phenotypes at a permissive temperature (24°C) when in trans with *fms*<sup>blue</sup> or *fms*<sup>l</sup>. Sequence analyses of *fms*<sup>ut.r4e174A</sup> (hereafter, *fms*<sup>174A</sup>)

identified a T→C transversion resulting in an amino acid substitution (Y185H) within the predicted second immunoglobulin-like domain of the protein, a region likely to be essential for ligand binding (Jiang et al., 2000) (Fig. 4A). Primer extension assays (Fig. 4B) confirmed cosegregation of this lesion and the temperature-sensitive phenotype (data not shown).

**Fig. 4.** Isolation of a temperature sensitive *fms* allele. (A) *fms*<sup>174A</sup> cDNA exhibits a tyr → his substitution within the second immunoglobulin-like domain. Grey, untranslated regions. Green, signal sequence and transmembrane domain. Red, split kinase domains. (B) Primer extension analysis for genotyping *fms*<sup>174A</sup> nucleotide substitution. (Upper trace) Wild-type (or *fms*<sup>l</sup>, *fms*<sup>blue</sup>) alleles result in the addition of 3 nucleotides (nt) to the extension primer. The peak at +0 nt represents excess extension primer without added nucleotides. (Lower trace) The *fms*<sup>174A</sup> allele results in addition of 2 nt to the extension primer; shown is a chromatogram for a heterozygous *fms*<sup>174A</sup>/*fms*<sup>+</sup> individual. (C-F) Homozygous *fms*<sup>174A</sup> individuals reared at 24°C (C,D) or 33°C (E,F). (C,D) At 24°C, hatchling larvae exhibit normal numbers of xanthophores (here evidenced by the yellow cast to the flank; C); adults exhibit melanophore stripes indistinguishable from wild-type (D). (E,F) At 33°C, hatchling larvae lack xanthophores (E) and adults both lack xanthophores and exhibit a severe disruption of melanophore stripes (F), resembling that seen in *fms*<sup>l</sup> or *fms*<sup>blue</sup> (Fig. 1B). (G-I) Molecular marker analyses reveal that *fms*<sup>174A</sup> conditionally affects the development of xanthophore precursors, as revealed by distributions of cells expressing the xanthophore lineage markers *fms* (G), *gch* (H), and *xdh* (I). (Upper panels) Presumptive xanthophore precursors are abundant over the myotomes of 60 h embryos at 24°C. (Lower panels) Presumptive xanthophore precursors are absent from over the myotomes of embryos reared at 33°C. Scale bars, (C,E) 600 μm, (D,F) 2 mm, (G-I) 40 μm.







and are becoming increasingly regular in their outlines, but prior to the development of adult scales, or the formation of secondary adult melanophore stripes (Quigley and Parichy, 2002) (D. M. P. and J. M. T., unpublished data). In contrast, in larvae upshifted at larger sites, we observed higher densities of residual xanthophores when formation of primary adult melanophore stripes was essentially completed and secondary melanophore stripes had started to develop (Fig. 6C; final xanthophore density vs. size when shifted, partial regression coefficient=5.13; s.e.=0.46;  $F_{1,92}=125.47$ ,  $P<0.0001$ ).

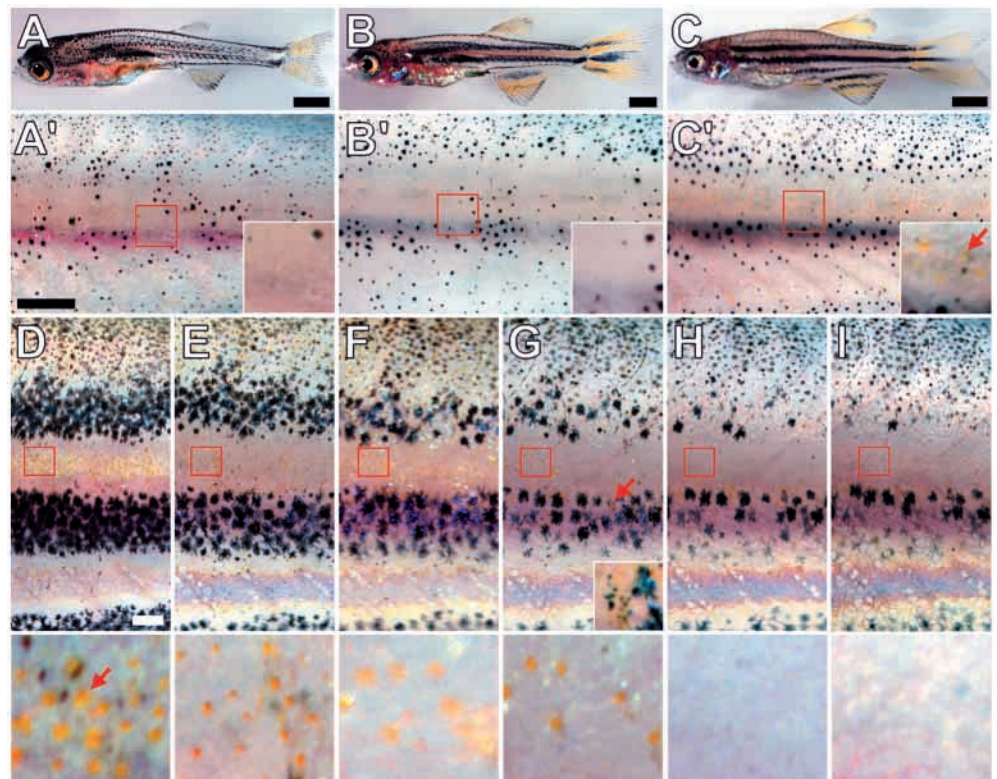
Correlated with the loss of xanthophores was a severe perturbation of adult melanophore stripes, with resulting phenotypes resembling the pigment pattern of *fms<sup>l</sup>* and *fms<sup>blue</sup>* mutants (e.g. Fig. 6A',B'). Melanophore stripes depend on both melanophore numbers and arrangements. Thus, to quantify effects on melanophore stripe morphology, we assessed both melanophore densities and organization. This analysis revealed an ~11% reduction in melanophore densities in upshifted individuals as compared to control siblings left at 24°C (respective means±s.d.=126±30.9, 142±30.8;  $n=95$ , 56;  $F_{1,148}=4.96$ ,  $P<0.05$ ). In contrast to xanthophores (above), we detected only a marginal difference in melanophore densities among individuals upshifted at different sizes, with a slightly more severe deficit in smaller individuals upshifted when smaller ( $P=0.06$ ; data not shown).

To assess melanophore organization quantitatively, we examined nearest neighbor distances among melanophores. Well-defined stripes are associated with low variability in the

distances between adjacent melanophores, whereas poorly defined stripes are associated with increased variability in the distances between adjacent melanophores; thus, coefficients of variation for melanophore nearest neighbor distances are a sensitive measure of melanophore distributions (D. M. P. and J. M. T., unpublished data). We examined coefficients of variation for mean melanophore nearest neighbor distances between individuals upshifted to 33°C and control siblings left at 24°C. This analysis revealed increased variability in melanophore positions in upshifted individuals compared to controls ( $F_{1,141}=99.8$ ,  $P<0.0001$ ; means±s.d.: 41.6±4.67, 31.0±7.56;  $n=93$ , 51). Given results of cell transplantation experiments that suggested a role for xanthophores in promoting the organization of melanophores in stripes, we further asked whether differences in xanthophore densities among upshifted individuals were associated with variation in melanophore spacing. Fig. 5C shows that lower xanthophore densities were directly related to increased coefficients of variation for melanophore nearest neighbor distances (partial regression coefficient=-0.11; s.e.=0.02;  $F_{1,90}=22.74$ ,  $P<0.0001$ ). Thus, continuous *Fms* activity is essential for maintaining normal numbers of melanophores, as well as the normal spacing of melanophores within stripes, in a manner that directly correlates with xanthophore densities.

During terminal stages of metamorphosis and during juvenile development (>10 mm SL), initial experiments resulted in a severe reduction of xanthophores, but not a

**Fig. 6.** Curtailing *Fms* activity eliminates xanthophores and perturbs melanophore stripes throughout development. (A-C) Examples of *fms<sup>174A</sup>* individuals reared at 24°C to the sizes indicated (upper panels) then shifted to 33°C until an adult pigment pattern had formed (lower panels). (A) Larva shifted during early pigment pattern metamorphosis (7.6 mm SL) loses xanthophores and fails to develop normal adult stripes (14.3 mm SL, A') after 28 days at 33°C. (B) Larva shifted during middle stages of pigment pattern metamorphosis (8.9 mm SL) loses xanthophores and initial melanophore stripes degenerate (15.6 mm SL, B') after 28 days at 33°C. (C) Individual that has already attained a juvenile pigment pattern (13.5 mm SL) retains some xanthophores and a partial stripe pattern with more variably spaced melanophores (14.9 mm SL, C') after 14 days at 33°C. (Insets) Higher magnification views of boxed regions showing absence of xanthophores (A',B') or residual xanthophores (C' arrow). (D-I) Prolonged rearing at 33°C results in a complete loss of xanthophores. Shown are sequential images of the same region on a representative *fms<sup>174A</sup>* individual that had developed a juvenile pattern of melanophore stripes (18 mm SL) at 24°C (D), with times after shifting to 33°C of (E) 3 days, (F) 6 days, (G) 8 days, (H) 12 days and (I) 20 days. (Upper images) Low magnification showing melanophore distributions. (Lower images) Higher magnification showing depletion of xanthophores (arrow). (Inset) in G, high magnification showing melanophore debris indicated by arrow. Scale bars, (A,B) 1 mm, (C) 2 mm, (A'-C') 500 µm, (D-I) 250 µm.

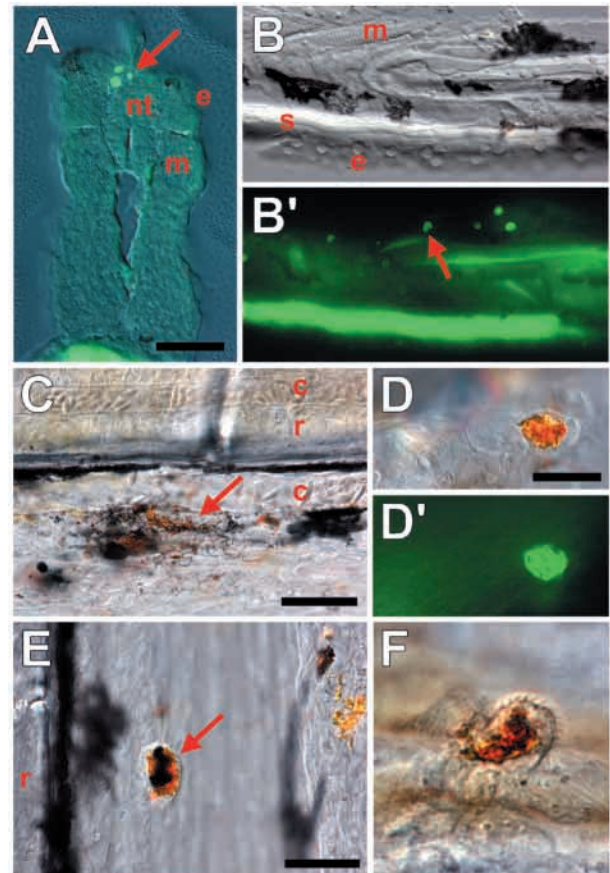


complete elimination of these cells (Fig. 5A, Fig. 6C). To assess whether these weaker phenotypes reflect a period of independence from Fms activity, we reared fish to adult stages (~18 mm SL) at 24°C, shifted these individuals to 33°C, and examined the patterns regularly for approx. 6 weeks. These individuals lost approx. 98% of xanthophores and exhibited a severe degeneration of melanophore stripes within 5 weeks of curtailing Fms activity (similar changes were not observed in *fms*<sup>174A/+</sup> individuals shifted at the same time; data not shown). Repeated imaging of individual fish revealed that loss of xanthophores occurred gradually over a period of weeks (Fig. 6D-G; for animated time-lapse images, see Supplemental Data: <http://dev.biologists.org/supplemental/>), with the onset of loss varying considerably among individuals.

These analyses show that Fms activity is essential for maintaining differentiated cells of the xanthophore lineage and for maintaining the striped arrangement of adult melanophores throughout development.

### Chromatoblast and chromatophore death when Fms activity is curtailed

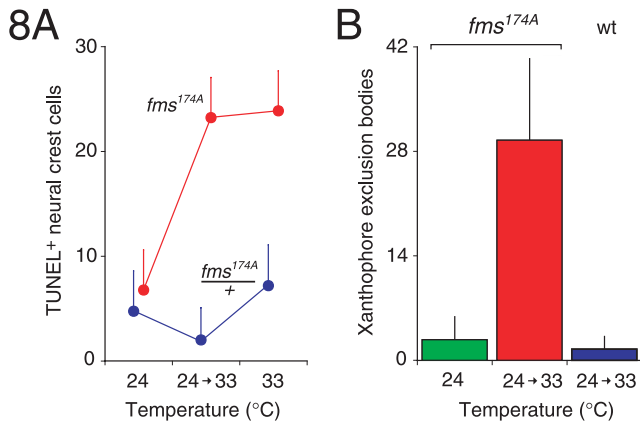
Given the loss of xanthophores and some melanophores upon shifting *fms*<sup>174A</sup> fish to a restrictive temperature, we asked whether these losses might be accounted for partly by cell death. In *fms*<sup>174A</sup> embryos shifted to 33°C, we observed a rapid (within ~2 hours) increase in unpigmented TUNEL<sup>+</sup> cells in neural crest migratory pathways (Fig. 7A, Fig. 8A). In *fms*<sup>174A</sup> juveniles, we did not observe a gross increase in xanthophore or melanophore death immediately upon shifting to 33°C, though unpigmented apoptotic cells were observed frequently within the dermis (Fig. 7B) in locations where *fms*-expressing cells normally are found (Parichy et al., 2000a) (D. M. P., unpublished data). Within 2-3 days of shifting fish to 33°C, however, we observed extensive xanthophore and melanophore debris within the fins of *fms*<sup>174A</sup> mutant but not wild-type individuals (Fig. 7C). This pigmented debris could also be identified within exclusion bodies that are extruded through the epidermis by unknown mechanisms (Parichy et al., 1999; Sugimoto et al., 2000; Sugimoto, 2002) (Fig. 7D-F). We confirmed that exclusion bodies contained xanthophore-derived pteridine pigments by their autofluorescence (Fig. 7D'). An analysis of the number of such extrusions in adult fish shifted to 33°C for 3 days revealed a dramatic increase in fins of *fms*<sup>174A</sup> mutants as compared to wild-type (Fig. 8B). Similar pigmented debris and exclusion bodies were observed on the trunk at lower frequencies (e.g., Fig. 6G, and data not shown). Since *fms* mutants are deficient in macrophages at embryonic stages (Herbomel et al., 2001), it might be argued that pigmented debris and exclusion bodies in upshifted *fms*<sup>174A</sup> individuals reflects an abnormal manifestation of normal pigment cell turnover, revealed by a loss of macrophages upon temperature upshift and a subsequent failure to clear dying cells. This appears not to be the case, however, since histological staining and time-lapse imaging reveals phagocytic and motile macrophages that persist even after temperature upshift of *fms*<sup>174A</sup> adults (D. M. P., unpublished data). Together, these findings suggest that Fms is required for maintaining pigment cell precursors, and that at least some differentiated xanthophores and melanophores that disappear when Fms activity is curtailed are lost by death rather than dedifferentiation.



**Fig. 7.** Chromatoblast and chromatophore death following Fms inactivation. (A) Two hours after shifting *fms*<sup>174A</sup> mutants from 24°C to 33°C, an increase in TUNEL<sup>+</sup> cells is observed in neural crest migratory pathways. Shown are superimposed fluorescence and bright-field images of TUNEL<sup>+</sup> cells (arrow) adjacent to the dorsal neural tube during the stages of neural crest cell migration in a 28 h embryo. nt, neural tube. e, epidermis. m, myotome. B,C,D,E,F are brightfield images; B' and D' are corresponding fluorescence images of B and D. (B) In juvenile fish shifted from 24°C to 33°C, unpigmented TUNEL<sup>+</sup> cells (arrow B') occur in the dermis where *fms*-expressing cells are found. s, scale. (C) Extensive chromatophore debris can be identified in the skin after shifting adult fish from 24°C to 33°C. Shown is a whole mount region of the caudal fin, with orange and black debris from xanthophores and melanophores, respectively. c, capillary. r, fin ray. (D) Extrusions in the superficial epidermis contain orange pigment. (D') Autofluorescence reveals presence of xanthophore-derived pteridine pigments. (E) Extrusion from the fin epidermis (arrow) contains debris of both xanthophores and melanophores. (F) Extrusion containing xanthophore-derived pigment on the fin of a wild-type adult. Scale bars: (A-C) 40 μm, (D,E) 20 μm.

### Late activation of Fms allows recovery of xanthophores and melanophore stripes through adult stages

Temperature shift experiments revealed an essential role for Fms in maintaining cells of the xanthophore lineage and maintaining the striped pattern of adult melanophores throughout development. These findings do not exclude the possibility that a critical period for Fms activity exists during early development; for example, Fms might be required to



**Fig. 8.** Quantitative analyses of cell death when Fms activity is curtailed. (A) Mean (+95% confidence intervals) numbers of TUNEL<sup>+</sup> cells observed in trunk neural crest migratory pathways of 24–28 h embryos ( $n=128$ ) maintained either at 24°C, transferred during these stages of neural crest migration from 24°C to 33°C for 2–3 hours, or maintained at 33°C. A dramatic increase in TUNEL<sup>+</sup> cells occurs in *fms*<sup>174A</sup> homozygous embryos exposed to the 33°C restrictive temperature, as compared to *fms*<sup>174A</sup> homozygotes at 24°C or heterozygotes at either temperature (genotype × temperature interaction for square root-transformed data:  $F_{2,122}=10.0$ ,  $P<0.0001$ ). (B) Mean numbers (+95% confidence intervals) of xanthophore pigment-containing exclusion bodies in caudal fins of adults ( $n=14$ ) maintained either at 24°C or transferred from 24°C to 33°C for 3 days. A sharp increase in the numbers of such exclusion bodies occurs in *fms*<sup>174A</sup> individuals transferred to 33°C as compared to *fms*<sup>174A</sup> maintained at 24°C or wild-type individuals transferred from 24°C to 33°C (square root-transformed data,  $F_{2,11}=28.04$ ,  $P<0.0001$ ).

establish a population of precursor cells during embryogenesis that is recruited to differentiate only much later, during pigment pattern metamorphosis. To investigate whether a critical period exists after which xanthophores and adult melanophore stripes can no longer be rescued, we reared homozygous *fms*<sup>174A</sup> individuals at 33°C and shifted them to 24°C at a range of sizes from embryo to early adult (SL=3.6–16.3 mm,  $n=67$ ). These analyses showed that shifting fish to 24°C allowed extensive recovery of xanthophores and melanophore stripes through late stages of metamorphosis (Fig. 5B, Fig. 9A,B; see below and Supplemental Data: <http://dev.biologists.org/supplemental/>). We did not observe marked recovery of xanthophores in individuals downshifted at larger sizes (>10 mm SL, but see below; final xanthophore density vs. size when shifted, partial regression coefficient=−15.67; SE=1.581;  $F_{1,63}=98.19$ ,  $P<0.0001$ ; Fig. 5B, Fig. 9C).

In addition to restoring xanthophores, providing Fms activity typically resulted in the recovery of an adult melanophore stripe pattern (Fig. 9A,B). Quantitation of melanophore densities and organization did not reveal a significant difference in melanophore densities between individuals downshifted at any stage and control individuals left at 33°C ( $P=0.9$ ), though variability in melanophore nearest neighbor distances was reduced in downshifted individuals with greater xanthophore densities (partial regression coefficient=−5.13; s.e.=0.46;  $F_{1,92}=125.47$ ;  $P<0.0001$ ; Fig. 5C). Thus, melanophores were more organized in individuals that recovered higher densities of xanthophores. Since some

downshifted individuals recovered only irregular and broken melanophore stripes, as an additional indicator of stripe morphology we assessed the frequencies of individuals that developed complete stripes (defined as stripes having no more than one break per side; e.g., Fig. 10). Fig. 5D shows that complete melanophore stripes were more frequent in individuals downshifted by middle metamorphic stages: whereas individuals reared either at 24°C or downshifted prior to 8.0 mm SL typically exhibited no more than one stripe break per side (mean=0.2 breaks, s.d.=0.53,  $n=95$ ), individuals downshifted at larger sizes exhibited from 3–10 breaks, or severely disrupted melanophore stripes resembling control fish maintained at 33°C. Thus, melanophore stripe organization is largely restored when Fms activity is provided at stages through middle to late metamorphosis and this restoration was associated with – and presumably caused by – the concomitant restoration of xanthophore densities.

We observed only partial recovery of xanthophore numbers and stripe morphology during late metamorphic stages and beyond (>8–10 mm SL; Fig. 5B,D, Fig. 9C). This difference from earlier stages could reflect either a critical point beyond which xanthophores and stripes can no longer be recovered fully; or simply insufficient time between temperature downshift and scoring of the resulting patterns for complete recovery to occur. To address these possibilities, we repeated the initial experiments by rearing fish to juvenile stages (~12 mm SL) at 33°C, shifting them to 24°C, and examining pattern development over several months. These individuals gradually recovered xanthophores over several weeks (Fig. 9D–I; for animated time-lapse images, see: Supplemental Data). New xanthophores appeared first in the fins and subsequently were found in ventral regions of the flank, and to a lesser extent on dorsal scales. Gradually, the distributional limits of these cells extended until they had completely covered the flank. As xanthophores occupied new regions, melanophores in these regions adopted increasingly spread morphologies and became increasingly well organized; partially formed adult melanophore stripes developed within approx. 6 weeks of temperature down-shift. Xanthophore distributions and melanophore patterns on the body were indistinguishable from wild-type by approx. 4 months (see: Supplemental Data: <http://dev.biologists.org/supplemental/>). Intriguingly, however, the orientations of fin stripes were haphazard (sometimes even within a fin) and were frequently perpendicular to the wild-type orientations. Such perturbations to fin stripes were evident in both caudal fins (Fig. 9J–L) and anal fins (data not shown). These results demonstrate that Fms activity is sufficient to recruit xanthophores (though not melanophores) and to rescue the organization of melanophore stripes through metamorphic and juvenile stages.

### Fms is not essential for establishing a population of chromatophore precursors during embryogenesis

Xanthophore recovery and pattern regulation in *fms*<sup>174A</sup> homozygotes following temperature down-shift (above) is consistent with a model in which Fms is not essential for establishing a population of chromatophore precursor during early development. Nevertheless, we reasoned that any residual activity by *fms*<sup>174A</sup> at the restrictive temperature might allow the escape of some otherwise Fms-dependent cells during embryogenesis, and these cells would then be able to

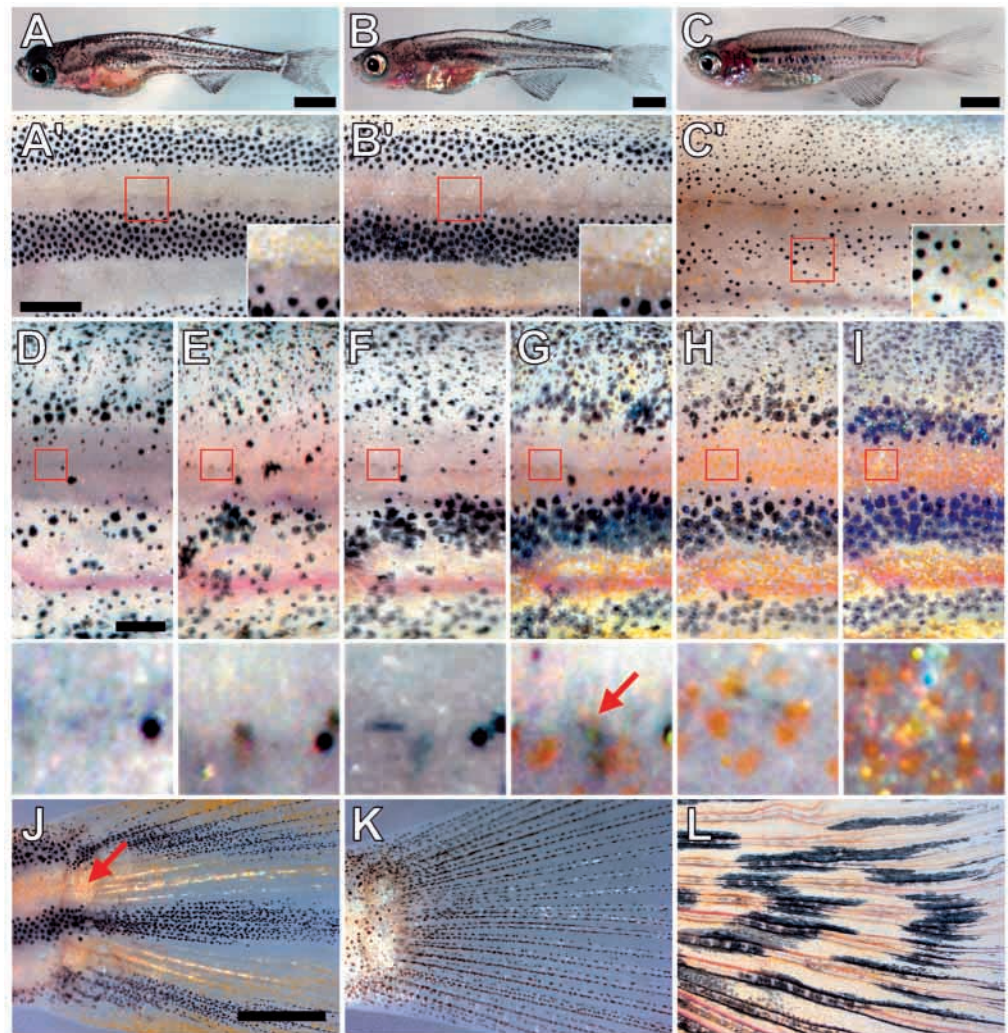
repopulate the flank. In light of this possibility, we repeated the temperature down-shift experiments using individuals transheterozygous for *fms*<sup>174A</sup> and *fms*<sup>blue</sup> [which is likely to act as a null allele (Parichy et al., 2000a)], and placed embryos at a higher initial temperature of 35°C until they had reached the feeding stage. When larvae were downshifted to 24°C, these individuals rapidly recovered approximately normal numbers of *fms*-expressing cells and xanthophores (similar results were obtained by knocking-down Fms activity in wild-type embryos with a morpholino oligonucleotide; data not shown). Interestingly, however, *fms*<sup>174A</sup>/*fms*<sup>blue</sup> transheterozygotes exhibited frequent breaks in adult stripes, far in excess of heterozygous *fms*<sup>174A/+</sup> or *fms*<sup>blue/+</sup> individuals reared initially at 35°C, or *fms*<sup>174A</sup>/*fms*<sup>blue</sup> individuals reared throughout development at 24°C (Fig. 10). These data do not support an essential early role for Fms in establishing precursor cells that are solely responsible for

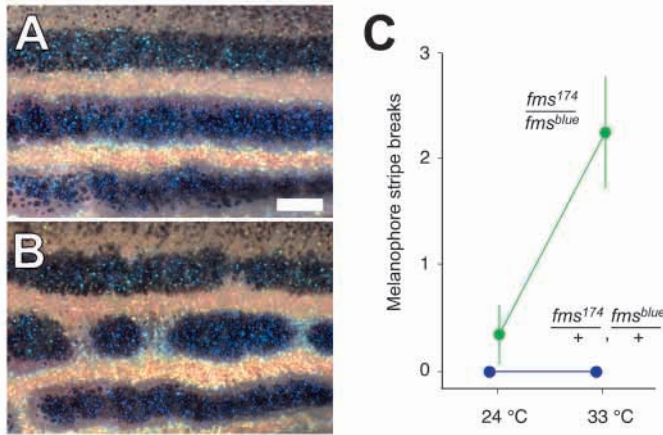
generating later adult stripes. Nevertheless, these findings are consistent with an initial reduction in Fms-dependent cells contributing to irregularities in the patterning of adult melanophore stripes.

## DISCUSSION

The results of this study provide new insights into the cellular basis for adult melanophore stripe development in zebrafish. These observations, and those of previous studies, suggest a model for how pigment cells are recruited and how these cells interact during the larval-to-adult transition (Fig. 11). During early pigment pattern metamorphosis, precursor stem cells begin to be recruited towards xanthophore and melanophore fates (as well as iridophores; not shown). For the xanthophore lineage, successful completion of this process requires *fms*

**Fig. 9.** Temperature downshift experiments reveal xanthophore recovery and pattern regulation after Fms activation. (A-C) Examples of *fms*<sup>174A</sup> homozygotes reared at 33°C to the size indicated (upper panels) then shifted to 24°C until an adult pigment pattern had formed (lower panels). (A) Larva shifted during early pigment pattern metamorphosis (6.5 mm SL) recovered xanthophores and a wild-type pattern of adult melanophore stripes (15.9 mm SL, A') after 34 days at 24°C. (B) Larva shifted during middle stages of pigment pattern metamorphosis (8.1 mm SL, B') after 34 days at 24°C. (C) Individual shifted when pigment pattern metamorphosis was essentially completed (12.3 mm SL) has recovered some xanthophores but not a normally organized stripe pattern (14.1 mm SL, C') after 14 days at 24°C. (Insets) Higher magnification views of boxed regions showing xanthophores. (D-I) Individuals reared initially at 33°C through late larval stages can regulate xanthophores and stripes after several weeks to months following shift to 24°C. Shown are sequential images of the same region on a representative *fms*<sup>174A</sup> individual (starting 12 mm SL) at 2 days (D), 10 days (E), 16 days (F), 22 days (G), 27 days (H), and 40 days (I) after temperature downshift. Note increasingly organized and spread melanophores as xanthophores populate the flank. (Upper images) Low magnification showing melanophore distributions. (Lower images) Higher magnification of boxed regions showing recovery of xanthophores (arrow). (J-L) Perturbation of fin patterning when Fms is activated late in development. (J) Normal horizontal stripes form in the caudal fin of *fms*<sup>174A</sup> individuals reared at 24°C. Xanthophore stripes on the fin extend caudally from stripes on the body (arrow). (K) Xanthophores and stripes are absent in the fins of *fms*<sup>174A</sup> individuals reared at 33°C. (L) Xanthophore recovery accompanied by stripe reorientation in *fms*<sup>174A</sup> when Fms is activated only late in development. Scale bars: (A,B) 1 mm, (C) 2 mm, (A'-C') 500 µm, (D-I) 250 µm, (J-L) 1 mm.





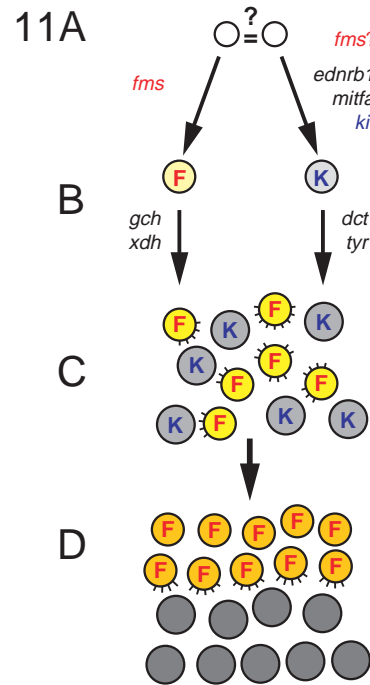
**Fig. 10.** Pattern regulation in *fms*<sup>174A</sup>/*fms*<sup>blue</sup> transheterozygotes. (A) Normal adult melanophore stripes develop in *fms*<sup>174A</sup>/*fms*<sup>blue</sup> individuals reared at 24°C. (B) Xanthophores and melanophore stripes are recovered in *fms*<sup>174A</sup>/*fms*<sup>blue</sup> individuals reared 35°C prior to feeding, and at 24°C thereafter. (C) More frequent breaks in adult melanophore stripes occur in *fms*<sup>174A</sup>/*fms*<sup>blue</sup> individuals reared at 33°C prior to hatching, as compared to *fms*<sup>174A</sup>/*fms*<sup>blue</sup> reared at 24°C prior to hatching, or *fms*<sup>174A</sup>/+ or *fms*<sup>blue</sup>/+ heterozygotes reared at either temperature (temperature × genotype interaction for square root-transformed data:  $F_{1,110}=6.33$ ,  $P<0.05$ ). Shown are unilateral mean numbers of breaks in melanophore stripes per individual (±95% confidence intervals). Scale bar: 500 μm.

activity. Subsequently, during middle stages of pigment pattern metamorphosis, *fms*-dependent cells of the xanthophore lineage influence cells of the melanophore lineage to form stripes. Finally, beginning during late stages of metamorphosis when stripes have formed and continuing thereafter, *fms*-dependent cells of the xanthophore lineage contribute to maintaining the organization of melanophore stripes. Below, we discuss various aspects of this model, as well as its relation to previous genetic analyses of *fms*- and *kit*-dependent melanophore populations.

**Independence of embryonic and metamorphic xanthophore populations revealed by modulation of Fms activity**

Many organisms undergo a metamorphosis in which an embryonic or larval morphology is transformed into that of an adult. Among vertebrates, such changes are especially pronounced in anuran amphibians; similar albeit less dramatic changes also occur in salamanders and teleosts. In zebrafish, a variety of traits are either altered, or develop apparently de novo during metamorphosis, including: fins (larval fin folds are lost and adult unpaired fins develop); hematopoietic, gut, sensory and nervous systems; skin (increased stratification and formation of adult scales); behavior and physiology; and the pigment pattern (e.g. Kirschbaum, 1975; Brown, 1997; Sire et al., 1997; Ledent, 2002). For zebrafish and other vertebrates, however, it remains largely unknown to what extent traits expressed both before and after metamorphosis share common cellular bases and genetic requirements (Parichy, 1998), though this issue is central to understanding the development and evolution of adult form.

With respect to pigment patterns, adult stripes could depend



**Fig. 11.** Model for pigment pattern metamorphosis in zebrafish. (A) Throughout metamorphosis new pigment cells appear from undifferentiated stem cells (see text for references). These cells (white) may be specified for one or another cell fate, or they may be pluripotent. Recruitment of stem cells to the xanthophore lineage (yellow cells, left) requires *fms*; in the absence of Fms activity these cells die, fail to advance through stages of xanthophore differentiation, or both. Stem cells also are recruited to melanophore fates (grey cells, right) under the influence of *ednrb1*, *mitfa* and *kit*. Although gene expression analyses reveal *fms* expression at early stages in some of these cells, a cell autonomous role for *fms* in promoting the development of early stages in the melanophore lineage has yet to be documented. (B) Terminal differentiation of chromatophores depends on genes encoding pigment synthesis enzymes that are likely to differ between xanthophores (e.g., *gch*; *xdh*) and melanophores (e.g., *dopachrome tautomerase*, *dct*; *tyrosinase*, *tyr*). During these stages, xanthoblasts express and require *fms* (F). A parallel requirement for *kit* is observed for fin melanoblasts, and likely body melanoblasts that also express *kit* (K). (C) During middle stages of pigment pattern metamorphosis and possibly prior to the terminal differentiation of chromatophores, *fms*-dependent cells of the xanthophore lineage influence *kit*-dependent cells of the melanophore lineage to form stripes. Although this interaction promotes melanophore competence for stripe formation, the directionality of these stripes depends on additional cues, possibly including initial asymmetries in chromatoblast or stem cell distributions, or other features of the extracellular environment. In the absence of Fms activity, xanthophores are not recruited and do not influence melanophore stripe formation. (D) During late stages of pigment pattern metamorphosis extending through adult life, *fms*-dependent xanthophores (or their precursors) contribute to maintaining melanophore stripes. In the absence of Fms activity, xanthophores die and melanophore stripes degenerate.

entirely on pigment cells that differentiate during embryogenesis, with cell migration, death and proliferation remodeling the early larval pigment pattern into that of the adult. Or, distinct populations of cells could contribute to patterns at different stages via the de novo differentiation of

new pigment cells during metamorphosis. The existence of mutants that ablate embryonic – but not adult – melanophores and iridophores demonstrates that distinct populations of these cells contribute to pigment patterns before and after metamorphosis (Johnson et al., 1995; Haffter et al., 1996; Kelsh et al., 1996; Parichy et al., 1999). This issue has remained unresolved for xanthophores, however, since mutants have yet to be identified that ablate these cells at one stage but not another. Results of temperature shift experiments in this study suggest that xanthophores comprising the embryonic early larval and adult pigment patterns in zebrafish represent temporally distinct populations: curtailing Fms activity during embryogenesis ablated embryonic xanthophores, but restoring Fms activity in these same individuals after embryogenesis permitted the recovery of adult xanthophores in approximately normal numbers. These findings imply that undifferentiated precursor cells are present through pigment pattern metamorphosis and adult development, and can be recruited to differentiate as xanthophores (Fig. 11A). These results and those of reciprocal temperature shift experiments (discussed below) further indicate that embryonic and adult xanthophores share a common dependence on *fms*. This requirement contrasts with *ednrb1* and some other loci that are essential for trait development at only one stage or another (Haffter et al., 1996; Parichy et al., 2000b) (D. M. P. and J. M. T., unpublished data). The extent to which phenotypes and gene activities are partitioned across metamorphosis thus appears to vary both across traits and across loci.

### Chromatophore stem cells during post-embryonic development

The existence of pigment stem cells at post-embryonic stages is indicated by results of this study, previous analyses of fish (Rawls and Johnson, 2001) (reviewed by Sugimoto, 2002), and recent findings from mammals (Grichnik et al., 1996; Kunisada et al., 1998; Nishimura et al., 2002). In zebrafish, however, the precise location and developmental potential of such cells remain unknown (Fig. 11A). Conceivably, independent populations of specified stem cells may contribute to xanthophore, melanophore and iridophore lineages. Alternatively, these cells may be multipotent and capable of contributing to multiple chromatophore lineages. Indeed, the existence of a common melanophore–xanthophore precursor during post-embryonic development would readily explain genetic analyses that revealed a role for *fms* in promoting the differentiation of normal numbers of melanophores (Parichy et al., 2000a), as well as the correlated melanophore and xanthophore deficits that resulted from curtailing Fms activity in this study. Nevertheless, both of these observations can also be interpreted to reflect a role for the *fms*-dependent xanthophore lineage in promoting the differentiation, survival, or proliferation of melanoblasts. Analyses of gene expression and cell lineage now underway at these stages should help to identify the locations and range of fates for post-embryonic pigment stem cells in zebrafish.

### *fms* requirement for recruiting and maintaining xanthophores throughout development

A major finding of temperature shift experiments in this study is a requirement for *fms* in recruiting cells of the xanthophore lineage throughout development (Fig. 11B): if Fms activity is

curtailed early, xanthophores and their precursors fail to develop; yet if Fms activity is provided late, these cells differentiate and can achieve roughly wild-type densities given sufficient time. This dependence could reflect several different requirements at the cellular level. For example, Fms could be required by fully differentiated xanthophores but not their precursors, by xanthophore precursors but not xanthophores, or by cells at all stages of specification and differentiation within the xanthophore lineage. Results of this study support the latter interpretation. Abrogating Fms activity resulted in the loss by apoptosis of unpigmented, presumptive neural crest-derived cells in embryos that are likely to include xanthophore precursors. [Useful markers of the xanthophore lineage are only now being developed for later stage larvae (D. M. P., unpublished data).] An essential role for Fms in maintaining xanthophore precursors during their differentiation is reminiscent of analyses of the *fms* homologue, *kit*, which is required for the survival of amniote melanoblasts (Cable et al., 1995; Okura et al., 1995; Ito et al., 1999) and during the differentiation of zebrafish melanoblasts into melanophores in the regenerating fin (Rawls and Johnson, 2001).

Our results also show that differentiated xanthophore require Fms activity, but their dependence is noticeably reduced. When juvenile fish were transferred to a restrictive temperature, a gradual loss of xanthophores is observed over a period of days to weeks. Similarly, abrogation of Fms activity results in more severe xanthophore deficits in the adult pattern when temperature shifts are performed during the early larval period or through the middle of pigment pattern metamorphosis, as compared to terminal stages of metamorphosis. Since xanthophore densities increase steadily during pigment pattern metamorphosis (D. M. P. and J. M. T., unpublished data), the persistence of xanthophores following temperature upshift at late stages coincides with a time when more of these cells have already differentiated. These observations suggest that xanthophores exhibit a reduced dependence on Fms activity as compared to xanthophore precursors. The eventual loss of xanthophores at a restrictive temperature could indicate that xanthophores require Fms only intermittently, or that the *fms*<sup>174</sup> allele exhibits sufficient residual activity even at a restrictive temperature to allow transient persistence of xanthophores. Overall these observations argue for a continued, but diminished requirement for Fms in differentiated xanthophores as compared to their precursors. The persistent requirement for Fms by fin xanthophores and body xanthophores contrasts with the eventual independence from Kit attained by fin melanophores (Rawls and Johnson, 2000; Rawls and Johnson, 2001) and possibly a subpopulation of body melanophores (Johnson et al., 1995).

### Dependence of adult melanophore stripes on the xanthophore lineage

Vertebrates exhibit a diversity of pigment patterns, yet we know little about how these patterns are generated. In zebrafish, a large number of genes essential for adult stripe development have been isolated as mutant lines and some of these genes have now been cloned (e.g. Lister et al., 1999; Parichy et al., 1999; Parichy et al., 2000a; Parichy et al., 2000b; Kawakami et al., 2000). Nevertheless, the cellular bases for stripe development remain largely unknown. For example, it is unclear to what extent patterns are generated by interactions

between chromatophores and cues in their extracellular environment, or interactions among chromatophore classes themselves. Results of chimera analyses in this study strongly suggest that *fms*-dependent cells of the xanthophore lineage are necessary to promote melanophore organization into adult stripes (Fig. 11C). In the absence of xanthophores and their precursors in *fms*<sup>-</sup> mutants, normal adult melanophore stripes do not form; however, when cells of the xanthophore lineage are provided by transplanting cells between *nacre*<sup>-</sup> and *fms*<sup>-</sup> mutant embryos, adult melanophore stripes are rescued. Similarly, greater xanthophore densities (provided by modulating Fms activity in temperature shift experiments) are associated with increasingly organized melanophore patterning.

The dependence of initial melanophore stripe formation on the xanthophore lineage could reflect several different underlying mechanisms. For example, direct interactions between melanophores and xanthophores or their precursors could generate stripes via contact inhibition of movement and contact stimulated migration (Tucker and Erickson, 1986b; Thomas and Yamada, 1992). Such interactions have been implicated in the formation of vertical bars and horizontal stripes in salamander larvae (Epperlein and Löfberg, 1990; Parichy, 1996a,b), and it is conceivable that homologous interactions are present during zebrafish adult stripe development. Differential adhesive properties could also contribute to a sorting out of these cell types (Steinberg, 1970); indeed xanthophores, but not melanophores, in the teleost *Oryzias latipes* express N-CAM and N-cadherin (Fukuzawa and Obika, 1995). Finally, an additional possibility is that interactions between melanophores and the xanthophore lineage are indirect, if xanthophores or their precursors provide signals that promote the competence of melanophores to receive other pattern-forming cues. For example, zebrafish melanophores express the Kit receptor, and xanthophores in salamanders express the Kit ligand, Steel Factor (N. Parker, personal communication); since Kit signaling modulates integrin expression and stimulates motility (Scott et al., 1994; Jordan and Jackson, 2000) (see also Grichnik et al., 1998), this type of interaction could contribute to organizing zebrafish melanophores.

Whatever the molecular mechanisms responsible for interactions between melanophores and the xanthophore lineage, these results suggest an important role for interactions among chromatophore classes in generating adult zebrafish stripes. This interpretation supports the conclusions of early analyses of fin stripe development and regeneration (Goodrich and Nichols, 1931; Goodrich et al., 1954). A role for melanophores in organizing iridophores has been suggested as well (Johnson et al., 1995), implying a cascade of interactions among chromatophores or their precursors: xanthophores → melanophores → iridophores.

Interactions between melanophores and the xanthophore lineage may be essential for generating stripes but may not be sufficient to determine the orientation of the pattern in some or all contexts. Evidence for this assertion comes from temperature downshift experiments in which late activation of Fms produced fin stripes perpendicular to their normal orientation. These observations suggest that although re-establishment of melanophore–xanthophore interactions contributes to stripe formation, other patterning cues with an early critical period are essential for setting the normal

directionality of these stripes. In the developing caudal fin, stripes appear initially as extensions of the body stripes (Rawls and Johnson, 2000), and melanophore–xanthophore interactions are likely to build upon this pre-existing pattern during fin growth. When xanthophores are produced in the fin only late in development, other cues presumably serve to set the directionality of these stripes, or stripe orientation is determined stochastically. On the body, late xanthophore regulation yielded stripes that resembled wild-type stripes, suggesting either that the same cues are present as at earlier stages, or that other cues are able to serve the same function late in development. A distinction between stripe generation and pattern directionality is also evident in larval salamanders, in which the positions of vertical bars depend on an apparently stochastic positioning of xanthophore aggregates along the neural tube (Epperlein and Löfberg, 1990; Parichy, 1996a). Similarly, horizontal stripes in most salamander larvae that have been examined depend on an initial interaction between melanophores and the lateral line sensory system that sets the directionality of the stripes, and these stripes are then enhanced by interactions between melanophores and xanthophores (Parichy, 1996a; Parichy, 1996b). It will be interesting to identify cues that set the directionality of horizontal stripes as well as vertical bars among *Danio* species (Fang, 2000; Quigley and Parichy, 2002), and whether interactions between melanophores and the xanthophore lineage are a shared pattern-forming mechanism for this group.

Finally, the results of this study reveal an essential role for Fms in maintaining melanophore stripes once formed (Fig. 11D). When Fms activity is curtailed after stripes have developed, melanophores are lost concomitant with xanthophores until a pattern resembling that of *fms* presumptive null alleles is obtained. These findings suggest that in addition to roles for the *fms*-dependent xanthophore lineage in promoting initial formation of melanophore stripes, these cells may also be required for melanophores to receive factors essential for maintenance of a differentiated state, survival, or both. This could indicate that xanthophores or their precursors provide maintenance signals to melanophores directly. Arguing against this notion is the normal death of melanophores that become surrounded by xanthophores in the developing melanophore-free interstripe regions of both body and fins (Goodrich et al., 1954; Goodrich and Greene, 1959; Parichy et al., 2000a) (D. M. P. and J. M. T., unpublished data). Alternatively, community effects among melanophores could promote the maintenance of these cells if melanophores present paracrine factors to one another that are essential for their support: local melanophore densities that fall beneath some critical threshold would then result in melanophore dedifferentiation or death, and a failure of normal stripe formation or maintenance (Parichy et al., 2000a; Aubin-Houzelstein and Panthier, 1999). The results of the present study are consistent with the idea that a loss of *fms*-dependent xanthophores allows melanophores to leave their initial positions in stripes and thereby to lose maintenance signals that otherwise would be received.

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