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CORRECTION

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There was an error published in *Development* **141**, 318-324.

The version of Fig. S1 originally published was incorrect (it was a duplication of Fig. S2). The correct Fig. S1 now appears in the supplementary data.

We apologise to the authors and readers for this mistake.

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RESEARCH ARTICLE

Involvement of Delta/Notch signaling in zebrafish adult pigment stripe patterning

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ABSTRACT

The skin pigment pattern of zebrafish is a good model system in which to study the mechanism of biological pattern formation. Although it is known that interactions between melanophores and xanthophores play a key role in the formation of adult pigment stripes, molecular mechanisms for these interactions remain largely unknown. Here, we show that Delta/Notch signaling contributes to these interactions. Ablation of xanthophores in yellow stripes induced the death of melanophores in black stripes, suggesting that melanophores require a survival signal from distant xanthophores. We found that *deltaC* and *notch1a* were expressed by xanthophores and melanophores, respectively. Moreover, inhibition of Delta/Notch signaling killed melanophores, whereas activation of Delta/Notch signaling ectopically in melanophores rescued the survival of these cells, both in the context of pharmacological inhibition of Delta/Notch signaling and after ablation of xanthophores. Finally, we showed by *in vivo* imaging of cell membranes that melanophores extend long projections towards xanthophores in the yellow stripes. These data suggest that Delta/Notch signaling is responsible for a survival signal provided by xanthophores to melanophores. As cellular projections can enable long-range interaction between membrane-bound ligands and their receptors, we propose that such projections, combined with direct cell-cell contacts, can substitute for the effect of a diffusible factor that would be expected by the conventional reaction-diffusion (Turing) model.

KEY WORDS: Delta/Notch signal, Pigment pattern, Turing mechanism

INTRODUCTION

How sequential and periodic structures form autonomously in organisms is a fundamental issue in developmental biology. One example of periodic structures is animal pigment patterns, which have long been studied both theoretically (Kondo and Miura, 2010; Meinhardt, 1982; Murray, 2002) and experimentally (Johnson et al., 1995; Parichy, 2007). The reaction-diffusion mechanism presented by A. M. Turing more than 60 years ago is the most common theoretical model for explaining various examples of biological pattern formation, including pigment patterning (Kondo and Miura, 2010; Meinhardt, 1982; Murray, 2002; Turing, 1952). This theory suggests that such patterns result from a stationary wave (Turing pattern or reaction-diffusion pattern) made by a combination of putative reactive and diffusible substances. Although the theory was much doubted initially, simulation studies have since demonstrated that this mechanism can lead to various pigment patterns commonly seen in nature (Kondo and Miura, 2010; Meinhardt, 1982; Murray, 2002) and also can predict dynamic pattern changes that occur during animal growth (Kondo and Asai, 1995; Yamaguchi et al., 2007). Molecular evidence supporting the hypothesis, however, has been sparse.

For testing the Turing model empirically, the zebrafish (*Danio rerio*) is especially useful. The stripe pattern of zebrafish is composed of three types of pigment cells: melanophores, xanthophores and iridophores (Johnson et al., 1995; Odenthal et al., 1996). Experiments using mutant zebrafish with altered pigment patterns have suggested that pigment patterns result from interactions between these three types of pigment cells (Maderspacher and Nüsslein-Volhard, 2003; Nakamasu et al., 2009; Parichy, 2003; Patterson and Parichy, 2013). Interactions specifically between melanophores and xanthophores were first demonstrated by genetic mosaic analyses using a xanthophore-deficient mutant for *colony stimulating factor 1* receptor (*csf1ra*, formerly *fms*); analyses of a temperature-sensitive allele, *csf1ra*^{utr4e174A}, further demonstrated that ablation of xanthophores resulted in the death of melanophores in the adult fish (Parichy and Turner, 2003). Subsequent experiments using laser ablation showed that at least two kinds of interactions between melanophores and xanthophores contribute to pattern formation (Nakamasu et al., 2009) (supplementary material Fig. S1C). In the first case, transient losses of a large number of xanthophores lead to the death of melanophores (supplementary material Fig. S1A), confirming the findings of Parichy and Turner. The functional distance of this interaction should be long because melanophores even in the middle of a black stripe, far from the nearest xanthophores, still died after xanthophore ablation. In the second case, when single melanophores or xanthophores are surrounded by cells of the other type, the isolated cell dies (supplementary material Fig. S1B). In this scenario, the two classes of pigment cells, melanophores and xanthophores, compete with each other for survival over short-range distances.

The network of short-range and long-range interactions deduced from these *in vivo* experiments is consistent with Turing's mathematical model (Nakamasu et al., 2009). Short-range repulsive interactions, in which xanthophore dendrites contact melanophores directly, have now been observed *in vitro* (Inaba et al., 2012). There is, however, little information concerning the long-range interaction. According to Turing's theory, long-range interactions are more important because they determine the width of stripes. Identifying

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the molecular bases of these interactions should, therefore, elucidate actual mechanisms underlying Turing pattern formation in a biological system. In the present article, we aimed to identify the molecular and cellular mechanism of long-range interaction.

RESULTS

Expression of Notch family members and their ligands in pigment cells

Previous *in vivo* experiments suggested that a survival signal is transmitted by xanthophores to melanophores (supplementary material Fig. S1A) (Nakamasu et al., 2009; Parichy and Turner, 2003). To identify candidate mediators of this interaction, we purified xanthophores and melanophores from fins and analyzed gene expression in these cells by microarray. Of the 15,500 genes surveyed, 482 were expressed at 2.5-fold higher levels (at least) in xanthophores than in melanophores. Among these, we selected seven genes encoding ligands or transmembrane proteins for further analysis. Using a reverse transcription-PCR (RT-PCR), we confirmed that six of these seven genes were expressed exclusively in xanthophores.

To screen these candidates functionally, we reasoned that an excess amount of any signal protein should alter the resulting patterns. Therefore, we expressed each of the six candidate genes ectopically in the melanophore lineage using the *mitfa* promoter (Dorsky et al., 2000; Inaba et al., 2012). In initial screening, we injected embryos with each transgene, and reared at least ten injected (mosaic) fish to adulthood (~3 months) for each candidate gene. For *deltaC*, two of 15 fish exhibited pattern alterations (supplementary material Fig. S2). For other candidate genes, no pattern differences were observed.

We performed *in situ* hybridization on late larval zebrafish and found that the *deltaC* is expressed in presumptive xanthophores, but not in melanophores (Fig. 1A). Confirming this idea, *deltaC* expression was not observed in the xanthophore-deficient, *csf1ra*^{j4e1} mutant (Parichy et al., 2000) (Fig. 1B). Next, by RT-PCR, we investigated whether genes encoding other Notch family members and their ligands are also expressed in pigment cells. Among the Notch ligands, we found that *deltaC* and *delta-like 4* were expressed robustly in xanthophores but not in melanophores. *deltaD* was expressed more weakly in xanthophores than *deltaC* or *delta-like 4* so we did not analyze it further. *deltaA* and *deltaB* were not expressed in either cell type (Fig. 1C). Among the Notch family genes, *notch1a* was expressed abundantly in melanophores. We also detected relatively low levels of *notch2* expression in both melanophores and xanthophores (Fig. 1D). These results suggested the possibility that a Delta/Notch signal (Kopan, 2010) is transmitted from xanthophores (*deltaC*, *delta-like 4* or both) to melanophores (*notch1a*, *notch2* or both) to promote melanophore survival in zebrafish skin.

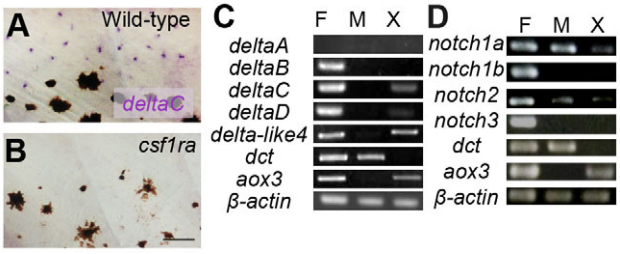

Fig. 1. Expression analysis of Notch receptors and their ligands. (A,B) *In situ* hybridization for *deltaC* in larval fish. Presumptive xanthophores in the inter stripe are stained in wild type (A) but these cells and *deltaC* staining is absent in the *csf1ra* mutant (B). (C,D) Expression by RT-PCR of major Notch ligands (C) and Notch receptors (D). F, fins; M, melanophores; X, xanthophores. *dct* and *aox3* are markers for melanophores and xanthophores, respectively. Scale bar: 50 µm.

Melanophore survival requires Notch signaling

To determine whether melanophore survival depends on the Delta/Notch signal, we incubated 1- to 1.5-month-old adult, wild-type fish with DAPT, which blocks cleavage of the Notch intracellular domain (NICD) fragment of the Notch receptor (Dovey et al., 2001; Geling et al., 2002). As shown in Fig. 2A,F, treatment with DAPT for 15 days decreased the number of melanophores to 60% of that before the treatment (xanthophore numbers were unaffected; supplementary material Fig. S3). In DAPT-treated fish, we observed melanin-containing debris, a hallmark of melanophore death (Lang et al., 2009; Parichy et al., 1999; Parichy and Turner, 2003) in the region from which melanophores had disappeared (Fig. 2D,E). Death of melanophores was not restricted to a specific position of the stripe, but seemed to occur randomly, with subsequent rearrangements of the remaining melanophores generating narrower black stripes (Fig. 2A). Upon removing DAPT, however, melanophore numbers recovered quickly (Fig. 2F; supplementary material Fig. S4), suggesting that melanophore precursors were unperturbed. These observations of melanophore death induced by DAPT are very similar to those following xanthophore-ablation experiments (Nakamasu et al., 2009; Parichy and Turner, 2003).

To confirm that DAPT-induced death of melanophores reflected an inhibition of Notch signaling, we generated stable transgenic lines expressing in the melanophore lineage either DeltaC [*Tg(mitfa:deltaC)*; derived from the fish shown in supplementary material Fig. S1] or the Notch1a intracellular domain [NICD1a; *Tg(mitfa:NICD1a)*]. We then tested for continued susceptibility to DAPT-induced melanophore death. We reasoned that ectopic expression of DeltaC should allow a Notch signal to be exchanged among melanophores, whereas expression of NICD1a should result in constitutive, autonomous activation of the pathway (Del Monte et al., 2007; Schroeter et al., 1998). In either case, we expected alterations in melanophore patterning. But, we also anticipated that if DAPT specifically blocks Notch signaling, then melanophores of *Tg(mitfa:deltaC)* fish would remain susceptible to DAPT-induced death despite Notch pathway activation, because such signals would still require NICD cleavage. By contrast, melanophores of *Tg(mitfa:NICD1a)* fish would be resistant to DAPT effects because NICD1a would be present in melanophores constitutively. As shown in Fig. 3, the stripes of melanophores in both transgenic lines were wider than those of the wild type, further supporting a role for Notch signaling in pattern formation. Moreover, we found that melanophores of *Tg(mitfa:NICD1a)* persisted even in the presence of DAPT, whereas melanophores of *Tg(mitfa:deltaC)* fish were lost during DAPT treatment (Fig. 2B,C,F), consistent with our predictions. These results confirm that melanophore death occurred specifically in response to Notch signaling inhibition.

Xanthophores are a source of the survival signal given to melanophores

Because xanthophores expressed Notch ligands and elimination of xanthophores caused the death of melanophores, xanthophores are the likely source of the Notch signal. To test this idea, we compared the effects of transient xanthophore loss between wild-type and *Tg(mitfa:NICD1a)* fish. Following laser ablation of xanthophores

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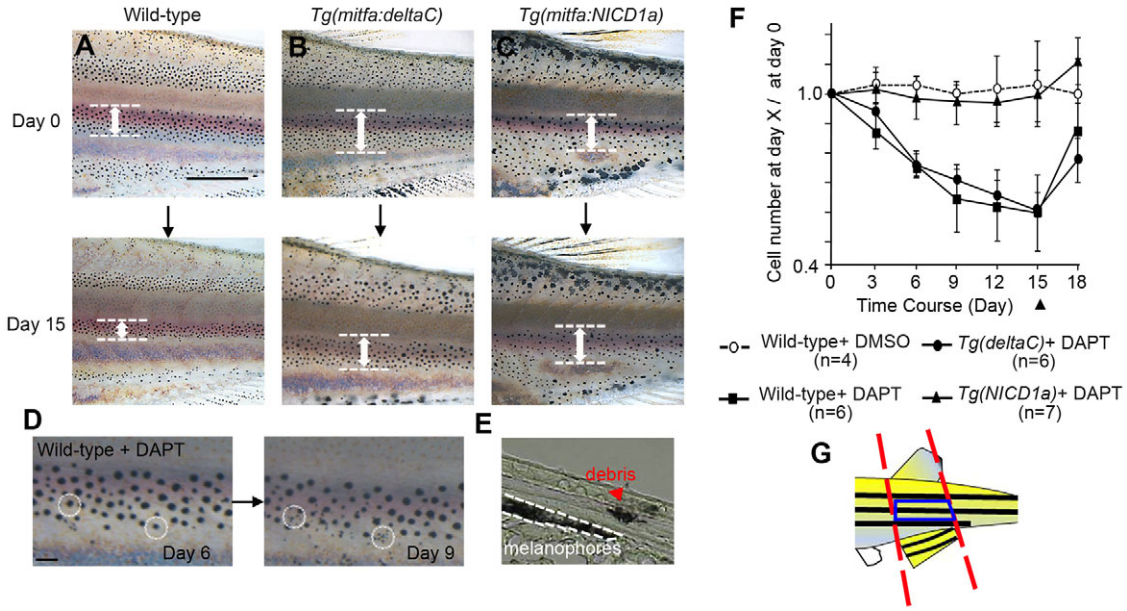

Fig. 2. Effect of Notch inhibitor on melanophores. (A-C) Altered melanophore distributions resulting from DAPT treatment over 15 days. Width of black stripes (white arrows) became narrowed in wild-type and *Tg(mitfa:deltaC)* fish, but remained unchanged in *Tg(mitfa:NICD1a)* fish. (D) Death of melanophores. Two cells (circled by dotted lines) present at day 6 (left) were lost by day 9 (right), though residual melanin-containing debris remains visible. (E) Cross-section through the skin of a DAPT-treated fish. A remaining melanophore is in the lowest layer of the skin (bounded by white dashed line). Melanin-containing debris from a dead cell was observed (red arrowhead) more superficially in the dermis. (F) Alteration of melanophore numbers (mean ± s.d.) during DAPT treatment, shown as the ratio of cells surviving at day X relative to the total number of cells at day 0. DAPT was applied at day 0 and removed at day 15 (black arrowhead). (G) Blue box shows the region in which melanophores were counted for analyses shown in F, defined as the middle stripe, delimited by anterior and posterior margins of the caudal and dorsal fins. Scale bars: 1 mm (A-C); 100 µm (D).

(Nakamasu et al., 2009), we found that 28% of melanophores died in wild-type fish (Fig. 4A), whereas no melanophores died in fish expressing NICD1a (Fig. 4B,C). Thus, Notch pathway activation autonomous to melanophores can substitute for xanthophores, consistent with the hypothesis that xanthophores promote melanophore survival through Delta/Notch signaling.

Melanophores extend long projections to touch xanthophores directly

Delta and Notch belong to families of membrane-bound proteins. Therefore, if a Delta/Notch survival signal is provided by xanthophores to melanophores, these cells must contact one another directly. As even melanophores distant from xanthophores died in response to xanthophore ablation, we anticipated that melanophores might contact xanthophores by cellular processes not normally apparent (Fig. 5A). To visualize even thin cellular projections, we used yohimbine (an alpha 2-adrenoceptor antagonist), which induces the peripheral dispersion of melanin-containing melanosomes. As shown in Fig. 5B, yohimbine caused the movement of melanosomes into long projections that extend towards xanthophores. These projections were visible at stripe edges but could not be discerned clearly within the interior of black stripes, because the region is totally filled with black pigment. To visualize the projection from single cells, we used a melanin pigment-lacking mutant, *brass*^{b2}, and injected them with a transgene in which *mitfa* drives expression of membrane-targeted enhanced green fluorescent protein, EGFP-CAAX (supplementary material Fig. S5D) (Gomes et al., 2003; Wright and Philips, 2006). In F0 fish expressing this transgene mosaically, processes of single melanophores even within the stripes are visible. For example, Fig. 5D shows three melanophores in a black stripe each of which extend long projections towards xanthophores. In Fig. 5E, a cluster of melanophores express EGFP-CAAX and most projections are oriented dorsoventrally, towards xanthophores above and below the stripe. Most of the melanophores extend projections and the direction is vertical to the stripe. Fig. 5F shows the cell projections of the melanophores at the region where the stripe terminates (illustrated in Fig. 5C). Even in wild-type fish, such a stripe gap occurs in ~5% of fish). In this case, the projection appeared to extend radially towards the xanthophore region. In *csf1ra* mutant fish, which lack xanthophores, long projections were rare (supplementary material Fig. S5A). We also checked the projection of xanthophores in wild-type zebrafish. Although there were some xanthophores that extended long projections towards melanophores, they were rare. Many xanthophores did not have long

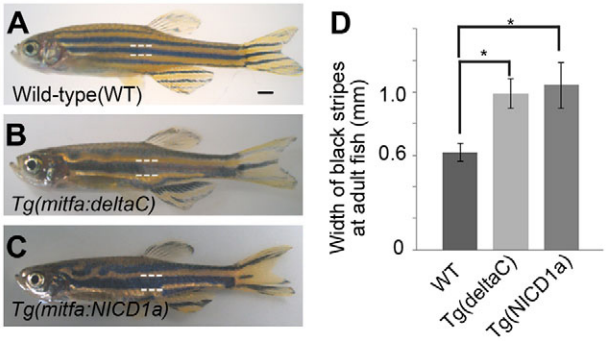

Fig. 3. Ectopic expression of DeltaC and Notch-NICD alter stripes. (A-C) Expression of DeltaC (B) and the Notch1a intracellular domain (C) in melanophores resulted in fewer, wider stripes than those in wild type (A). (D) Width of black stripes in transgenic fish [wild type, n=12; *Tg(mitfa:deltaC)*, n=12; *Tg(mitfa:NICD1a)*, n=13]. *P<0.0001 (Student's t-test). Error bars represent s.d. Scale bar: 1 mm.

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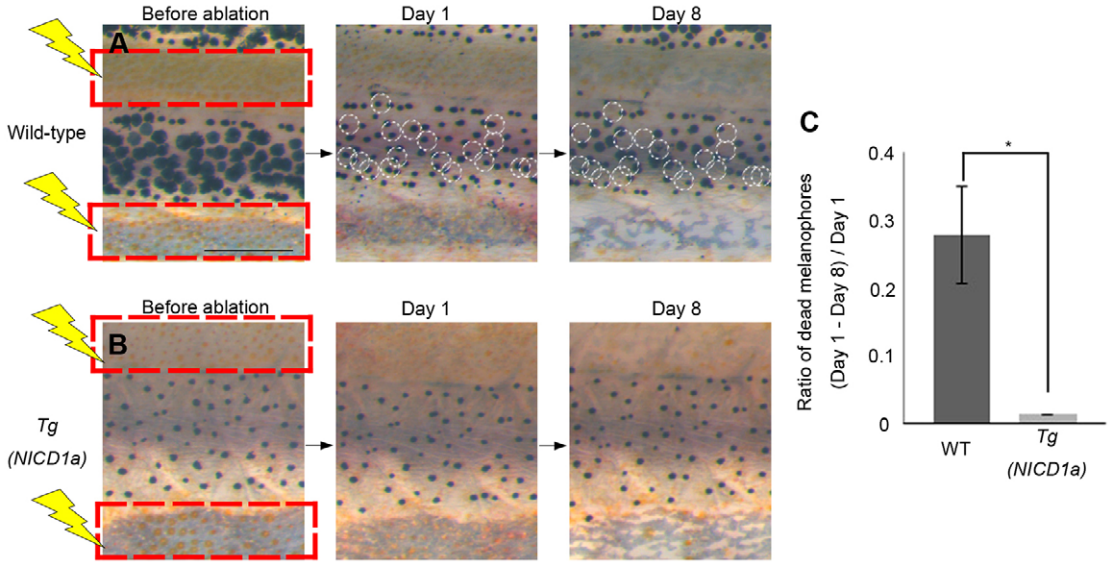

Fig. 4. Melanophore survival is independent of xanthophores in *Tg(mitfa:NICD1a)* transgenic fish. (A,B) Effects on melanophores after ablation of xanthophores is shown for wild type (A) and *Tg(mitfa:NICD1a)* (B). Red squares indicate the areas in which xanthophores were ablated. White circles indicate melanophores present on day 1 but lost by day 8. (C) Proportion of melanophores that died in wild-type and *Tg(mitfa:NICD1a)* fish (n=5, respectively). **P*<0.0001 (Student's *t*-test). Error bars represent s.d. Scale bar: 500 µm.

projections (supplementary material Fig. S5B,C). These projections are often greater than three times the length of the cell body and nearly half of the width of stripes. We further confirmed by electron microscopy that the melanophore projections directly touched xanthophores passing through the layer of iridophores (supplementary material Fig. S6).

DISCUSSION

In this article, we showed that xanthophores express Delta ligands (*deltaC* and *delta-like 4*), a family of membrane-bound ligands, and that melanophores express Notch receptors (*notch1a* and *notch2*). Next, we showed that Delta/Notch signaling promotes melanophore survival, and that this signal originates with xanthophores. Finally,

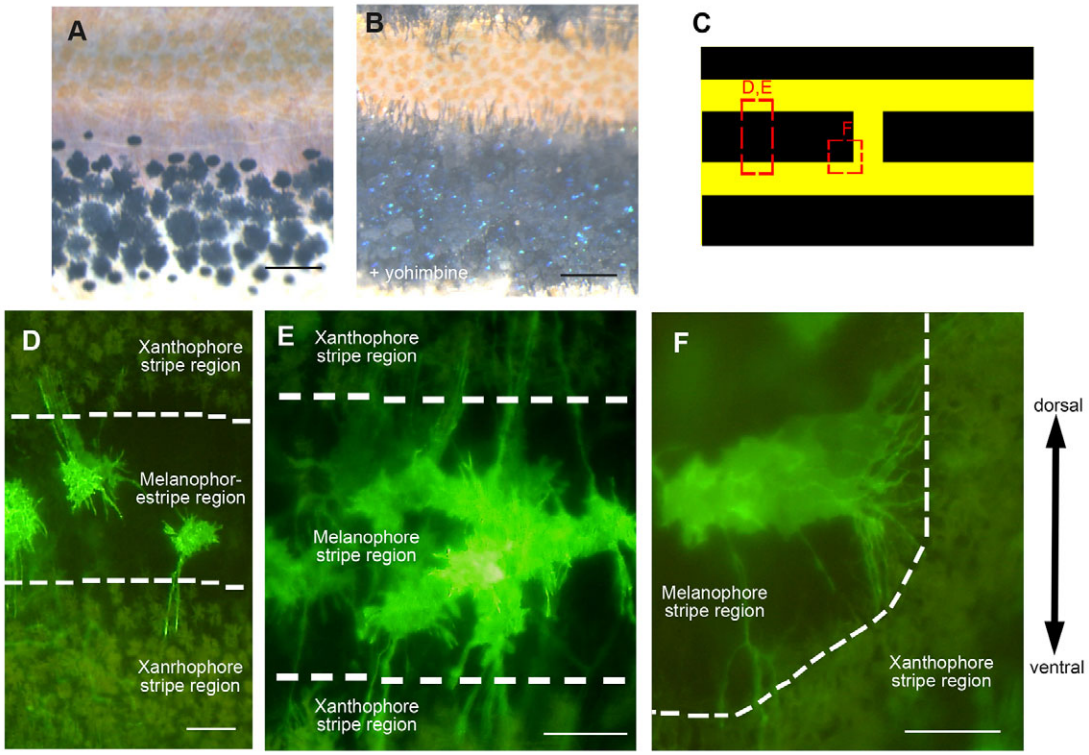

Fig. 5. Melanophores extend long projections. (A,B) Black stripe region of wild-type fish in the normal condition (A) and after addition of yohimbine (B), which reveals projections extending towards xanthophores. (C) Regions imaged in D-F. (D-F) Fluorescent images of melanophore membranes revealed by *mitfa*: EGFP-CAAX expression in *brass*^{b2} mutants. (D) Three melanophores in the black stripe extend long projections towards xanthophores. (E) A cluster of melanophores, each of which exhibits long projections. (F) Melanophores adjacent to a break in the stripe extend long projections radially. Scale bars: 100 µm.

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we found that melanophores in the black stripes extend long projections towards xanthophores that may enable pigment cells to transfer these long-range signals, despite association of Notch and Delta with the cell membrane. Although we have not confirmed that the Delta/Notch signal is transduced specifically at the sites of such projections, we propose this as a working model for future exploration.

There are at present few data pertaining to cellular mechanisms that control melanophore survival. Although our observations suggest the involvement of Delta/Notch signaling, this could be just part of a more complex mechanism; indeed, melanoblast survival depends on signaling through the *kit receptor a*, *endothelin receptor B1a* and the immunoglobulin superfamily member *igsf11* (Budi et al., 2011; Cooper et al., 2009; Eom et al., 2012). It is possible that Delta/Notch synergizes with such pathways to promote melanophore survival.

The maximum length of melanophore projections is about half the width of the adult stripe. This suggests an interesting hypothesis to explain how the width of a stripe could be formed and stably maintained (supplementary material Fig. S7). When the growth of a fish makes the stripes wider than twice the projection length, projections of melanophores in the middle of a stripe would no longer reach xanthophores and the melanophores would die. This effect could divide the transiently wider stripe, returning it to its original width. Indeed, bifurcation of stripes has been observed in a tropical fish (*Pomacanthus imperator*) (Kondo and Asai, 1995), in zebrafish expressing overexpressing a mutant form of the gap junction protein *connexin 41.8* (Watanabe and Kondo, 2012) and in very old and big wild-type zebrafish (Asai et al., 1999). This hypothesis also explains the altered pigment patterns of *deltaC* and NICD1a transgenic fish. In these fish, melanophores should be less sensitive to losing a survival signal from xanthophores, thereby allowing for persistence at sites more distant from xanthophores in the yellow stripes, and resulting in the wider melanophore stripes we observed. Although our hypothesis is simple and useful to explain patterning, it remains to be confirmed by further experiments. Particularly important will be to test if Delta/Notch signaling occurs specifically at the tips of melanophore projections and the extent to which such interactions occur not only in the adult, but also during larval stages when the pattern is first developing.

It has been proposed that a reaction-diffusion mechanism underlies pigment pattern formation because such a mechanism can predict pigment pattern changes in growing fish (Kondo and Asai, 1995; Yamaguchi et al., 2007). However, experimental investigations have indicated that the true mechanisms are considerably different. We showed previously that short-range signals are transmitted to melanophores not by diffusion of a diffusible molecule, but rather by relatively short processes of xanthophores contacting melanophores directly (Inaba et al., 2012). Here, we presented the possibility that a long-range signal is likewise transmitted by direct contact between melanophores and distant xanthophores. There is, so far, no diffusible molecule demonstrated to be involved in pigment pattern formation. If diffusion of chemicals really does not occur during pattern formation, how could a simulation based on a reaction-diffusion mechanism generate and predict the pigment pattern correctly? We guess this is because signaling via long projections can mimic the effect of diffusion in the conventional Turing (reaction-diffusion) model. Gierer and Meinhardt noted that essential conditions for autonomous pattern formation are the combination of short-range activation and long-range inhibition (Gierer and Meinhardt, 1972). ‘Diffusion’ itself is dispensable and can be replaced by any other means that can transmit signals over the appropriate spatial distance. Therefore, even using a mechanism very different from diffusion, a pattern can emerge by a similar dynamic.

Recent studies have shown that signal transduction via cellular projections is important in several developmental contexts (e.g. Sanders et al., 2013), and include intercellular communication via the Delta/Notch pathway (e.g. Cohen et al., 2010; Fitzgerald and Greenwald, 1995; Renaud and Simpson, 2001). We guess that the projection mechanism may participate in other morphogenetic events to which the involvement of a reaction-diffusion mechanism is suspected.

MATERIALS AND METHODS

Fish stocks

Studies used wild-type and *brass*^{b2} mutant (Link et al., 2004) zebrafish supplied from the Zebrafish Stock Center at the Max Planck Institute (Tübingen, Germany) as well as *csf1ra*^{j4e1} (Parichy et al., 2000). Breeding and the standard methods for fish manipulation were as described (Westerfield, 2000).

Microarray analysis

We cut caudal and anal fins from 1000 adult fish, and dissociated the pigment cells. Fins were incubated in a solution containing 2.5 mg/ml trypsin, 1.2 mg/ml bovine serum albumin and 1 mM EDTA for 12 hours at 4°C. After incubation, fins were washed five times with PBS for 5 minutes with shaking at 1000 rpm at 28°C, and incubated with 0.1% collagenase I for 60 minutes at 28°C. Then, cells were collected by the method described by Inaba et al. (Inaba et al., 2012).

Total RNA was isolated from the purified pigment cells using an RNeasy Mini Kit (Qiagen). We compared gene expression between melanophores and xanthophores with the Gene-Chip Zebrafish Genome Array (Affymetrix). All microarray data are available in the NCBI Gene Expression Omnibus under accession number GSE42129.

In situ hybridization

In situ hybridization of late larval [~3.5 weeks post fertilization; ~10 mm standardized standard length (Parichy et al., 2009)] fish was performed according to Larson et al. (Larson et al., 2010). Riboprobe was made from full-length 2635-bp cDNA for *deltaC*, and hydrolyzed to ~500 nt before use. Hybridizations were performed at 68°C on wild-type and *csf1ra*^{j4e1} mutant larvae.

RT-PCR

Assessment of mRNA expression by pigment cells was carried out as described previously (Eom et al., 2012) using primers that spanned the intron. *dct* and *aox3* were used as markers of melanophores and xanthophores, respectively (Kelsh et al., 2000; Parichy et al., 2000). PCR primers and the parameters used in this experiment are shown in supplementary material Table S1.

DAPT treatment

A stock solution (10 mM) of the γ-secretase inhibiter *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine-*t*-butyl ester (DAPT; Wako) was prepared in DMSO. The stock solution was dissolved in 50 ml fish water and adjusted to a final concentration of 10 μM. Fish water containing DAPT was changed every 3 days. After 15 days of incubation in DAPT, fish were moved to normal water. We counted the number of melanophores in the region of the middle trunk shown in supplementary material Fig. S2G and calculated the ratio of surviving melanophores (cell number at Day X/cell number at Day 0).

Generation of transgenic fish

To express *deltaC* and NICD1a ectopically, we used the Tol2 transposon-based transgenesis system (Urasaki et al., 2006). Total RNA was isolated from fins using an RNeasy kit (Qiagen) and reverse transcription-PCR was performed using the Super Script III First-Strand Synthesis System (Invitrogen). Primer sets used to generate *deltaC* and NICD1a cDNA clone

are as follows: *deltaC*-F, 5′-CCCAAGCTTATGGCTCGTGTTTTATTA-3′ and *deltaC*-R, 5′-CGCCATGGCTATACCTCAGTAGCAAA-3′ (this primer set amplifies whole *deltaC* open reading frame); NICD1a-F, 5′-ATGCAAGCTTATGGTGGTCGTCTCCAGGAAGAGG-3′ and NICD1a-R, 5′-GCATAGTCGACCTACTTGAAGGCTTCTGGAA-3′ [this primer set amplifies the intracellular domain (1745V~2437K) of *notch1a* gene (Del Monte et al., 2007)].

Vector construction and insert position of the transgene are as described (Inaba et al., 2012). We used a 1.3-kb region upstream of the melanophore-specification gene *microphtalmia-a* (*mitfa*) to drive expression in melanophores (Lister et al., 1999). In our previous reports (Inaba et al., 2012; Watanabe and Kondo, 2012), we showed that identical promoter could rescue the melanophore defect. In Fig. 5 of this article, we observed the strong EGFP signal only in melanophores, further confirming the specificity of the promoter in adult fish. The same construct was used for both F0 screening and for further analysis with F1 fish.

The CAAX motif (Casey and Seabra, 1996) was fused with EGFP (Gomes et al., 2003; Wright and Philips, 2006) and introduced into pTol2-*mitfa* plasmid as illustrated in supplementary material Fig. S5D.

Plasmid DNA plasmid (30 ng/μl) and Tol2 mRNA (25 ng/μl) were co-injected to wild-type (*deltaC*/*NICD1a*) or *brass*^{b2}, *csf1ra*^{j4e1} and wild-type (EGFP-CAAX) fertilized eggs at the one-cell stage. To confirm transgene insertions, we performed genomic PCR using specific primer sets.

Laser ablation of melanophores

Details of the laser ablation method have been described (Nakamasu et al., 2009). Before ablation, fish were anesthetized with 0.01% methyl-methanesulfonate (Westerfield, 1995) and mounted on a dish with water. Ablation was performed by 440-nm multiple light-pulse lasers from the MicroPoint pulse laser system (Photonic Instruments).

Yohimbine treatment

To disperse melanin-containing melanosomes into peripheral dendrites of melanophores, fish were placed in a black tank containing 100 μM yohimbine (Sigma-Aldrich) for 30 minutes before analysis.

Electron microscopy

Zebrafish (adult; ~25 mm standard length) were anesthetized with MS-222 and cut into small pieces. The specimens were then fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer for 60 minutes at room temperature. The fish pieces were then treated with 2% osmium in 0.1 M cacodylate buffer containing 4% sucrose for 60 minutes at room temperature. Then, samples were dehydrated through a graded ethanol series and embedded in Quetol-812 resin. Ultrathin sections were obtained with an Ultramicrotome (Reichert-Jung Ultracut E), counterstained with uranyl acetate and lead citrate, and examined on a transmission electron microscope (JEM-1200 EX, JEOL, Japan) with an acceleration voltage of 80 or 90 kV. Micrographs were recorded with a CCD camera (Morada) using the iTEM software (Olympus Soft Imaging Solutions).

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Competing interests

The authors declare no competing financial interests.

Author contributions

H.H. conceived and planned the study with the help of S.K. and M.W.; H.H. performed the experiments and wrote the paper with the help of S.K.; M.W. performed the microarray analysis. H.E.L. performed the *in situ* hybridization with the help of D.M.P. T.N. and T.H. performed the electron microscopy analysis.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.099804/-/DC1

References

Asai, R., Taguchi, E., Kume, Y., Saito, M. and Kondo, S. (1999). Zebrafish leopard gene as a component of the putative reaction-diffusion system. *Mech. Dev.* **89**, 87-92.

Budi, E. H., Patterson, L. B. and Parichy, D. M. (2011). Post-embryonic nerve-associated precursors to adult pigment cells: genetic requirements and dynamics of morphogenesis and differentiation. *PLoS Genet.* **7**, e1002044.

Casey, P. J. and Seabra, M. C. (1996). Protein prenyltransferases. *J. Biol. Chem.* **271**, 5289-5292.

Cohen, M., Georgiou, M., Stevenson, N. L., Miodownik, M. and Baum, B. (2010). Dynamic filopodia transmit intermittent Delta-Notch signaling to drive pattern refinement during lateral inhibition. *Dev. Cell* **19**, 78-89.

Cooper, C. D., Linbo, T. H. and Raible, D. W. (2009). Kit and foxd3 genetically interact to regulate melanophore survival in zebrafish. *Dev. Dyn.* **238**, 875-886.

Del Monte, G., Grego-Bessa, J., González-Rajal, A., Bolós, V. and De La Pompa, J. L. (2007). Monitoring Notch1 activity in development: evidence for a feedback regulatory loop. *Dev. Dyn.* **236**, 2594-2614.

Dorsky, R. I., Raible, D. W. and Moon, R. T. (2000). Direct regulation of nacre, a zebrafish MITF homolog required for pigment cell formation, by the Wnt pathway. *Genes Dev.* **14**, 158-162.

Dovey, H. F., John, V., Anderson, J. P., Chen, L. Z., de Saint Andrieu, P., Fang, L. Y., Freedman, S. B., Folmer, B., Goldbach, E., Holsztynska, E. J. et al. (2001). Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *J. Neurochem.* **76**, 173-181.

Eom, D. S., Inoue, S., Patterson, L. B., Gordon, T. N., Slingwine, R., Kondo, S., Watanabe, M. and Parichy, D. M. (2012). Melanophore migration and survival during zebrafish adult pigment stripe development require the immunoglobulin superfamily adhesion molecule Igsf11. *PLoS Genet.* **8**, e1002899.

Fitzgerald, K. and Greenwald, I. (1995). Interchangeability of Caenorhabditis elegans DSL proteins and intrinsic signalling activity of their extracellular domains in vivo. *Development* **121**, 4275-4282.

Geling, A., Steiner, H., Willem, M., Bally-Cuif, L. and Haass, C. (2002). A gamma-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. *EMBO Rep.* **3**, 688-694.

Gierer, A. and Meinhardt, H. (1972). A theory of biological pattern formation. *Kybernetik* **12**, 30-39.

Gomes, A. Q., Ali, B. R., Ramalho, J. S., Godfrey, R. F., Barral, D. C., Hume, A. N. and Seabra, M. C. (2003). Membrane targeting of Rab GTPases is influenced by the prenylation motif. *Mol. Biol. Cell* **14**, 1882-1899.

Hirata, M., Nakamura, K., Kanemaru, T., Shibata, Y. and Kondo, S. (2003). Pigment cell organization in the hypodermis of zebrafish. *Dev. Dyn.* **227**, 497-503.

Inaba, M., Yamanaka, H. and Kondo, S. (2012). Pigment pattern formation by contact-dependent depolarization. *Science* **335**, 677.

Johnson, S. L., Africa, D., Walker, C. and Weston, J. A. (1995). Genetic control of adult pigment stripe development in zebrafish. *Dev. Biol.* **167**, 27-33.

Kelsh, R. N., Schmid, B. and Eisen, J. S. (2000). Genetic analysis of melanophore development in zebrafish embryos. *Dev. Biol.* **225**, 277-293.

Kondo, S. and Asai, R. (1995). A reaction-diffusion wave on the skin of the marine angelfish Pomacanthus. *Nature* **376**, 765-768.

Kondo, S. and Miura, T. (2010). Reaction-diffusion model as a framework for understanding biological pattern formation. *Science* **329**, 1616-1620.

Kopan, R. 2010. *Notch Signaling*. London: Academic Press.

Lang, M. R., Patterson, L. B., Gordon, T. N., Johnson, S. L. and Parichy, D. M. (2009). Basonuclin-2 requirements for zebrafish adult pigment pattern development and female fertility. *PLoS Genet.* **5**, e1000744.

Larson, T. A., Gordon, T. N., Lau, H. E. and Parichy, D. M. (2010). Defective adult oligodendrocyte and Schwann cell development, pigment pattern, and craniofacial morphology in puma mutant zebrafish having an alpha tubulin mutation. *Dev. Biol.* **346**, 296-309.

Link, B. A., Gray, M. P., Smith, R. S. and John, S. W. (2004). Intraocular pressure in zebrafish: comparison of inbred strains and identification of a reduced melanin mutant with raised IOP. *Invest. Ophthalmol. Vis. Sci.* **45**, 4415-4422.

Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L. and Raible, D. W. (1999). nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development* **126**, 3757-3767.

Maderspacher, F. and Nüsslein-Volhard, C. (2003). Formation of the adult pigment pattern in zebrafish requires leopard and obelix dependent cell interactions. *Development* **130**, 3447-3457.

Meinhardt, H. (1982). *Models of Biological Pattern Formation*. London: Academic Press,.

Murray, J. D. (2002). *Mathematical Biology*, 3rd edn. New York, NY: Springer.

Nakamasu, A., Takahashi, G., Kanbe, A. and Kondo, S. (2009). Interactions between zebrafish pigment cells responsible for the generation of Turing patterns. *Proc. Natl. Acad. Sci. USA* **106**, 8429-8434.

Odenthal, J., Rossnagel, K., Haffter, P., Kelsh, R. N., Vogelsang, E., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Hammerschmidt, M. et al. (1996). Mutations affecting xanthophore pigmentation in the zebrafish, Danio rerio. *Development* **123**, 391-398.

Parichy, D. M. (2003). Pigment patterns: fish in stripes and spots. *Curr. Biol.* **13**, R947-R950.

Parichy, D. M. (2007). Homology and the evolution of novelty during Danio adult pigment pattern development. *J. Exp. Zool.* **308B**, 578-590.
Parichy, D. M. and Turner, J. M. (2003). Temporal and cellular requirements for Fms signaling during zebrafish adult pigment pattern development. *Development* **130**, 817-833.
Parichy, D. M., Rawls, J. F., Pratt, S. J., Whitfield, T. T. and Johnson, S. L. (1999). Zebrafish sparse corresponds to an orthologue of c-kit and is required for the morphogenesis of a subpopulation of melanocytes, but is not essential for hematopoiesis or primordial germ cell development. *Development* **126**, 3425-3436.
Parichy, D. M., Ransom, D. G., Paw, B., Zon, L. I. and Johnson, S. L. (2000). An orthologue of the kit-related gene fms is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in the zebrafish, Danio rerio. *Development* **127**, 3031-3044.
Parichy, D. M., Elizondo, M. R., Mills, M. G., Gordon, T. N. and Engeszer, R. E. (2009). Normal table of postembryonic zebrafish development: staging by externally visible anatomy of the living fish. *Dev. Dyn.* **238**, 2975-3015.
Patterson, L. B. and Parichy, D. M. (2013). Interactions with iridophores and the tissue environment required for patterning melanophores and xanthophores during zebrafish adult pigment stripe formation. *PLoS Genet.* **9**, e1003561.
Renaud, O. and Simpson, P. (2001). scabrous modifies epithelial cell adhesion and extends the range of lateral signalling during development of the spaced bristle pattern in Drosophila. *Dev. Biol.* **240**, 361-376.
Sanders, T. A., Llagostera, E. and Barna, M. (2013). Specialized filopodia direct long-range transport of SHH during vertebrate tissue patterning. *Nature* **497**, 628-632.
Schroeter, E. H., Kisslinger, J. A. and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382-386.
Turing, A. M. (1952). The chemical basis of morphogenesis. *Philos. Trans. R. Soc. B* **237**, 37-72.
Urasaki, A., Morvan, G. and Kawakami, K. (2006). Functional dissection of the Tol2 transposable element identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. *Genetics* **174**, 639-649.
Watanabe, M. and Kondo, S. (2012). Changing clothes easily: connexin41.8 regulates skin pattern variation. *Pigment Cell Melanoma Res.* **25**, 326-330.
Westerfield, M. (1995). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish* (Danio Rerio) (ed. M. Westerfield). Eugene, OR: University of Oregon Press.
Westerfield, M. (2000). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish* (Danio Rerio) (ed. M. Westerfield). Eugene, OR: University of Oregon Press.
Wright, L. P. and Philips, M. R. (2006). Thematic review series: lipid posttranslational modifications. CAAX modification and membrane targeting of Ras. *J. Lipid Res.* **47**, 883-891.
Yamaguchi, M., Yoshimoto, E. and Kondo, S. (2007). Pattern regulation in the stripe of zebrafish suggests an underlying dynamic and autonomous mechanism. *Proc. Natl. Acad. Sci. USA* **104**, 4790-4793.

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CORRECTION

Involvement of Delta/Notch signaling in zebrafish adult pigment stripe patterning

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There was an error published in *Development* **141**, 318-324.

The version of Fig. S1 originally published was incorrect (it was a duplication of Fig. S2). The correct Fig. S1 now appears in the supplementary data.

We apologise to the authors and readers for this mistake.

Development

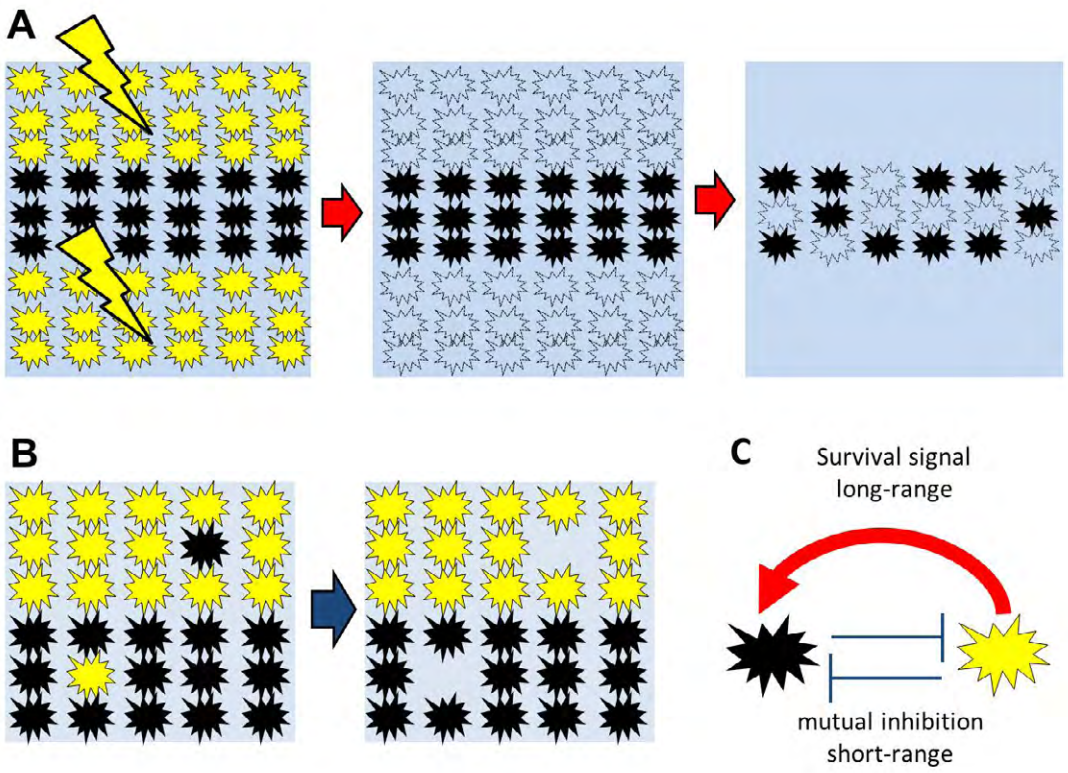

Fig. S1. Summary of our previous studies. (A) A transient loss of a large number of xanthophores leads to the death of melanophores. Melanophores adjacent to xanthophores died but so too did melanophores originally at a distance from xanthophores. **(B)** When a single melanophore or xanthophore is surrounded by the other cell type, it dies in a few days. **(C)** Interaction network suggested from the experiments

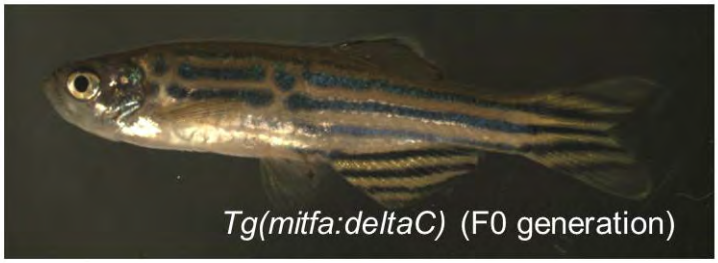

Fig. S2. Pattern change observed during primary screening of candidate genes. 2/15 F0 fish mosaic for the *mitfa:deltaC* transgene showed abnormal pigment patterns. No transgenic fish for other candidate gene exhibited such stripe abnormalities. Presence of the transgene was confirmed by genomic PCR.

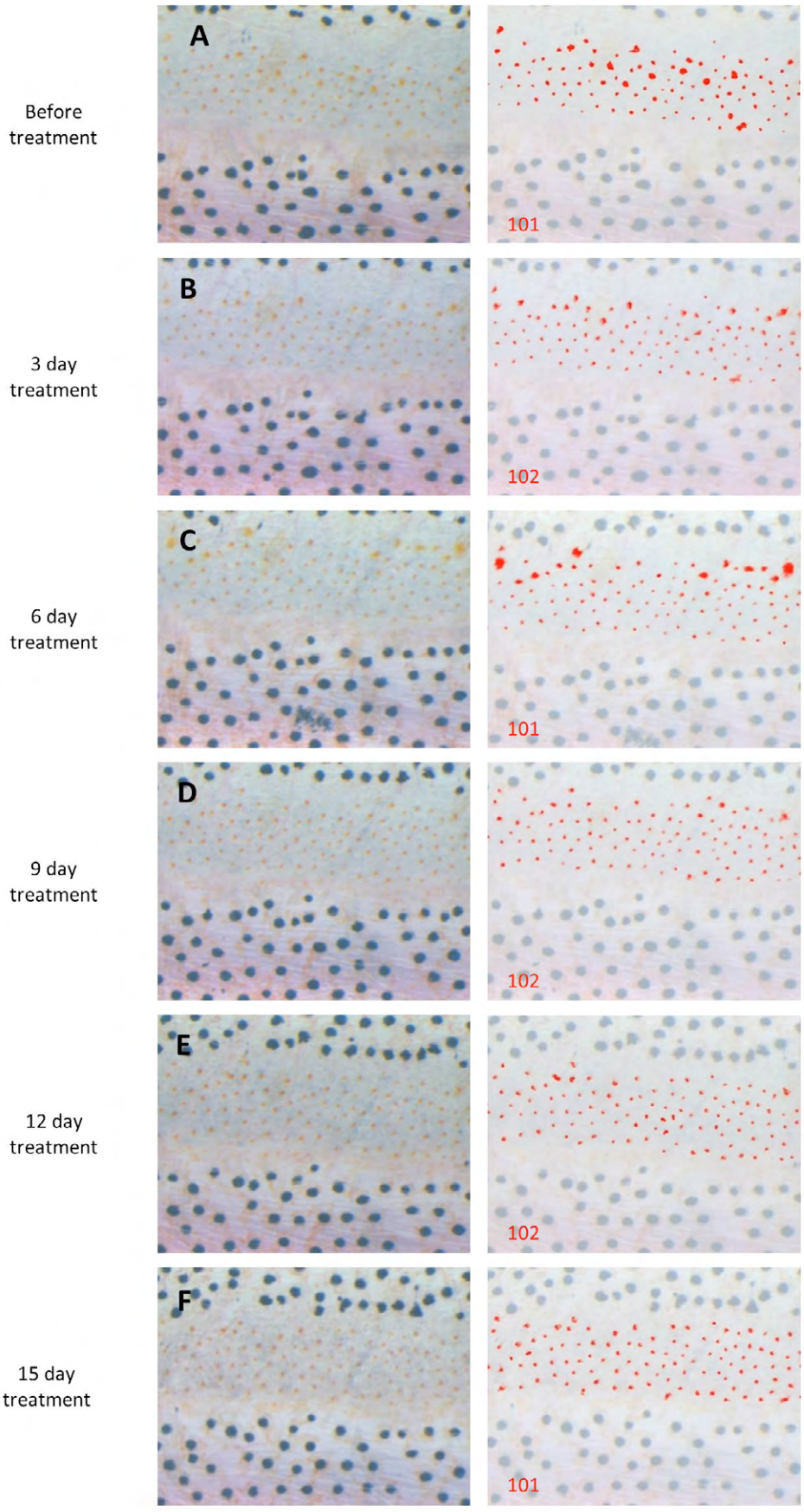

Fig. S3. Xanthophores were not affected by DAPT treatment. (A-F) Left panels are original images. In right panels, xanthophores we counted are marked by red overlay. The number of xanthophores is shown at the bottom-left of each panel. Scale bar: 500 μm.

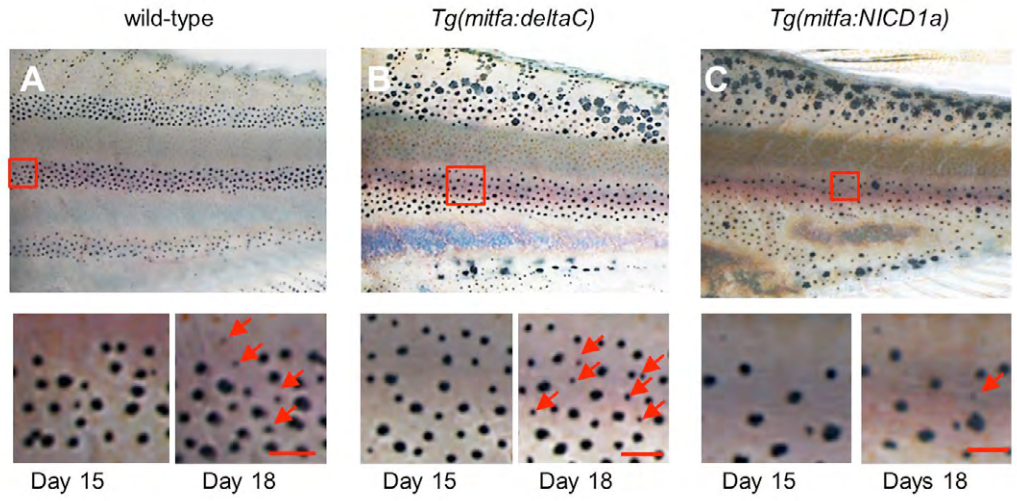

Fig. S4. Recovery of melanophores after the removal of DAPT. (A-C) After 15 day DAPT treatment, fish were moved to normal water. To assess the recovery, melanophore distributions were compared between day 15 and day 18. Red arrows show newly differentiated melanophores. In wild-type and *Tg(mitfa:deltaC)* transgenic fish, new melanophores developed rapidly, whereas no changes were observed for *Tg(mitfa:NICD1a*) transgenic fish. Scale bars: 100μm.

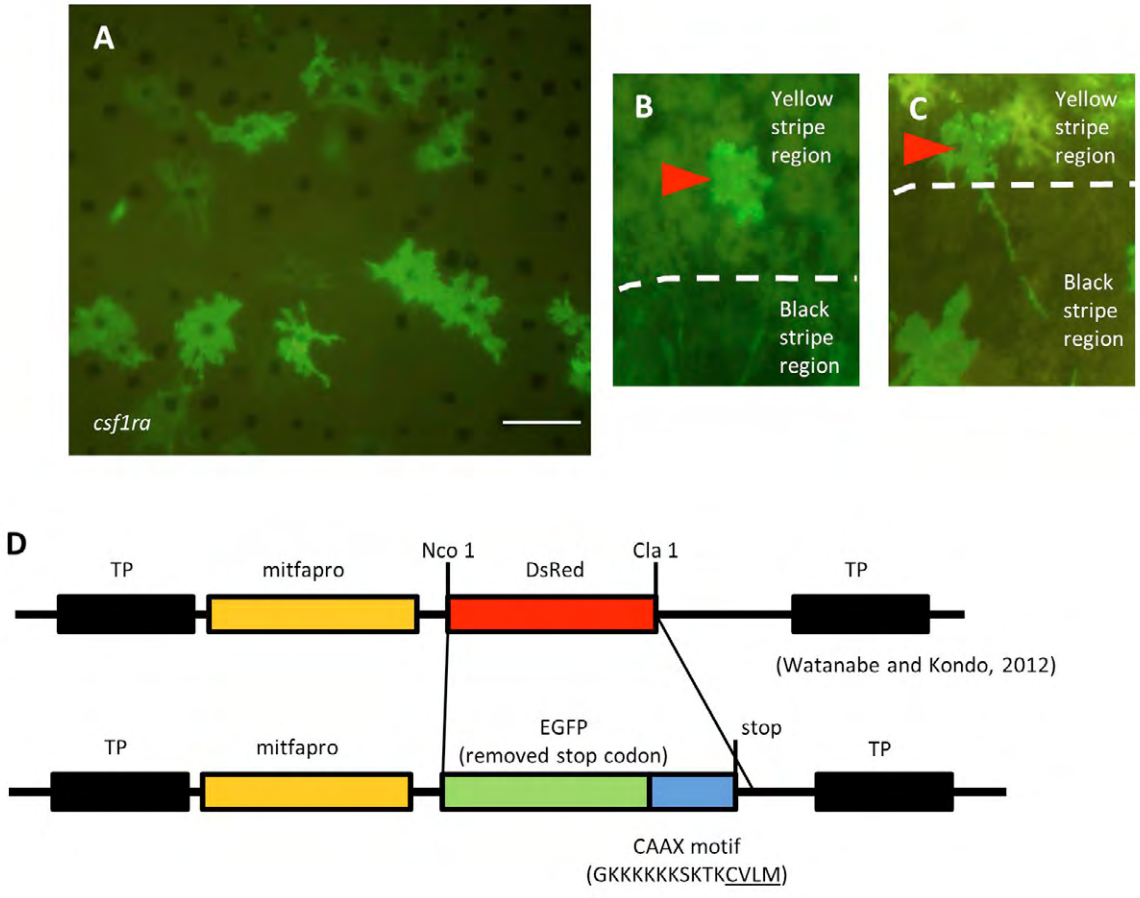

Fig. S5. Projections in melanophores in *csf1ra* mutant and xanthophores in wild-type. (**A**) Long projections of melanophores were rarely observed in csf1ra mutant, which lacks xanthophores. (**B, C**) As there is a leak expression of Mitfa promoter in F0 fish, xanthophore membrane is also visible with the same construct. Most of xanthophores do not have long projections in wild type fish(B). We rarely found xanthophores with long projection(C). (**D**) Design of plasmid for the EGFP-CAAX. Upper line indicated the plasmid which used in (Watanabe and Kondo, 2012). Lower line indicated the plasmid which used in this study.

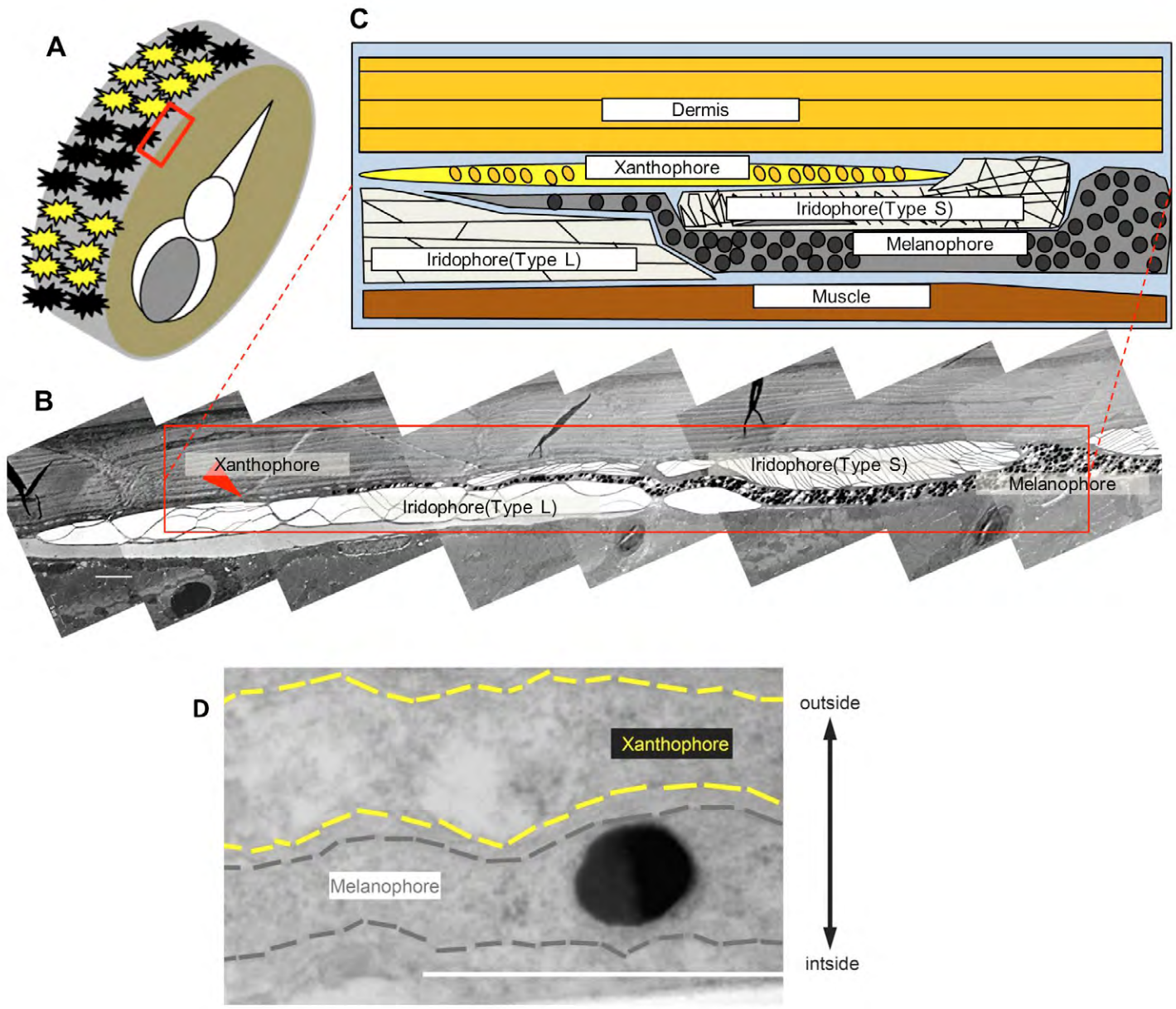

Fig. S6. Electron microscopy of zebrafish skin showing the direct contact between melanophores and xanthophores. (A) Region of the skin examined by transmission electron microscopy (red square). **(B)** Low magnification image showing different pigment cell classes. **(C)** Schematic rendering of boxed region in (B). **(D)** Magnified image of melanophore projection tip in other sample. In the previous report of electron microscopy analysis, we showed that melanophores and xanthophores are generally separated by the insertion of iridophores (Hirata et al., 2003). We found, however, that melanophore membrane (black dashed line) and xanthophore membrane (yellow dashed line) were close proximity to one another at the stripe boundary. Scale bar = 5μm in B, 1μm in D.

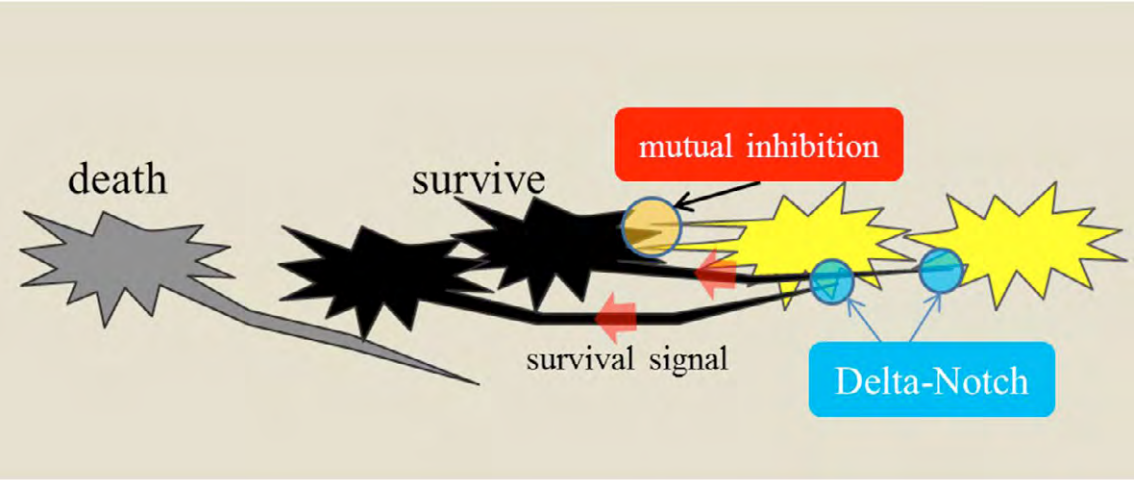

Fig. S7. Interactions between two kinds of pigment cells. Our studies have shown that there are two interactions, short-range and long-range, between melanophores and xanthophores. Short-range interactions result in mutual inhibition and depend on Kcnj13 (formerly Kir7.1). Long-range interactions transmit a survival signal, involving Delta-Notch, from xanthophores to melanophores. The effective range of this Delta-Notch signal may contribute to determining the width of black stripes.

Table S1. Primer sets and parameter for RT-PCR. All RT-PCR were performed 45cycles for Notch receptors, and 40 cycles for DSL family at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

Gene name		Primer set	Cycle number
dct	F	ATCAGCCCGCGTTCACGGTT	45 for Fig. 1C 40 for Fig.1D
	R	ACACCGAGGTGTCCAGCTCTCC	
aox3	F	AGGGCATTGGAGAACCCCCAGT	45 for Fig. 1C 40 for Fig.1D
	R	ACACGTTGATGGCCCACGGT	
notch1a	F	GTCTGCTGGAGTCGTGCGCC	40
	R	ACAGCAGCCGCCCAGTGAAG	
notch1b	F	ACCAGACAGACCGCACGGGT	40
	R	ATGCCCTCGACCGCCAGTCT	
notch2	F	CTGCACTGGGCTGCTGCTGT	40
	R	TCCAGGACGCATGCCGAGGA	
notch3	F	CAGGCCGTGAGACGCGCATT	40
	R	GCTGTGTTCACGCTTGCGGC	
deltaA	F	CGGCATCCACGTCGGCTTGT	45
	R	GCCTACAGGCCACCACTGCC	
deltaB	F	CTGCCGGCCGGGATTTGGAG	45
	R	GTCGGTGGGCATCGGCAGAC	
deltaC	F	GACCGGTGCAGCAGTGACCC	45
	R	TGTGCCCATGAAGCCTGCCG	
deltaD	F	AGCGACGGCGACAAAAACGGA	45
	R	TGTGGCGTTACACCTCGGTTGC	
delta-like4	F	AGTGTGACAGCAGCCCACGC	45
	R	CTGGCCAGTGAAGCCCGCTC	
β-actin	F	CGGTTTTGCTGGAGATGATG	45 for Fig. 1C 40 for Fig.1D
	R	CGTGCTCAATGGGGTATTTG	