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Zebrafish pigmentation mutations and the processes of neural crest development

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SUMMARY

Neural crest development involves cell-fate specification, proliferation, patterned cell migration, survival and differentiation. Zebrafish neural crest derivatives include three distinct chromatophores, which are well-suited to genetic analysis of their development. As part of a large-scale mutagenesis screen for embryonic/early larval mutations, we have isolated 285 mutations affecting all aspects of zebrafish larval pigmentation. By complementation analysis, we define 94 genes. We show here that comparison of their phenotypes permits classification of these mutations according to the types of defects they cause, and these suggest which process of neural crest development is probably affected. Mutations in eight genes affect the number of chromatophores: these include strong candidates for genes necessary for the processes of pigment cell specification and proliferation. Mutations in five genes remove part of the wild-type pigment pattern, and suggest a role in larval pigment pattern formation. Mutations in five genes show ectopic chromatophores in distinct sites, and may have implications for chromatophore patterning and proliferation. 76 genes affect pigment or morphology of one or more chromatophore types: these mutations include strong candidates for genes important in various aspects of chromatophore differentiation and survival. In combination with the embryological advantages of zebrafish, these mutations should permit cellular and molecular dissection of many aspects of neural crest development.

Key words: melanophore, xanthophore, iridophore, specification, proliferation, survival, differentiation

INTRODUCTION

The questions of how cells become specified and patterned within the embryo are fundamental to developmental biology. The vertebrate neural crest is an attractive model system for studying these questions because it gives rise to a great diversity of different cell fates which after migration form highly patterned structures, including much of the craniofacial skeleton, peripheral nervous system and pigment patterns (Hörstadius, 1950; Weston, 1970, 1991; Le Douarin, 1982; Eisen and Weston, 1993).

Neural crest cell development involves at least five general processes, which overlap in their timing. (1) Specified precursors must be generated from initially multipotent neural crest cells (Bronner-Fraser and Fraser, 1988). (2) These precursors must be patterned within the embryo. Neural crest patterning involves two steps: choice of migration pathway and choice of site of localisation. Two major migration pathways are used by neural crest cells (Weston, 1963; Le Douarin, 1982). The lateral migration pathway lies between the skin and the somites, and is used predominantly by pigment cells, whereas the medial pathway lies between the neural tube and the somite, and is used by most or all derivatives (Le Douarin, 1982). Cells of each derivative migrate to characteristic locations at which they form stereotypical arrangements of cells. (3) Neural crest cells are usually highly proliferative (e.g. Fraser and Bronner-Fraser, 1991; but compare Raible and Eisen, 1994). (4) Neural crest cells are dependent on various trophic factors for their survival (e.g. Stemple et al., 1988; Morrison-Graham and Weston, 1993). (5) Cells of each derivative must express characteristic differentiation products.

Although in vivo and in vitro studies have revealed much about the biology of these different processes (see reviews by Le Douarin, 1982; Weston, 1991; Anderson, 1993; Selleck et al., 1993; Stemple and Anderson, 1993), key molecular regulators involved are largely unknown. We have undertaken a genetic approach to the dissection of these processes. Studies of mouse mutations that affect coat pigmentation have identified several genes crucial for certain steps of pigment cell development (Silvers, 1979; Lyon and Searle, 1989; Jackson, 1994).

Thus, *Dominant spotting* (*W*) and *Steel* (*Sl*) have been shown to have multiple effects on proliferation (Reid et al., 1993), survival (Morrison-Graham and Weston, 1993), and probably also the choice of migration pathway (Wehrle-Haller and Weston, 1995) of melanoblasts. In addition, mouse pigmentation mutations have been important in understanding melanocyte differentiation (Jackson, 1994). For example, in *dilute* (*d*) mutant mice, melanocytes appear less dendritic (Silvers, 1979). *d* encodes a novel myosin protein that may be involved in dendrite formation or in the transport of melanosomes, the membrane-bound pigment-containing organelles of melanocytes, into dendrites (Mercer et al., 1991; Jackson, 1994). These studies suggest that extensive screening for mutations disrupting pigmentation would be a powerful approach to the identification of key regulators of neural crest development.

The advantages of zebrafish as a genetic model organism have been discussed elsewhere (Kimmel, 1989; Mullins and Nüsslein-Volhard, 1993; Driever et al., 1994; Henion et al., 1995). Analysis of zebrafish neural crest has revealed many parallels with other vertebrates, in terms of the range and types of derivatives, their migration routes, and their embryonic distribution (Raible et al., 1992; Schilling and Kimmel, 1994). Furthermore, the ability to follow individual cells in living embryos has been elegantly exploited to characterise neural crest fate specification (Raible et al., 1992; Eisen and Weston, 1993; Raible and Eisen, 1994; Schilling and Kimmel, 1994).

Zebrafish offer unique opportunities for addressing the mechanisms underlying neural crest cell development. Their larvae display a rich, reproducibly patterned arrangement of three distinct pigment cell types (chromatophores): melanophores, xanthophores and iridophores (Milos and Dingle, 1978; Kimmel et al., 1995; this work). Since these cells are naturally labelled, readily observed under the dissecting microscope, and complete development of the larval pigment pattern within 6 days post-fertilisation, zebrafish provide a useful system for the identification of mutations affecting all aspects of their development. A number of larval pigmentation mutations have been described previously (Streisinger et al., 1981, 1986), and the genetic basis of adult stripe formation is being studied using these and other mutations (Johnson et al., 1995). A small-scale screen for zebrafish neural crest mutations has recently been described (Henion et al., 1995).

As part of a large-scale screen for embryonic/early larval zebrafish mutations (Haffter et al., 1996), we isolated many mutations affecting pigment cell development. In this paper we present detailed descriptions of pigment mutant phenotypes. We relate these to aspects of neural crest development that may be involved, and distinguish mutations affecting early processes of crest development (e.g. specification) from those affecting later processes (e.g. differentiation). Many of the advantages of zebrafish pigmentation as a neural crest model system apply also to the zebrafish craniofacial skeleton, and many mutations affecting these structures were isolated in the same screen (Piotrowski et al., 1996; Schilling et al., 1996). This work forms the foundation for a detailed analysis of the development of particular neural crest derivatives.

MATERIALS AND METHODS

Fish breeding and maintenance is described by Mullins et al. (1994), and identification, isolation and complementation analysis of mutations is described in an accompanying paper (Haffter et al., 1996). Stocks of previously described pigmentation mutations (*albino* (*alb*), *golden* (*gol*), *brass* (*brs*), *sparse* (*spa*) and *rose* (*rse*); Streisinger et al., 1981, 1986; Johnson et al., 1995) were maintained similarly.

Examination of pigment phenotypes

We kept all mutations that affected pigmentation by changing chromatophore cell number, pattern, morphology or intensity of pigmentation, but which were without severe defects in early development. Mutations with pigment defects believed to be secondary effects of another aspect of the phenotype were not kept unless they fitted criteria described for the other phenotype (see accompanying papers). Many mutations with severe melanophore degeneration phenotypes are necrotic by the sixth day (all stages as defined by Kimmel et al., 1995) and were also not kept.

During reidentification from outcross families, pigmentation phenotypes were reexamined to get consistent descriptions. Embryos were examined, during the hatching period and on the sixth day, with a Wild stereo microscope with 0.8×, 2×, 4× and 8× objectives, using both transmitted and incident light. The latter was vital for examining iridophore and xanthophore phenotypes. Observations of pigment cell morphologies were made with a Zeiss Axiophot during the photography of selected phenotypes.

Embryos were generally kept in the light on a white background. Due to background adaptation, melanophores in 6-day-old larvae show their pigment organelles (melanosomes) clustered tightly around the cell centre, and so appear smaller than at earlier stages. Hence, mutants in which this process of background adaptation was hindered were easily found, by the much larger appearance of their melanophores.

Neural crest cell counts

To count crest cells migrating on the lateral migration pathway (Raible et al., 1992), living embryos were mounted in embryo medium (Mullins et al., 1994) under a No. 1 coverslip, using No.1 coverslips as spacers. Migrating neural crest cells were identified by their characteristic morphology and location immediately below the periderm (Raible et al., 1992). Cells were counted on one side over somites 5-15 in each embryo, between the 26-somite and prim-24 stages of development (22-35 hours of development at 28.5°C; stages as defined by Kimmel et al., 1995). Individually identified embryos were

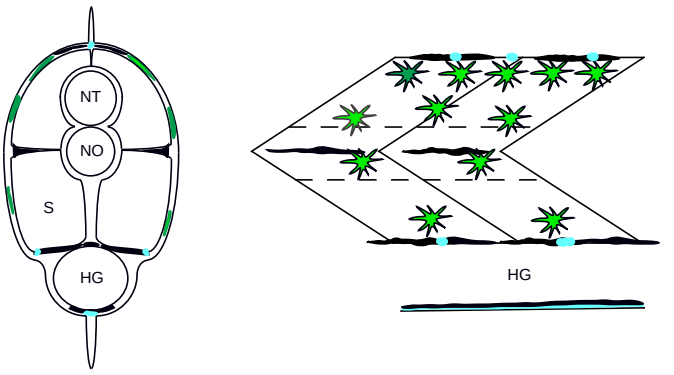

Fig. 1. Schematic drawings of sixth day larval pigment pattern in posterior trunk. The left panel shows a transverse section (dorsal to the top), whilst the right panel shows a lateral view (dorsal to the top, rostral to the left), both to the same scale. Melanophores are shown as black shapes and form four stripes, the dorsal, lateral, ventral and yolk sac stripes in dorsoventral order. Iridophores (pale blue) are associated with the dorsal, ventral and yolk sac stripes. Xanthophores (green) populate the sides of the body, and are more abundant in dorsal regions. NT, neural tube; NO, notochord; S, somite; HG, hindgut.

then raised overnight in embryo medium and their phenotype identified.

Photography

Embryos/larvae were mounted in 2% methyl cellulose without a coverslip or between No. 1 coverslips, using 2 (pharyngula - hatching periods) or 3 (sixth day) No. 1 coverslips as spacers. Phenotypes were photographed in living fish with a Zeiss Axiophot microscope with 5×, 10×, 20× and 40× (water immersion) objectives, using Ektachrome 64T film. Embryos were illuminated with incident light from a fibre-optic tungsten light source to photograph iridophores, and the orientation of larvae with respect to the light source, as well as the exposure time, were comparable.

RESULTS

The larval zebrafish pigment pattern consists of an array of three neural crest-derived pigment cell types that is essentially complete by the sixth day (Figs 1, 2A,C). The pattern consists of four longitudinal stripes of black, melanin-expressing melanophores (Milos and Dingle, 1978). The dorsal stripe and the ventral stripe extend from the head to the tail tip; the yolk sac stripe extends from under the heart to the anus; and the lateral stripe runs in the horizontal myoseptum of somites 6-26 out of the normal 30-34 somites (with some variation in the boundaries between individuals). Iridophores appear silver under incident light, or gold when viewed through overlying xanthophores (Fig. 2C). They are associated with three of the melanophore stripes, and have a characteristic pattern in each of them: a medial array in the dorsal stripe, a series of bilateral pairs and a pair of lateral patches (the large patches above the yolk sac) in the ventral stripe, and a dense band in the yolk sac stripe (Fig. 2L). Individual xanthophores are difficult to distinguish (Fig. 2J,K), even on the sixth day, but together they give a strong yellow cast to the larval body, most prominent dorsally, and rather weaker ventrally. They are not visible ventral to the ventral stripe.

Zebrafish embryos begin to develop pigmentation visible under the stereo microscope at around 25 hours of development

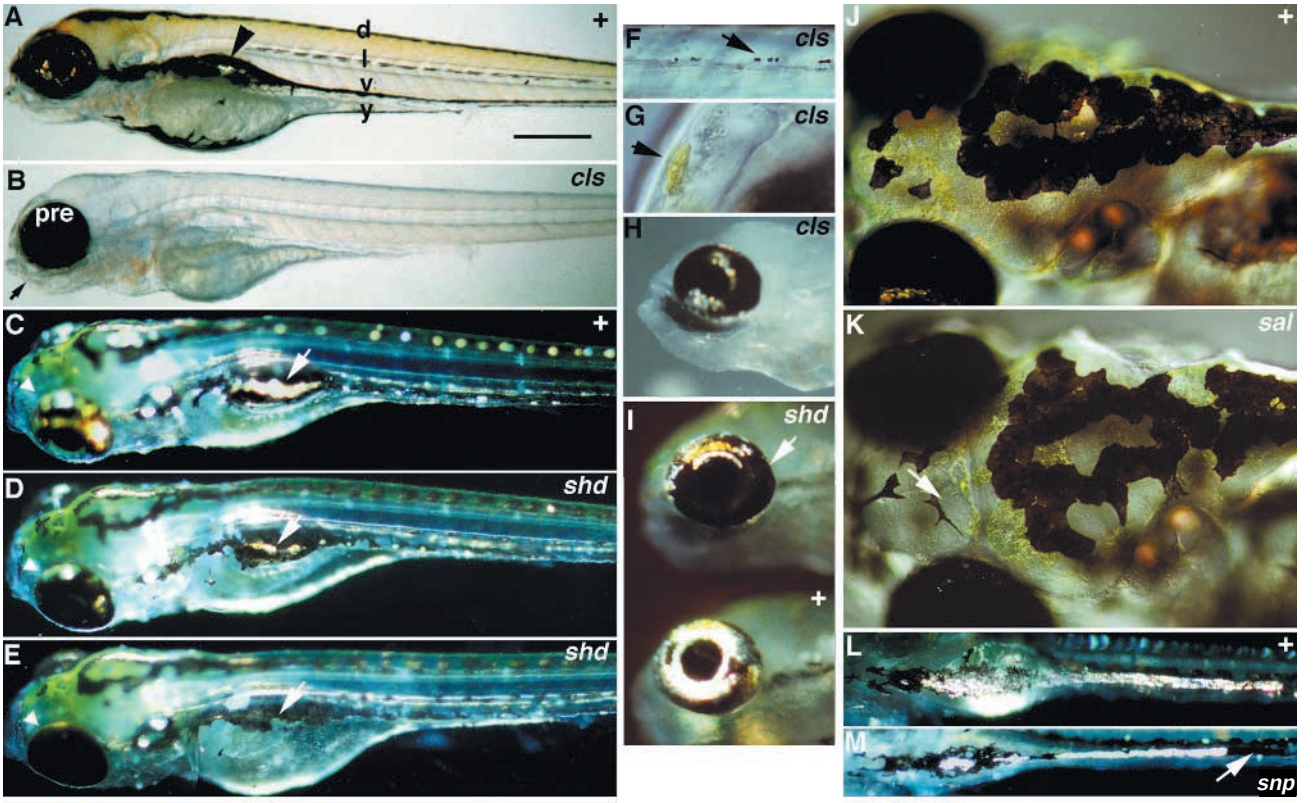

Fig. 2. Sixth day phenotypes of decreased cell number (Classes I and II) mutants. Panels show wild-type control sibling together with homozygous individuals for each of *cls* (A,B,F-H), *shd* (C-E,I), *sal* (J,K) and *snp* (L,M). Chromatophores are prominent in wild-type larvae (four melanophore stripes are labelled, yellow xanthophore pigmentation is clear dorsally, and iridophores are just visible in the eye and the lateral patches (arrowhead)) (A), but absent in *cls* mutants (B). Note wild-type pigmented retinal epithelium and jaws and arches (arrow) in *cls* mutants. Occasional chromatophores found in *cls* include abnormally tiny melanophores (F), but normal xanthophores (G) and iridophores (H). *shd* mutants (D,E) have fewer iridophores, but where present they look normal. Iridophores in the lateral patches (arrow) and dorsal eye (arrowhead) are highlighted. *shd* alleles show a phenotypic series: shd^{ty70} (D) is weaker than shd^{ty82}(E). An intermediate strength *shd* allele, shd^{tm46}, shows iridophores over 30% of the outer eye (arrow, I). Ventral views of the yolk sac stripe in *snp* mutants (M) show gaps in the normally complete (compare L) stripe of iridophores (arrow). The continuous sheet of xanthophores shown in a dorsal view of the head (J) is interrupted in intermediate strength *sal* alleles (K) by cell-free areas which lack the characteristic granularity and yellow colour of xanthophores (arrow). C-E are dorsolateral views. C-E,I,L and M were photographed with incident light, H with a mix of incident and transmitted light. In this, and all subsequent figures, fish are oriented dorsal up, rostral left, and are photographed with transmitted light, unless otherwise noted. d, dorsal stripe; l, lateral stripe; pre, pigmented retinal epithelium; v, ventral stripe; y, yolk sac stripe. Scale bars, 400 μm (A-E), 75 μm (F), 150 μm (G), 325 μm (H), 250 μm (J,K) and 350 μm (L,M).

Table 1. Summary of phenotypes and complementation analysis

Mutant category	Number of genes defined	Number of alleles	Average allele frequency	Unresolved mutations (N)
Reduced chromatophore numbers				
I: no chromatophores	2	3	1.5	0
II: missing cell-type	4	26	6.5	0
III: reduced melanophores	2	10	5	0
Abnormal chromatophore distribution				
IV: abnormal pigment pattern	5	7	1.4	0
V: ectopic chromatophores	5	14	2.8	0
Reduced chromatophore pigmentation				
VI.A: pale melanin	7	24	3.4	2
VI.B: delayed melanophore differentiation	3	13	4.3	3
VI.C: melanophore degeneration	4	13	3.3	3
VI.D: melanophore degeneration, xanthophores pale	2	5	2.5	2
VI.E: melanophore degeneration, dull iridophores	7	22	3.1	8
VI.F: pale melanin, dull iridophores	1	1	1	2
VI.G: pale xanthophores, dull iridophores	5	13	2.6	6
VI.H: melanophore degeneration, pale xanthophores, dull iridophores	12	17	1.4	1
VI.I: dull iridophores	7	8	1.1	3
VI.J: pale xanthophores	15	49	3.3	2
Abnormal chromatophore morphology				
VII.A: spindly melanophores	3	3	1	0
VII.B: small melanophores	1	1	1	0
VII.C: stellate chromatophores	2	12	6	0
VII.D: no background adaptation	7	9	1.3	3
Total	94	250	2.7	35

at 28.5°C (Kimmel et al., 1995; see also Milos and Dingle, 1978). Melanin is visible first in the retinal epithelium, and within 2 hours is visible within migrating melanophores in the anterior trunk and posterior head regions. This pigmentation becomes stronger throughout the second day. Xanthophores and iridophores become visible during the hatching period. Xanthophores appear as a faint yellow cast to the head, especially dorsally. Iridophores become visible as tiny silver spots on the eye and in the dorsal tail region. Over the next 3 days all three chromatophore types continue to appear and become more strongly pigmented, and the larval pattern matures.

We identified 285 mutations with pigment phenotypes which promise to include mutations in key genes of all processes of chromatophore development. 250 (88%) of these mutations have been assigned to 94 complementation groups and these all fall within seven major phenotypic classes (Table 1). We later use this classification to suggest testable hypotheses regarding the process of neural crest development that may be affected in each class. A few genes affect pigment cell number, either all three pigment cell types (Class I), or just a single type (Classes II and III). Chromatophore distribution is affected in two ways: (1) part of the wild-type pattern is absent (Class IV), or (2) the pattern is complete but ectopic chromatophores are also present (Class V). The majority of mutations affect the degree of pigmentation of one or more cell types (Class VI). Mutations altering chromatophore morphology (Class VII) were identified by their effects on melanophores; two of these also affect xanthophore morphology. No mutations affecting iridophore morphology were found.

All mutations are described briefly in Tables 2-6, and in more detail in the next sections and Figs 2-13. Many mutations show additional phenotypes, and affected structures are noted in the tables and the papers referenced. The genes thus defined have from 1-12 alleles; 54 (58%) have only a single allele, although some of the unresolved mutations (Table 1) may be allelic with these complementation groups.

Class I: no chromatophores

Mutations in two genes result in dramatic reductions of all chromatophore cell types (Table 2; Fig. 2). *white tail (wit*) mutants show melanophores with normal morphology and pigmentation, but their number is reduced and they are absent posterior to the midtrunk (see Jiang et al., 1996). *wit* affects neurogenesis and mutant embryos die during the hatching period (Jiang et al., 1996). Hence defects in xanthophores and iridophores cannot be evaluated.

This phenotype is very different to that of *colourless* (*cls*), a gene originally defined by a spontaneous mutant allele, cls^{t3}, found in our *rose* stock, and represented in this screen by two alleles. *cls* mutants have a normal eye and a fully pigmented retinal epithelium, but are otherwise without pigmentation (Fig. 2B). Close examination reveals a few chromatophores of each type in some mutant individuals: isolated xanthophores in the head, patches of iridophores on the eye and/or in the lateral patches or dorsal stripe, and tiny melanophores in the dorsal stripe (Fig. 2F-H). These few remaining cells are all in normal positions. Mutant embryos survive until the eleventh day with normal morphology.

To test whether *cls* mutants affect chromatophore precursors at an early stage we examined crest cells on the lateral migration pathway. Crest cells using this route are fated to become chromatophores (Raible and Eisen, 1994). Hence we wanted to know if the number of such cells was decreased in

cls mutants. Counts of lateral pathway neural crest cells revealed a 95% reduction in the number of such cells in *cls* embryos compared with their wild-type siblings, consistent with the general effect on chromatophore number that we observe. Hence, *cls* affects the development of neural crest-derived chromatophores prior to their migration on the lateral pathway. In addition to the chromatophore phenotype, *cls* has an early effect on ear development (Whitfield et al., 1996).

Class II: missing cell-type

13 mutations, defining two complementation groups, *shady* (*shd)* and *stolen pearls* (*snp*), result in reduced numbers of iridophores, with no effect on the intensity of pigmentation (Table 2; Fig. 2). The *shd* complementation group form a clear phenotypic series. Mutants in the strongest alleles have no more than a few iridophores on the inside face of the eye only (Fig. 2E). The weakest alleles show an approximately 50% reduction in the extent of iridophores in the yolk sac stripe, but a much weaker reduction elsewhere. Intermediate phenotypes show iridophores in the lateral patches and on all surfaces of the eye (Fig. 2D,I), but relatively few in the yolk sac stripe. Some alleles are homozygous adult viable (see Odenthal et al., 1996a). Unlike *shd* mutants, *snp* mutants cannot be identified until the sixth day (Fig. 2M). The phenotype is weaker than that of any *shd* allele and is most evident as a variable reduction in the number of iridophore spots in the ventral stripe and in the completeness of the yolk sac stripe.

Mutants of *salz* (*sal*) and *pfeffer* (*pfe*) show partial absence of xanthophores in the dorsal head and have spaces between remaining xanthophores (Fig. 2K; see Odenthal et al., 1996b). We find an allelic series with homozygotes for strong alleles showing almost no xanthophores. These mutations seem to be distinguishable from those affecting xanthophore differentiation (Class VI.J), since mutants in the latter process show pale yellow, whitish, or simply granular cells in xanthophore positions, usually without normal-looking cells interspersed (Odenthal et al., 1996b; see also Figs 9,12). Like the analogous iridophore mutations, all *sal* and *pfe* alleles are homozygous adult viable and show a phenotypic series of adult phenotypes (see Odenthal et al., 1996a).

Class III: reduced melanophores

We found mutants in which essentially all xanthophores or iridophores are absent (Class II). Surprisingly, however, we found no mutants showing an analogous absence of melanophores, despite their being the most obvious cell type

Table 2. Reduced chromatophore number mutations

Class	Gene name (symbol)	Alleles	d	Pigment phenotype	Other phenotypes	Other references
I:						
All pigment cells absent; pigmented retinal epithelium wt	*colourless (cls)*	*tw2, tw11*	1	No chromatophores	Ear and otoliths	a
	white tail (wit)	*ta52b*	1	Chromatophore number reduced; none in tail	Abnormal somite; brain; small eye; tail short	b,c
II:						
One pigment cell type reduced, essentially absent in strong alleles	*pfeffer (pfe)*	*tc227b, te220, tg17, tg283a, tm236b, tq211*	2	Reduced xanthophore number	Adult pigment pattern	d,e
	salz (sal)	*tb213c, tf34, tf238b, tl241, tm246b, tp71c tt254a*	2	Reduced xanthophore number	Adult pigment pattern	d,e
	shady (shd)	*tc205, te295*(s) *te300, tf238c, th219*(w), *ti263c, tj229e, tm46a, tp218*(s), *ty9, ty70, ty82*(s)	2	Reduced iridophore number	Adult pigment pattern	e
	stolen pearls (snp)	*tq280*	5	Reduced iridophore number		
III:						
Reduced melanophore number, melanophores collect by ear	*sparse (spa)*	*te237*(s), *th35c*(w) *(lost), tj245*(w), *tm18b*(w), *tm63a, tm102c, tm228*(s), *to75b, tp44*	1	Melanophore number reduced	Adult pigment pattern	e,f
	sparse-like (slk)	*tc244b*	1	Melanophore number reduced		

In this and all subsequent tables, the column labelled d indicates the stage (days postfertilisation) at which the phenotype is first visible.

All phenotypes are recessive unless noted otherwise.

Alleles with relatively strong (s) or weak (w) phenotypes are indicated.

All pigmentation mutations that were not assigned to a complementation group (‘unresolved mutations’ in Table 1) are listed under the appropriate class in Tables 2-6.

References: a, Whitfield et al. (1996); b, Jiang et al. (1996); c, van Eeden et al. (1996a); d, Odenthal et al. (1996b); e, Haffter et al. (unpublished); f, Streisinger et al. (1986).

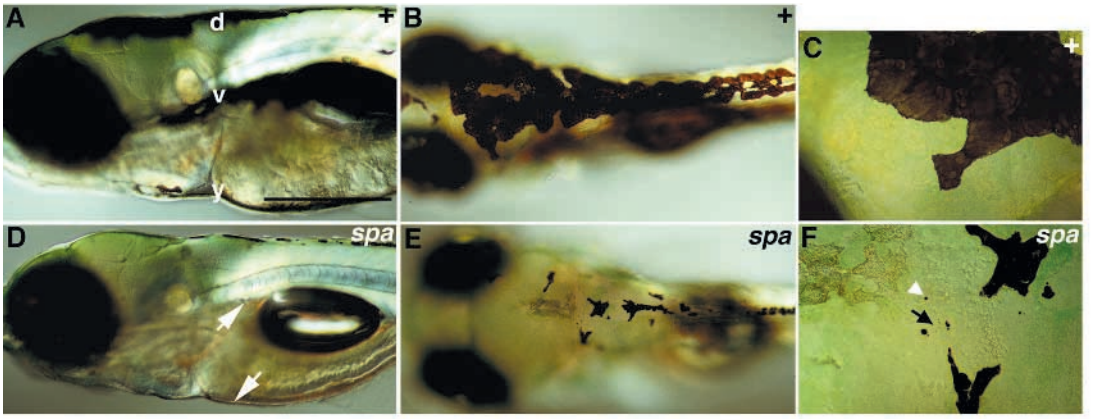

Fig. 3. Sixth day *spa* mutant phenotype (Class III). Wild-type control (A-C) and *spa* homozygote sibling (D-F) are shown. Melanophore number is decreased, weakly dorsally (D,E), and more strongly ventrally (arrows in D). Melanophores are abnormally shaped and many are seen as small spots (F, arrowhead) or fragmented (arrow). Xanthophores fill in gaps in the dorsal stripe, giving general yellow colouration in F (compare Fig. 6D). B,C,E and F are dorsal views, of the head and rostral trunk (B,E) or of dorsal stripe melanophores (C,F). Abbreviations as Fig. 2. Scale bars, 400 μm (A,D), 500 μm (B,E) and 125 μm (C,F).

we examined. Although a number of mutants with reduced numbers of morphologically normal melanophores were found, even the strongest alleles show no more than 50% reduction in cell number on the second day.

We found ten mutants with a phenotype similar to *sparse* (*spa*) (Table 2; Fig. 3) (Streisinger et al., 1986). The embryonic phenotype consists of a striking reduction in melanophore number, most pronounced in the anterior head, in more ventral stripes, and, within any stripe, in more posterior regions (Fig. 3D,E). During the hatching period melanophores accumulate behind the otic vesicle, but disappear by the sixth day. Melanophores initially look normal, but by the sixth day they are abnormal in shape and size, and some contain just an isolated spot of melanin (Fig. 3F). Nine of these are new *sparse* alleles and show a range of phenotypic strengths. Alleles that have been tested are homozygous adult viable, as is the original allele. The tenth mutation complements *spa*, but is phenotypically indistinguishable from a strong *spa* allele; we have named this locus *sparse-like* (*slk*).

Class IV: pigment pattern

Mutations in five genes cause defects in pigment pattern. In these, chromatophores are morphologically normal and appear at normal times, but part of the wild-type pattern is absent or forms incorrectly (Table 3; Fig. 4). Most striking is the *choker* (*cho*) mutant allele, which causes two pigment pattern defects: deletion of the lateral stripe (Fig. 4E) and an ectopic band of melanophores forming a partial collar in the posterior hindbrain region (Fig. 4D). *cho* mutants show a somite defect (Fig. 4E). Other mutants with more severe somite defects e.g. *no tail* (*ntl*) (Halpern et al., 1993; Odenthal et al., 1996a), also have no lateral stripe. Together, these observations suggest that the horizontal

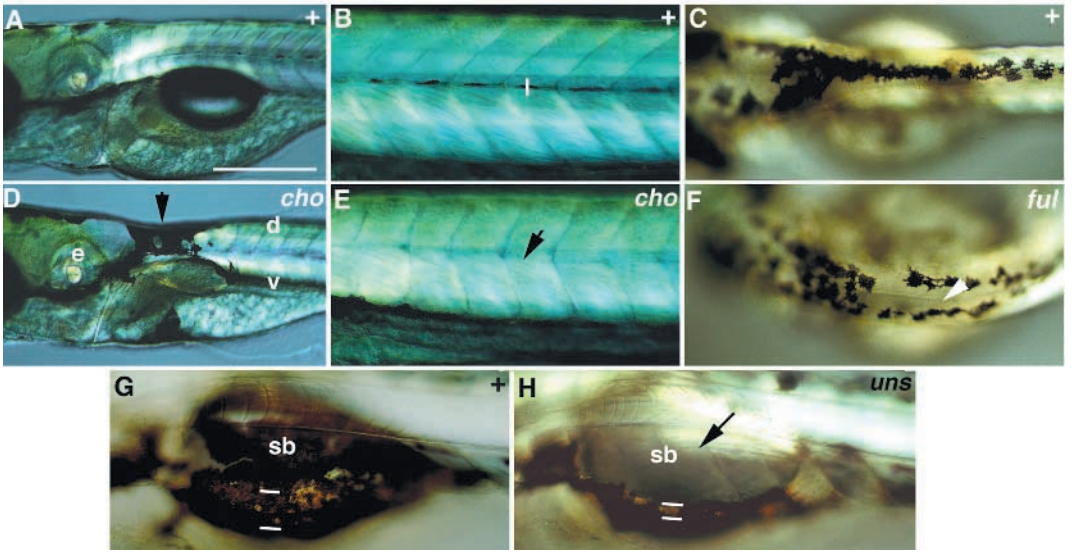

Fig. 4. Phenotypes of pigment pattern mutations (Class IV). Wild-type siblings are compared with homozygous mutants for *cho* (A,B,D,E) on the sixth day, *ful* on the third day (C,F) and *uns* on the sixth day (G,H). *cho* mutants show ectopic melanophores (arrow) in a half collar joining the dorsal and ventral stripes caudal to the ear (D) and have no lateral stripe (arrow, E). *ful* mutants (F) show laterally displaced dorsal stripe melanophores in the anterior trunk (arrowhead). Close-up view of ventral stripe of wild-type larva (G) shows melanophores covering dorsal surface of swim bladder and large mediolateral extent of lateral patches (flanked by white lines). In *uns* mutants this region is devoid of melanophores (arrow) and mediolateral extent of lateral patches (white lines) is narrowed (H). C,F are dorsal views of anterior trunk dorsal stripe. Abbreviations as in Fig. 2. e, ear; sb, swimbladder. Scale bars, 300 μm (A,C,D,F) and 150 μm (B,E,G,H).

myoseptum is important in lateral stripe formation, and that the pigment pattern defect in *cho* may be more direct than in other somite mutations (see Fig. 4E and van Eeden et al., 1996a).

Mutants of the single *fullbrain* (*ful*) allele show fewer lateral stripe melanophores. Also melanophores in the dorsal and ventral stripes of the anterior trunk are displaced laterally to leave the dorsal midline devoid of pigment cells (Fig. 4F; see also Jiang et al., 1996). *ful* mutants have a complex phenotype and die during the hatching period. It is not clear to what extent these defects are merely secondary effects of an axial defect.

Part of the ventral stripe directly overlying the swimbladder is absent in mutant *unsaddled* (*uns*) alleles (Fig. 4H). Both melanophores and some iridophores of the lateral patches are affected. Initially, individual melanophores may be seen in this region; presumably these subsequently migrate away or die.

The variable absence of melanophores in the posterior yolk sac stripe characterises the single mutant allele of *lost trail* (*los*).

Finally, both mutant *mercedes* (*mes*) alleles result in a lateral duplication of the ventral part of the tail fin (see Hammerschmidt et al., 1996), and also in duplication of the ventral stripe associated with this fin.

Class V: ectopic chromatophores

Another group of mutants also have chromatophores in normal positions, but show additional chromatophores in ectopic locations (Table 3; Fig. 5).

Mutants in one large complementation group, *moonshine* (*mon*), show overabundant iridophores in the dorsal stripe, post-anal ventral stripe and the yolk sac stripe, where they extend into the adjacent median fins (Fig. 5C,D). *mon* mutations cause no defect in parts of the pigment pattern that are not associated with median fins. Moreover, all mutant alleles show distinct reductions in the median fins (Fig. 5D), and the strongest seven alleles result in reductions in the number of circulating blood cells (see Ransom et al., 1996).

Three mutations, defining two different genes, *tiger* (*tig*) and *parade* (*pde*), cause transient accumulations of chromatophores on the medial migration pathway. *tig* mutants combine a strong ventral curvature of the tail with collected melanophores oriented dorsoventrally on this pathway in the trunk and tail. Both phenotypes are transient, but the pigment phenotype disappears more rapidly than the tail curvature. The two *pde* alleles cause a pigmentation phenotype like *tig*, but it appears one day later than in *tig* and is not associated with tail defects. Ectopic cells in *pde* mutants seem to contain pigment organelles characteristic of both iridophores (iridosomes) and melanophores (melanosomes) (Fig. 5H-K).

Finally two complementation groups, *floating head* (*flh*)

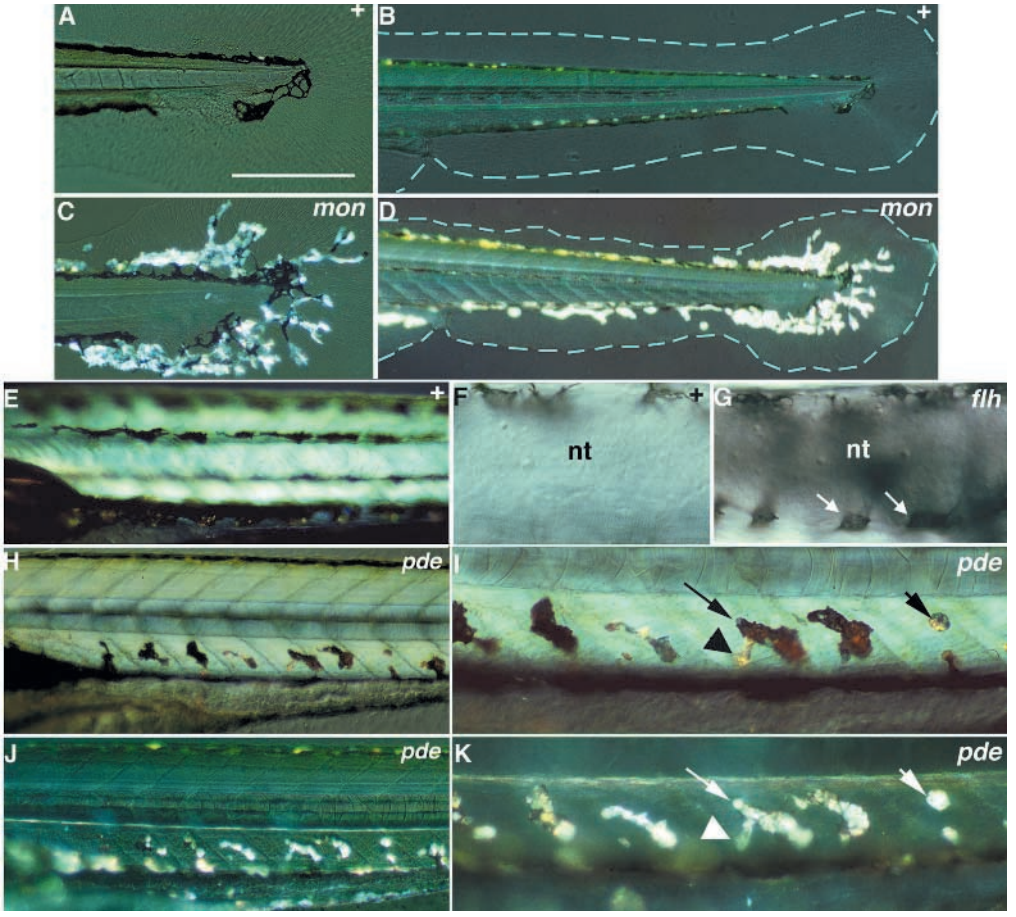

Fig. 5. Phenotypes of ectopic chromatophore mutations (Class V). Wild-type siblings are compared with homozygous mutants for *mon* (A-D), *pde* (E,H-K) and *flh* (F,G). *mon* larvae display on the sixth day large numbers of iridophores extending into the medial fins (C,D), while medial fins (blue outline) are irregular in shape and reduced in area (D). During the pharyngula period, melanophores are normally found in close association with the neural tube in a dorsal position (F), but in *flh* mutant embryos they completely encircle the neural tube (melanophores on the ventral surface of the tube; arrow, G). On the sixth day, *pde* larvae show dorsoventrally oriented chromatophores ventral to the notochord and dorsal to the ventral stripe (H, and at higher magnification, in I), which are not present in the wild type (E). Most of these chromatophores look like melanophores under transmitted light, but have large regions (see arrowhead, I) with the appearance of iridophores. When viewed with incident light (J,K), most of these chromatophores (e.g. long arrow, K) look like iridophores. The morphology of these regions strongly implies that the same cell contains both melanosomes and iridosomes. Some of these chromatophores (short arrow, I and K) appear to be pure iridophores. B-D and I-K were photographed with incident light. nt, neural tube. Scale bars, 200 μm (A,C), 400 μm (B,D), 225 μm (E,H,J), 70 μm (F,G) and 110 μm (I,K).

Table 3. Abnormal chromatophore distribution mutations

Class	Gene name (symbol)	Alleles	d	Pigment phenotype	Other phenotypes	Other references
IV:						
Part of larval pigment pattern absent	*choker (cho)*	*tm26*	2	No lateral stripe, hindbrain collar	Minor somite and hindbrain defects	a,b
	fullbrain (ful)	*tq279*	1	Trunk melanophores displaced laterally, number in lateral stripe decreased	Brain; rough skin	b,c
	lost trail (los)	*ty85b*	5	Melanophore number reduced in posterior yolk sac stripe		
	mercedes (mes)	*tm305, tz209*	1	Duplicated ventral stripe	Split ventral fin; adult phenotype	d,e,f
	unsaddled (uns)	*tm142, tp212*	3	No ventral stripe melanophores above swim bladder, lateral patches reduced medially		
V:						
Ectopic pigment cells	*floating head (flh)*	*tk241, tm229*	0	Melanophores around neural tube on d1	No notochord; fused somites	a,g,h,i
	momo (mom)	*th211*	0	Melanophores around neural tube on d1	As *flh*	a,g,i
	moonshine (mon)	*tb222b, tc239b*(w), *tc246b, te345*(s), *tg234, tu244b, ty57*(s), *tz276*	1	Increased iridophore number dorsal and ventral stripes; ectopic iridophores in medial fin	Reduced blood cells; irregular medial fins	e,j
	parade (pde)	*tj262, tv212*	3	'Mixed chromatophores' accumulate below notochord		
	tiger (tig)	ta23	2	Melanophores accumulate below notochord; recovered d5	Curly tail down	k

References: a, van Eeden et al. (1996a); b, Jiang et al. (1996); c, Schier et al. (1996); d, Hammerschmidt et al. (1996); e, van Eeden et al. (1996b); f, Haffter et al. (unpublished); g, Odenthal et al. (1996a); h, Talbot et al. (1995); i, Granato et al. (1996); j, Ransom et al. (1996); k, Brand et al. (1996).

and *momo* (*mom*), mutants of which show dramatic axial defects (see Odenthal et al., 1996a), also have minor pigmentation defects. These mutations cause temporary accumulations of ectopic melanophores around the neural tube during the pharyngula period (Fig. 5G). In contrast, wild-type embryos show pigmented melanophores adjacent to the neural tube only in a dorsal position (Fig. 5F). The fusion of the somites below the neural tube in these mutants, and the consequent obstruction of the medial migration pathway, fully explains the phenotype. These cells subsequently disappear, suggesting that they dedifferentiate, die in this position, or are able to migrate away on the lateral migration pathway.

Class VI: chromatophore pigmentation

Mutants showing reduced levels of pigmentation within each cell account for the majority of the mutations we found. This large group can be readily subdivided into ten subclasses (VI.A-J) according to the combination of pigment cell types that are affected. Three classes of melanophore differentiation phenotypes could be distinguished by the precise appearance of the melanophores; incomplete melanogenesis (Class VI.A), delayed melanogenesis (Class VI.B), or melanophore degeneration (Class VI.C). Effects on iridophores and xanthophores (Classes VI.G, VI.I and VI.J) could not be subdivided at this level of analysis and await further study. Where mutations affect melanophores in addition to another pigment cell-type (Classes VI.D, VI.E, VI.F and VI.H), melanophore morphology is used as a preliminary evaluation of the mutations' effect on all the affected pigment cell types.

(VI.A) Melanin-synthesis mutations

Mutations affecting only melanophore pigmentation cause one of three general phenotypes (Classes VI.A-C). The first is characteristic of mutations in melanin-synthesis genes and appears to be directly analogous to the *albino (c)* locus of mice and humans. Seven such genes are defined in our collection: *albino* (*alb*), *sandy* (*sdy*), *mustard* (*mrd*), *golden* (*gol*), *nickel* (*nkl*), *lead* (*led*) and *pewter* (*pew*) (Table 4; Fig. 6). Two of these loci, *alb* and *gol*, have been described previously (Streisinger et al., 1986). In the embryo, both the pigmented retinal epithelium and melanophores themselves are evenly pale or unpigmented, and melanophore size and number are normal. Furthermore, for *alb* and *mrd* mutants melanin is synthesised upon addition of DOPA; in contrast, *sdy* mutants do not make melanin in this assay (Odenthal et al., 1996a). Hence, *sdy* shows no tyrosinase activity, and is thus a strong candidate for a fish tyrosinase locus (Odenthal et al., 1996a). The adult phenotypes of these zebrafish mutations are described elsewhere (Odenthal et al., 1996a).

Mutants for strong alleles of *alb*, *sdy*, and *mrd* show no trace

Table 4. Reduced chromatophore pigmentation I

Class	Gene name (symbol)	Alleles	d	Other phenotypes	Other references
VI.A:					
Melanin pale to absent	*albino (alb)*	*tf31, ti9*(s), *ti225, tj20e, tm83a, tr282*	1	Adult pigmentation	a,b
	golden (gol)	*tg271, ty213*	1	Adult pigmentation	a,c
	lead (led)	*ts32*	2		
	mustard (mrd)	*ta229c*(s), *tg16, tj204, tk68b, tn215*(w), *tp72i*(s), *tv41*	1	Adult pigmentation	c
	nickel (nkl)	*tm40*	2		
	pewter (pew)	*tm79b*	2		c
	sandy (sdy)	*te326, tk20*(s), *tm118*(s), *to1, to102*(w), *ty79*	1	Adult pigmentation	c
	Unresolved	*ti205, tz249*			
VI.B:					
Melanophores tiny, pale, low number, later recovers	*landing (lnd)*	*tf21a*	1		
	slow tan (sln)	*tg208*	2		
	touch-down (tdo)	*tg243, th208, tk49, tm267e, tp2, tp8*(w), *tp31, tq277, tt245, tv60a , tz310c*(s)	1	Reduced touch response; eye; adult body shape	d,e
	Unresolved	*tc218, to253, ti201*			
VI.C:					
Melanophores initially normal; later pale, fragmented or spot-like, collect abnormally below ear, around eye, on hindgut, and in piles in dorsal stripe	*delayed fade (dfd)*	*tj241a*	3	Eye; brain degeneration	e,f,g
	fade out (fad)	*tc7b, tg14*(lost), *tk224*(w, lost), *tm63c*(s), *tp94c*(s)	2	Retinal degeneration	e
	fading vision (fdv)	*th236a*	1	Retinal degeneration; adult pigmentation and eye	c,e
	quasimodo (qam)	*ta81, tb244c, tf208, tm138b, tw25a, ty41,*	1	Pigmented retinal epithelium pale; undulated notochord	b
	Unresolved	*tx216, to275a, tu46*			
VI.D:					
Melanophores degenerate, xanthophores pale	*cold-light (cot)*	*tv205b, tz264*(lost)	2		
	polished (pol)	*ti224, ty32b, ty124d*	2	Motility	
	Unresolved	*tc249, tz294*			
VI.E:					
Melanophores degenerate, dull iridophores; pigmented retinal epithelium usually pale	*blurred (blr)*	*tk12, tm297*(s), *tp223a, tp90*(s), *tq262b, tu29c*(w), *tx3*(s)	1	Retinal degeneration	e
	freckles (frk)	*th35d*	2		
	ivory (ivy)	*tm271a, tp30*	1	Retinal degeneration; brain degeneration	e
	pepita (pep)	*th204a*(lost), *tl23*	1		
	pile-up (pup)	*te256*(lost), *tv215, tk62, tm9a, tm88a*(lost)	1		
	punkt (pun)	*te380, tp219d*(s), *tl39, tk22*	1	Notochord degeneration; grey yolk	b
	speckled (spe)	*tq213a*	2		
	Unresolved	*tv49a, tf241a, tg203b, tg306, ts236, tq1b, te374b, tm79d*			
VI.F:					
Pale melanin, dull iridophores	*brassy (bry)*	*tm111*	1	Adult pigmentation	c
	Unresolved	*tt249a, tv201a*			

References: a, Streisinger et al. (1986); b, Odenthal et al. (1996a); c, Haffter et al. (unpublished); d, Granato et al. (1996); e, Heisenberg et al. (1996); f, Furutani-Seiki et al. (1996); g, Trowe et al. (1996).

of melanin even on the sixth day (or later), but even these can be distinguished from mutants with a reduced number of melanophores (Class III). Melanin synthesis mutants show unpigmented gaps in the dorsal head xanthophore pattern that mimic the characteristic wild-type melanophore pattern and suggest the presence of normally patterned, unpigmented melanophores (Fig. 6). In contrast, mutations affecting melanophore number cause no such gaps: xanthophores surround the remaining (pigmented) melanophores, but 'fill in' the positions where melanophores are absent (see *spa* in Fig. 3; also Fig. 8D).

(VI.B) Delayed melanophore differentiation mutations

Two mutations [defining two loci, *landing* (*lnd*) and *slow tan* (*sln*)] do not affect the pigmented retinal epithelium, but result in most melanophores during the pharyngula period appearing only as tiny black dots (Table 4; Fig. 7C). Other melanophores are very pale, but of normal size (Fig. 7F). Melanophore number appears reduced, but by the sixth day melanophore number, size and pigmentation are almost indistinguishable from wild type (Fig. 7L). These mutations thus appear to cause delayed melanophore pigmentation.

Eleven *touch-down* (*tdo*) alleles cause a similar phenotype,

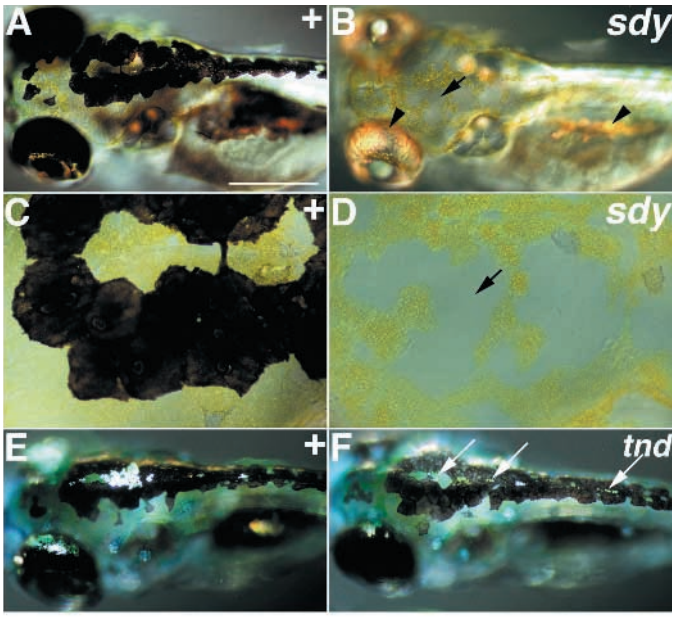

Fig. 6. Sixth day phenotypes of no melanin synthesis and dull iridophore mutations (Classes VI.A and VI.I). Wild-type siblings are compared with homozygous mutants for *sdy* (A-D) and *tnd* (E-F). In *sdy* mutants, gaps (arrow) in the xanthophore pattern correspond to positions of melanophores in wild type (B,D). Both the pigmented retinal epithelium and melanophores are affected. Iridophores are strikingly prominent, as seen in the eye and lateral patches (arrowheads, B). In *tnd* mutants, iridophores are present in the normal places (arrows, F), but are duller than in wild type. Scale bars, 400 μm (A,B,E,F) and 100 μm (C,D).

but there is a reduced touch response (Granato et al., 1996) and the melanophore number seems to be reduced. Mutants in strong alleles initially show almost all melanophores as tiny spots and the apparent number is highly reduced (Fig. 7B,E). They are similar in size and appearance to the few melanophores seen in *cls* mutants, but are clearly more abundant, and more widely distributed. Unlike *lnd* and *sln*, the phenotype is only partially recovered by the sixth day. Xanthophores 'fill-in' the gaps in the melanophore pattern (Fig. 7K), thus suggesting that, in contrast to melanin synthesis (Class VI.A) mutations, melanophore number is decreased at this stage. In mutants of a weaker allele (tdo^{tp8}), most melanophores are initially merely pale and by the sixth day the number of normally pigmented melanophores is almost wild type. These mutations seem therefore to combine delayed pigmentation with a reduced survival of melanophores. It should also be noted that the touch-response defect shown by *tdo* mutations is a transient phenotype and may be explained by delayed differentiation of the touch-response circuitry.

(VI.C) Melanophore degeneration mutations

One group of melanophore mutations (13 alleles, representing four distinct genes; Table 4; Fig. 8) causes a third melanophore phenotype, progressive deterioration of melanophores.

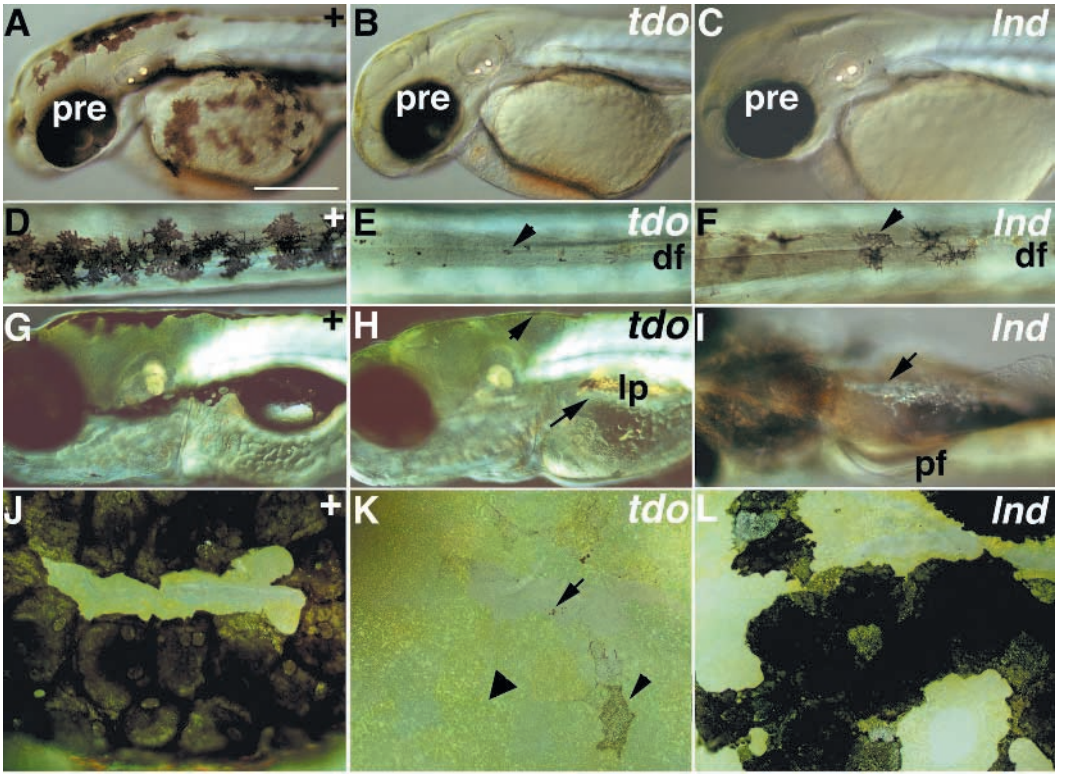

Fig. 7. Delayed melanophore differentiation phenotypes (Class VI.B). Wild-type siblings (A,D,G,J) are compared with homozygous mutants for *tdo* (B,E,H,K) and *lnd* (C,F,I,L) on the third day (A-F) and the sixth day (G-L). In both *tdo* and *lnd* mutants (B and C) the pigmented retinal epithelium is normal, but melanophores are all tiny (*tdo*, E) or a mixture of tiny and pale cells (*lnd*, C,F). On the sixth day, *tdo* mutants now have a few larger, but still pale melanophores (short arrow, H; small arrowhead, K), in addition to tiny melanin spots (arrow, K), although large areas still lack visible melanophores (e.g. none visible around lateral patches: long arrow, H). Xanthophores fill the regions of the dorsal stripe normally occupied by melanophores (large arrowhead, K). In contrast, *lnd* mutants are now almost fully recovered. Some melanophores are still a little paler (L), and they are still invisible in the yolk sac stripe in some individuals (arrow, I). D-F and J-L are dorsal views of the dorsal stripe in the midtrunk (D-F) or the dorsal head (J-L). I is a ventral view of the trunk. Abbreviations as Fig.2; df, dorsal fin; lp, lateral patch; pf, pectoral fin Scale bars, 250 μm (A-C,G-I) and 100 μm (D-F,J-L)

Mutations in these genes result in additional phenotypes; in *fade out* (*fad*), *fading vision* (*fdv*) and *quasimodo* (*qam*) mutants, melanin in the pigmented retinal epithelium also becomes paler, and retinal degeneration occurs in *fad* and *fdv*. Remarkably, one such allele, *fdv*^{th236a}, is homozygous adult viable, and results in fish with pale melanophore stripes and lacking eyes. As these fish age, large tumours grow from the eye sockets (Odenthal et al., 1996a).

Unlike mutants with melanin synthesis (Class VI.A) or delayed pigmentation (Class VI.B) phenotypes, melanophores are initially normal in these melanophore degeneration mutants (Class VI.C). Then, at a time specific to particular alleles, but ranging between the second and fourth days, melanophore pigmentation changes. Some paler melanophores, or others having only small, black melanin spots (larger than the tiny spots in *ton*), become visible (Fig. 8B). These phenotypes become progressively more severe: by the sixth day mutants in strong alleles show melanophores that are almost all small and spot-like in appearance (Fig. 8D). These small, spot-like melanophores collect in abnormal locations and arrangements: ventrolateral to the ear, on the hindgut, and in clusters in the dorsal stripe (Fig. 8D,F,H).

(VI.D) Mutations affecting melanophores and xanthophores

Mutations in two genes [*polished* (*pol*) and *cold-light* (*cot*)] cause the melanophore degeneration phenotype exhibited by Class VI.C mutants, but also result in pale xanthophores (Table 4; Fig. 9). We cannot examine xanthophore morphology in the same detail as that of melanophores. Hence we are uncertain whether xanthophores also degenerate.

(VI.E) Mutations causing melanophore degeneration and abnormal iridophore pigmentation

Eight genes defined by 23 mutations affect the pigmentation of melanophores and iridophores (this and the following section; Table 4; Fig. 10). Mutations in seven complementation groups combine melanophore degeneration phenotypes (as in Class VI.C) with dull iridophores (Fig. 10E-H). Mutations in six additionally cause a pale pigmented retinal epithelium, and mutants in two of these show retinal degeneration by the sixth day (see Heisenberg et al., 1996). Mutants in the single allele defining *freckles* (*frk*) have normally pigmented retinas (Fig. 10E), and may affect only the survival of the neural crest-derived melanophores themselves.

(VI.F) Mutations affecting melanin synthesis and iridophore pigmentation

Mutants in one complementation group, *brassy* (*bss*), have a melanophore phenotype (Fig. 10D) similar to the melanin-synthesis mutants (as in Class VI.A). Additionally, iridophores are duller and whiter in appearance than in wild types, but occupy approximately the normal extent (see, for example, the eye and lateral patches in Fig. 10C). Thus, both melanophore and iridophore numbers appear normal, but pigment synthesis or processing is abnormal. The phenotype of *bss* is similar to that of *brass* (Streisinger et al., 1986); no *brass* allele was found in our screen.

(VI.G) Mutations affecting xanthophores and iridophores

Five genes, defined by 13 mutations, affect only xanthophore and iridophore pigmentation; mutants in these genes all share at least a slight reduction in eye size. It is striking that mutations in two of these genes, *mlk* and *pio*, show jaw/arch defects (Table 5; Fig. 11; see Schilling et al., 1996). The combination of defects in distinct neural crest derivatives (chromatophores and craniofacial skeleton) is particularly interest-

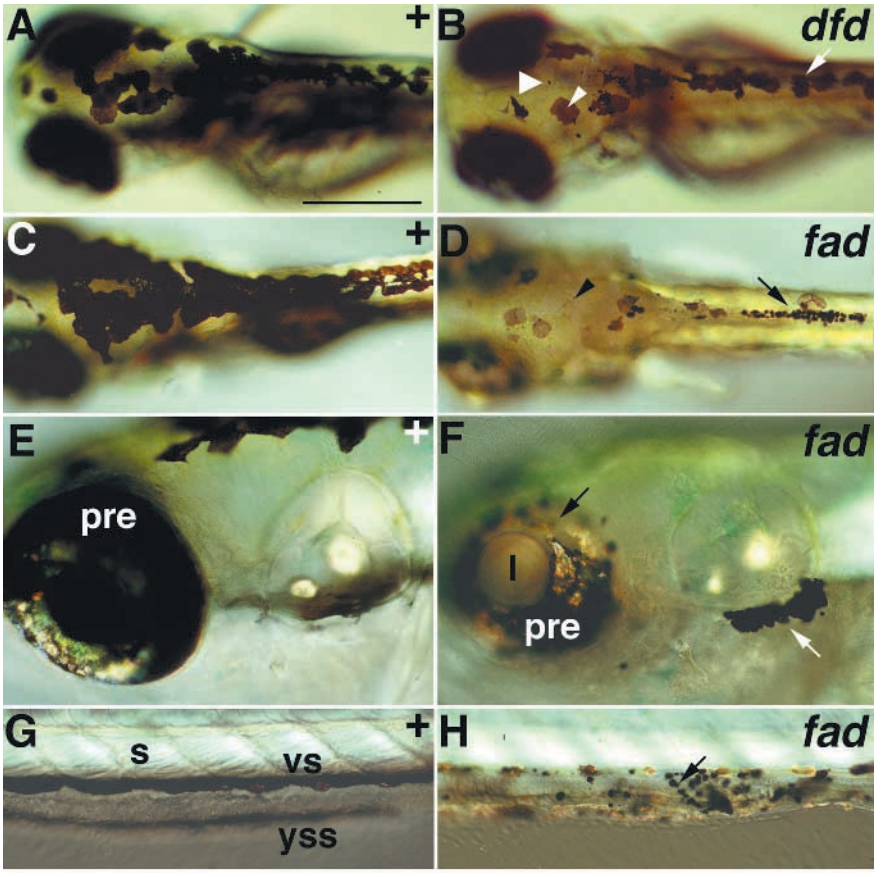

Fig. 8. Melanophore degeneration phenotypes (Class VI.C). Wild-type siblings are compared with homozygous mutants for *dfd* on the fourth day (A,B), and *fad* on the sixth day (C-H). Abnormal melanophores are first seen mixed in with normal ones (arrow, B) on the fourth day in *dfd*, as both large, pale cells (small arrowhead, B) and small spots (large arrowhead, B). Abnormal melanophores are first seen on the third day in *fad*, and by the sixth day the majority are abnormal, being either small and spot-like (arrow, D), pale or apparently fragmented (arrowhead, D). Spot-like melanophores collect abnormally, in piles in the dorsal stripe (arrow, D), in collections ventrolateral to the ear (white arrow, F) and on the hindgut (arrow, H). In *fad* the pigmented retinal epithelium depigments (D,F) and the eye degenerates (note small size in D,F), although the lens remains normal (F). Abbreviations as Fig. 2; s, somite; l, lens. Scale bars, 350 μm (A-D), 200 μm (E,F) and 125 μm (G,H).

Table 5. Reduced chromatophore pigmentation II

Class	Gene name (symbol)	Alleles	d	Other phenotypes	Other references
VI.G:					
Pale xanthophores, dull iridophores	*cookie (coo)*	*tm130f, tw33*	2	Eye	a
	choco (cco)	*ta87, ty86b*	2	As *coo*	a
	milky (mlk)	*te1a, tg2, tv10*	2	Head, including eyes and arches reduced	a
	pistachio (pio)	*tb202, tc276c, tk17, to75a, ts41*	2	As *mlk*	a,b
	vanille (van)	*ty6d*	2	Eye; brain degeneration	a,c
	Unresolved	*tq262a, ty105g, tu235b, tz284, tj266c, tm107*			
VI.H:					
Melanophores degenerate, pale xanthophores, dull iridophores	*blanched (bch)*	*ti282b*	2	Reduced touch response; eye; brain degeneration; strong body retarded by d5	a,d
	bleached (blc)	*th204b, ts23, ty89*	1		a,c,d
	bleich (bli)	*tl240c*			
	pech (pch)	*ts292e*	1	Retinal degeration; ear	d
	puzzle (puz)	*tp49c* (distinct phenotype: see text)	2	Ear; jaws/arches; general necrosis by d5	b,d
	sahne (sah)	*tq251e*	1	Eye; ear	a,d
	sallow (*sll*)	*tu43*	1	Retinal epithelium pale; brain degeneration; retinal degeneration; ear; body retarded by d5	a
	stone-washed (stw)	*tw29c*	2	Ear and otoliths; body retarded by d5	d
	sunbleached (sbl)	*to4*	2	Delayed pigmentation of retinal epithelium; eye; jaw retarded	a,b
	u-boot (ubo)	*tp39* (distinct phenotype: see text)	1	Abnormal somites, motility and fins	e,f
	weiss (wei)	*to11*	1	Brain degeneration; ear; body slightly retarded	d
	washed-out (wsh)	*tg248a, tr255, tm132e, tm141*	2	Delayed pigmentation of retinal epithelium	
	Unresolved	*tc233b*			
VI.I:					
Dull iridophores	*bedimmed (bed)*	*to240*	3	Adult pigmentation	g
	dimmed (dim)	*te214*	2		
	heirloom (hei)	*tl225a*	3		
	lacklustre (lcl)	*ta229d*	3		
	matt (mat)	*ti280*	3	Xanthophores ‘fibrous’	
	tarnished (tnd)	*tl33*(lost), *tf222c*	2		
	toned-down (tod)	*tz262a*	5		
	Unresolved	*tg283c, tw212, tz224*			
VI.J:					
Xanthophores pale	*bressot (bst)*	*tp223b*	2		h
	clorix (clx)	*tj244*	2	Otoliths	d,h
	edison (edi)	*tc245c, tl35, tk232a, tl245, to255b, tp62, tp67, tr276, tt232, tv4*(lost), *tz253*(lost)	2		h
			3	Retinotectal projection	h,i
	esrom (esr)	*tb241a, te250, te275, te279, te376, tf4z, th36b, th222, tg5, tg265, tj236, tp203, tn207, ts208*			
	feta (fet)	*ty107*	2	Retinotectal projection	h,i
	kefir (kef)	*ta65b, tf229*(lost)	2		h
	non blond (nob)	*tt288*	2	Otoliths; retarded	d,h
	brie (bri)	*tg211b, tj226a, tm42c, tu269*	2		h
	quark (qua)	*tc276, tg239d, tk236, to241b, tp72g, tv46*	3		
	ricotta (ric)	*tb212*	3		h
	tartar (tar)	*td09*	3	Adult pigmentation	g,h
	tilsit (til)	*ty130b*	2		h
	tofu (tof)	*tq213c*	2		h
	yobo (yob)	*tc251, tk13, ty44d*	2	Maternal effect	h
	yocca (yoc)	*tm86*	3		h
	Unresolved	*ta53b, tn14*			

References: a, Heisenberg et al. (1996); b, Schilling et al. (1996); c, Furutani-Seiki et al. (1996); d, Whitfield et al. (1996); e, van Eeden et al. (1996a); f, Granato et al. (1996); g, Haffter et al. (unpublished); h, Odenthal et al. (1996b); i, Karlstrom et al. (1996).

Table 6. Abnormal chromatophore morphology

Class	Gene name (symbol)	Alleles	d	Pigment phenotype	Other phenotypes	Other references
VII.A:						
Spindly chromatophores	*gossamer (ger)*	*tc11c*	2	Melanophores thin, elongated or spot-like	Brain degeneration, jaw, arches necrosing	a
	petroglyph (pet)	*tm65*	1	Homozygotes: melanophores spindly, pale; Heterozygotes: melanophores weakly spindly and pale; both phenotypes later recover		
	tinte (tin)	*tq266a*	1	Homozygotes: melanophores and xanthophores pale, melanophores uni or bipolar; Heterozygotes: melanophores pale and spindly; later recover	Homozygotes: brain degeneration	
VII.B: Small melanophores	*obscure (obs)*	*to2b*	4	Smaller melanophores	Adult pigmentation	b
VII.C: Stellate chromatophores	*stars-and-stripes (sas)*	*tc21, tc318a, th274*(lost), *tl25, tm57a, tm235a, tm245*(lost), *tr254b, tu247*(lost), *ty120, tz227b*	1	Melanophores small, stellate with pale spot; xanthophores smaller, more discrete		
	union jack (uni)	*tn16*	1	Melanophores small, stellate with pale spot		
VII.D:						
No background adaptation	*dropje (drp)*	*tr256*	4	No background adaptation	Adult pigmentation	b
	fata morgana (fam)	*te267*	4	No background adaptation; later recovers		
	lakritz (lak)	*th241*	4	No background adaptation		
	melancholic (mac)	*tj19e*	4	No background adaptation		
	noir (nir)	*tc22, tp89*	5	No background adaptation	Motility defect	c
	submarine (sum)	*tr6*	5	No background adaptation	Motility defect	c
	zwart (zwa)	*tj213, tp93a*	4	No background adaptation	Motility defect	c
	Unresolved	*tc327c, tj218, tm97c*				

References: a, Schilling et al. (1996); b, Haffter et al. (unpublished); c, Granato et al. (1996).

ing since it suggests roles for these genes in the development of multiple neural crest fates.

We suspect that some of these mutations may cause xanthophore and iridophore degeneration in a manner analogous to the melanophore degeneration mutations (Class VI.C), but xanthophore and iridophore phenotypes vary in severity between alleles, and are difficult to evaluate (see above). However, there is no sign of the normal-looking cells characteristic of mutations with decreased xanthophore or iridophore cell number (Class II). Iridophores are duller than wild type in all these mutants, but whether the number is reduced is difficult to say without better markers; in *pio* mutants there are fewer by the sixth day (e.g. in the lateral patches, Fig. 11H). Xanthophore defects in *pistachio* (*pio*), *milky* (*mlk*), *cookie* (*coo*) and *choco* (*cco*) mutants may be due to poor xanthophore differentiation, since in most alleles some faintly pigmented xanthophores are visible in the dorsal head. Thus, whereas there may by the sixth day be an effect on xanthophore and iri-

Table 7. Pigmentation mutation classes suggest which processes of neural crest development are affected

Process	Expected pigment phenotypes	Relevant Classes[1]
Specification	Cell number very strongly decreased; remaining cells normal in position and appearance; pigmented retinal epithelium unaffected	I; II
Patterning	Subset of pigment pattern absent, remaining cells in normal positions and of normal appearance; pigmented retinal epithelium unaffected	IV
Proliferation	Cell number decreased; remaining cells normal in position and appearance; pigmented retinal epithelium unaffected	III
Survival	Cell number and appearance initially normal, but later cells die	VI.C-E, G-J
Differentiation	Pigment levels reduced; pigmented retinal epithelium affected (melanophore differentiation mutants); or cell morphology abnormal	VI.A,B,F,G,I,J;VII

[1]The Class II mutation, *snp*, is unlikely to represent an iridophore specification mutation, but the process affected by this mutation is unclear (an iridophore-specific proliferation defect is possible). Xanthophore and iridophore phenotypes could not be described in the same detail as melanophore phenotypes; hence classes VI.G, VI.I and VI.J are included under 'Survival' and 'Differentiation'. Although not indicated in this table, some mutations (e.g. *cls* and *spa*) are likely to affect more than one aspect of pigment cell development (see main text).

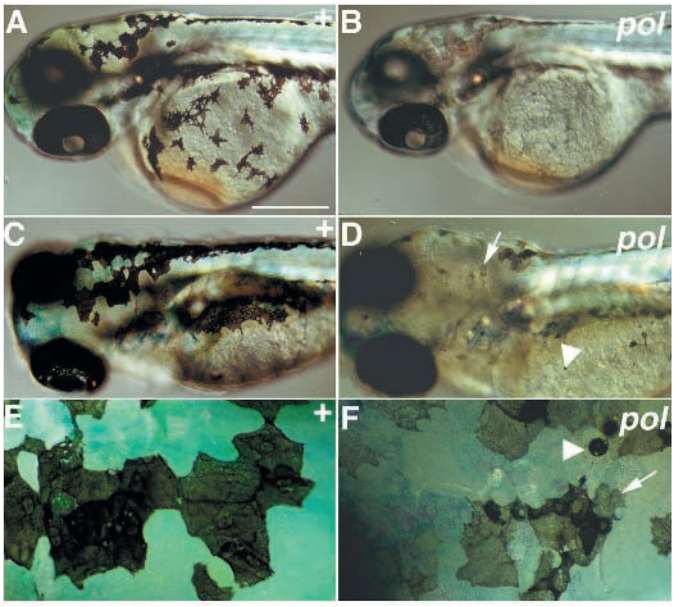

Fig. 9. Phenotype of melanophore degeneration and pale xanthophore mutants (Class VI.D). Wild-type siblings are compared with homozygous mutants for *pol* on day 2 (A,B) and day 5 (C-F). Melanophores on the third day are of normal size, but are very pale (pigmented retinal epithelium is also pale) (B). By the sixth day many melanophores are abnormal in shape (arrow, D,F) and some are small and spot-like (arrowhead, D,F). Xanthophore pigmentation is paler (B,D,F): note that blue colour of xanthophores is due to their taking up methylene blue from the medium. A-D are dorsolateral views; E-F are dorsal views of the head dorsal stripe. Scale bars, 250 μm (A,B), 175 μm (C,D) and 45 μm (E,F).

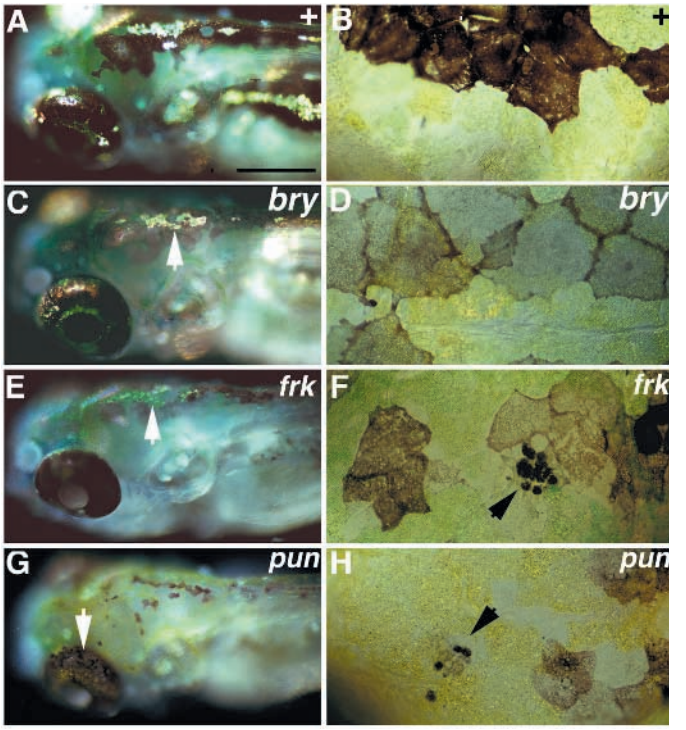

Fig. 10. Sixth day phenotypes of abnormal melanophore and dull iridophore mutations (Classes VI. E and VI.F). Wild-type siblings (A,B) are compared with homozygous mutants for *bry* (C,D), *frk* (E,F) and *pun* (G,H). In *bry* mutants, iridophores are slightly dull (arrow, C), and melanophores are pale (D). Unlike *bry* mutants, mutants in *frk* and *pun* combine melanophore degeneration phenotypes (arrows, F and H) with very dull iridophores (E,G). The strength of the iridophore and melanophore phenotypes varies between mutations: *pun* has a stronger effect than *frk*, and indeed iridophores can only be seen in the eye (arrow, G). Note in F and H that xanthophores occupy the regions of the dorsal stripe that are without melanophores, thus implying a decrease in melanophore cell number. A,C,E and G are dorsolateral views with incident light; B,D,F and H are dorsal views of the head dorsal stripe. Scale bars, 300 μm (A,C,E,G) and 75 μm (B,D,F,H).

dophore cell number, there is also always a defect in pigmentation itself in the remaining cells.

The xanthophore and iridophore mutations (Class VI.G), like the melanophore degeneration mutations (Class VI.C), also cause an eye defect. However, whereas in the melanophore degeneration mutants the eye degenerates, losing pigmentation as it becomes smaller (Fig. 8D,F), in the xanthophore and iridophore mutants there is no sign of degeneration; the eye simply fails to grow as large as in the wild type (Fig. 11D,G; see Heisenberg et al., 1996).

(VI.H) Mutations affecting all three cell types

12 genes (17 alleles) affect intensity of pigmentation of all three pigment cell types (Table 5; Fig. 12). Mutants in ten of these genes show the melanophore degeneration phenotype described in Class VI.C. The xanthophore and iridophore phenotypes, as in Classes VI.D-F, vary in strength, and are difficult to evaluate. We have been unable to correlate the severity of the xanthophore and iridophore defects with the melanophore defect.

Whereas no other obvious phenotypes (except an uninflated swimbladder on the sixth day) are caused by mutations in two genes, *washed-out* (*wsh*) and *bleich* (*bli*), all other mutations in this class cause further defects. In several cases, e.g. *blanched* (*bch*) and *sallow* (*sll*), these other defects are believed to be characteristic of a general retardation of larval development. All these mutants show severe melanophore degeneration phenotypes and may also have relatively severe defects in xanthophore and iridophore pigmentation. These mutants show a range of eye defects from severe eye degeneration (e.g. *bleached* (*blc*) and *pech* (*pch*); Fig. 12L) to slightly reduced size (e.g. *bch*). There is some correlation between severity of eye defect and that of the pigmentation phenotype, as in other mutants with melanophore degeneration (Classes VI.C and E).

An eleventh gene, *puzzle* (*puz*), has a single mutant allele. Mutants have slowly differentiating melanophores but, unlike delayed melanophore differentiation mutants (Class VI.B), they never appear as tiny spots. Unlike the melanin-synthesis mutants (Class VI.A), however, there appears to be an initially reduced number of melanophores in ventral stripes (compare *spa*, Fig. 3), but by the fifth day this defect is essentially recovered. Mutant individuals are too necrotic to examine xanthophore and iridophore phenotypes carefully, but iridophores appear reduced in number by the fifth day.

Finally, *u-boot* (*ubo*), also with a single allele, has a melanophore phenotype like *puz*, but additionally lacks lateral stripe melanophores. The latter aspect is a secondary

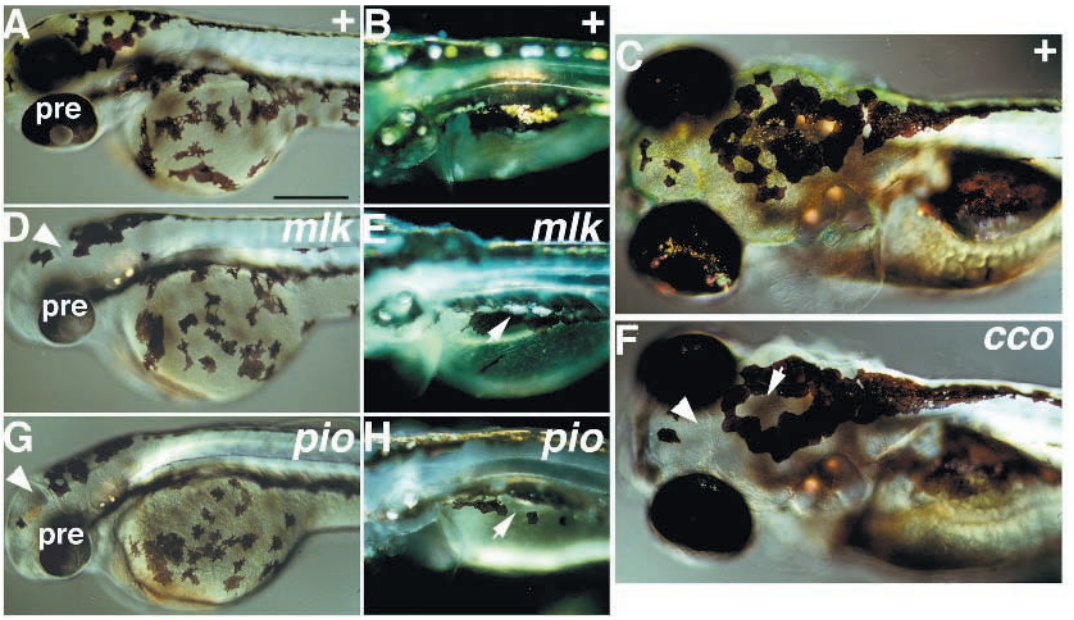

Fig. 11. Phenotypes of pale xanthophores and dull iridophores mutations (Class VI. G). Wild-type siblings (A-C) are compared with homozygous mutants for *mlk* (D,E), *pio* (G,H) and *cco* (F) on the third day (A,D,G) and the sixth day (B,C,E,F,H). Xanthophore pigmentation is not visible on the third or sixth days (arrowheads, D,F,G). Iridophores are dull (arrow, E,F) or apparently strongly decreased in number (arrow, H). Eye is small, but normally pigmented, in *mlk* and *pio* (D,G). All panels show dorsolateral views. Abbreviations as Fig. 2. Scale bars, 250 μm (A,C,D,F,G) and 325 μm (B,E,H).

effect of the somite defect shown by this mutation (see van Eeden et al., 1996a). In *ubo* mutants xanthophores and iridophores, like melanophores, are reduced in apparent number early, but have largely recovered by the sixth day. Pigmentation in the head appears normal even on the second day, so that the areas showing pigment and somite defects correlate well. However, this pigment defect is not shown by other mutants with a similar somite phenotype (see van Eeden et al., 1996a).

(VI.I) Dull iridophore mutations

A group of mutations cause a dull iridophore phenotype, but no other defects (Table 5; Fig. 6E,F). Further study will be required to evaluate the basis of the phenotype.

(VI.J) Pale xanthophore mutations

This large class of mutations (Table 5) are discussed in detail elsewhere (Odenthal et al., 1996b).

Class VII: chromatophore shape and size

Finally, a small set of mutations cause defects in chromatophore size and/or shape (Table 6; Fig. 13). Two of these, *tinte* and *petroglyph*, are of further interest because they show semi-dominance.

(VII.A) Spindly chromatophores

Three mutations, defining three genes, result in pale, spindly melanophores. The *tinte* allele, (*tin*^{tq266a}) (Fig. 13B,C) is semi-dominant: homozygous mutants have small, spot-like melanophores with short single or double projections, whilst presumed heterozygous individuals have larger melanophores of intermediate appearance between those of homozygotes and wild types. In homozygotes, xanthophores are also paler. Whereas homozygotes die by the fifth day, heterozygotes are viable, and are indistinguishable from wild type by the sixth day. The single *petroglyph* (*pet*) allele is also semi-dominant. Homozygous mutants have paler, more spindly, but multipolar, melanophores; melanophores of heterozygotes are initially paler (but normal in shape) than in normal embryos (Fig. 13E,F). All embryos look normal by the sixth day. The mutation *gossamer* (*ger*^{tc11}) has a similar phenotype to *tin*, except that there is no semi-dominant phenotype and no effect on the xanthophores; the phenotype is less severe, and homozygotes survive until the sixth day.

(VII.B) Small melanophores

One mutation, defining the gene *obscure* (*obs*), has a subtle embryonic phenotype, with melanophores being slightly smaller than in wild types.

(VII.C) Stellate chromatophores

A large set of mutations all cause a consistent phenotype of small, rather stellate melanophores in which a bare central area presumably reflects the position of the nucleus (Fig. 13D,H,I). All except one of these have a similar effect on the xanthophores (Fig. 13H). These 11 alleles define the gene *stars-and-stripes* (*sas*). These alleles complement the remaining mutation, in which mutants have no xanthophore phenotype, and which defines a second gene *union jack* (*uni*; Fig. 13I).

(VII.D) No background adaptation

Around the fifth day of development, zebrafish larval melanophores respond to the general tone of the background

surface by altering the distribution of melanosomes within the cell. This process, known as background adaptation, is apparently defective in a group of nine alleles, defining seven genes: *noir* (*nir*), *zwart* (*zwa*), *melancholic* (*mac*), *submarine* (*sum*), *fata morgana* (*fam*), *dropje* (*drp)* and *lakritz* (*lak*) (Table 6). Mutants in these genes show fully expanded melanophores, even on the sixth day. Four of these mutations show other defects, in three cases a motility defect (see Granato et al., 1996).

DISCUSSION

The development of neural crest derivatives is a highly complex process and is expected to involve many genes. We have focussed here on the identification of mutations affecting development of chromatophores, three crest derivatives that are easily scored. This is reflected in the large proportion of mutations in this collection that affect pigmentation (see Haffter et al., 1996). These mutations may identify most genes involved in chromatophore development that can mutate to a phenotype visible under the conditions of the screen (see Haffter et al., 1996).

The wide range of phenotypes seen, plus the readiness with which most mutations (and genes) could be classified into particular classes, suggested that these classes might reflect the process of neural crest development that is affected in the mutants. A summary of these processes, the predicted phenotypes of mutations affecting each, and the pigmentation mutation classes that might fit these predictions are given in Table 7 and discussed below. As expected, the majority of genes identified are clearly involved in the differentiation or survival of pigment cells. A small number of complementation groups have the phenotypic characteristics expected for genes involved in specification, proliferation and patterning, and are candidates for mutations affecting these processes. These functions need not be mutually exclusive: there are several examples in which the phenotype suggests at least two distinct roles for a particular gene.

Neural crest specification and pigment cell fates

We have identified several candidates for genes involved in specification of pigment cells from the neural crest (Classes I and II). Mutations causing defects in chromatophore specification were expected to cause very severe pigment phenotypes, affecting one or more chromatophore types, and in mutants for strong alleles, to show a total absence of that cell-type(s). Furthermore, we expected no effect on melanin synthesis in the non-neural crest-derived pigmented retinal epithelium in these mutants. Although *wit* mutants have a very severe pigment defect, they also have clear central nervous system defects and may disrupt neural crest formation rather than pigment cell specification (see Jiang et al., 1996).

cls is the only candidate for a gene involved in the specification of all pigment cell types. *cls* mutants have the most severe pigmentation defect, with essentially no pigment cells, but the pigmented retinal epithelium is normal and other neural crest derivatives (e.g. craniofacial skeleton) are unaffected. The interpretation of *cls* as a gene important in chromatophore specification is supported by our counts of migrating neural crest cells in mutant embryos. The 95% reduction in their number is consistent with the loss of chromatophores and suggests a defect at some early stage of crest development, prior to migration.

By similar criteria, *sal* and *pfe* are the best candidates for genes important in xanthophore specification, and *shd* may be necessary for iridophore specification. None of these putative specification mutations show an increase in one chromatophore type corresponding to a decrease in another. This suggests that no one chromatophore fate is a default state, and implies that each pigment cell-type has to be actively specified. We found no melanophore equivalent of the *shd* or *sal* and *pfe* genes that specifically disrupts melanophore fate. Melanophore specification could be a further role of *cls* itself.

The phenotype of *cls* mutants suggests that *cls* has multiple roles in melanophore development. Residual melanophores in *cls* mutants show almost no melanin. In contrast, remaining xanthophores and iridophores are phenotypically normal. The similarity of the early melanophore phenotype in *tdo* mutants (Class VI.B), which we attribute to delayed differentiation, with that of *cls* mutants is consistent with an inability of *cls* melanophores to differentiate fully. Hence, *cls* has an additional role in melanophore differentiation. Analogous multiple functions are already assigned to *W* (c-kit) and *Sl* (SCF) genes in mice, which have roles in the proliferation, survival and perhaps also patterning of melanocytes (Reid et al., 1993; Morrison-Graham and Weston, 1993; Wehrle-Haller and Weston, 1995).

Chromatophore patterning

Neural crest cell patterning involves control of cell migration and localisation. Mutants in genes affecting pigment cell localisation were expected to have phenotypically normal chromatophores in normal numbers, except in discrete regions. Mutations in five genes show defects fitting these criteria (Class IV). Whereas each of these has effects on a different part of the wild-type pattern, only one mutation results in the total absence of a stripe. Assuming no interactions between them, these genes alone cannot account for all of the wild-type pattern: the sum of all the pattern defects would not remove all of the wild-type pattern. It seems likely that mutations causing similar pattern defects affecting other aspects of the pattern were overlooked (see below).

These pattern mutants and other phenotypes we describe suggest a working hypothesis of zebrafish larval pigment pattern formation, in which localised cues direct melanophores and iridophores to particular positions, and then xanthophores occupy remaining sites in the skin. Pattern mutations disrupt parts of an individual stripe, and imply localised cues in the environment to which migrating pigment precursors respond. For example, in the case of the lateral stripe it is clear that any mutation with a severe effect on the horizontal myoseptum results in loss of that stripe, and hence we believe that some component(s) of that myoseptum is required for localisation of melanophores to the lateral stripe. *chk* may affect that component more directly: it certainly has the least severe somite defect of all mutants that lack the lateral stripe. In addition, local patterning influences between chromatophore cell types are suggested by observations of xanthophore distribution in wild types and mutants. The exclusion of xanthophores from dorsal regions occupied by melanophores is seen most clearly in melanin synthesis mutations (Class VI.A). Where

melanophore number is decreased, e.g. in *spa*, xanthophores are found throughout these regions as if they are filling in spaces normally occupied by melanophores. Possible interactions between xanthophores and melanophores have been described in adult zebrafish tail regeneration and in axolotl body pigment patterning (Goodrich and Nichols, 1931; Goodrich et al., 1954; Epperlein and Löfberg, 1990). In axolotl, melanophores migrate first and spread evenly throughout the skin, while xanthophores form spaced clusters in the premigratory crest. As the latter cells then migrate adjacent melanophores are repelled, thus generating the pattern of alternating dorsoventral bars of melanophores and xanthophores. Furthermore, formation of the wild-type iridophore stripes in the adult may be dependent on correct patterning of the melanophores (Johnson et al., 1995). Our mutations give no evidence for interactions between other combinations of chromatophore types being important in larval pigment pattern formation.

Pigment pattern formation must include control of neural crest cell migration. Two mutations producing ectopic chromatophores are candidates for genes affecting this aspect of neural crest development. Phenotypes of both *tig* and *pde* mutations (Class V) suggest the temporary interruption of chromatophore migration on the medial pathway. Although in *tig* this phenotype is associated with a strong tail curvature, the two phenotypes may not be causally linked since (1) other mutations causing tail curvatures do not give pigment phenotypes (Brand et al., 1996) and (2) there is no such correlation in *pde*. Elucidation of the basis for these phenotypes might reveal mechanisms involved in controlling pigment cell migration in zebrafish.

Pigment cell proliferation

We suggest that *spa* and *slk* (Class III) are candidates for mutations affecting the proliferation of melanoblasts. The degree of pigment cell proliferation that occurs in the first few days of development can be estimated from published studies of zebrafish neural crest clone sizes (Raible and Eisen, 1994). Premigratory melanophore precursors undergo on average just one division. Hence we predict a twofold reduction in melanophore number in mutations preventing melanoblast proliferation over this time period. This melanoblast proliferation estimate is consistent with the approximately twofold melanophore decrease by the third day in the strongest *spa* and *slk* alleles (Class III). A further role in melanophore survival or differentiation seems likely, since in both cases the originally normal-looking melanophores become morphologically abnormal and decrease in number. The Raible and Eisen (1994) study also suggests that, in the absence of proliferation, the xanthophore/iridophore number should be reduced by up to 75%. *cls* and *shd* mutants have many fewer chromatophores, suggesting that they affect specification, rather than proliferation, of pigment cell precursors. Effects on the general proliferation of the early crest are more difficult to eliminate, but might be expected to affect other neural crest derivatives as well as chromatophores. We see no evidence of this. For example, the jaw and arch elements and fin mesenchyme are normal in these animals.

The *mon* phenotype is particularly intriguing. Iridophore proliferation seems to be greater than normal, but only in regions adjacent to the median fin fold. The possible relationship between fin development and iridophore number remains to be elucidated.

Chromatophore survival

Genes involved in survival of neural derivatives and melanocytes are known from mice (Stemple et al., 1988; Morrison-Graham and Weston, 1993). We propose that a large number of our mutations produce phenotypes consistent with affects on survival of all three chromatophore types. We distinguish three classes of melanophore phenotype. In the melanophore degeneration mutations (Class VI.C), the progressive loss of pigment and frequent decrease in apparent cell number suggests melanophore death, although this interpretation must be tested by cytological studies. We have identified mutations in 24 genes that give this phenotype (Classes VI.C, VI.D, VI.E and most of VI.H). Often, mutations affecting melanophores and one or more other chromatophores show such a melanophore phenotype, and in some cases, e.g. *blr*, degeneration of these other pigment cell types seems likely. We suggest, therefore, that this group represents genes important in chromatophore survival. Since these mutations often produce additional phenotypes (e.g. *sunbleached* (*sbl*); Tables 4 and 5) some may have equivalent roles in other tissues, including other crest derivatives such as the branchial arches. However, some of these mutations might not be affecting a normal selection step in chromatophore development. Instead the degeneration phenotypes might be caused by the abnormal accumulation of a cytotoxic gene product. For instance, the *Light* allele of the mouse gene *brown*, B^{lt}, causes melanocyte death due to a missense mutation in a melanin-synthesis gene (Johnson and Jackson, 1992).

Chromatophore differentiation

The remaining genes in classes VI and VII are in many cases clearly affecting chromatophore differentiation, either the expression of pigment (e.g. melanin mutations, Class VI.A) or cell morphology (Class VII). Since proteins used in the synthesis of multiple organelles are known from a series of mouse mutations that show effects on both lysosome and melanosome synthesis (Jackson, 1994), there may be mutations affecting organelle formation among our mutations affecting the differentiation of multiple chromatophores (Classes VI.D-H). Their further characterisation should then reveal aspects of the genetic control of organelle synthesis. Melanophore morphology mutants (Classes VII.A-C) may be analogous to mouse mutations such as *d*, and will also be interesting in terms of the genetic control of cell morphology or organelle motility. The melanogenesis genes (Class VI.A) will be especially useful as cell markers e.g. in transgenic zebrafish (see Odenthal et al., 1996a). In contrast to melanin synthesis, pathways of pigment synthesis in xanthophores are poorly understood, although pteridine synthesis is assumed to utilise pathways similar to those characterised in *Drosophila* (Bagnara and Hadley, 1973). The xanthophore mutations (Class VI.J; Odenthal et al., 1996b) will be a useful resource for studying the biochemistry of vertebrate pteridine synthesis. Iridophore pigmentation is dependent on precise orientation of

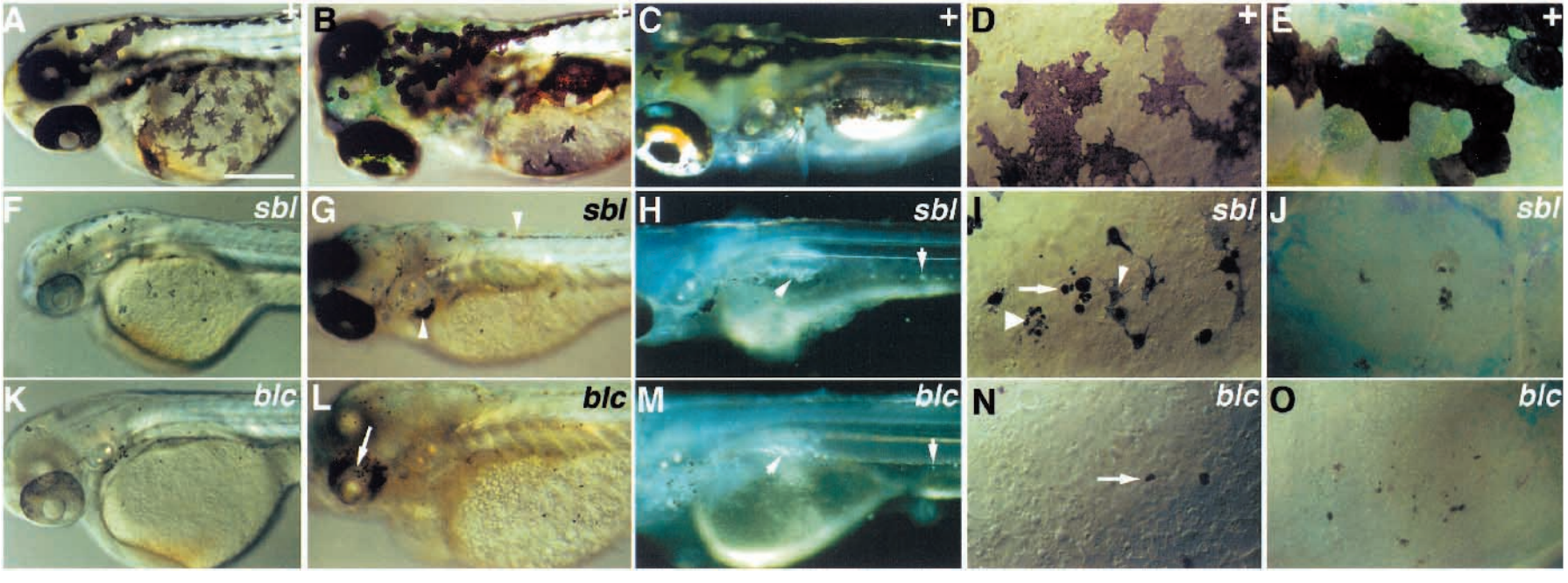

Fig. 12. Phenotypes of mutations affecting pigmentation of all chromatophores (Class VI.H). Wild-type siblings (A-E) are compared with homozygous mutants for *sbl* (F-J) and *blc* (K-O) on the third day (A,D,F,I,K,N) and the sixth day (B,C,E,G,H,J,L,M,O). Melanophore degeneration begins early (F,I,K,N) and pale (small arrowhead, I), spot-like (arrows, I,N) and fragmenting (large arrowhead, I) melanophores are seen. Melanophore degeneration phenotypes are extreme by the sixth day (G,J,L,O), numbers appear strongly decreased (G,L) and collect abnormally (arrowheads, G). In *sbl* the eye is small and pale (F), whereas in *blc* the pigmented retinal epithelium is pale on the third day (K) and largely degenerated by the sixth day (arrow, L). Xanthophores are very pale (F,G,J) or almost unpigmented (K,L,O). Iridophores are dull (arrows, H,M). Panels C,H and M were photographed with incident light. A-C, F-H and K-M are dorsolateral views; D,I and N are dorsolateral views of melanophores on the yolk sac; E,J and O show dorsal views of the head dorsal stripe. Scale bars, 250 μm (A-C,F-H,K-L) and 60 μm (D,E,I,J,N,O).

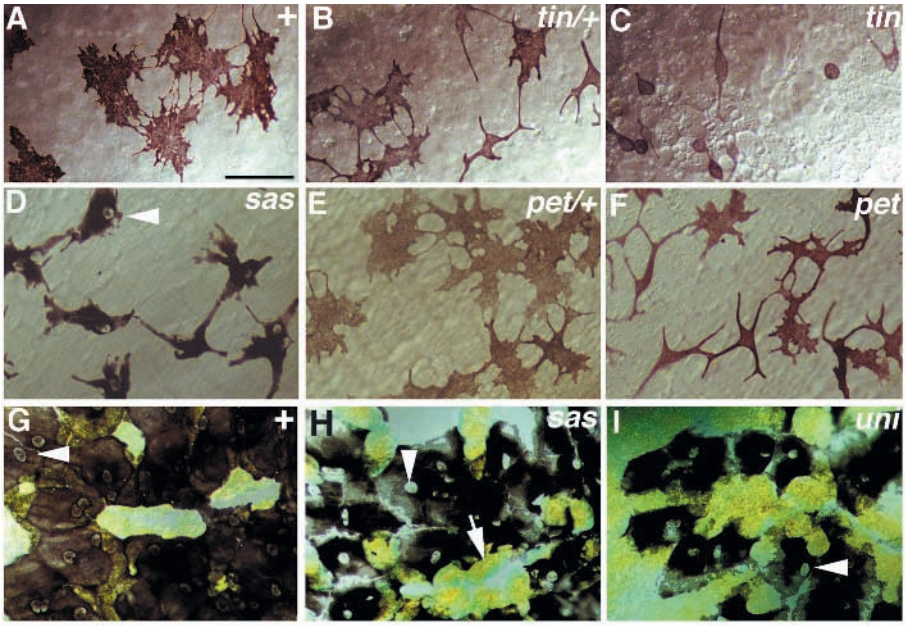

Fig. 13. Abnormal chromatophore morphology phenotypes (Class VII). Wild-type siblings (A,G) are compared with heterozygous *tin* (B), homozygous *tin* (C), heterozygous *pet* (E), homozygous *pet* (F), and homozygous *sas* (D,H) and *uni* (I) mutants on the third day (A-F) and the sixth day (G-I). Melanophores are pale and spindly (*tin*/+, B) or spot-like and uni- or bipolar (*tin/tin*, C) in *tin* mutants. Melanophores are pale (*pet*/+,E) or pale and spindly, but multipolar (*pet/pet*, F) in *pet* mutants. *sas* mutants show small, stellate melanophores with a pale central area (arrowhead, D,H). Melanophores remain small on the sixth day, and xanthophores now have a similar phenotype (arrow, H). *uni* mutants share the melanophore phenotype (arrowhead, I), but xanthophores are unaffected (I). A-F are dorsolateral views of yolk sac melanophores; G-I are dorsal views of head dorsal stripe. Scale bars, 60 μm (A-F) and 100 μm (G-I).

iridosomes; their shininess depends upon these organelles being oriented in parallel to each other. The dull iridophore mutations (Class VI.I) may therefore include genes affecting organelle orientation. Background adaptation of chromatophores depends on hormonally controlled responses to ambient lighting (Bagnara and Hadley, 1973). Some components of this behavioural response should be revealed by study of the background adaptation mutations (Class VII.D) identified in this collection.

As noted above, we believe that we have identified most of the genes important in chromatophore development that can mutate to a visible phenotype. We expect that further complementation testing of the unresolved mutations will show some to be allelic to the genes we have defined, and that the proportion of single hit genes will decrease. However, as expected in such a screen, certain phenotypic classes are probably not saturated. This is expected for a number of reasons. Firstly, mutant phenotypes may be too subtle to be readily found. This is especially true for two classes (pigment pattern (Class IV) and dull iridophore mutations (Class VI.I)). Secondly, mutant phenotypes may be very obvious, but fewer alleles will be found where genes are physically small, or where only unusual (e.g. neomorphic) alleles give the phenotype. This may be the case for *cls* and *slk*. Finally, in the class affecting pigmentation of all chromatophores (Class VI.H), the high proportion of single hits is a result of criteria used to decide which mutants to keep in the primary screen. Many similar mutants, but showing severe necrosis on the sixth day, were discarded. Hence mutations in this class may be relatively weak, hypomorphic alleles. Whatever the explanation in particular cases, for all these phenotypic classes further genes await identification.

We expect that among this collection of pigmentation genes are homologues of at least some of the previously characterised mouse mutations. As mentioned above, *sdy* is likely to be a tyrosinase (*c* locus) homologue. Such ideas can be readily tested by sequencing candidate genes from mutant embryos (Schulte-Merker et al., 1994) and by locating the mutation and the candidate gene on the zebrafish genetic map (Postlethwait et al., 1994; Talbot et al., 1995).

The mutations described in this paper provide a wealth of material for the analysis of all stages of neural crest development, in an organism that can be manipulated and analysed at the single cell level. They should eventually lead to a molecular genetic understanding of many aspects of the development of this uniquely vertebrate tissue.

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Note added in proof

Mutations in the genes *colourless* (this paper) and *golas* (Malicki et al., 1996) fail to complement each other and thus are to be considered allelic. Future references to this gene should use the name *colourless*.

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