Melanophores in the stripes of adult zebrafish do not have the nature to gather, but disperse when they have the space to move

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Summary

Animal skin pattern is one of the good model systems used to study the mechanism of pattern formation. Molecular genetic studies with zebrafish have shown that pigment cells play a major role in the mechanism of stripe formation. Among the variety of cellular events that may be involved in the mechanism, aggregation of melanophores has been suggested as an important factor for pattern formation. However, only a few experimental studies detected the migration ability of melanophores in vivo. Here, we tried to determine whether melanophores really have the ability to aggregate in the skin of zebrafish. Melanophores in the adult stripes are packed densely and they rarely move. However, when the neighboring pigment cells are killed, they move and regenerate the stripe pattern, suggesting that melanophores retain the migration ability. To analyze the migration, we ablated a part of the melanophores by laser to give free space to the remaining cells; we then traced the migration. Contrary to our expectation, we found that melanophores repulsed one another and dispersed from the aggregated condition in the absence of xanthophores. Apparent aggregation may be forced by the stronger repulsive effect against the xanthophores, which excludes melanophores from the yellow stripe region.

Introduction

Many vertebrates have a variety of color patterns on their skin. How these beautiful patterns benefit the natural selection of animals is one of the major questions of evolutionary biology. On the other hand, these patterns are also of interest from the perspective of spatial pattern formation in animal development. Animal skin patterns are generally not similar at all to the shape of the tissues under the skin. In the cases of some tropical fish, pigment patterns in the skin regenerate when they are perturbed by the growth of the fish or artificial manipulation of the pigment cells (Kondo and Asai, 1995; Yamaguchi et al., 2007), suggesting that these patterns form autonomously in the skin without any prepattern. As most of the known patterning mechanisms identified in early development are dependent on the 'prepattern', the discovery of a mechanism of skin pattern formation may lead to finding a new principle that controls the late-stage morphogenesis where the positional information derived from the egg becomes less important.

Among the model organisms used in molecular genetic studies, only zebrafish has a very clear pigment pattern in its skin and fins. Recent studies of skin patterning are thus mainly focused on zebrafish. The stripes of zebrafish are composed of three types of pigment cells (melanophores, xanthophores, and iridophores) localized in the hypodermis of the skin. The black stripe mainly consists of melanophores and thin iridophores. The yellow stripe consists of xanthophores and thick iridophores (Hirata et al., 2003, 2005).

Screening of artificially induced mutants or the pet shop lines has identified many kinds of mutant lines

having different pigment patterns, and the responsible genes have been cloned. These mutant genes are classified into two categories: those with a defect in the development of pigment cells and those with normal development of pigment cells at the embryonic stage but a disrupted pattern in adulthood. Molecular genetic studies of the mutants in the first category have shown that *nacre* (*mitf*; Lister et al., 1999), *sparse* (*kit*; Parichy et al., 1999), and *rose* (*ednrb1*; Parichy et al., 2000a) are required for the development of melanophores, and that *panther* (*fms*; Parichy et al., 2000b) is required for the development of xanthophores. Interestingly, when either type of pigment cell 'mis-develops', other pigment cells fail to localize to their normal positions. This fact suggests that the interaction between pigment cells plays a critical role in the generation of the skin pigmentation pattern (Maderspacher and Nüsslein-Volhard, 2003). Mutants of *leopard* (Kirschbaum, 1975) and *jaguar/obelix* (Haffter et al., 1996) belong to the second category. In these mutants, both types of pigment cells develop normally, but the arrangement of the cells is changed from stripes to spots (*leopard*) or wider stripes (*jaguar/obelix*), suggesting that these genes are required specifically for pattern formation. Recently, positional cloning of these mutants revealed that the responsible genes code a gap junction (Connexin41.8; Watanabe et al., 2006), and an inwardly rectifying potassium channel 7.1 (Kir7.1; Iwashita et al., 2006), respectively. However, little is known about their functions in the interactions between the pigment cells.

Parichy and Turner (2003a) utilized the temperature specific allele of the *panther* gene and killed both melanophores and xanthophores temporally. They have observed that the pigment cells re-developed to form the stripe pattern in the caudal fin. Recently, Yamaguchi et al. (2007) also found a similar regeneration phenomenon in the body trunk using laser ablation of the pigment cells. Interestingly, in both cases, the regenerated patterns are not always in the original direction, whereas the spacing between the lines is identical to the original. These facts suggest that the stripe pattern is not fixed, but that the pigment cells in the adult fish retain the ability to generate the stripe pattern. Therefore, by investigating the behavior of the pigment cells in the adult fish, it should be possible to identify the cellular mechanisms involved in pattern formation.

From the observation of melanophore behavior in the wild-type and mutant lines, it has been suggested that the migration of melanophores may play an important role in pattern formation (McClure, 1999; Kelsh, 2004). In larval pigment pattern formation, Svetic et al. (2007) have shown *Sdf1a*, which is an attractant for melanophore, is necessary for normal pattern formation. At the early stage of adult pattern formation, melanophores are distributed almost randomly in the body trunk. Then dorsal melanophores tend to move ventrally, and ventral melanophores tend to move dorsally, to form the initial two black stripes. Once the melanophores are integrated in the black stripes, migration of the cell becomes rare. Parichy and Turner reported that this movement is impaired in *fms* (which has dispersed melanophores all over the trunk) and *puma* (which has mottled pattern), suggesting that the defect of such directed migration caused their abnormal patterns (Parichy et al., 2000b; Parichy and Turner, 2003b). Maderspacher and Nüsslein-Volhard (2003) also pointed the importance of the migration from their chimera experiments and proposed a hypothetical mechanism that includes the autonomous aggregation activity of melanophores. However, these suggestions are mainly from the simple observation of the resulting pattern in normal and chimera fish. To determine the migration activity of the pigment cells, a more extensive elucidation is required.

In this research, we analyzed the migration of melanophores to determine whether they have the ability to aggregate. As Yamaguchi et al. (2007) have shown in their laser experiment, pigment cells in the adult stripe retain the ability to generate the pigment pattern. If they still retain the migration ability, it should be possible to assess the migration of the melanophore by eliminating the surrounding cells to give space to move to. Using laser ablation, we performed a series of experiments to determine if the melanophore can move and, if they can, to which direction. The result was contrary to our expectation and previous suggestions. We found that melanophores in the adult skin repulsed one another and dispersed from the aggregated condition.

Results

Migration of melanophore during adult pigment pattern formation

First, we observed melanophore migration during the early formation of adult stripe by tracing the position of individual cells (Figure 1). In zebrafish, the transition from larval pigment pattern (Milos and Dingle, 1978a,b; Milos et al., 1983) to adult pigment pattern began about 2 week post-fertilization. At the onset [14–21 day post-fertilization (dpf)], only melanophores were distributed almost randomly over the flank. One week later (21–28 dpf), xanthophores developed around the horizontal myoseptum to form the first yellow stripe that contains the isolated melanophores. At the dorsal and ventral regions of the yellow stripe, xanthophores did not appear, but melanophores continuously developed, resulting in two black stripes composed of only melanophores. One-fourth (14 of 54) of the melanophores remained in the yellow stripe and died soon after. However, three-fourths (40 of 54) of them survived and migrated dorsally or ventrally to join the black stripes (Figure 1). Some of the melanophores migrated along the distance of a half stripe width (Figure 1 cell numbers 1, 2, 3, 7 and 11), implying that there is some long-range attracting effect that gathers melanophores.

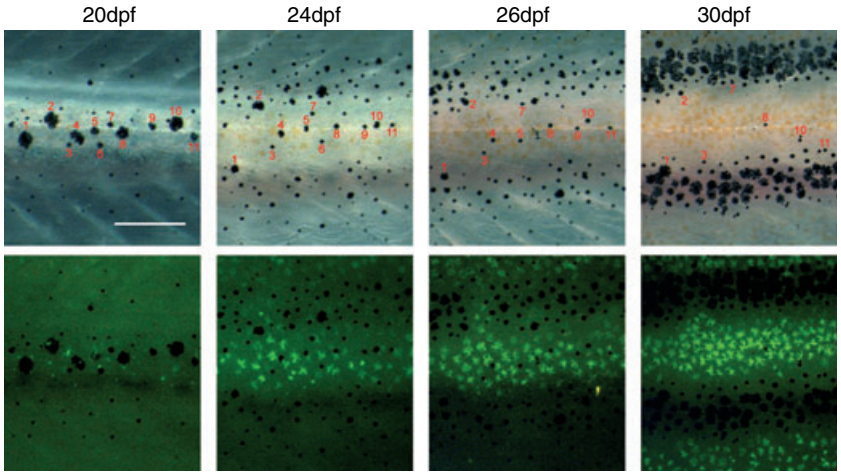


Figure 1. Movement of the melanophores during the normal stripe formation. Time course of the stripe development in the body trunk of wild type zebrafish was recorded under the normal lighting (upper panels) and UV dark field lighting (lower panels). Melanophores existed in the 20 dpf fish were labeled by the number (1∼11) and traced in the afterwards images. Xanthophores were visible by auto-fluorescence. The daily images and the movie to show the migration trait are in the supplementary data (Figure S1 and Movie S1). To compensate the growth of the fish, each image was rescaled before the stacking. See the Method section and Figure S5 for the procedure of rescaling. (20 dpf) melanophores were differentiated almost the all over the flank. Xanthophores are developing in the prospective yellow stripe region. (24–30 dpf) Melanophores (numbered) in the yellow stripe region migrated to join the black stripe (40/54) or disappeared (14/54).

Isolated melanophore does not migrate toward the cluster of melanophores

To test whether the isolated melanophore really has the tendency to migrate to the cluster of the same cell type, we ablated both melanophores and xanthophores to set up a situation where the isolated cell was in the vicinity (about half the distance of a stripe width) to the cluster of melanophores (Figure 2). If the cluster of melanophores has some attracting effect, the isolated melanophore should move toward the cluster. Contrary to what has been observed in normal stripe development (Figure 1), the isolated cell moved little and did not approach the cluster (Figure 2). Instead, some of the melanophores in the cluster migrated toward the vacant space where the pigment cells were ablated. This result suggested that the melanophores do not have the ability to gather, but that they actively disperse when they have a space to move to.

Melanophores tend to disperse from a crowded condition

To confirm that the movement of the melanophores observed in Figure 2 is not by the attracting effect from the isolated cells, we performed a similar experiment without the isolated cell (Figure 3). We made a vacant space at the horizontal and vertical regions of the black stripe to see whether this movement is related to the overall directionality of the fish body or not. As shown in Figure 3, the melanophores always dispersed into the vacant space regardless of the directionality in the fish body. This result showed that the melanophores simply have the tendency to increase the distance from the neighboring melanophores.

Melanophores migrate more actively when they are in crowded condition

In the results shown in Figures 2 and 3, the isolated melanophore moved little, whereas the melanophores in the cluster moved actively. From this observation, we expected that the migration activity of melanophores is enhanced when they are in a crowded condition. To confirm this hypothesis, we compared the movement of melanophores set in high and in low cell densities (Figure 4A–D, O). Five melanophores were selected in the middle of a black stripe, and the pigment cells surrounding the selected cells were ablated by laser to make a space where they can move. Then we traced the migration of all cells for 7 day and measured the total length of the migration path (Figure 4O). In Figures 4A, C, the selected cells are pointed in the white circles. The path of each cell is highlighted in Figures 4B, D. As shown in Figures 4B, O, the high-density cells moved actively to be apart from the other cells. On the other hand, the movement of the low-density cells was less active and did not have any directionality at all (Figure 4D, O).

Using the same method, we also analyzed the melanophore migration in two mutant lines, *leopard* (*leo*; Figure 4M) and *jaguar/obelix* (*jag/obe*; Figure 4N). The *leopard* fish have spotted patterns. The *jaguar/obelix* fish have wider stripes in which the melanophore density is less than that of the wild type. Close to the anal fin of the *jaguar/obelix* fish, there is a region in which the melanophores and xanthophores are distributed in a mixed manner. The experimental results with the *leopard* fish were identical to those with the wild type, suggesting that melanophore migration is not affected by

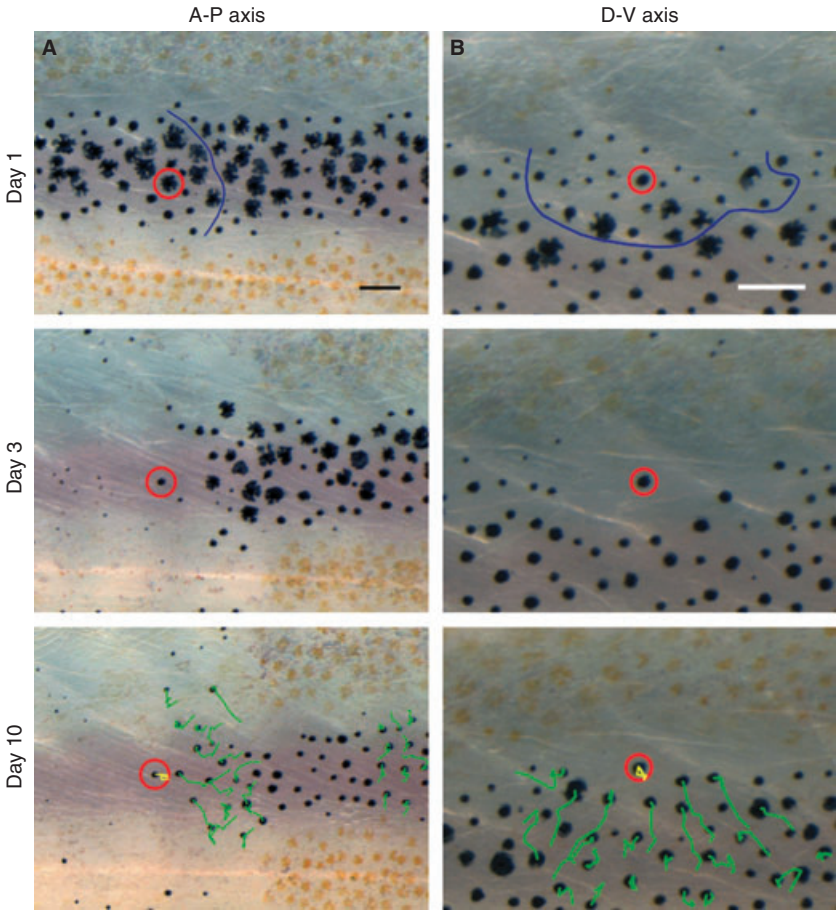


Figure 2. In vivo migration of the isolated melanophore. We ablated all pigment cells that were left of the blue line (A) or above the blue line (B), except the circled cell and made a situation where the isolated cell (red circle) was in the vicinity of the cluster of melanophores (day 3). The yellow and green lines show the migration path of the isolated cell and the cells in the cluster, respectively. Daily images are in Figure S2. Each image was rescaled to compensate the fish growth by the method described in Method section and Figure S5.

the *leopard* mutation (Figure 4E–H, O), whereas the migration activity of melanophores in the *jaguar/obelix* fish was apparently diminished in both high-density and low-density conditions (Figure 4I–L, O), suggesting that the migration ability of melanophores are restricted by the mutation.

Xanthophores may exclude melanophores from the yellow stripe region

As shown above, melanophores do not have ability to gather but have the tendency to go away from nearby melanophores when they have a space to move into. This conclusion apparently contradicts the observation with respect to natural stripe formation (Figure 1) in which the isolated melanophores come to join the cluster of melanophores in the black stripes. This difference may be due to the existence of xanthophores. If the repulsive effect between xanthophore–melanophore is stronger than that between melanophore–melanophore, the exclusion of melanophores may cause to move the isolated melanophore into the black stripe.

To test this hypothesis, we used the day 30 fish in which the initial melanophores remained in the xanthophore stripe and ablated the surrounding xanthophore to see whether the xanthophores affect melanophore migration. As we expected, the melanophores in the prospective yellow stripe region remained there when the surrounding xanthophores were lost, whereas the melanophores in the control region went into the black stripe (Figure 5). This result shows that xanthophores affect the migration of melanophores and may exclude the melanophores from the yellow stripe region.

Discussion

Melanophores in the adult stripe are distributed thickly and rarely move during normal growth. However, our study has shown that they cannot stay in a crowded condition when there is a room to migrate to and they move to widen the distance from the neighboring melanophores. Although this conclusion is contrary to that predicted in previous studies (Maderspacher and Nüsslein-Volhard, 2003), a similar nature of melanophore (melanocytes) has been reported in other experiments. Milos and Dingle (1978a,b) have shown that melanophores in zebrafish larvae have the natural tendency to avoid the close proximity of the neighboring cells. Melanoblasts or young melanophores isolated from the amphibian skin repel one another in culture condition (Twitty and Niu, 1948, 1954). This repulsive property of

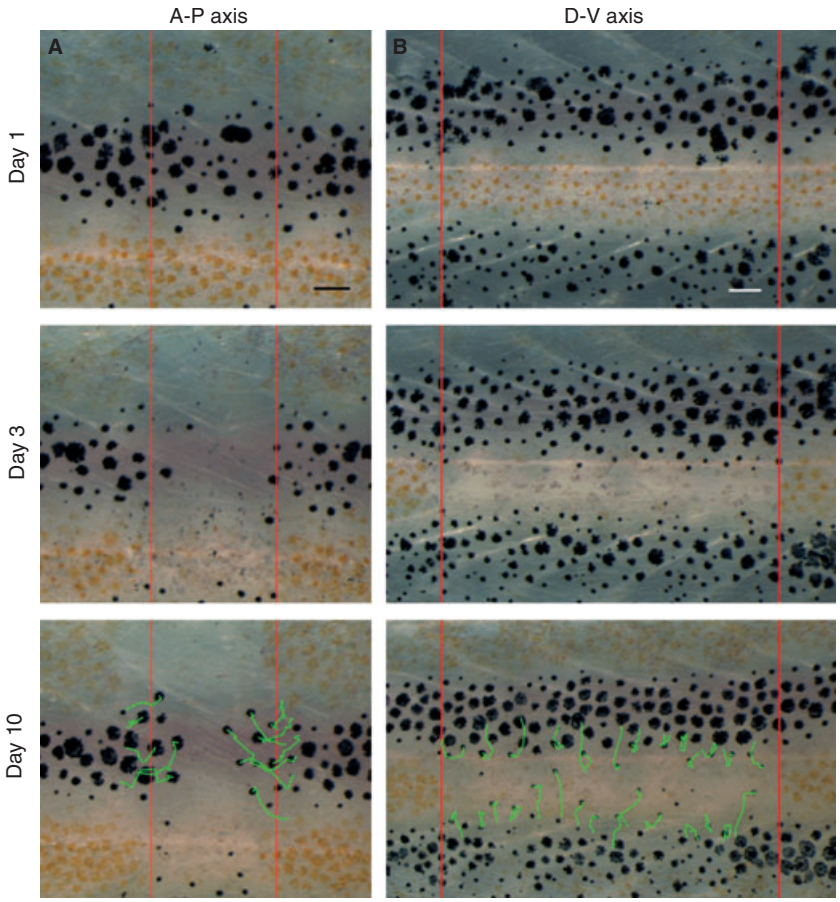


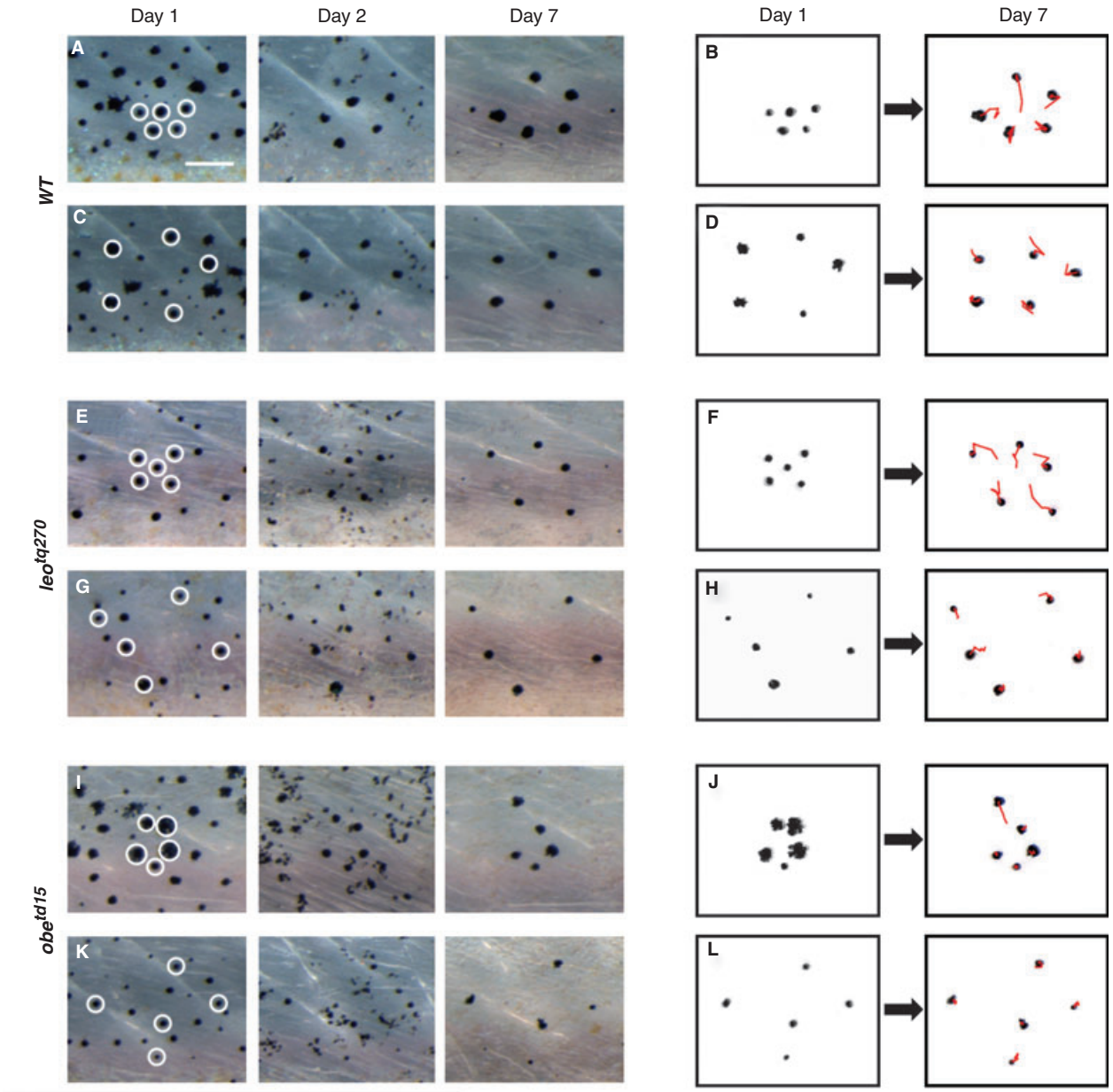
Figure 3. Migration of the melanophore located at the edge of the cluster. To give the space to move, we ablated a group of melanophores (A) or xanthophores (B) and recorded the migration of the melanophores located near the vacant space. Green lines show migration path. Daily images are in Figure S3. Rescaling of the images was performed as described in Method section and Figure S5.

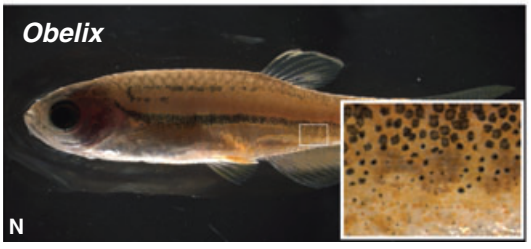
the pigment cells may be functionally reasonable because this effect can give rise to a uniform distribution in the two-dimensional spaces. Holbrook et al. (1989) have shown that the melanocytes in the epidermal sheets of fetuses appear to be regularly distributed without making contact with one another. The uniform distribution of melanocytes in the skin should be a benefit in terms of protection from UV light.

In spite of their nature to disperse, melanophores in the adult stripes stay unmoved in a crowded condition. This suggests that there is some external force that packs melanophores densely in the black stripe region. From observations that melanophores moved to the yellow stripe region when xanthophores were absent (Figure 3B), and that the isolated melanophore joined the black stripe when xanthophores were present (Figure 5), we surmise that the yellow stripe acts as the wall to pack the melanophores into the black stripes. There are at least two kinds of simple mechanisms that explain the wall effect of xanthophores.

The first is that melanophores were excluded by the stronger repulsion between melanophores and xanthophores. In the completed adult pattern, the gaps between black stripe and yellow stripe look larger than those between homogeneous cells (Figure 6A). We measured the distances between the center of cells and found that the distance between melanophore–xanthophore (82 μm) is much larger than that of melanophore–melanophore (50 μm) or xanthophore–xanthophore (36 μm; Figure 6B, C). Therefore, there should some gaps between the melanophores and xanthophores. In a detailed study using electron microscopy, Hirata et al. (2003) found that some remaining xanthophores exist in the adult black stripe. However, a layer of iridophores always exists between the melanophores and xanthophores to preclude direct interaction between them, suggesting that the direct contact of these two cell types is not preferable in the skin. A similar repulsive effect between melanophores and xanthophores has also been reported in the culture cell from larval axolotl. Lehman (1957) showed that both melanophores and xanthophores in cultures tended to avoid close contact with a different cell type. These observations also suggest the strong repulsion between melanophores and xanthophores.

The second possibility is that melanophores were sorted out via strong aggregation of the xanthophores. In *Oryzias latipes*, N-CAM and N-cadherin are specifically expressed in xanthophores but not in melanophores or iridophores (Fukuzawa and Obika, 1995).





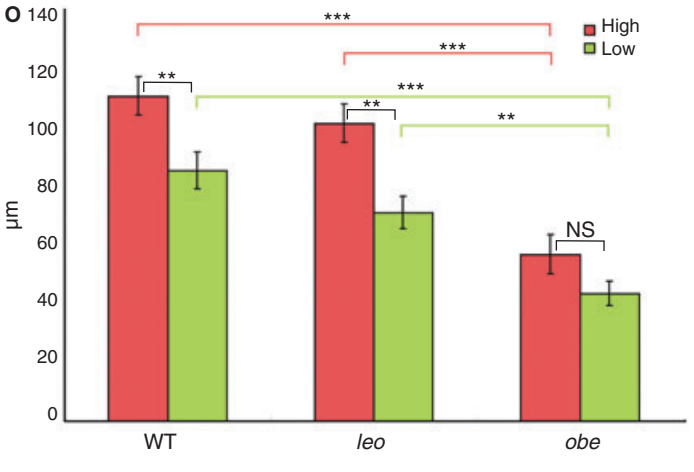


Figure 4. Repulsive effect among the melanophores in the cluster. (A–L) Migration of five melanophores those distributed in a crowded (A, E, I) or dispersed (C, G, K) condition were recorded after the ablation of the surrounding pigment cells. B, D, F, H, J and L show the original distribution and the migration path of each melanophore. For the details of the path recording, see the Method section and Figure S5. (M, N) Pigment Pattern of *leo* mutant and *jag/obe* mutant, respectively. (O) The total length of the migration path (green lines) were measured and represented in the graph. Values are the means of the 19 (for wild type) or 20 (for *leopard* and *jag/obe*) cells. Statistical analyses were performed by *t*-test (one-tailed) in WT and *leo*, and by Welch's t test (one-tailed) in *obe* (**P < 0.01 ***P < 0.001 NS: not significant). To assess the difference in migration activity among WT, *leo,* and *obe*, we performed multiple comparisons by Tukey's test (**P < 0.01 ***: P < 0.001). Daily images of this experiment are in Figure S4. For the rescaling of the images to compensate the fish growth, see the Method section and Figure S5. Scale bar: 100 μm. Error bars represent ±SEM.

Therefore, xanthophores can gather by these binding molecules and, as result, exclude the neutral melanophores. However, there are some circumstantial evidences that deny this possibility. Our detailed electron microscopy analysis (Hirata et al., 2003, 2005) did not detected overlaps or tight junctions between two adjacent xanthophores, suggesting that aggregation of xanthophores is unlikely. The wider gap between the black stripes and yellow stripes cannot be explained by this xanthophore aggregation effect either.

Temporarily, we cannot exclude one of these possibilities as we have not succeeded to test the cell–cell affinity in vitro. However, sorting derived from the difference in cell–cell affinity is common in animal development (Steinberg, 2007) and a highly possible mechanism may be involved in the segregation of different cells.

As migration of pigment cells is expected as one of the important factors in the development of pigment patterns, the resulting pattern may change when the migrating ability is affected by mutation. In this study, we found that the motility of melanophores is reduced in the *jaguar/obelix* mutant (Figure 4I–L, O), which has wider stripes. The gene responsible for the mutation was identified as *kir7.1* (Iwashita et al., 2006), and it was shown through mosaic analysis that gene activity is required in the melanophores (Maderspacher and Nüsslein-Volhard, 2003). Loss of the ion channel may change

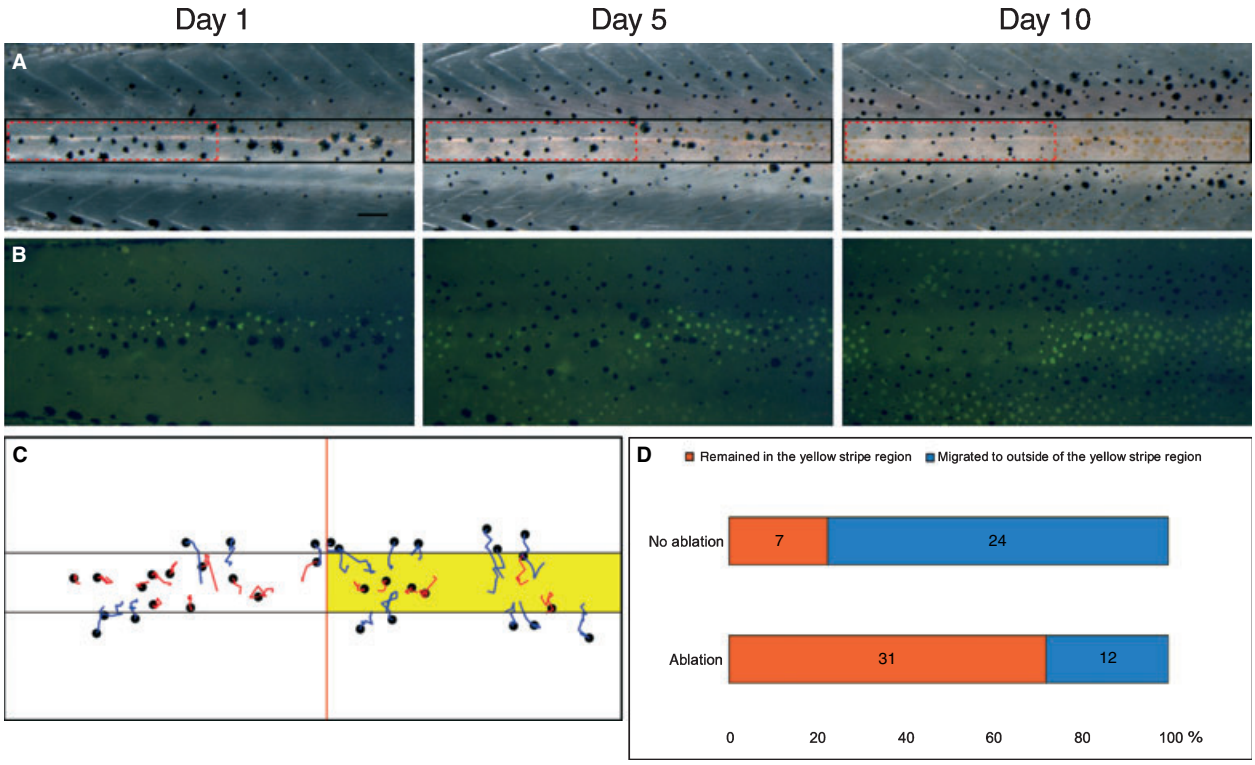


Figure 5. Migration of melanophores in the presence or absence of xanthophores. (A, B) Xanthophores in the red square were ablated, and the migration of melanophores with or without the xanthophore was recorded by bright field lighting (A) and UV lighting (B). (C) The migration paths of the melanophores recorded from the (A) and (B). In the region where xanthophores were removed many melanophores remained in the yellow stripe region. On the other hand, with the xanthophores, most of the melanophores migrated into the black stripes. (D) The proportion of cells migrated into the black stripe region and those remained in the yellow stripe region. Values are sum of the melanophores from five individuals. The statistical significance of differences between each group was assessed by chi-squared test (P < 0.0001). We rescaled the images: day 5 (95%), day 10 (84%). Scale bar: 100 μm.

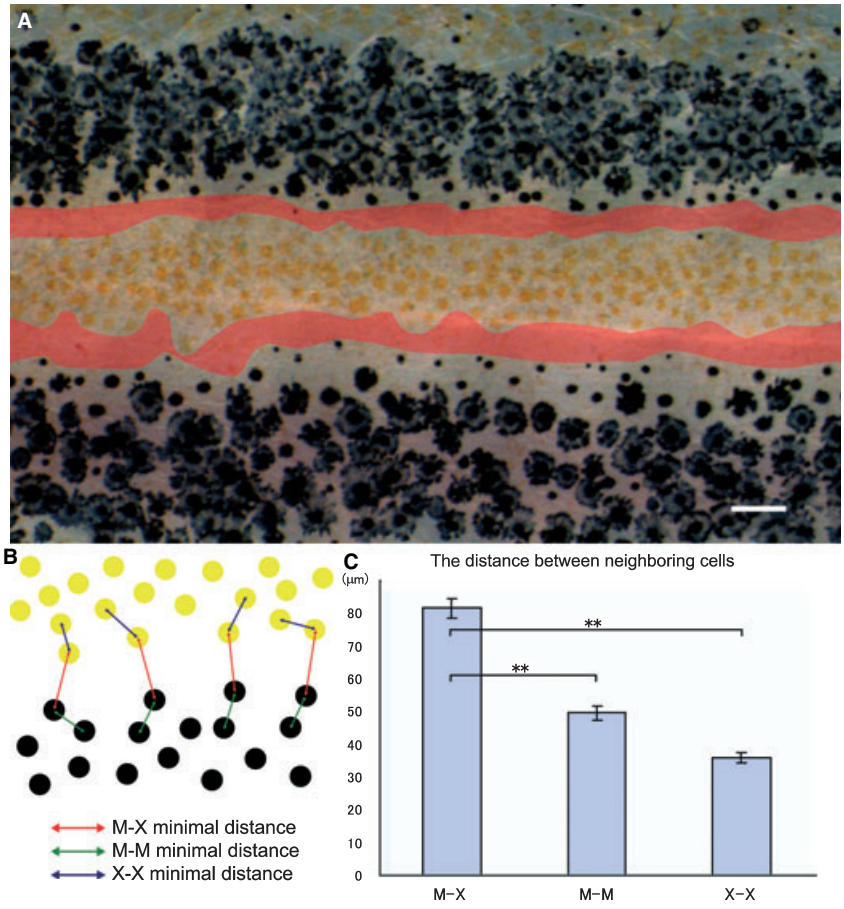


Figure 6. Distance between melanophore–melanophore, xanthophore–xanthophore and melanophore–xanthophore. (A) An image from body trunk of adult (3 months) zebrafish. There are gaps between the black and yellow stripes. (B) The procedure to measure the distances between the pigment cells. (C) The average distances between the center of neighboring cells (M: melanophore X: xanthophore). The distance between M–X is much larger than that of M–M or X–X, suggesting the existence of gap between the melanophores and xanthophores. To assess the difference of distances between homogeneous cells (M–M or X–X) and heterogeneous cells (M–X), we performed Welch's *t*-test (one-tailed; $^{**}P < 0.001$).

the cellular response to external signals that induce the migration of melanophores. In the body trunk of the *jaguar/obelix* fish, there are some regions where both melanophores and xanthophores exist in a mixed condition. The reduced motility of melanophores could be one of the causes of this incomplete segregation.

In this research, we have identified the migration ability of melanophores, which is related to the mechanism of stripe formation. However, this should only be a part of the mechanism and many other cellular behaviors remain to be elucidated. The most important is the behavior of the xanthophores. In contrast to melanophores, xanthophores mainly emerge at the prospective yellow stripe region and rarely move, even when there are vacant spaces (this study and Goodrich et al., 1954). This fact implies that xanthophore migration is not involved in the pattern-forming mechanism. If this is so, how can the xanthophores find the position to make the yellow stripe? This is a question that needs to be addressed. In *nacre* mutants that lack the melanophores, xanthophores cannot form the stripes, suggesting that melanophores are required for the correct positioning of the xanthophores. As xanthophores do not migrate, the influence from melanophores should be related to the differentiation and cell death of mature xanthophores. As well as cell migration, locally controlled cell differentiation and death are the major factors that affect stripe formation. Identification of such an interaction will uncover the mechanism that specifies the pigment pattern, and we expect that our experimental methods used in this study can be utilized for such purpose.

Methods

Zebrafish stock

In this research, the wild-type strain used was Tübingen. They were maintained under standard laboratory conditions (Westerfield, 2000). The age of the fish used in the experiments was about 40 day after hatching.

Laser ablation of pigment cells

Before ablation, the fish were anesthetized by using 0.01% MMS (ethyl-*m*-aminobenzoate methanesulfonate; Sigma, Munich, Germany) and were laid on a slide glass. During ablation, the fish were always maintained under moist condition. Ablation was performed by using a 337-nm pulse laser (wavelength was changed to 365 nm by dye BPBD) from the VSL-337ND-S Laser Systems (Spectra-Physics, Mountain View, CA, USA), which was focused to a ×20 objective on a microscope.

We could confirm which cells were ablated to death or not by some different methods. Before doing the ablation, fish were subjected to epinephrine to make the pigment cells compact. After ablation, we put the fish in the dark environment (~60min) to make

the living melanophore disperse the melanosome. The living melanophore became dendritic while the dead melanophores remained in the round shape. More conveniently, we could detect some signs of cell damage when doing the ablation. At the instant of cell ablation, the ablated cell slightly shrunk. Moreover, the damaged point in the cell was visible for approximately 10 s after the ablation. Ablated cell showing these two signs died without exception. As the multiple image recording frequently kills the fish, we did the melanosome dispersion test only when we were not sure whether the targeted cells were dead or not.

Time-lapse recording of cell migration

Migrations of melanophores were analyzed by image series that were recorded every day by CCD camera (LeicaDFC300FX; Leica, Wetzlar, Germany) mounted on a stereoscopic microscope (Leica MZ16FA; Leica) and software Leica Application Suite Version2.4.0 R1; Leica. To better visualize distinct melanophores, we treated fish with two to three drops of 40 μM epinephrine, causing movement of melanosomes to the cell centers prior to viewing. The images were transferred to Adobe Photoshop for analysis.

As the fish grows during the experiment, rescaling of each image is required to determine the migration path of each pigment cell. We took the horizontal myoseptum and non-ablated melanophores those located out of the ablated region as the reference points, which were used for rescaling and positioning of each image. [The explicit method is explained in the Figure S5. This method was also described in other papers, (Parichy et al., 2000b; Maderspacher and Nüsslein-Volhard, 2003; Parichy and Turner, 2003b; Quigley et al., 2005)]. To make sure the accuracy of this method, we applied the method to the normal growing zebrafish, and found all the pigment cells looked unmoved compared to the movement observed in the ablation experiments. Therefore, this method should be reliable enough to determine the migration path recorded in this report.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Daily images of the experiment shown in Figure 1.

Fig. S2 Daily images of the experiment shown in Figure 2.

Fig. S3 Daily images of the experiment shown in Figure 3.

Fig. S4 Daily images of the experiment shown in Figure 4.

Fig. S5 The method for scaling the images.

Movie. S1 Normal pigment pattern formation (20-30 dpf).

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