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Formation of the adult pigment pattern in zebrafish requires *leopard* and *obelix* dependent cell interactions

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SUMMARY

Colour patterns are a prominent feature of many animals and are of high evolutionary relevance. In zebrafish, the adult pigment pattern comprises alternating stripes of two pigment cell types, melanophores and xanthophores. How the stripes are defined and a straight boundary is formed remains elusive. We find that mutants lacking one pigment cell type lack a striped pattern. Instead, cells of one type form characteristic patterns by homotypic interactions. Using mosaic analysis, we show that juxtaposition of melanophores and xanthophores suffices to restore stripe formation locally. Based on this, we have analysed the pigment pattern of two adult specific mutants: *leopard* and *obelix*. We demonstrate that *obelix* is required in melanophores to promote their aggregation and controls boundary integrity. By contrast, *leopard* regulates homotypic interaction within both melanophores and xanthophores, and interaction between the two, thus controlling boundary shape. These findings support a view in which cell-cell interactions among pigment cells are the major driving force for adult pigment pattern formation.

Key words: Melanophore, Xanthophore, Zebrafish, Pigment pattern

INTRODUCTION

Many animal species employ colour patterns for interacting with their biotic environment. They are involved in diverse behaviours such as mate choice, camouflage and deterrence, and thus are probably subject to extensive selection. Additionally, colour patterns can vary dramatically even between closely related taxa, and hence seem to undergo rapid evolutionary change. Studying the evolutionary transformation of colour patterns should ultimately allow for understanding how diversity in adult morphology is generated. In recent years, some basic principles of pigment pattern formation have been elucidated in several organisms. For example, the eyespots on butterfly wings (reviewed by Beldade and Brakefield, 2002) appear to be generated by readout of positional information provided by an organiser, the eyespot focus (Nijhout, 1980), that expresses morphogens such as Hedgehog (Keys et al., 1999). Cells in the wing epidermis presumably develop their type of pigmentation differently according to their distance from the focus (Brunetti et al., 2001). Another mechanism is exemplified in *Drosophila* wings where local deposition of a pigment-synthesizing enzyme and the supply of precursors generate a pigment pattern (True et al., 1999).

The pigment patterns of many fish and amphibians are comprised of alternating arrays of different neural crest-derived pigment cell types. Often, these cells are already differentiated as the pattern forms, making a mechanism as described above unlikely. Characterisation of these patterns mainly carried out in amphibian larvae has implicated a variety of cell behaviours such as migration, cell-substrate and cell-cell interactions in the generation of pigment patterns (e.g. Epperlein and Claviez, 1982; Epperlein and Löfberg, 1990; Macmillan, 1976; Parichy, 1996a; Parichy, 1996c; Tucker and Erickson, 1986; Twitty, 1945). However, the identification of specific factors involved in pigment pattern formation is difficult in non-genetic model systems.

The emergence of zebrafish as a model system has resulted in a collection of mutants affecting various aspects of pigment cell development and physiology. One major class of these mutants, such as *colourless* (Dutton et al., 2001), *nacre* (Lister et al., 1999), *sparse* (Parichy et al., 1999), *rose* (Parichy et al., 2000a) and *fms* (Parichy et al., 2000b), shows a pigment cell phenotype already during early larval stages. Molecular analysis of some of these mutants has revealed a high degree of functional conservation with genes previously implicated in melanocyte development in mice (Lister, 2002). Another class of mutants, such as *asterix*, *obelix* (Haffter et al., 1996b), *leopard* (Kirschbaum, 1975), *puma* (Parichy and Turner, 2003b; Parichy et al., 2003) and *hagoromo* (Kawakami et al., 2000), shows a late phenotype during formation of the adult pigment pattern. So far, only one of these genes, *hagoromo* (*hag*), has been cloned. *hag* encodes a dactylin homologue (Kawakami et al., 2000) and perturbs the pattern locally upon mutation.

In zebrafish, the adult pigment pattern (Goodrich and Nichols, 1931) (reviewed by Quigley and Parichy, 2002) consists of alternating stripes formed by melanin-bearing melanophores and pteridine containing xanthophores (Bagnara, 1998). The third major class of chromatophores in

zebrafish, silvery iridiophores, is likely to be irrelevant for stripe formation, as tissues devoid of iridiophores also display a striped pattern. At the onset of adult development, this pattern evolves through the alignment of newly differentiating pigment cells with a lateral melanophore stripe that persists during larval stages (McClure, 1999; Milos et al., 1983). One major question is therefore how the cells become organised into these domains. In principle, this might be accomplished by filling in a prepattern that is set up independent of the pigment cells. Alternatively, mutual interactions between the pigment cells might define the striped domains. Other crucial characteristics of this pattern are the strict separation between regions occupied by either cell type and the straight boundary between them. How these features are generated and which cell behaviour underlies their formation remains largely obscure. In this study we show, using mutants that abolish formation of either melanophores or xanthophores, that presence and juxtaposition of both pigment cell types is necessary and sufficient for stripe formation. Thus, the domains appear to be largely defined by short-range interactions among pigment cells. Based on the analysis of patterns formed in the presence of only one cell type, we classify the cell behaviours during stripe formation into homo- and heterotypic interactions. In light of this distinction we analyse the phenotypes of two mutants, *leopard* (*leo*) and *obelix* (*obe*), that specifically alter the adult pigment pattern in a qualitative way and assay their cell type specific requirements by mosaic analyses. *leo* and *obe* affect different subsets of these cell behaviours and constitute central components of the stripe-forming system.

MATERIALS AND METHODS

Zebrafish maintenance and genetics

Zebrafish were bred and maintained under standard laboratory conditions (Brand et al., 2002). Double mutants between adult specific phenotypes, such as *leo* and *obe*, and mutants with an early larval phenotype such as *nac* and *fms* were generated by intercrossing homozygous adult carriers. Subsequently, F2 individuals homozygous for the adult phenotype were incrossed and larvae displaying the larval phenotype were raised. The following alleles were used in this study: *nacre*^{w2} (Lister et al., 1999), *leopard*^{tw28}, *leopard*^{tq270}, *obelix*^{td15} (Haffter et al., 1996b), *golden*^{b1} (Streisinger et al., 1986). Mutants in *salz*^{tl41a} and *pfeffer*^{tm36b}, previously assigned to two complementation groups, (Odenthal et al., 1996) were used in this study. Both, *salz*^{tl41a} and *pfeffer*^{tm36b}, fail to complement the *panther*^{j4blue} allele, carry mutations in the RTK *fms* (Parichy et al., 2000b) and will, therefore, be referred to as *fms* in the following. In *salz*^{tl41a}, codon 187 within the second IG domain is changed from TGC(Cys) to TGA. In *pfeffer*^{tm36b}, a conserved Phe (TTT) at position 579 is changed to a Ser (TCT). The transgenic line expressing EGFP under the control of a 5.3 kb fragment of the zebrafish β-Actin promoter (bpeGFP) was a gift of K. Poss.

Cell-transplantation

For all transplantations, donor embryos were homozygous for the β-Actin GFP transgene (bpeGFP) in combination with various mutant genotypes. For *leo* and *obe*, the two strongest alleles, *tq270* and *td15*, were used. Cell transplantations were performed essentially as described previously (Kane and Kishimoto, 2002). Surviving larvae were sorted on day 2 according to presence of pigment cell clones and raised to adulthood for analysis.

Image acquisition and analysis

For images of the developmental series, juvenile fish of defined genotype, age and size were maintained individually. Fish were anaesthetised in 0.04% Mesab and mounted on a glass slide in 3% methylcellulose. At 1 day intervals, body size was measured and pictures of the same region of the flank posterior to the dorsal fin were captured using a Zeiss AxioCam mounted on a dissecting microscope. Images of xanthophore autofluorescence and GFP fluorescence were taken with the same setup, using UV epiluminescence and a GFP filter. For images of the adults, male fish were sacrificed and fixed overnight in ice-cold 4% PFA. Scales and pectoral fins were removed and fish were mounted in 0.5% Agarose. All images were processed using Adobe Photoshop 6.0. Direct comparisons of cell positions on at 24-hour intervals were made by overlaying images manually to a maximum overlap of cell positions and marking cells that had changed position.

RESULTS

Formation of the striped pattern requires short-range interaction between melanophores and xanthophores

As the pigment pattern of adult zebrafish (Fig. 1A,D) consists of alternating stripes of two cell types, melanophores and xanthophores, one intriguing issue is whether either of these is capable of forming stripes in the absence of the other. This could indicate a prepattern provided by underlying tissues that is filled in independently by the pigment cells. Alternatively, the striped domains might not be predefined but rather generated by mutual interactions among pigment cells. Mutants that specifically abolish formation of one pigment cell type provide an ideal tool to address this point.

We first analysed the distribution of melanophores in the absence of xanthophores. Homozygous mutants for the kit-related RTK *fms* (Parichy et al., 2000b) display a strong reduction of xanthophores during larval stages and completely lack this cell-type in the adult (Fig. 1B,E). At the onset of adult pigment pattern formation in *fms* mutants (Fig. 1I), the initial pattern of melanophore differentiation is indistinguishable from wild type (Fig. 1G,H), with melanophores scattered throughout the flank. At around 25 days of age, melanophores start to aggregate in a similar fashion as in the presence of xanthophores. Later, melanophores fail to be cleared from the region around the horizontal myoseptum, which corresponds to the position of the first xanthophore stripe in wild type. In addition, melanophores do not clear from the more ventral regions, suggesting that the absence of xanthophores negatively influences their tendency to migrate towards the horizontal myoseptum region (Parichy et al., 2000b). In *fms* mutant adults (Fig. 1B,E), melanophores are found in circular clusters of about 10-20 cells. A substantial fraction of melanophores is interspersed individually between the clusters and no larger regions completely devoid of melanophores can be observed.

To study the pattern formed by xanthophores in the absence of melanophores, we used mutants for *nacre* (*nac*; *mitfa* – Zebrafish Information Network) (Lister et al., 1999), a zebrafish homologue of the mammalian microphthalmia gene (Hemesath et al., 1994; Hodgkinson et al., 1993). *nac* mutants are homozygous viable and completely lack melanophores in larval as well as adult stages. During larva to adult transition,

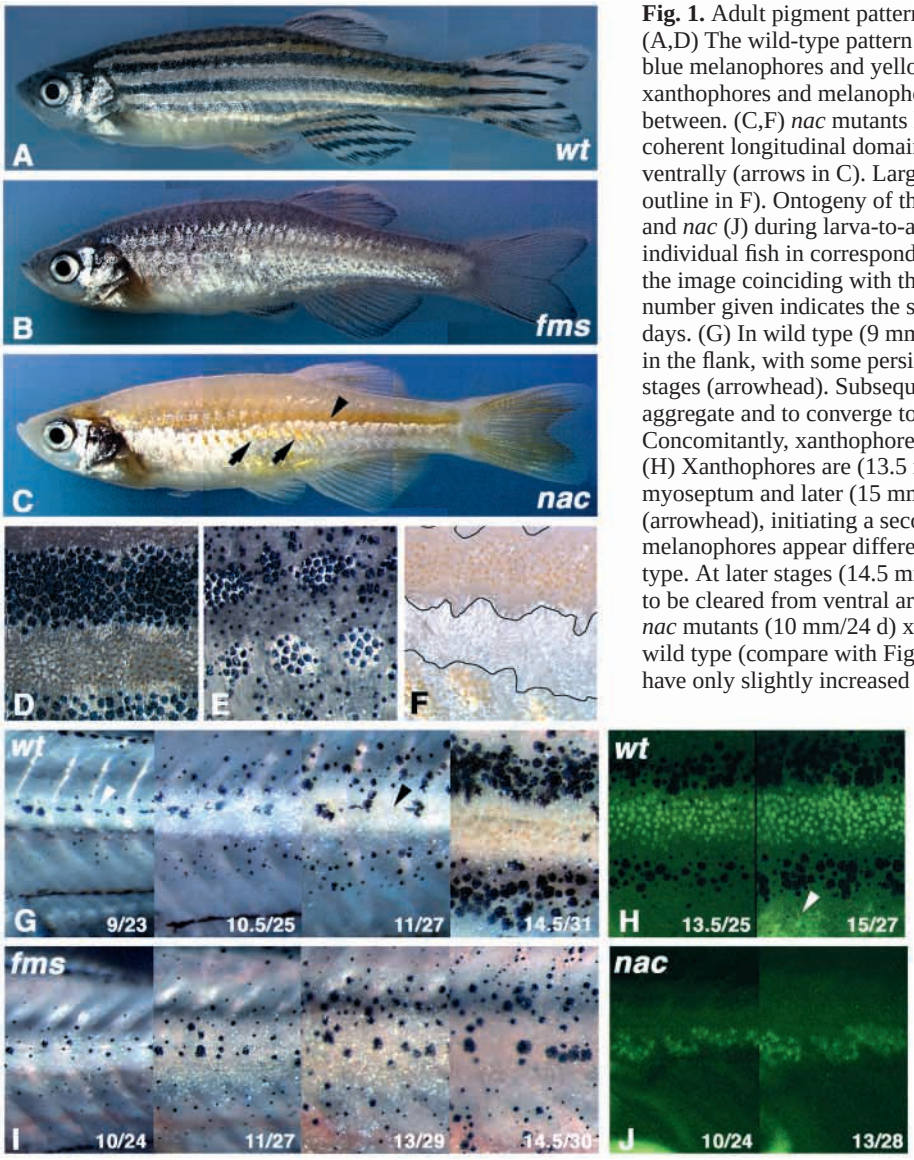

Fig. 1. Adult pigment pattern in mutants lacking one pigment cell type. (A,D) The wild-type pattern consists of four or five alternating stripes of blue melanophores and yellow xanthophores. (B,E) *fms* mutants lack all xanthophores and melanophores occur in clusters as well as scattered in between. (C,F) *nac* mutants lack melanophores and xanthophores form a coherent longitudinal domain (arrowhead in C) as well as clusters more ventrally (arrows in C). Large areas are devoid of xanthophores (black outline in F). Ontogeny of the pigment pattern in wild type (G,H), *fms* (I) and *nac* (J) during larva-to-adult transition. Images were taken from individual fish in corresponding regions of the body with the anterior edge of the image coinciding with the anterior edge of the dorsal fin. The first number given indicates the size in millimetres and the second the age in days. (G) In wild type (9 mm/23 d), melanophores initially appear scattered in the flank, with some persisting in the horizontal myoseptum from larval stages (arrowhead). Subsequently (11 mm/27 d), melanophores start to aggregate and to converge towards the horizontal myoseptum. Concomitantly, xanthophores appear (11 mm/27 d, arrowhead). (H) Xanthophores are (13.5 mm/25 d) seen in a stripe around the horizontal myoseptum and later (15 mm/27 d) start appearing in more ventral regions (arrowhead), initiating a second xanthophore stripe. (I) In *fms* (10 mm/24 d), melanophores appear differentiating throughout the flank, similar to wild type. At later stages (14.5 mm/30 d) melanophores start aggregating but fail to be cleared from ventral areas and around the horizontal myoseptum. (J) In *nac* mutants (10 mm/24 d) xanthophores appear in the same position as in wild type (compare with Fig. 2B). Later (13 mm/28 d), the xanthophores have only slightly increased in number.

xanthophores in *nac* mutants (Fig. 1J) first appear adjacent to the horizontal myoseptum in essentially the same position as in wild type. However, their appearance is strongly delayed, and a fraction (4/9) of the individuals analysed showed no xanthophores by day 30. Xanthophores appear in small clusters of only a few cells and seem to expand their domain much slower than in wild type. In *nac* adults (Fig. 1C,F), xanthophores occupy a coherent longitudinal field of cells straddling the horizontal myoseptum and irregular patches further ventrally. Between these domains, large areas are completely devoid of xanthophores.

We next asked whether the altered positioning of pigment cells in *nac* and *fms* mutants is solely due to the absence of one cell type and thus reflects their intrinsic positioning behaviour. To this end, we generated genetic mosaics by transplanting wild-type blastula cells marked by constitutive GFP expression (bpeGFP) into homozygous *nac* and *fms* mutants. Embryos resulting from these experiments were raised to adulthood and patches (‘clones’) containing the pigment cell type normally absent from the respective mutant were analysed.

Upon transplantation of bpeGFP blastula cells into homozygous *nac* mutants, all melanophores observed in transplanted fish are of donor origin, as *nac* has been shown to be autonomously required in melanophores (Lister et al., 1999), whereas xanthophores are both donor as well as host derived. In all animals analysed (n=26) the melanophores within such clones formed stripes that alternate with xanthophore stripes, resulting in a locally rescued pattern that is indistinguishable from wild type (Fig. 2C). The xanthophore stripes within such clones contain donor-derived, GFP-positive xanthophores as well as host derived ones. This shows that *nac* mutant xanthophores retain the ability to organise into stripes. Only xanthophores that are in contact or close proximity to melanophores will contribute to the striped pattern, whereas donor as well as host derived xanthophores further away from the melanophore clone assume a distribution as seen in untransplanted *nac* mutants (Fig. 2D). This indicates that indeed the absence of melanophores causes the altered xanthophore distribution in *nac* mutants. Moreover, the fact that xanthophores will form stripes only in the vicinity of melanophores suggests a short-range mode of interaction between melanophores and xanthophores during stripe formation. The cells transplanted at blastula stages will give rise to a variety of tissues in the adult. However, we found that striped patterns always occur where xanthophores and melanophores are in proximity, irrespective of the origin of the underlying tissue.

In the complementary experiment, we introduced bpeGFP cells into homozygous *fms* mutant embryos. To allow for distinction of donor derived melanophores from host-derived ones, the donor embryos were additionally homozygous for the

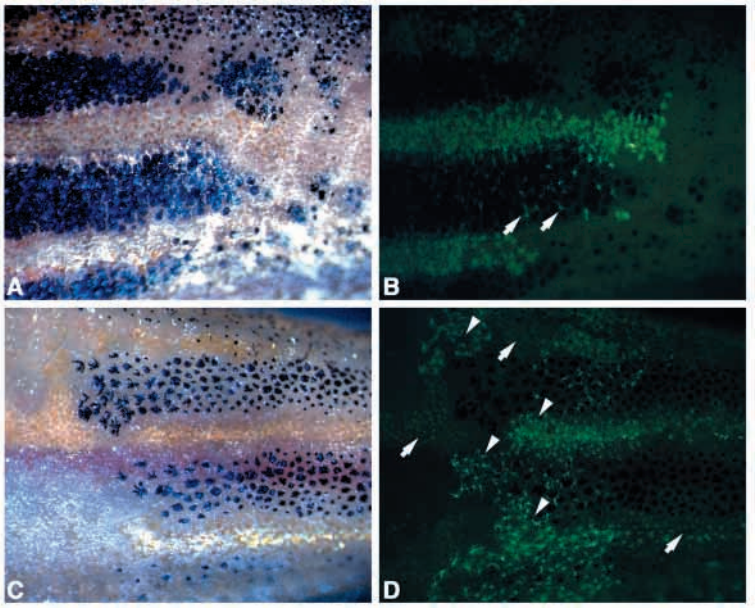

Fig. 2. Reintroduction of the missing cell type restores stripes in *fms* and *nac*. (A,B) Transplantation of β-Actin GFP expressing *golden* mutant cells into *fms* hosts. (A) Normally pigmented, donor-derived *fms* mutant melanophores form a stripe pattern if they are in proximity of xanthophores. Melanophores further away from the xanthophore clone form the typical melanophore clusters seen in *fms* mutants. (B) UV-illumination shows GFP+, wild-type xanthophores forming stripes with *fms* mutant melanophores. The xanthophores form a coherent domain; however, several xanthophores have invaded the melanophore stripe (arrows). Some of the GFP signal within the melanophore stripe is due to transplanted cells underlying the melanophore stripe. (C,D) Transplantation of bpeGFP cells into *nac* hosts. (C) Melanophores form stripes of relatively normal position and size. (D) Fluorescence image of the same sample. Host-derived xanthophores (arrows) display pteridine autofluorescence around their nuclei. GFP+ xanthophores (arrowheads) show fluorescence in the entire cell. In the proximity of melanophores, both donor- and host-derived xanthophores organise into stripes, whereas outside the melanophore clone, xanthophores form the *nac* mutant pattern.

golden (*gol*) mutation (Streisinger et al., 1986), which results in hypopigmented melanophores. Adults carrying clones (n=24) displayed a normally striped pattern within the clone. Most xanthophores were GFP positive, i.e. donor derived, whereas most of the melanophores were normally pigmented (*gol*+), i.e. host derived (Fig. 2A,B). This indicates that melanophores lacking *fms* function still can form stripes and thus the melanophore pattern in *fms* mutants is exclusively caused by the lack of xanthophores. In about one-fifth of the mosaic fish a completely rescued pattern in the entire flank was obtained, a situation we never observed with *nac* mutant mosaics. This indicates a strong proliferative capability of xanthophores. Notably, in such clones also GFP-negative host-derived xanthophores were present (data not shown), which suggests a partial non-autonomous function of *fms*. In cases where the striped clone bordered a region devoid of xanthophores, the xanthophores within the stripes were forming one coherent domain, and melanophores outside the clones assumed the distribution normally seen in *fms* mutant adults.

Taken together, these findings show that juxtaposition of melanophores and xanthophores is necessary and sufficient for stripe formation.

obe and *leo* affect stripe integrity and shape respectively

The experiments outlined above indicate that stripe formation requires interactions between the two pigment cell types. To look for factors that might govern these interactions we investigated two mutants, *leopard* (*leo*) (Kirschbaum, 1975) and *obelix* (*obe*) (Haffter et al., 1996b), that alter the adult pigment pattern. Four dominant alleles of *obe* were isolated in a large-scale mutagenesis screen (Haffter et al., 1996a) (F.M. and C.N.-V., unpublished). Heterozygous adults display only two to three melanophore stripes that are wider than in wild type and frequently interrupted by xanthophores (Fig. 3B,L). This is most likely a dosage-dependent effect of *obe*, as it is also observed in heterozygotes for the c7 deletion that covers the *obe* locus (data not shown). In *obe* homozygous adults (Fig. 3A,E) melanophores are found in two broad longitudinal domains flanking the horizontal myoseptum. Strikingly, the melanophore domains always contain interspersed xanthophores, indicating that *obe* mutants lack the strict separation between the two cell types seen in wild type.

The four alleles of *leo* (Haffter et al., 1996b) can be ordered into a series of increasing phenotypic strength. Heterozygotes for weak alleles, such as *tw28*, show a rather normal striped pattern, but the boundaries between melanophores and xanthophores are undulating. In leo^{tw28} homozygotes (Fig. 3M) the stripes are always undulating and sometimes break up into spots. Mutants for the strongest allele leo^{tq270} show undulating stripes and spots already in the heterozygous condition and in homozygous individuals (Fig. 3C,H) the stripes are entirely transformed into a spotted pattern with melanophore spots of about 10-20 cells surrounded by xanthophores. Unlike in *obe*, the two cell types still occupy spatially separated domains. The spotting phenotype and the undulating stripes in weaker alleles can be interpreted as decreased *leo* function that results in an increased boundary length between the melanophore and xanthophore populations. Double homozygotes for *leo* and *obe* (Fig. 1F,D) display single melanophores surrounded by xanthophores, suggesting that *leo* and *obe* act in parallel to control length and integrity of the boundary between melanophores and xanthophores.

We next examined the ontogeny of the pigment pattern in individual *leo* and *obe* mutant fish over the course of 14 days during larva to adult transition. At the onset of adult pigment pattern formation in wild type (Fig. 1G,H), melanophores are differentiating in broad regions of the flank dorsally and ventrally of the horizontal myoseptum, which bears melanophores of the larval lateral stripe. Subsequently, xanthophores appear around the horizontal myoseptum. Concomitantly, the melanophores in the flank begin to converge towards the horizontal myoseptum by migration (Parichy et al., 2000b), whereas melanophores within the xanthophore stripe are eliminated. Thus, a xanthophore stripe

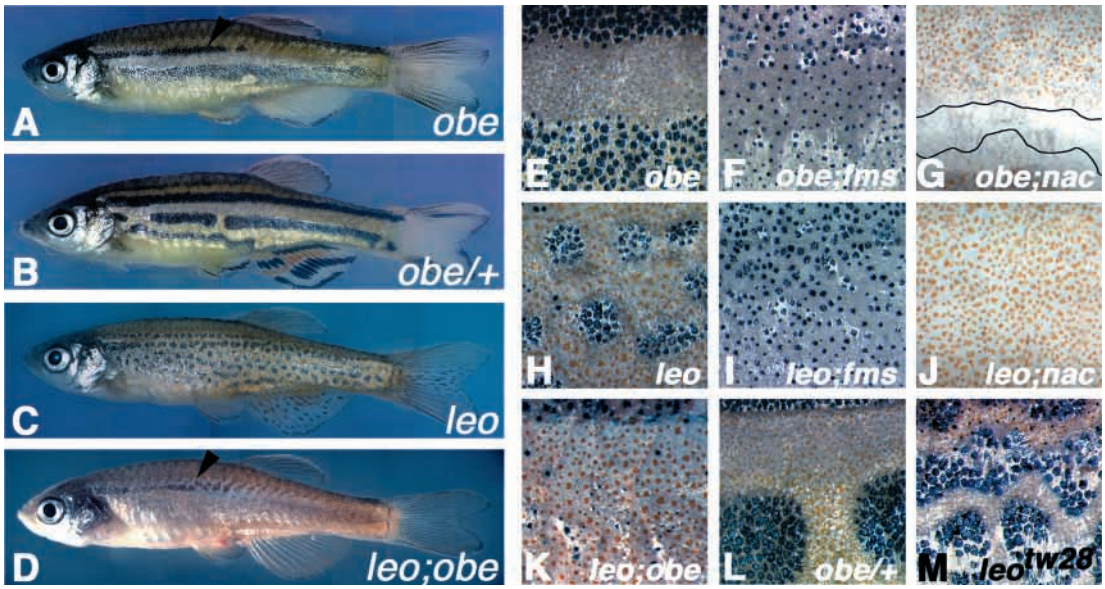

Fig. 3. Mutants affecting the adult pigment pattern in zebrafish. (A,E) Homozygous *obe* mutants display fewer and wider stripes of loosely clustered melanophores, which contain intermingled xanthophores. Partially, the melanophore stripes show a higher density of cells (arrowhead in A). (B,L) *obe* heterozygotes display fewer and wider stripes and melanophore stripes are interrupted. (C,H) In homozygous mutants for a strong *leo* allele, melanophores form spots that are surrounded by xanthophores. (D,K) In an *obe*;*leo* double mutant, melanophores are singled out between xanthophores, apart from minor melanophore stripe remainders (arrowhead in D). (M) In mutants for a weak *leo* allele (*tw28*), melanophore stripes are undulating and interrupted. (F) *fms*;*obe* double mutants lack the melanophore clusters seen in *fms* single mutants (Fig. 1C,D). (G) *obe*;*nac* double mutants still contain xanthophore-free areas (black outline) similar to *nac* single mutants (Fig. 1E,F). (I) In a *leo*;*fms* double mutant, the melanophore clustering is lost. (J) In *leo*;*nac* mutants xanthophore-free areas are absent.

flanked by two melanophore stripes has formed. As melanophores are cleared from dorsal and ventral regions, new xanthophores appear, continuing the alternation of the pattern.

In *obe* mutants, the larval pattern (not shown) and the initial appearance of melanophores at 21 days are similar to wild type (Fig. 4A). During appearance of the xanthophores, melanophores fail to cluster and remain scattered instead. Furthermore, melanophores do not converge towards the horizontal myoseptum and persist in more ventral areas as well as within the xanthophore stripe. The xanthophores in *obe* mutants (Fig. 4B) appear initially in the same position as in wild type, but later start to emerge between the scattered melanophores, giving rise to the mixing pattern.

In addition, *leo* mutants display a normal larval pattern (not shown) and a normal initial distribution of early adult melanophores (Fig. 4C). Similar to *obe*, melanophores fail to undergo clustering and convergence towards the horizontal myoseptum and remain scattered in the flank as well as within the xanthophore stripe. Initially, *leo* mutant xanthophores appear in a stripe straddling the horizontal myoseptum (Fig. 4D), as in wild type, and later start to populate the space left by the scattered melanophores. These observations suggested that one prime cause of the altered pigment patterns in *leo* and *obe* might be the failure of melanophores to aggregate into stripe-like domains. To investigate if this defect is caused by the inability of mutant melanophores to change position, we directly compared images captured at 24 hour intervals during emergence of the adult pattern from the same region of the trunk (Fig. 5). This comparison revealed that both *obe* and *leo* mutant melanophores are still able to translocate, albeit in the case of *obe*, to a lesser extent than in wild type. Therefore, the *obe* and *leo* mutant patterns are not caused by immobility of melanophores.

obe and *leo* have similar effects on melanophore behaviour but affect xanthophores differently

As melanophores in *fms* and xanthophores in *nac* mutants retain their ability to form stripes, their distribution most probably reflects interactions also employed normally during stripe formation. Therefore, we used these mutants to study the effects of *leo* and *obe* on melanophore and xanthophore patterns. In double homozygotes for *fms* and *obe* (Fig. 3F), melanophore clusters are lost and all melanophores seem more or less evenly scattered instead. Strikingly, *fms*;*leo* double homozygotes (Fig. 3I) also show the same effect of loss of melanophore clustering and are basically indistinguishable from *fms*;*obe* double mutants. We next characterised the effect of *leo* and *obe* on the behaviour of xanthophores in *nac* mutants. The distribution of xanthophores in *nac*;*obe* double mutants (Fig. 3G) is essentially the same as in *nac* single mutants, with xanthophore free regions interspersed. Conversely, in *nac*;*leo* double mutants (Fig. 3J), xanthophores tend to fill up the entire flank and leave no patches devoid of xanthophores, an effect that is already discernible upon visual inspection of double mutant adults. The spacing and density of the cells however appear similar as in wild type or *nac* single mutants.

Taken together, these data indicate that both *leo* and *obe* have a similar effect on melanophore behaviour, whereas only *leo* also affects xanthophore behaviour.

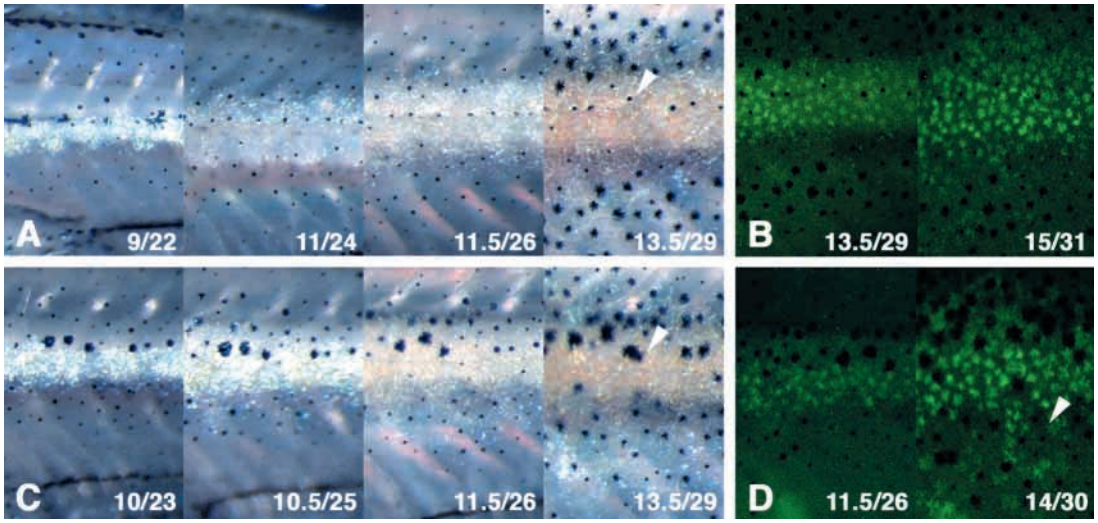

Fig. 4. Ontogeny of the pigment pattern in *obe* (A,B) and *leo* (C,D) during larva to adult transition. (A) In *obe*, the initial distribution of melanophores at the onset of stripe formation (9 mm/22 d) is similar to the wild-type situation. Later (11 mm/24 d), melanophores fail to aggregate and remain evenly scattered. After the onset of xanthophore differentiation (11.5 mm/26 d), melanophores fail to be cleared from within the xanthophore stripe (13.5 mm/29 d, arrowhead). (B) The early (13.5 mm/29 d) distribution of xanthophores in *obe* is similar to wild type. Later (15 mm/31 d), differentiating xanthophores appear in between the scattered melanophores, giving rise to a mingled pattern. (C) *leo* mutants show a similar early (10 mm/23 d) melanophore pattern as in wild type. Later (11.5 mm/26 d), melanophores fail to cluster and converge towards the horizontal myoseptum and remain within the xanthophore domain (13.5 mm/29 d, arrowhead). (D) The early positioning of xanthophores in *leo* is similar to wild type, but later (14 mm/30 d) xanthophores start to differentiate within the melanophore domains, with some xanthophores encircling several melanophores (arrowhead).

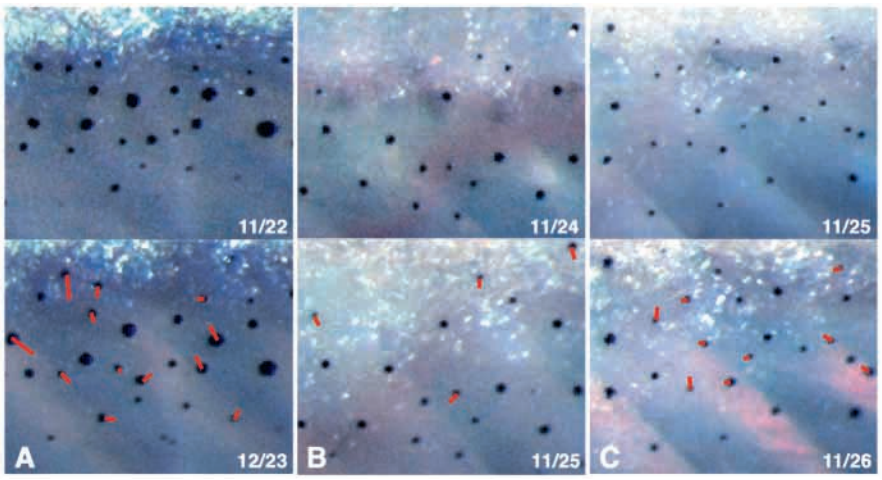

Fig. 5. Comparison of melanophore movements in a region ventral to the horizontal myoseptum in 1 day intervals. In the lower panels, cells that changed their position are marked with red bars, indicating the direction and extent of movement. (A) In wild type, 12 out of 26 melanophores changed position, some for over one cell-diameter. (B) In *obe* only 4/18 melanophores changed their position, indicating that *obe* mutant melanophores still are able to translocate. (C) In addition, *leo* mutant melanophores are able to move, as 9/24 melanophores changed their position.

obe function is required by melanophores

To assay the cell-type-specific requirements for *leo* and *obe*, we juxtaposed wild-type melanophores with mutant xanthophores and vice versa. This was accomplished by transplanting mutant or wild-type cells into *nac* single or double mutants, because in *nac* mosaics all melanophores will be donor derived. These mosaics could then be scored for formation of a wild-type or mutant pattern. As only one of the cell types will be mutant in a given mosaic condition, the type of pattern generated in that condition indicates the cell types in which the formation of the respective gene is required. Additionally, this experiment should also allow to determine whether the genotype of the pigment cells themselves or rather that of the underlying tissue is decisive for the type of pattern formed. In order to confirm that *obe* or *leo* mutant patterns could be reconstituted in mosaic experiments, we transplanted *obe* or *leo* mutant cells into *nac*;*obe* or *nac*;*leo* double mutant hosts, respectively, and found that in both cases the mutant patterns were faithfully reproduced (data not shown). Furthermore, all mosaic conditions for *leo* and *obe* generated consistent patterns within the clones, irrespective of whether the underlying tissue was wild type or mutant for *obe*, thus establishing a requirement for both genes within the pigment cells.

We analysed the cell type specific requirements for *obe* by transplanting bpeGFP cells that are wild type for *obe* into *nac*;*obe* double mutants (Fig. 6A,B). To distinguish host and donor cells, the donor cells were marked by the *gol* mutation

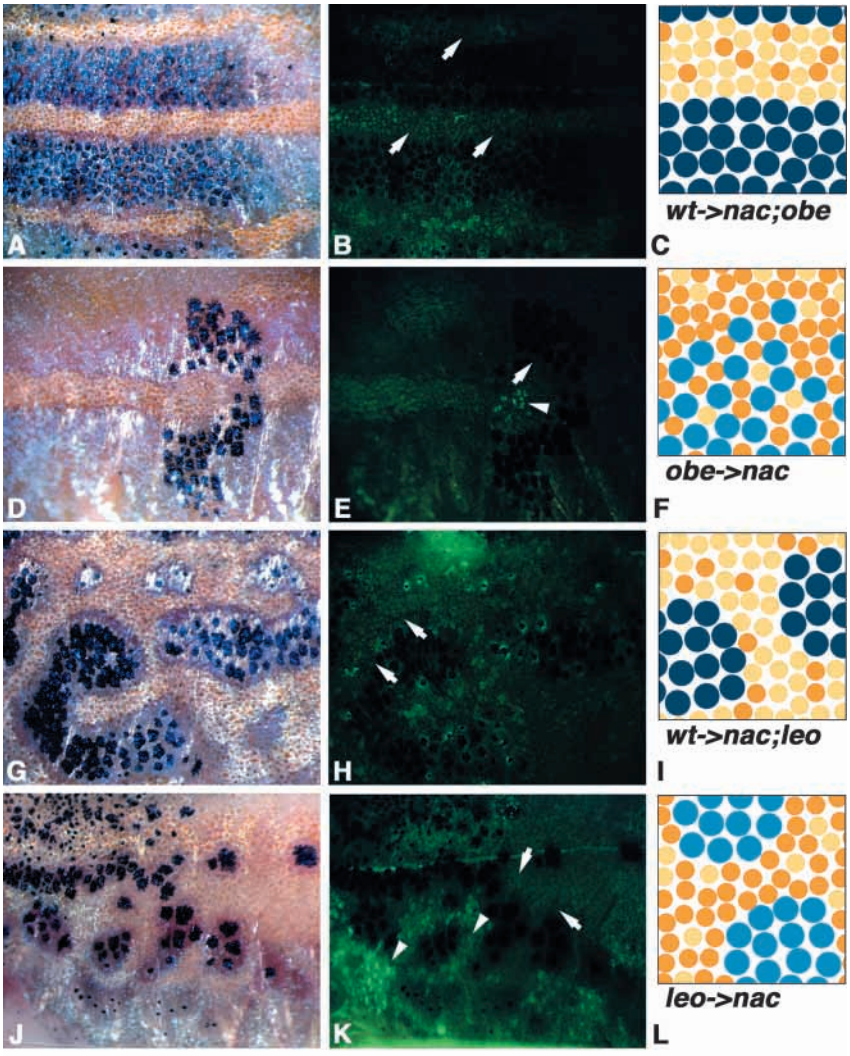

Fig. 6. Cell-type-specific requirements for *obe* and *leo*. (C,F,I,L) Diagrammatic representation of the outcomes of the mosaic analyses. Melanophores are represented in blue, xanthophores in yellow. Wild-type cells are shaded darker than mutant ones. (A-C) Transplantation of golden bpeGFP cells into *nac*;*obe* double mutant hosts generates a wild-type pattern. (A) A clone of wild-type melanophores organising the surrounding xanthophores into stripes of wild-type appearance. (B) A variety of cell types displaying GFP expression in the vicinity of the clone, but the majority of xanthophores is GFP negative and thus mutant for *obe* (arrows). (D-F) Upon transplantation of *obe*;bpeGFP cells into *nac* mutants, an *obe* like pattern is formed. (D) A melanophore clone with loosely clustered melanophores and intermingled xanthophores. (E) Only a few xanthophores are GFP positive (arrowhead), whereas the majority only displays autofluorescence (arrow) and is thus wild type for *obe*. (G-I) Transplantation of bpeGFP cells that are wild type for *leo* into *nac*;*leo* hosts generates a *leo* like pattern. (G) A clone of wild-type melanophores displaying key features of the *leo* pattern such as undulating interrupted stripes and spots. (H) Many different GFP positive cells are discernible around the clone, but most of the xanthophores are GFP negative (arrows) and thus mutant for *leo*. (J-L) Transplantation of *leo*; bpeGFP cells into *nac* single mutant hosts results in a *leo* like pattern. (J) A clone of *leo* mutant melanophores forming spots and undulating stripes. (K) Both GFP positive *leo* mutant xanthophores (arrowheads) as well as GFP-negative, wild type xanthophores (arrows) participate in formation of *leo* like pattern elements.

and GFP expression. In the mosaic adults analysed (n=24) melanophore clones always formed a wild-type-like stripe pattern with clear separation between melanophores and xanthophores (Fig. 6A). This was also the case when the vast majority of xanthophores within a clone was host derived and thus mutant for *obe* (Fig. 6B), showing that wild-type melanophores can form a regular boundary with *obe* mutant xanthophores. In the complementary experiment, *obe* mutant bpeGFP cells were transplanted into *nac* single mutant hosts, melanophore clones in all mosaics analysed (n=18) formed broad irregular domains, similar to the *obe* mutant pattern (Fig. 6D,E). In cases where most of the xanthophores were wild type, they mingled with *obe*$^{-}$ melanophores. Thus, both mosaic conditions indicate that the genotype of the melanophores determines whether a wild type or an *obe* mutant pattern is formed. Hence, *obe* function is required within the melanophores for spatial separation between the pigment cell types.

leo is required within both melanophores and xanthophores

We performed the analogous experiments for the *leo* mutant. First, bpeGFP cells that are *leo*+ were transplanted into a *nac*;*leo* host (Fig. 6G,H). None of the adults bearing wild-type melanophore clones (n=22) showed an entirely wild-type melanophore pattern within the clone. Inspection of melanophore clones mainly surrounded by GFP-negative (and hence *leo* mutant xanthophores) showed that *leo* mutant xanthophores form roundish, irregular boundaries with wild-type melanophores. This indicates that wild-type melanophores are unable to impose a wild-type striped pattern with straight boundaries onto *leo* mutant xanthophores. Notably, even though the strongest leo allele *leo*^{tq270} was used in all mosaic experiments, the clones never consisted entirely of spots, but rather showed an intermediary phenotype, with undulating stripes and spots.

With the complementary mosaic condition, transplantation of *leo*;bpeGFP cells into a *nac* single mutant host, a similar result was obtained (Fig. 6J,K). Again, none of the *nac* adults containing melanophore clones (n=18) showed a clear wild-type pattern in the clone. Instead, similar undulating stripes and spots were observed as for the inverse mosaic condition. In addition, *leo* mutant melanophores predominantly surrounded by wild-type xanthophores were forming spots, and hence a *leo*-like pattern. Thus, *leo* mutant melanophores can organise wild-type xanthophores into a *leo*-like spotted pattern. These

findings indicate that *leo*, unlike *obe*, is required by both pigment cell types, and that compromising *leo* function in either melanophores or xanthophores alone is sufficient to generate a *leo* like pattern.

DISCUSSION

Formation of the striped pattern requires interaction between pigment cell types

Our phenotypic and mosaic analysis of the mutants lacking one pigment cell type shows that juxtaposition of both cell types is necessary and sufficient for formation of a striped pattern. The mosaic analysis corroborates recent findings (Parichy and Turner, 2003a), which were obtained under slightly different experimental conditions. Thus, mutual interactions between the pigment cells play a predominant role during establishment of the striped domains, even though few features of the pattern can be formed independently (see below). A strict prepattern of stripes that would be formed independently of the pigment cells and populated differentially by them is therefore unlikely. Such prepatterns have been shown to dominate the generation of larval pigment patterns in amphibians (reviewed by Epperlein et al., 1996; Parichy, 1996b). In these systems, the underlying tissue plays an instructive role in defining the territories occupied by the different pigment cell types, whereas cell-cell interactions are likely to play a minor role. This apparent difference might indicate different mechanisms operating in the formation of larval versus adult patterns. Indeed, also in zebrafish the position of the larval melanophore pattern coincides with anatomical structures such as the horizontal myoseptum (Milos et al., 1983). Moreover, different mechanisms might be operating in different taxa.

In addition, the analysis of *nac* and *fms* mutants also reveals which aspects of cell behaviour in particular depend on the presence of the other cell types. The delayed appearance of xanthophores in *nac* mutants could indicate an requirement of melanophores during adult development for correct differentiation and/or proliferation of xanthophores. Alternatively, this could be due to a direct requirement for *nac* in xanthophore development, as in the embryo some xanthophore precursors have been shown also to express *mitf* (Parichy et al., 2000b). Melanophores can aggregate independently of xanthophores, but they fail to converge towards the horizontal myoseptum and also persist in areas from which they are normally cleared (Parichy et al., 2000b). This indicates an attractive effect of xanthophores on distant melanophores, which promotes their alignment on either side of the xanthophore stripe. Conversely, within their domain, xanthophores might repel melanophores.

The exact cellular mechanisms that underlie these interactions are still unclear. It can be assumed that it involves an ability of the cells to recognise each other and to aggregate. As pigment cells outside clones cannot be organised into stripes at a distance, stripe formation appears to involve mainly short-range interaction or even direct cell-cell contact. Indeed, histological examination of adult dermis revealed intimate cell contact both among and between cells of one type (F.M. and C.N.-V., unpublished). One intriguing possibility is that differential affinities (McNeill, 2000; Steinberg, 1970) between pigment cells account for the spatially distinct domains of the pattern. Clearly, cells in *Drosophila* wing imaginal disks display differential affinities (Dahmann and Basler, 1999; Dahmann and Basler, 2000; Milan et al., 2001) during establishment of compartment boundaries. Given the intimate contact and the aggregative behaviour of pigment cells in the adult, one could envisage that affinities of melanophores and xanthophores for cells of the same type could establish separation, whereas affinity for cells of the other type might generate contact at the boundary.

Early features of the adult pattern can be formed in the absence of one cell type

Apart from interacting with cells of the other cell type (heterotypic interaction), pigment cells of one type also interact with each other (homotypic interaction). This is evident from the fact that neither xanthophores nor melanophores are randomly distributed in the absence of the other cell type. Rather, cell-type-specific patterns are formed that can be seen as the outcome of homotypic interactions reflecting the intrinsic positioning behaviour of the cells. One key feature of these single cell type patterns is the cells tendency to aggregate. The clustering of a fraction of melanophores involves translocation of cells from an initially scattered distribution towards the clusters formed during the early adult period (Parichy et al., 2000b). Even though xanthophores appear to change their position very little, if at all, they also remain in coherent areas during expansion of the xanthophore domain. Thus, both cell types must be able to establish and maintain cell-cell contact either directly or indirectly.

Although formation of the hallmarks of the adult pigment pattern depends on presence and interaction of melanophores and xanthophores, early features of the wild-type pattern can be generated independently. The xanthophores in *nac* mutants appear first close to the horizontal myoseptum, which corresponds to the position of the first xanthophore stripe in wild type. In addition, the initial position in which melanophores appear in *fms* mutants is similar to wild type. This indicates that at least the initial positioning of pigment cells during adult pattern formation is largely independent of the presence of the other cell type. Instead, initial positioning might be defined by anatomical landmarks, such as the horizontal myoseptum. Notably, mutants in *fss*/Tbx24 (Nikaido et al., 2002; van Eeden et al., 1996) disrupt horizontal myoseptum integrity, and display locally interrupted stripes as adults (F.M. and C.N.-V., unpublished). The function of the horizontal myoseptum as a starting point for stripe formation may be indirect, as it also governs migration of the lateral line primordium, which has been implicated in pigment pattern formation in salamanders (Parichy, 1996a; Parichy, 1996c).

obe controls boundary integrity by regulating melanophore aggregation

Among the few mutants known to affect stripe formation in adult zebrafish, *leo* and *obe* are probably central regulators of this process, as they perturb stripe formation in all parts of the body. Furthermore, they disrupt qualitative aspects of the pattern: the spatial separation of melanophores and xanthophores (*obe*), and boundary shape (*leo*). We believe that theses effects are not due to altered pigment cell numbers, because mutants in genes known to affect the numbers of pigment cells, such as *sparse* or heterozygosity for some semi-

dominant alleles of *fms* (Haffter et al., 1996b; Odenthal et al., 1996) do not affect boundary integrity or shape in such a dramatic way as *leo* or *obe*.

For *obe*, our analysis indicates that this gene is required in the melanophores to control their homotypic clustering. As xanthophores mingle with melanophores in *obe* mutants, melanophore clustering appears to be a major prerequisite for establishing spatial separation between melanophore and xanthophore stripes. The fact that in *obe* xanthophores also appear within the melanophore domains indicates an intrinsic affinity of xanthophores for the proximity of melanophores. This can overcome the tendency of xanthophores to remain clustered and to leave substantial regions of the flanks unoccupied, which occurs in the absence of melanophores. Maintaining melanophore clustering may not only be a way to establish separation, but might also regulate stripe width, as *obe* heterozygotes display wider melanophore stripes. The reduced melanophore clustering in this condition might result in a larger area occupied by more loosely clustered melanophores, whose clustering is just strong enough to prevent xanthophores from invading the melanophore domain.

At present, we can only speculate about which cell-biological aspect of clustering behaviour might exactly be controlled by *obe*. It is apparent from the ontogeny of wild-type pigment patterns (McClure, 1999; Parichy et al., 2000b) that melanophore clustering involves extensive translocation of melanophores and thus requires an ability of these cells to migrate, to recognise each other and to maintain contact. Notably, *obe* mutant melanophores neither cluster nor converge individually towards the horizontal myoseptum, nor do they disappear from within the xanthophore domain, suggesting that all of these processes are dependent on *obe*. This defect might either be caused by a reduced ability to recognise an attractive signal or by impaired motility. One obvious candidate for an attractive signal guiding melanophore aggregation is the kit ligand Steel (Copeland et al., 1990; Huang et al., 1990; Zsebo et al., 1990), which stimulates melanocyte migration in the mouse (Jordan and Jackson, 2000; Kunisada et al., 1998). Although a Steel homologue has not yet been identified in zebrafish, *sparse* adults that are mutant for the Steel-receptor still display melanophores aggregated in stripes (Parichy et al., 1999) (F.M. and C.N.-V., unpublished), thus making a role for Steel-kit as essential regulators of melanophore aggregation unlikely. Molecular analysis of the *obe* gene will certainly shed light on its exact function.

leo affects multiple aspects of cell behaviour

In contrast to the relatively restricted role of *obe*, *leo* affects homotypic interactions of both melanophores and xanthophores, as revealed by double mutant and mosaic analysis. The expansion of the xanthophore domain in *leo; nac* double mutants is unlikely to be caused by hyperproliferation of xanthophores, because in this condition the differentiation of xanthophores is also strongly delayed (data not shown). Instead, loss of *leo* function might weaken the cohesion between xanthophores and allow for insertion of newly differentiating xanthophores, thus expanding the domain. Interestingly, the disruptive effect of *leo* on melanophore clustering is highly similar to *obe*. Nevertheless, melanophores and xanthophores are still spatially separated in adults, raising the question of why melanophores and xanthophores do not mingle in *leo* in a similar fashion as in *obe*. This could be explained by the concomitant alteration of homotypic xanthophore behaviour, which might counteract the loss of melanophore clustering and re-establish separation, albeit in a spotted pattern. However, when confronted with *leo* mutant melanophores in mosaic experiments, xanthophores that are wild type for *leo* do not mingle. Therefore, the effect of *leo* on melanophore clustering must be quantitatively or qualitatively different from that of *obe*.

Additionally, *leo* function is required in both melanophores and xanthophores for formation of a straight boundary, as loss of *leo* function in one cell type can impose a *leo* pattern on the other. This notion is corroborated by the fact that if only one cell type is mutant for *leo*, the pattern formed by mosaics displays a weaker *leo* phenotype (undulating stripes and spots) than the *leo* mutant. These findings strongly suggest that *leo* also affects heterotypic interactions between both cell types. In light of this idea, it is important to note that the straight interface between melanophore and xanthophore stripes can be interpreted as minimised contact and that reducing *leo* function gradually increases contact between melanophores and xanthophores by rounding the boundary between them. The *leo* gene is thus a central component of the stripe-forming system in that it controls both homotypic and heterotypic interactions of melanophores and xanthophores. This pleiotropy might be achieved by *leo* regulating several downstream components that control subsets of cell interactions. Alternatively, *leo* could favour homotypic interaction among melanophores and xanthophores at the expense of heterotypic ones between the two. Loss of *leo* function could then result in an increased tendency of either cell type to contact the other and hence to increase the boundary length between the two.

A model of cell interactions during stripe formation

Based on our findings, a model of interactions the adult pigment pattern in zebrafish can be formulated (Fig. 7). The process is initiated by the differentiation of melanophores at about 3 weeks of development (Fig. 7A) and influenced by size and age of the fish. Depending on the presence of melanophores, xanthophores start differentiating in a relatively restricted domain straddling the horizontal myoseptum (Fig. 7B). Positioning of this initial xanthophore stripe is presumably directed by anatomical landmarks such as the horizontal myoseptum. Concomitantly, melanophores start aggregating in an *obe*- and *leo*-dependent fashion, and align with the xanthophore stripe. Melanophore aggregation prevents further differentiation of xanthophores within the melanophore domain, thus establishing spatial separation and also regulates melanophore stripe width. Additionally (Fig. 7C), xanthophores exert a repulsive effect on melanophores present within their domain. The straight interface between melanophores and xanthophores is subsequently formed by *leo*-dependent regulation of both homo- and heterotypic cell interactions (Fig. 7D).

Clearly, *obe* and *leo* will not be the only factors in this complex process, but the detection of adult pattern mutants is currently limited by the types of screens conducted so far. Novel screening approaches should therefore result in a more complete collection. Even though the exact cellular mechanisms guiding formation of the adult pigment pattern remain elusive, it will be interesting to see to what extent the

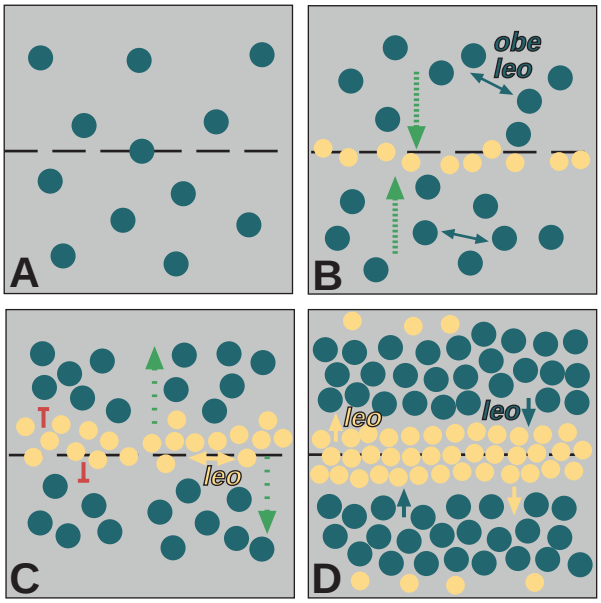

Fig. 7. A model of cell behaviours underlying formation of the adult pigment pattern in zebrafish. Melanophores are depicted in blue, xanthophores in yellow. Double headed arrows indicate homotypic interactions and single headed arrows heterotypic ones. The horizontal myoseptum is represented by broken lines. (A) At the onset of pattern formation, melanophores are scattered. (B) Melanophores start aggregating by *obe*- and *leo*-dependent homotypic interactions and also exert a positive effect on xanthophore differentiation (green arrow). (C) Xanthophores that also display *leo*-dependent homotypic interactions positively attract melanophores over a distance (green arrows), but repel them in short range (red bars). (D) The boundary between melanophores and xanthophores is shaped by *leo*-dependent heterotypic interactions.

findings presented here are applicable to other species (e.g. Parichy and Johnson, 2001), especially as stripes, either longitudinal or vertical, are a central feature of the pigment patterns of many animals.

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