Iridophores and their interactions with other chromatophores are required for stripe formation in zebrafish

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SUMMARY

Colour patterns of adult fish are produced by several types of pigment cells that distribute in the dermis during juvenile development. The zebrafish, Danio rerio, displays a striking pattern of dark stripes of melanophores interspersed by light stripes of xanthophores. Mutants lacking either cell type do not form proper stripes, indicating that interactions between these two chromatophore types are required for stripe formation. A third cell type, silvery iridophores, participates to render a shiny appearance to the pattern, but its role in stripe formation has been unclear. Mutations in rose (rose) or shady (shad) cause a lack or strong reduction of iridophores in adult fish; in addition, the melanophore number is drastically reduced and stripes are broken up into spots. We show that rose and shad are autonomously required in iridophores, as mutant melanophores form normal sized stripes when confronted with wild-type iridophores in chimeric animals. We describe stripe formation in mutants missing one or two of the three chromatophore types. None of the chromatophore types alone is able to create a pattern but residual stripe formation occurs with two cell types. Our analysis shows that iridophores promote and sustain melanophores. Furthermore, iridophores attract xanthophores, whereas xanthophores repel melanophores. We present a model for the interactions between the three chromatophore types underlying stripe formation. Stripe formation is initiated by iridophores appearing at the horizontal myoseptum, which serves as a morphological landmark for stripe orientation, but is subsequently a self-organising process.

KEY WORDS: Iridophores, Pigment pattern formation, shady, rose, Chimeras

INTRODUCTION

Colour patterns are prominent features of many animals; they have important functions in protection against UV irradiation, camouflage, kin recognition, shoaling and sexual selection. Colour patterns in birds and mammals are generated by melanocytes, which produce melanin and transfer it to the tissues of fur or plumage. In fish, amphibia and reptiles, chromatophores retain their pigment and it is their distribution in the dermis that determines the pattern (Kelsch, 2004).

The characteristic stripe pattern of zebrafish is composed of black melanophores, yellow xanthophores and silvery iridophores containing light-reflective purine platelets; these are responsible for the shiny appearance of the pattern. All chromatophores, except the retinal melanocytes, originate from the neural crest, a transient pluripotent embryonic cell population. In the zebrafish embryo, neural crest cells and their progeny differentiate directly into chromatophores that form the larval pattern (Eisen and Weston, 1993; Raible and Eisen, 1994). During metamorphosis, the adult pattern is generated by new chromatophores that emerge in the dermis. There is increasing evidence that these cells are produced by neural crest-derived stem cells that have been set aside in distinct niches, such as the ganglia of the peripheral nervous system or the base of the fins (Budi et al., 2008; Budi et al., 2011; Hultman et al., 2009; Hultman and Johnson, 2010; Tryon et al., 2011; Tu and Johnson, 2011; Dooley et al., 2013).

Adult chromatophores localise in different tissue layers. At the most superficial level, chromatophores are organised in the dorsal epidermis on scales and around scale pockets (Kirschbaum, 1975). Other pigment cells lie deep in the body, e.g. the dense sheet of iridophores covering the viscera and the melanophores associated with blood vessels. The chromatophores that form the horizontal pigment stripes characteristic for zebrafish distribute in the dermis. This pattern starts to develop with a light stripe (interstripe) in the region of the horizontal myoseptum. Subsequently, dark stripes appear dorsally and ventrally to this interstripe, and more interstripes and stripes are added as the fish grows (Kirschbaum, 1975; Takahashi and Kondo, 2008; Parichy et al., 2009). Melanophores are restricted to the dark stripes, whereas a dense sheet of xanthophores supported by xanthophores form the light interstripes. A thin layer of iridophores spreads over the melanophores (Fig. 1A). These are S-iridophores, whereas another iridophore type, L-iridophores, is located underneath the melanophore stripes (Hirata et al., 2003).

Several genes involved in the formation of the adult pigment pattern have been identified by mutations causing a strong reduction in number or the complete absence of one or more of the chromatophore types (Johnson et al., 1995; Haffter et al., 1996; Kelsh et al., 1996; Lister et al., 1999; Parichy et al., 1999). The nacre (nac) gene encodes the transcription factor Mitfa (Lister et al., 1999). nac mutants lack both larval and adult melanophores. In larvae, the pattern of iridophores and xanthophores is normal. In adult fish, however, iridophores and xanthophores do not form proper stripes (Maderspacher and Nüsslein-Volhard, 2003). The pfeffer/panther (pfe) gene encodes the receptor tyrosine kinase CsF1ra/Fms (Parichy et al., 2000a). In pfe mutants, the development of xanthophores is strongly suppressed in larvae and abolished in adults (Haffter et al., 1996; Odenthal et al., 1996). Additionally, in adults the number of melanophores is reduced (Parichy et al., 2000a). Iridophores and melanophores are normal in larvae, but are...
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not able to maintain the striped organisation in mutant adults. In both nac and pfe mutants, introduction of the missing cell type by blastula transplantation restores normal stripe formation, thus indicating that nac and pfe act cell-autonomously in melanophores and xanthophores, respectively (Maderspacher and Nüsslein-Volhard, 2003; Parichy and Turner, 2003). These results show that the striped pattern depends strongly on the interactions between melanophores and xanthophores.

The process of stripe formation displays properties of self-organising systems based on Turing-type interactions (Turing, 1952; Gierer and Meinhardt, 1972; Meinhardt and Gierer, 1980). In zebrafish, stripe formation has been modelled based on interactions between melanophores and xanthophores observed in normal development and during regeneration (Asai et al., 1999; Yamaguchi et al., 2007; Nakamasu et al., 2009).

Several mutations are known to cause a reduction of iridophores (Johnson et al., 1995; Haffter et al., 1996; Kelsh et al., 1996; Lang et al., 2013). shady (encoding leukocyte tyrosine kinase) mutants lack iridophores both in larvae and in adults whereas in rose (encoding endothelin receptor b1a) mutants only the adult pattern is affected (Parichy et al., 2006b; Lopes et al., 2008). In both mutants, strikingly, the melanophore numbers are also strongly reduced and the stripes are broken up into spots. A similar phenotype is displayed in transparent (tra) mutants (Walker and Streisinger, 1983; Krauss et al., 2013), bnc2 (Lang et al., 2009) and roy (White et al., 2008). Strikingly, mutants lacking iridophores display a normal striped pattern in the anal and tail fins. Therefore, iridophores have been regarded not to be active players in the process of stripe formation (Maderspacher and Nüsslein-Volhard, 2003; Parichy and Turner, 2003; Nakamasu et al., 2009). Alternatively, stripe formation in the fins may share some but not all properties of the mechanism working in the dermis of the trunk.

In this study, we show that iridophores play a crucial role in melanophore stripe formation in the dermis. We present the phenotypes and the development of the pigment pattern of rse and shd mutants. By creating chimeric animals we show that both act cell-autonomously in iridophores, whereas the reduction in melanophore number and stripe formation is caused by the absence of iridophores. To elucidate the interactions between iridophores and the other two chromatophore types, we analyse the development of the pigment pattern in mutants lacking either melanophores or xanthophores, nac and pfe, as well as in double mutants, in which two types of chromatophores are absent. We deduce several short- and long-range interactions between iridophores, xanthophores and melanophores and present a model for the interactions underlying stripe formation in zebrafish. Finally, we identify the horizontal myoseptum, which is lacking in the choker mutants, as a prepattern for the first interstripe, whereas the subsequent formation of the pattern of alternating stripes and interstripes depends on the mutual interaction between all three chromatophores.

MATERIALS AND METHODS

Zebrafish maintenance and genetics
We investigated zebrafish (Danio rerio) of the following genotypes: nac<sup>E12</sup>, pfe<sup>pat2</sup>, chot<sup>pat2</sup> (ZFIN database), shd<sup>W6</sup> (Lopes et al., 2008) and Tg(TDL358:gfp) (Levesque et al., 2013). We describe two new n-ethyl-n-nitrosourea (ENU)-induced rse alleles, rse<sup>JA107</sup> and rse<sup>LF802</sup> (strong), which we will refer to as rse<sup>e</sup> and rse<sup>e</sup> or simply rse. The phenotype of the stronger allele resembles that of the published amorphic allele (Parichy et al., 2006b). Zebrafish were maintained as described by Brand et al. (Brand et al., 2002). Metamorphic fish were staged [AR, anal fin ray/6.2 mm standardised standard length (SSL); PB, pelvic fin bud/7.2 mm SSL; PR, pelvic fin ray/8.6 mm SSL; SP, squamation onset posterior/9.6 mm SSL; J, juvenile/11.0 mm SSL; J+, juvenile+/13 mm SSL]; the size of the registration mark is not to scale. Melanophore stripe formation was scored in the dermis of the caudal fins as described by Parichy and Turner (2003).

Fig. 1. Adult phenotypes of iridophore mutants. (A) Wild-type fish. To denote individual stripes, we follow the nomenclature of Parichy et al. (Parichy et al., 2009), which we have extended by naming the interstripes, the central interstripe being X0. Additional stripes and interstripes added dorsally and ventrally during development are numbered according to their sequence of appearance. (B,C) rse mutants. Weak (B) and strong (C) rse alleles show a reduction of iridophores and melanophores. In strong rse mutants (Fig 1C), the dense S-iridophore zone in X0 is lacking, and S-iridophores spread thinly from X0 dorsally and ventrally over the melanophores. In the weaker allele, a small ridge of dense S-iridophores persists (B). There are at most four melanophore stripes compared with five in a typical wild-type adult. Stripe 2D is reduced or absent, 2V is better preserved and 1V stays more strictly in a wild-type position parallel to X0 in rse compared with five in a typical wild-type adult. (D,E) shd mutants. The white arrow in E displays an escaper S-iridophore patch (Lopes et al., 2008). In close proximity, the number of melanophores is significantly increased. (F) Double mutant rse;shd. The phenotype is indistinguishable from that of shd mutants.
Cell transplantation
Chimeric animals were generated by transplantation of blastula cells into embryos of the same stage essentially as described by Kane and Kishimoto (Kane and Kishimoto, 2002). The number of transplanted cells was estimated to be in the range of 30-50. Animals were raised to adulthood and analysed for donor-derived chromatophores.

Image acquisition and analysis
Adult fish were briefly anaesthetised with 0.004% MS-222 (Sigma) and imaged with Canon D5MarkII/MACRO 100 (Figs 1, 3). Fish fixed in 4% parafomaldehyde/0.08% glutaraldehyde (Sigma) (Fig. 2) were photographed under a Leica MZ1 stereomicroscope. Metamorphic fish were anaesthetised, embedded in low melting point agarose with 4.5 mg/ml ± epinephrine (Sigma) for melanosome contraction, and photographed under a Leica M205 FA stereomicroscope with a Leica DCF300 FX camera using the software LAS V4.1 to allow multifocus images. An illumination was chosen to display iridophores optimally while xanthophore visibility is poor. Photographs were processed in Adobe Photoshop.

RESULTS

Melanophore stripe formation is affected in the iridophore mutants rse and shd
In zebrafish, melanophores are restricted to the stripes whereas iridophores and xanthophores are present in both stripes and interstripes (Fig. 1A; Fig. 2A). Two types of iridophores can be distinguished in the trunk. Superficial S-iridophores form a dense zone in the interstripe (‘dense S-iridophores’) and spread as a thin net-like layer (‘blue S-iridophores’) over the melanophores of the stripe. Xanthophores are located on top of S-iridophores. L-iridophores form a homogeneous silvery sheet below melanophores (Hirata et al., 2003). The colour of the iridophores may appear silvery, golden, brownish or blue, depending on the illumination (Fig. 1A; Fig. 2).

Mutations in the genes rse and shd result in similar recessive adult phenotypes of varying strengths, displaying (1) a reduction (rse) or absence (shd) of S-iridophores, (2) a reduced number of melanophores and (3) fewer stripes (Fig. 1B-D). With increasing loss of iridophores, the stripes dissolve into a series of spots. The central stripes 1D and 1V, adjacent to the first interstripe X0, remain most prominent, whereas the stripes added later are more strongly reduced. The interstripe regions become wider as S-iridophores are lost from the interstripes. In the weak rse allele (Fig. 1B) a thin ridge of S-iridophores is present in X0, and the adjacent stripes are fairly well preserved. By contrast, in the absence of dense S-iridophores (Fig. 1C,D; Fig. 2B,C) the melanophores are reduced and broken up into spots. Xanthophores cover the regions between melanophores (Fig. 2B’,C’). In strong mutants (shd), neither S- nor L-iridophores develop in the dermal trunk, both 2D and 2V stripes are strongly reduced or absent (Fig. 2C) and the horizontal alignment of 1V spots may be lost (Fig. 2C, grey arrows).

Iridophores are also reduced in other regions of the body: shd mutants show a strong reduction of iridophores in the eye, whereas in rse mutants this phenotype is more subtle (Fig. 1C,D). The operculum and gut are sparingly covered by iridophores, rendering the gills, the intestine and the melanophore-covered blood vessels along the myosepta visible through the skin (Fig. 1B-E; Fig. 2B,C). Along the dorsal aspect of the fish, the numbers of iridophores and melanophores are reduced, and the melanophores appear less aggregated (supplementary material Fig. S1). The iridophores in the anal and tail fins are reduced but the striped pattern of the fins is not affected (Fig. 1B-F).

The double mutant rse;shd (Fig. 1F) does not show a significant increase in the strength of the phenotype when compared with the strongest single mutant, i.e. a mutation in rse does not enhance the mutant effect of shd. This indicates that in both mutants the same cell type(s) are primarily affected.

Mutants lacking xanthophores and melanophores display abnormal iridophore distribution
To elucidate the dependencies between iridophores, xanthophores and melanophores, we analysed the adult phenotypes of pfe and nac mutants, as well as those of the double mutants nac; pfe, shd; pfe and shd;nac.

No xanthophores
In pfe mutants (Fig. 3A,A’), melanophore stripes are broken up into spots, separated by dense S-iridophore regions extending into the stripes. Melanophores also appear ectopically as single cells within the interstripes. Adult pfe mutant fish show an increase in S-iridophores and a reduction of melanophore number although stripes appear to be of normal width, at least in anterior regions where the striped organisation is better preserved (Fig. 3A).
No melanophores

*nac* mutants lack melanophores completely. In addition, the number of xanthophores is variable. A prominent interstripe of xanthophores and dense S-iridophores with irregular borders forms in the region of X0, accompanied by spots ventrally (Fig. 3B,B’). Xanthophores strictly colocalise with the dense S-iridophores. X0 interstripe and X1V spots are separated from regions composed of a thin net of blue S-iridophores, as well as L-iridophores (Fig. 3B’). We regard these regions as rudimentary 1V stripes (lacking melanophores) and interpret this pattern as a form of residual stripe formation. In those *nac* individuals that display a reduced number of xanthophores, the dense iridophores of the first interstripe may expand dorsally and ventrally, like in *nac;pfe* mutants (see below) (Lister et al., 1999; Maderspacher and Nüsslein-Volhard, 2003).

No melanophores and xanthophores

In the double mutant *nac;pfe* (Fig. 3C,C’), a dense layer of S-iridophores covers the entire trunk region, which is replaced towards the anterior area by L-iridophores.

No iridophores and xanthophores

In *shd;pfe* double mutants, melanophores are dispersed over the flank and they also cover the region of the first interstripe. Their density gets lower with age, in particular towards the ventral aspect of the fish (Fig. 3D’).

No melanophores and iridophores

Xanthophores homogeneously fill most of the flanks in a dense layer in *shd;nac* fish (data not shown).

In conclusion, none of the three chromatophore types alone is capable of forming a pattern. In the presence of two of the three chromatophore types, irregular and incomplete stripes are formed. Lack of iridophores, and to a lesser extent of xanthophores, is correlated with a strong reduction of melanophore numbers (10-15% reduction in *rse* and *shd* mutants, 50% reduction in *pfe* mutants, compared with wild type; see Fig. 7).

**shd** and **rse** are required in iridophores but not in melanophores

As both iridophores and melanophores are affected in *rse* and *shd* mutants, we investigated in which cell types the gene products of *rse* and *shd* are required. We generated chimeric animals of various mutant combinations by transplantations of blastula donor cells into host embryos. Resulting adult fish were analysed for regions displaying a striped pattern. First, we used wild-type donor embryos, which carried the transgenic GFP marker *Tg(TDL358:gfp)* (Levesque et al., 2013) to label iridophores, for transplantation into *rse* mutant hosts. The resulting chimaeras displayed reconstituted stripe patterns in regions where GFP-positive iridophores developed (Fig. 4A,A’). This result shows that *rse* is required in iridophores.

In transplantations of *nac;pfe* cells into *rse* or *shd* mutant hosts, the resulting chimeric fish display large regions of restored stripe formation. Iridophores derived from the *nac;pfe* donor form interstripes of normal width, and the stripes contain normal numbers of melanophores that must be mutant for *rse* or *shd* as the *nac;pfe* donors are unable to produce melanophores and xanthophores (Fig. 4B,C). In the reciprocal experiment, we transplanted cells from *rse* or *shd* mutants into *nac;pfe* double mutants. These chimaeras develop regions containing donor-derived melanophores and xanthophores organised with host iridophores into stripes of wild-type pattern (Fig. 4D; data not shown). These results demonstrate that melanophores and xanthophores mutant for *rse* and *shd* restore stripe formation when confronted with iridophores provided by the *nac;pfe* host. Thus, *rse* and *shd* are not required in melanophores or xanthophores. When we transplanted cells reciprocally between *rse* and *shd* mutants, we did not observe rescue of stripe formation (0/124 with *rse* hosts, 0/87 with *shd* hosts), confirming that both genes are autonomously required in the same cell type, iridophores.

To conclude, the complex phenotypes, including the strong reduction in melanophore numbers in *shd* and *rse* mutants, are caused by the absence or reduction of iridophores.
In *rse* mutants, the initial development of iridophores resembles wild type except that their number is strongly reduced (Fig. 5E,F). During stage PR, iridophores spread over the neighbouring melanophore regions in a similar manner to wild type (Fig. 6F) and the iridophore density of X0 is reduced suggesting a migration of cells from X0 into the periphery. This results in a rather homogeneous distribution of bluish iridophores over interstripes and stripes (Fig. 6F-H) and the loss of the dense ridge of iridophores in X0. *shd* mutants are completely devoid of reflective iridophores on their lateral flanks (Fig. 6I-L) and Tg(TDL358:gfp) labelling of dermal iridophores is absent (data not shown).

### L-iridophores

During juvenile development at around stage J, L-iridophores begin to appear underneath the ventral melanophore stripes. They form first anteriorly behind the head and extend into more posterior and dorsal regions (data not shown). *shd* mutants lack this cell type.

### Melanophores

In the posterior trunk of wild-type fish, melanophores begin to appear during stage PB. The first metamorphic melanophores arise dorsally and ventrally to X0 (Fig. 6A). During subsequent stages of metamorphic development, melanophores increase in number and localize closer to X0, i.e. in the region of the prospective 1V and 1D (Fig. 6B).

In *rse* and *shd* mutants, metamorphic melanophores become first visible during stage PB, comparable to wild type (Fig. 6E,J). During the following stages, however, fewer melanophores emerge and they remain more homogeneously dispersed over the entire flank, i.e. there is not much accumulation towards the prospective stripe regions (Fig. 6F,G,J,K). We describe the melanophores of 1V, ventral to X0, because in this region no epidermal scale melanophores complicate the observation of stripe formation in the dermis. Homogeneously distributed melanophores start to aggregate into patches that are now located in the region of the prospective 1V stripe (Fig. 6H,L). In *shd* mutants, fewer melanophores emerge and they remain more homogeneously dispersed over the entire flank, i.e. there is not much accumulation toward the prospective stripe regions (Fig. 6F,G; supplementary material Fig. S2). Melanophores can form patches anywhere in the space ventral to X0 (Fig. 6K,L). Melanophores located in rather ventral positions often associate with melanophores of the larval ventral stripe (Fig. 6L). These aggregates can give rise to vertical stripe-like arrangements surrounded by a layer of xanthophores.

### Xanthophores

In wild type, metamorphic xanthophores become visible in interstripe X0 during stage PB, and the intensity of pigmentation and density of cells increases in subsequent stages. Appearance of xanthophores in *rse* and *shd* mutants is delayed in X0. In the ventral region of wild type, xanthophores follow the appearance of iridophores in interstripes. In *rse* and *shd* mutants, they fill the space between melanophore aggregates (data not shown).

In the mutants, there is a remarkable variation between individuals. One of five *rse* mutant fish had formed stripes already at stage PR, comparable to wild type. One of five *shd* mutant fish displayed a homogeneous distribution of melanophores ventrally to X0 until stage J++.

The number of melanophores per ventral hemisegment in wild type, *rse* and *shd* mutants was calculated by counting cells in the ventral stripes of the eight hemisegments above the anal fin. In *shd* and *rse* mutants during metamorphosis we observe ~30%, and in the adults only 10-20% of the normal melanophore number (Fig. 7).
Development of the adult pattern in xanthophore and melanophore mutants

We analysed the development of the pigment pattern of pfe and nac mutants, as well as double mutants missing two of the three chromatophore types.

No xanthophores
In pfe mutants, iridophores start to appear in the X0 region, similar to wild type (Fig. 8A-D). Occasionally, iridophores arise even slightly earlier than in wild type, during stage CR (data not shown), and the iridophore region is expanded. The accumulation of melanophores in 1D and 1V during the early metamorphic stages is less pronounced compared with wild type (supplementary material Fig. S2). During further development, S-iridophores ingress into the territory of 1D and 1V, thereby splitting the melanophore field into patches (Fig. 8B,C). From stage SP onwards, melanophore and iridophore patches mix in the ventral half of the flank (Fig. 8C).

No melanophores
In nac mutants, a prominent interstripe of xanthophores and iridophores with irregular borders forms in the region of X0 (Fig. 8E-H). During SP, the ventral region is covered by a thin blue iridophore sheet. In juvenile stages, dense iridophore patches covered with xanthophores mark the appearance of X1V.

No melanophores, no xanthophores
In nac;pfe double mutants, iridophores initially form a X0 interstripe between stages AR and PB (Fig. 8I), as in wild type. However, even more pronounced than in nac and pfe single mutants, S-iridophores increase in number and expand into dorsal and ventral regions in subsequent stages (Fig. 8J,K). Finally, they cover the entire lateral region (Fig. 8L).

No iridophores, no xanthophores
In the double mutant shd;pfe, melanophores appear rather evenly distributed in numbers that are slightly increased compared with shd or pfe single mutants (Fig. 8M-P). Whereas the melanophores in shd aggregate during the juvenile stages, a homogeneous distribution which decreases in density towards the ventral aspect of the fish persists in shd;pfe (Fig. 8P).

A prepattern for self-organising stripe formation
The mutant choker (cho) was identified for its defects in pigmentation and somite formation. Mutant larvae lack the horizontal myoseptum and the stripe of larval melanophores associated with it (Kelsh et al., 1996; van Eeden et al., 1996; Svetic et al., 2007). We found that cho mutant animals occasionally survive to adulthood. cho fish develop a peculiar pigment pattern. Stripes and interstripes of normal width are formed in a parallel arrangement, but they are heavily curved, sometimes branched and often interrupted, and they also may run in a vertical rather than horizontal orientation, unique in each mutant fish (Fig. 9A,B). During metamorphosis, the appearance of iridophores is considerably delayed. Anteriorly they are first visible during stage AR, rather...
than in CR (data not shown). In the posterior trunk, the first iridophores become apparent during stage PR (Fig. 9D). Melanophores, however, are not delayed; they populate the medium and posterior trunk in a rather homogeneous fashion in stage PB (Fig. 9C). During stage PR, more melanophores are added, initially intermingled with newly arising iridophores (Fig. 9D). The iridophores accumulate in patches with arbitrary positions and orientations (Fig. 9E). In the centre of the new iridophore patches, xanthophores start to appear during stage PR, and melanophores aggregate into stripes and disappear from the iridophore patches. At stage J+, melanophore and iridophore areas are largely separated. The arbitrary nature of the orientation of stripes found in adults indicates that during normal development the appearance of iridophores at the horizontal myoseptum serves as a morphological landmark for stripe orientation, but subsequent stripe formation as well as stripe width are determined in an autonomous manner.

**DISCUSSION**

**Cell-autonomous requirement of shd and rse in iridophores**

In this article, we investigate zebrafish mutants defective in individual chromatophores with the aim of elucidating interactions between the pigment cell types during stripe pattern formation. Regarding melanophores and xanthophores, chimeric animals have shown that nac and pfe are required autonomously in the two cell types, respectively (Maderspacher and Nüsslein-Volhard, 2003; Parichy and Turner, 2003) (data not shown). We conclude that the mutants are primarily affected in a single cell type and that phenotypic consequences observed in the patterning of the remaining cell types indicate chromatophore interactions.

To determine the role of iridophores in pattern formation, we investigated the cell-type specificity of the mutants rse and shd. Cell transplantations show that rse and shd cells can give rise to normally behaving melanophores and xanthophores, which form regular stripes when confronted with wild-type iridophores. This indicates that rse and shd are cell-autonomously required only in iridophores and that the adult melanophore phenotype is caused by the lack of iridophores. The same result has been obtained for tra (Krauss et al., 2013). shd may be required for the specification of iridophores (Lopes et al., 2008), rse possibly for the expansion of the iridophore population (J.K., unpublished), and in tra mutants iridophores do not survive (Krauss et al., 2013). Despite different functions in iridophore development, these three genes have similar effects on melanophores. We speculate that a positive signal emanates from iridophores in the dermis sustaining melanophores during stripe pattern formation and maintenance.

Iridophores differentiate into two distinct forms. We consider L-iridophores that are located under the melanophore stripes to be dispensable for stripe formation, as they appear late in juvenile development after the basic striped pattern has been established. Further, they are absent in shd and tra, but present in rse mutants, with very similar consequences on melanophore numbers (Fig. 7). L-iridophores might, however, play a role in stripe maintenance. S-iridophores differentiate into a dense form in the interstripes, spreading into a thin, bluish layer covering the melanophores in the striped regions. These ‘blue iridophores’ are present in rse but absent in shd mutants. A subtle difference we observe between the rse and shd phenotypes is that in shd (and aged tra) adults the stripes almost always break up into spots, whereas in rse mutants more frequently a coherent thin stripe is maintained (Fig. 1). This may be attributed to...
the blue S-iridophores, as they are absent in *tra* and *shd* mutant fish. Because in both *rse* and *shd* mutants the melanophore numbers are reduced to the same extent (Fig. 7), it seems that the dense S-iridophores of the interstripes exert a long-range effect on the aggregation and support of melanophores in the neighbouring stripes.

We conclude that stripe formation is predominantly based on interactions between S-iridophores, xanthophores and melanophores, which are eliminated in the mutants *shd*, *pfe* and *nac*, respectively.

**Stripes do not form with only one chromatophore type**

Because *shd*, *pfe* and *nac* act cell-autonomously in the respective cell types, the mutants allowed us to investigate the behaviour of any two chromatophore types in the absence of the third, and the potential of each cell type left on its own in double mutants.

In double mutants, each cell type is capable of filling the entire space; in other words, neither of them needs another cell type to expand. However none of them is able to form a pattern in the absence of the other two. In the double mutant *nac*: *pfe*, iridophores cover the flank of the fish in a dense silvery layer (Fig. 3C); likewise in *shd*: *nac* the xanthophores spread evenly in the dermis. This is remarkable because in wild type, xanthophores are always resting on top of iridophores. In the absence of both iridophores and xanthophores (*shd*: *pfe*), melanophores are also capable of distributing evenly, but they do so at a lower density than in wild type. This indicates that, in contrast to iridophores and xanthophores, melanophores have a tendency to avoid each other and depend on iridophores or xanthophores in their neighbourhood to aggregate into stripes or spots. In support of this idea, Takahashi and Kondo (Takahashi and Kondo, 2008) describe in regeneration experiments that melanophores in the absence of xanthophores do not aggregate but spread out into the available space, maximising their distance from each other.

These observations indicate that pigment pattern formation requires the interaction of at least two cell types. We observe residual stripe formation in each of the single mutants lacking only one of the three chromatophore types. The phenotypes of single and double mutants are schematically illustrated in Fig. 10.

**Defective stripe formation with two pigment cell types**

In *pfe* mutants, devoid of xanthophores, iridophores display a strong tendency to spread and invade melanophore regions. Melanophores fail to maintain a coherent stripe organisation, which is interrupted by invasions of iridophores. Whereas in wild type melanophores are separated from dense iridophores by a transitional zone, in *pfe* the melanophores appear immediately adjacent to dense S-iridophore regions. Single melanophores are even observed on top of the interstripe iridophores. However, a horizontal alignment of melanophore spots maintaining approximately normal width of stripes is preserved in *pfe* mutants and in many individuals three stripe-like melanophore arrangements are discernible.

In *nac* mutants, in the absence of melanophores, a prominent X0 interstripe region composed of dense S-iridophores and xanthophores occupies the lateral side of the fish. Ventrally, ‘stripe’ regions composed of blue S-iridophores but devoid of melanophores, and interstripe regions are added. The boundary between these regions is ragged, and the width of both ‘stripe’ and interstripe is narrow. We note that xanthophores and dense S-iridophores attract each other as they strictly colocalise; furthermore, the presence of xanthophores prevents the spreading of dense S-iridophores into ventral regions, which occurs in the double mutant *nac*: *pfe*.

The lack of iridophores causes a severe reduction of melanophore numbers, as observed in the *rse* and *shd* phenotypes (Fig. 7). Nevertheless, a pattern composed of dense melanophore spots or thin stripes is maintained in which xanthophores fill the space left by the melanophores. In comparison to *shd*: *pfe*, it appears that the presence of xanthophores is required for melanophores to aggregate into stripes or spots. Melanophores in *shd* mutants show a weaker tendency to aggregate into a stripe region close to X0, compared with *rse* mutants (Fig. 2B,C). The distance between dorsal and ventral melanophore spots or stripes is considerably enlarged. Thus, although xanthophores and melanophores are perfectly capable of segregating into different regions, the alignment into stripes requires blue S-iridophores present in *rse*, but absent in *shd* and *tra* mutants.

**Model of the interactions between the three chromatophore types**

Based on the analysis of single and double mutants, we deduce the following interactions between different pigment cell types (Fig. 11).

S-iridophores support the production and maintenance of high numbers of melanophores in their neighbourhood and cause their aggregation into stripe regions. As locally melanophores are strictly separated from dense S-iridophores, this strong positive interaction occurs over a distance of several cell diameters. This support is apparent in the melanophore reduction observed in *shd* and *rse* mutants, as well as in transplantation experiments in which melanophore stripes of normal width appear in these mutants when supplied with wild-type iridophores (Fig. 4B,C). Further, the spontaneous iridophore patches that are sometimes present in *shd* mutants are always surrounded by aggregations of melanophores (Fig. 1E). One might ask whether the effect of iridophores on melanophores is exerted via xanthophores. But the accumulation of
melanophores in 1D and 1V in pfe mutants (Fig. 3A; Fig. 8C) indicates that iridophores alone can cause the aggregation of melanophores in their immediate environment. The mutual exclusion of stripe and interstripe suggests a short-range repulsion between iridophores and melanophores.

Dense S-iridophores and xanthophores exhibit mutual attraction. This is seen in wild type, with xanthophores being embedded in the sheet of S-iridophores from the beginning of stripe formation. The mutual attraction is most prominently apparent in the nac phenotype, in which xanthophores strictly colocalise with dense S-iridophores. We also observe that in cho mutants xanthophores follow the distribution of iridophores. Xanthophores, however, do not require iridophores for spreading, as seen in the free distribution in shd mutants, filling the space between melanophores. Xanthophores might exert a positive signal sustaining melanophores at a distance, as melanophore number is reduced in adult and to a

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**Fig. 8. Iridophore development in the posterior trunk of xanthophore, melanophore and double mutants.** *pfe* (A-D), nac (E-H), nac;pfe (I-L) and shd;pfe (M-P) mutants. In *pfe*, as well as in nac and nac;pfe mutants, X0 is more prominent than in wild type; it expands to cover the entire flank in nac;pfe mutants. In shd;pfe mutants, melanophores distribute evenly also in the territory normally occupied by X0. Stages: PB, pelvic fin bud; PR, pelvic fin ray. SP, squamation onset posterior; J+, juvenile+. Scale bars: 250 μm.

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**Fig. 9. The choker phenotype.** *(A,B)* Adult individuals, displaying parallel stripes with arbitrary orientation. *choker* adults can reach normal sizes and are fertile. *(C-E)* Metamorphic development. In the absence of the horizontal myoseptum, melanophores appear before iridophores and xanthophores, evenly dispersed over the flank (C). Iridophores and xanthophores emerge in patches interspersed with melanophores (D), but then aggregate into stripe-like arrangements, and separate from melanophore regions (E). The aorta is visible as dark horizontal shade in C. Scale bars: 250 μm.
Development 140 (14)

Formation of horizontal stripes requires a prepattern

The first interstripe, X0, is formed by iridophores emerging at the horizontal myoseptum followed by xanthophores. This interstripe appears in both pfe and shd mutants, suggesting that either iridophores or xanthophores alone can form the first interstripe. We propose that this origin provides a prepattern, as has been suggested previously (Maderspacher and Nüsslein-Volhard, 2003).

Consistent with the notion that the horizontal myoseptum acts as a morphological prepattern, we observe that in cho mutants, which lack the horizontal myoseptum, chromatophore-dependent stripe-forming processes act at arbitrary orientations. In cho mutants, melanophores appear during metamorphosis evenly distributed in the dermis at normal time points, whereas iridophores are delayed. This is consistent with the finding that melanophores do not emerge to the skin through the horizontal myoseptum, but migrate along spinal nerves through the myotomes and appear in the dermis at locations dorsal or ventral to the horizontal myoseptum (Dooley et al., 2013). The arbitrary position of the later-appearing iridophores suggest that they, in the absence of a horizontal myoseptum, find their way to the dermis by following paths taken by melanophores.

Conclusions

So far only melanophores and xanthophores, but not iridophores, have been proposed to play an essential role in stripe formation (Asai et al., 1999; Yamaguchi et al., 2007; Nakamasu et al., 2009). The phenotype of mutants lacking iridophores and the residual stripe/interstripe formation in nac and pfe mutants shows, however, that iridophores play an essential role in stripe formation; thus, interactions between all three chromatophore types contribute in overlapping ways (Fig. 11). Both iridophores and xanthophores exert short-range and long-range interactions with melanophores. S-iridophores attract melanophores in high numbers, induce their aggregation into the prospective stripe area (positive long-range effect) (1) and are to some extent able to exclude them from the interstripe (negative short-range effect) (2). Xanthophores have a minor effect on melanophore aggregation (positive long-range effect) (3), but keep melanophores out from interstripe regions resolutely (negative short-range effect) (4). Interactions between iridophores and xanthophores are required to confine the shape of interstripes (5,6). Although in cho mutants a primary signal to orient lesser extent in juvenile pfe mutants. A positive long-range and a negative short-range effect of xanthophores on melanophores has also been postulated from regeneration experiments (Nakamasu et al., 2009). Xanthophores prevent iridophores from spreading, as deduced from the pfe mutants in which S-iridophores invade the melanophore territory, as well as in the double mutant nac;pfe.

Xanthophores and melanophores mutually repel each other. In pfe mutants, melanophores can be seen ectopically in the interstripe region, which is never observed in wild-type adults. In the absence of iridophores in shd or rse mutants, the interstripes, filled with xanthophores, may be considerably enlarged with a sharp boundary between xanthophore and melanophore regions. Iridophores may only have a minor local repulsive interaction with melanophores, by contrast; although the dense iridophore sheet is excluding melanophores, the vicinity appears to be required for dense melanophore stripe formation (see above). We therefore propose that xanthophores and iridophores support aggregation of melanophores by reducing their tendency to avoid each other.

Fig. 11. Scheme of interactions between chromatophore types. Red curved arrows, long-range interactions; black arrows, short-range interactions. For further details, see text.

Fig. 10. Schematics of the central striped region in wild-type and mutant zebrafish.

The iridophore cell bodies are not discernible in live fish. Dense S-iridophores are symbolised with grey dashes on a white background, whereas the thin blue iridophore sheet covering the melanophores in wild type and pfe mutants are indicated in light blue. Fish skin background devoid of pigment cells is indicated in pink. In contrast to the wild type, the mutant phenotypes are highly variable; the drawings illustrate the characteristic features. M, melanophores (black circles); I, iridophores; X, xanthophores (yellow circles).

Melanophores appear in the dermis dispersed dorsally and ventrally to the horizontal myoseptum. While melanophores are increasing in number, they aggregate close to X0 into 1V and 1D. The dense S-iridophores of the interstripe expand dorsally and ventrally to produce a field of blue iridophores that spreads thinly over the melanophore stripes. A parallel copy of an interstripe is formed from a new accumulation of dense iridophores within the blue region (Fig. 6C; Fig. 8C,D,H). In wild type, blue iridophores are associated with melanophores and dense iridophores with xanthophores.

For further details, see text.
the stripes is missing, the cho phenotype produces patterns of parallel stripes and interstripes, indicating that stripe width is autonomously controlled by cell-cell interactions. Melanophores, in conjunction with blue iridophores, may contribute to control the width and continuity of stripes.

Acknowledgements
We thank Brigitte Walderich for help with the transplantation experiments; Iris Koch for help with Fig. 10; and Ajey Singh, Uwe Irion, Andrey Fadeev, Alessandro Mongera, Christian Stöllner and Patrick Müller for discussions and critical reading of the manuscript.

Funding
This work was supported by the Max-Planck-Gesellschaft, FRG. Deposited in PMIC for immediate release.

Competing interests statement
The authors declare no competing financial interests.

Author contributions
H.G.F. discovered the role of iridophores in stripe formation, isolated the new rse alleles, provided the figures of the mutants during metamorphosis and performed the melanophore counts. J.K. and H.G.F. provided the figures of adult mutants. J.K. isolated the TDL 358 transgenic line and discovered the cho phenotype. H.-M.M. performed the transplantations under the supervision of H.G.F. C.-N.V. coordinated the investigations, finalised the model (Figs 10 and 11) and wrote the manuscript together with H.G.F.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.096719/-/DC1

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