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The Developmental Genetics of Vertebrate Color Pattern Formation: Lessons from Zebrafish

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Abstract

Color patterns are prominent features of many animals; they are highly variable and evolve rapidly leading to large diversities even within a single genus. As targets for natural as well as sexual selection, they are of high evolutionary significance. The zebrafish (*Danio rerio*) has become an important model organism for developmental biology and biomedical research in general, and it is the model organism to study color pattern formation in vertebrates. The fish display a conspicuous pattern of alternating blue and golden stripes on the body and on the anal and tail fins. This pattern is produced by three different types of pigment cells (chromatophores) arranged in precise layers in the hypodermis of the fish. In this essay, we will summarize the recent advances in understanding the developmental and genetic basis for stripe formation in the

ISSN 0070-2153
http://dx.doi.org/10.1016/bs.ctdb.2015.12.012
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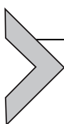
zebrafish. We will describe the cellular events leading to the formation of stripes during metamorphosis based on long-term lineage imaging. Mutant analysis has revealed that a number of signaling pathways are involved in the establishment and maintenance of the individual pigment cells. However, the striped pattern itself is generated by self-organizing mechanisms requiring interactions between all three pigment cell types. The involvement of integral membrane proteins, including connexins and potassium channels, suggests that direct physical contacts between chromatophores are involved, and that the directed transport of small molecules or bioelectrical coupling is important for these interactions. This mode of patterning by transmitting spatial information between adjacent tissues within three superimposed cell layers is unprecedented in other developmental systems. We propose that variations in the patterns among *Danio* species are caused by allelic differences in the genes responsible for these interactions.



1. INTRODUCTION

In most animal species, the body is colored. A primary function of colors is easy to comprehend: dark pigments and reflecting structural colors prevent harmful radiation from damaging vital tissues (Brenner & Hearing, 2008). Strikingly, coloration is often displayed in beautiful patterns that are composed of several pigments, as well as various kinds of nanostructures producing a wide range of colors in the skin and its appendages such as bristles, scales, hairs, and feathers. Although the significance of these patterns for the animal is not always obvious, in many instances color patterns have important functions in the communication among individuals of a species, for example, recognition and selection of mating partners, or attraction between many individuals to form large groups. Color patterns are also instrumental in prey–predator interactions by allowing adaptation to the environment, but also serve as deceptive or attractive signals that are recognized by individuals of different species (Protas & Patel, 2008). Often, patterns are highly variable and evolve rapidly, which leads to large diversities in coloration, even within a single genus. In other cases, evolutionary convergence can lead to remarkable similarities in the color patterns of distant genera. In short, color patterns are of high evolutionary relevance as targets of natural as well as sexual selection. Understanding the mechanisms that underlie pigmentation and color pattern formation is an important step toward comprehending the evolution of biodiversity.

In insects, pigments are produced by epidermal cells and shed into the extracellular cuticle, bristles, and hairs. Frequently, color patterns follow morphological landmarks, such as segment boundaries, or wing veins that

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serve as prepatterns. In these instances, color pattern formation is explained as a readout of positional information in a two-dimensional sheet of cells, singling out epidermal cells to produce the enzymes required to synthesize the pigments (Wittkopp & Beldade, 2009). Well-known developmental signaling systems have been shown to be involved in this mode of pattern formation (Simpson, 2007; Werner, Koshikawa, Williams, & Carroll, 2010). In contrast, vertebrate color patterns are composed of specialized pigment-producing cells that undergo extensive cell movements and cell–cell interactions to form the final pattern. These pigment cells originate from the neural crest, a transient primordium of multipotent cells located at the dorsal neuroectodermal ridge from which progenitor cells migrate out into the periphery to develop a variety of structures and tissues including the peripheral nervous system, glia, and pigment cells (Le Douarin & Dupin, 2003; Le Douarin & Kalcheim, 1999). Birds and mammals have mainly one pigment cell type, the melanocyte, producing the pigment melanin (although in different shades, brownish-black eumelanin and reddish pheomelanin) that is secreted into the skin or the integumentary appendages, feathers, and hairs. Furthermore, birds display carotenoid-based colors and structural colors (Caro, 2013; Roulin & Ducrest, 2013; Uong & Zon, 2010). In contrast, basal vertebrates such as fish, amphibia, and reptiles develop several pigment cell types—chromatophores—producing different colors (Bagnara & Matsumoto, 2007). In fishes in particular, beautiful patterns displaying a wide spectrum of colors arise as multilayered mosaics of chromatophores distributed in the hypodermis of the body, and the epidermis of scales and fins (see a few examples in Figs. 1 and 2).

Although color pattern formation has fascinated scientists since the beginning of modern biology (Darwin, 1871; Poulton, 1890), it is a field that still is dominated by theories rather than detailed knowledge of the molecular, cellular, and developmental events leading to the striking display of different colors in the integument and its appendages of many vertebrate species. Genes affecting the production of pigments and colors have been identified by mutations in many species (Hubbard, Uy, Hauber, Hoekstra, & Safran, 2010); however, long developmental times and increasing body sizes make it technically challenging to study the development of color patterns in any vertebrate. Therefore, it is necessary to concentrate on organisms, in which individuals can be readily observed during the periods when patterning occurs, in which there is both, natural and induced variation, interbreeding is possible and transgenic and imaging methods are applicable. These features are uniquely combined in the zebrafish, *Danio rerio*,

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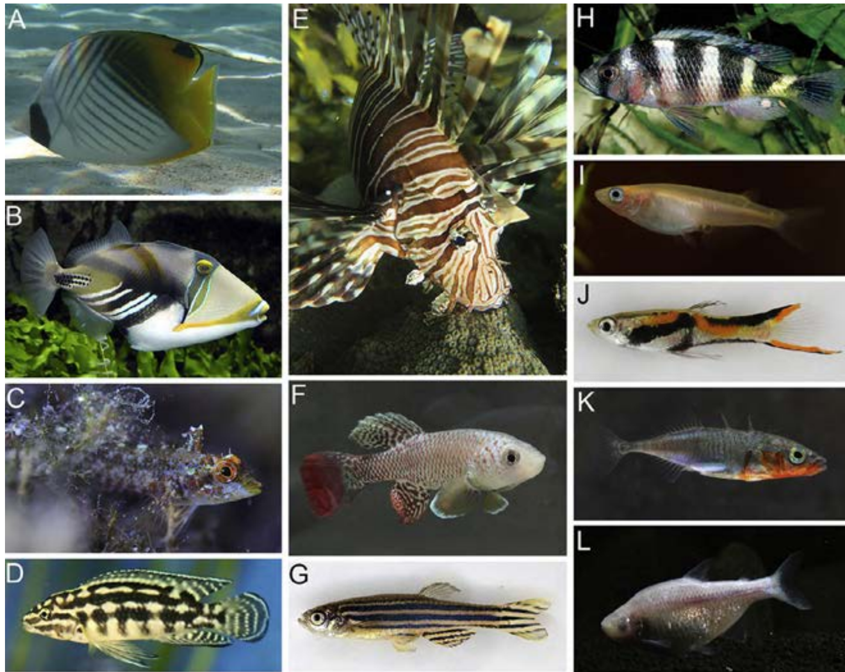

Figure 1 Color pattern diversity in fish. Fish show a great diversity of color patterns mainly by different combinations of stripes and spots. Some patterns are used to attract mates (e.g., in guppies or sticklebacks), others to deter predators (lion fish); cave fish are often blind and not colored. (A) Threadfin Butterfly fish (*Chaetodon auriga*), (B) Picasso fish (*Rhinecanthus aculeatus*), (C) Black-faced blenny (*Tripterygion delaisi*), (D) Checkered Julie (*Julidochromis marlieri*), (E) Common Lionfish (*Pterois spec.*), (F) Turquoise killifish (*Nothobranchius furzeri*), (G) Zebrafish (*Danio rerio*), (H) Cichlid (*Haplochromis latifasciatus*), (I) Japanese rice fish (*Oryzias latipes*), (J) Endler's Guppy (*Poecilia wingei*), (K) Three-spined stickleback (*Gasterosteus aculeatus*), and (L) Mexican cavefish (*Astyanax mexicanus*). Several of these fish are used as model organisms for biomedical research. *Panel (A): Image courtesy of Isabelle Côté. Panel (B): Image source—Wikipedia. Panel (C): Image courtesy of Nico Michiels. Panel (E): Image courtesy of Isabelle Côté. Panel (F): Image courtesy of Nils Hartmann. Panel (H): Image courtesy of Walter Salzburger. Panel (I): Image courtesy of Matthew Harris. Panel (J): Image courtesy of Verena Kottler and Christine Dreyer. Panel (L): Image courtesy of Nicolas Rohner.*

owing its name to the striking stereotypic pattern of longitudinal blue and golden stripes on the flanks and on the anal and tail fins (Figs. 2 and 3A) (Kelsh, Harris, Colanesi, & Erickson, 2009; Parichy & Spiewak, 2015; Singh & Nüsslein-Volhard, 2015; Watanabe & Kondo, 2015). The adult striped pattern of zebrafish is composed of melanophores, iridophores, and xanthophores arranged in superimposed layers in the skin

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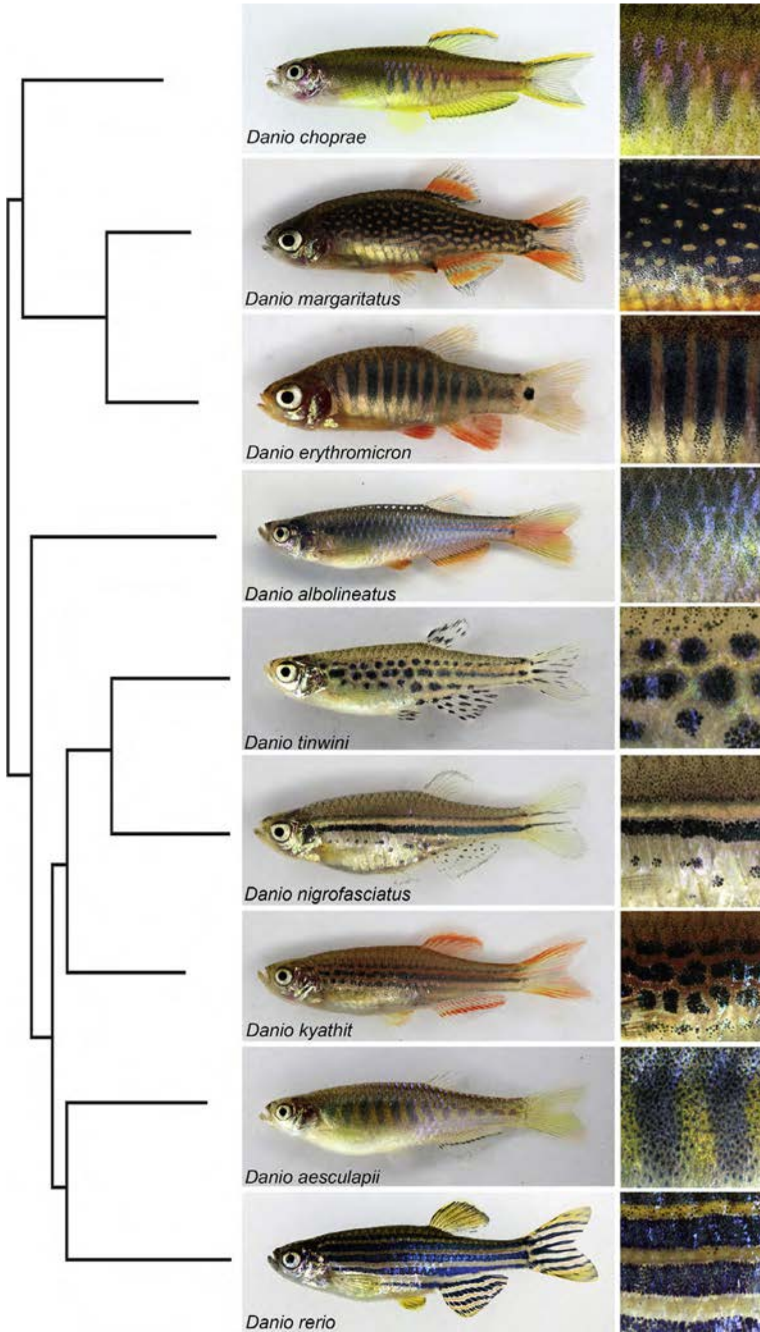

Figure 2 See legend on next page.

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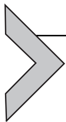
(Fig. 3A and B) (Hirata, Nakamura, Kanemaru, Shibata, & Kondo, 2003; Hirata, Nakamura, & Kondo, 2005). Development takes place outside the mother, allowing direct inspection at all stages. Most importantly, an increasing collection of spectacular adult-viable mutants with altered patterns has been accumulated (Table 1). These genes provide the basis of our understanding of the molecular mechanisms of color pattern formation. Novel approaches of long-term imaging of individual fish have been developed, which resulted in a detailed insight into the cellular and molecular background of stripe formation (Frohnhöfer et al., 2013; Singh & Nüsslein-Volhard, 2015; Singh, Schach, & Nüsslein-Volhard, 2014). The color patterns in related *Danio* species are amazingly different (Fig. 2) (McClure, 1999; Parichy, 2007; Parichy & Johnson, 2001); their variation offers a great opportunity to investigate the genetic and developmental basis of color pattern evolution in vertebrates, starting from detailed molecular and cellular investigations in one model species, *D. rerio*.



2. THE STRIPED COLOR PATTERN OF ZEBRAFISH

A common feature of the color patterns of basal vertebrates—fish, amphibians, and reptiles—is the layered organization of pigment cells in the skin, with xanthophores/erythrophores in the outermost layer primarily absorbing short-wavelength light, reflective iridophores/leucophores in the middle layer, and melanophores in the basal layer absorbing light across the full spectrum of wavelengths (Bagnara, Taylor, & Hadley, 1968; Grether, Kolluru, & Nersissian, 2004). Modifications of this standard color-forming unit lead to the particularly rich variation of color patterns in fishes. The striped pattern of the zebrafish body is composed of three distinct types of

Figure 2 Color pattern diversity within the genus *Danio*. The cladogram on the left shows the evolutionary relationship among different *Danio* species. Color patterns vary greatly even between closely related species; they range from almost uniformly distributed melanophores with a posterior red stripe on the trunk of *D. albolineatus*, over dark spots in *D. tinwini*, or stripes and spots on the trunk of *D. nigrofasciatus*, to vertical bars on the trunk of *Danio choprae* and *D. erythromicron* and light spots on a dark background in *D. margaritatus*. *D. rerio* (female) displays sharp boundaries between light and dark stripes. In *D. kyathit*, stripes are broken; *D. aesculapi*, the closest relative of *D. rerio*, has a pattern of irregular vertical bars dissolving into spots. Note the distinct motifs of fin patterning in several *Danio* species such as in *D. choprae* and *D. margaritatus*; the contiguous striped pattern of body and fins as seen in *D. rerio* is an exception. Rightmost panes show enlarged portion of the trunk skin. *Adapted from McCluskey and Postlethwait (2015).*

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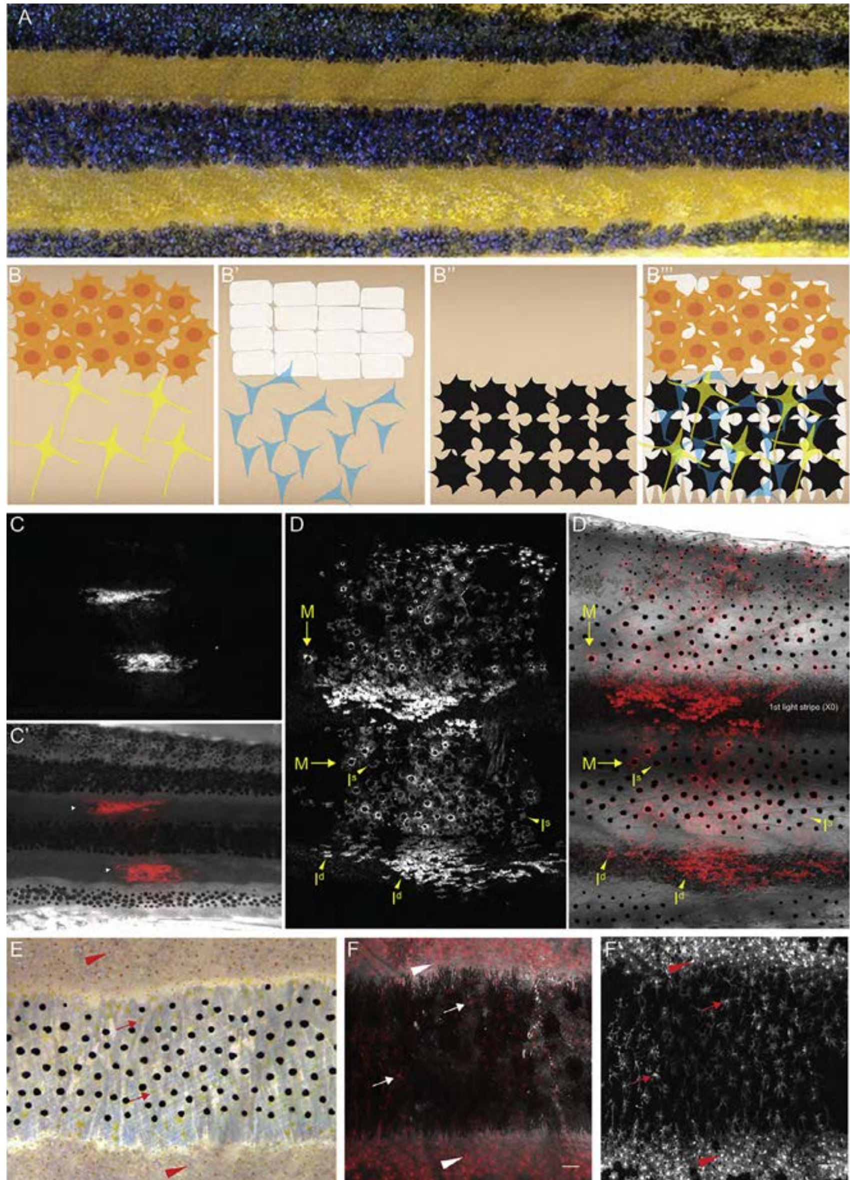

Figure 3 Layered organization of pigment cells in the trunk skin of zebrafish. (A) Close-up view of the lateral trunk of adult zebrafish displaying blue dark stripes and golden light stripes. (B–B‴) Schematic representation of the layered arrangement of pigment cells in the skin of the lateral trunk of zebrafish. The light stripe region consists of dense iridophores (white) covered by dense xanthophores (dark orange). In the stripe

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pigment cells—xanthophores, iridophores, and melanophores; leucophores are only present in the dorsal and tail fins. Electron microscopic analysis and observation of the zebrafish pigmentation using fluorescent markers reveal distinct differences in the layered organization of these chromatophores between the light and the dark stripe regions (Fig. 3) (Hirata et al., 2003, 2005; Mahalwar, Walderich, Singh, & Nüsslein-Volhard, 2014; Singh et al., 2014). In the light stripe region, compact and bright yellow/orange xanthophores cover a densely packed monolayer of iridophores. Such a layered arrangement imparts a golden iridescent appearance to the light stripes. In the dark stripes, the pigment cells are layered in the following fashion from top to bottom: stellate and faint yellow xanthophores, loose and bluish iridophores, black melanophores, and shiny L-iridophores (Fig. 3B). This arrangement of pigment cells provides a glittering blue coloration with a yellow hue to the dark stripe regions. Whereas xanthophores and iridophores exist in different shapes depending on their position, melanophores of the stripe occur in only one compact shape such that they fill the space almost entirely leaving little room between the cells (Fig. 3C–F) (Frohnhöfer et al., 2013; Hirata et al., 2003, 2005; Mahalwar et al., 2014; Singh et al., 2014). The exact superposition of the differently shaped chromatophores is crucial for the contrast and brightness of the pattern. In the fins, the stripe organization is simpler and essentially composed of alternating melanophores and compact xanthophores. Iridophores do not participate in the fin stripes, but line the fin rays.

Figure 3—Cont'd region, scattered xanthophores (stellate, yellow) and bluish reflective iridophores are present above a compact layer of black melanophores. Beneath melanophores, there is a layer of L-iridophores (white elongated cells in B‴). (C–C′) Overview of a *Tg(sox10:ER^{T2}Cre)*-induced clone (induced on 5 dpf) shows clonally restricted organization of iridophores in the adult skin. Clonally related iridophores exhibit characteristic spreading along the dorsoventral axis. (D–D′) Confocal image of a clone showing detailed organization of labeled pigment cells in the skin with dense iridophores (arrowheads; I^{d}) in the light stripe region, loose iridophores (arrowheads; I^{s}) and melanophores (arrows) in the dark stripe region. (E) Yellow–orange xanthophore pigment in the dark stripe (arrows) and light stripe regions (arrowheads) of the adult skin. Melanophores, compacted due to treatment with epinephrine, display a regular spacing in the dark stripe region. In (F–F′), melanophores are expanded. Xanthophores appear compact and densely packed in the light stripes (arrowheads) and appear stellate and loose in the dark stripe region (arrows).

Table 1 Genes Affecting the Color Pattern of Zebrafish

Gene (Protein)	Function	Required for/in	Refs.
Genes affecting one chromatophore type			
albino (Slc45a2) *golden* (Slc24A5) *sparse* (Kit a) *sparse-like* (Kit ligand a) *nacre* (Mitfa)	Solute carrier Solute carrier Receptor Ligand Transcription factor	Melanophores	a, b, c, d, e
pfeffer (Csf1ra)	Receptor	Xanthophores	f, g
shady (Ltk) *rose* (Endothelin receptor Ba) *rose-like* (Endothelin 3) *karneol* (Ece 2) *transparent* (Mpv17)	Receptor Receptor Ligand Enzyme Mitochondrial protein	Iridophores	h, i, j, k, l, m
Genes affecting stripe width and integrity			
leopard (Connexin 41.8)	Gap junction protein	Melanophores, xanthophores	n, o
luchs (Connexin 39.4)	Gap junction protein	Melanophores, xanthophores	p
schachbrett (Tjp 1A, ZO-1)	Tight junction protein	Iridophores	q
seurat (Igsf11)	Cell adhesion protein	Melanophores, xanthophores	r
obelix (Kcnj13)	Inwardly rectifying potassium channel	Melanophores	n, s
dali (Tetraspanin3c)	Transmembrane protein	Unknown	t
asterix (Notch 2)	Receptor	Melanophores	f, m
Genes affecting surrounding tissue			
idefix (Spermidine synthase)	Enzyme	Unknown	m
mau (Aqp3a)	Water channel	Unknown	u
bonaparte (Bnc2)	Zinc finger protein	Unknown	v, w
choker (Meox)	Transcription factor	Myotome	x, y
picasso/hypersensitive (Erbb3b)	Receptor	Dorsal root ganglia, PNS	z

a, Parichy, Rawls, Pratt, Whitfield, and Johnson (1999); b, Lister, Robertson, Lepage, Johnson, and Raible (1999); c, Lamason et al. (2005); d, Dooley, Mongera, Walderich, and Nüsslein-Volhard (2013); e, Dooley, Schwarz, et al. (2013); f, Haffter et al. (1996); g, Parichy, Ransom, Paw, Zon, and Johnson (2000); h, Parichy, Mellgren, et al. (2000); i, Lopes et al. (2008); j, Frohnhöfer, Krauss, Maischein, and Nüsslein-Volhard (2013); k, Krauss, Astrinidis, Fröhnhofer, Walderich, and Nüsslein-Volhard (2013); l, Krauss et al. (2014); m, Frohnhöfer et al. (submitted); n, Maderspacher and Nüsslein-Volhard (2003); o, Watanabe et al. (2006); p, Irion, Frohnhöfer, et al. (2014); q, Fadeev, Krauss, Fröhnhofer, Irion, and Nüsslein-Volhard (2015); r, Eom et al. (2012); s, Iwashita et al. (2006); t, Inoue, Kondo, Parichy, and Watanabe (2014); u, Eskova et al. (in preparation); v, Lang, Patterson, Gordon, Johnson, and Parichy (2009); w, Patterson and Parichy (2013); x, van Eeden et al. (1996); y, Nguyen et al. (2014); and z, Budi, Patterson, and Parichy (2008).

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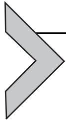

3. STRIPE FORMATION, A SELF-ORGANIZING PROCESS INVOLVING THE INTERACTION OF ALL THREE CELL TYPES

The precise longitudinal orientation of the stripes in adult zebrafish depends on the presence of the horizontal myoseptum serving as a morphological prepattern. In *choker* mutants, which lack the horizontal myoseptum, a meandering pattern of parallel dark and light stripes with random orientations but normal width and composition is formed (Fig. 4) (Frohnhöfer et al., 2013). This indicates that the horizontal myoseptum orients the stripes, whereas stripe formation is a self-organizing process depending on interactions between the pigment cells.

Based on the assumption that iridophores are not necessary for stripe formation, as they are not required in the striped fins, and present in both the dark and light stripes of the body, Turing-type models have been proposed describing stripe formation based on interactions between

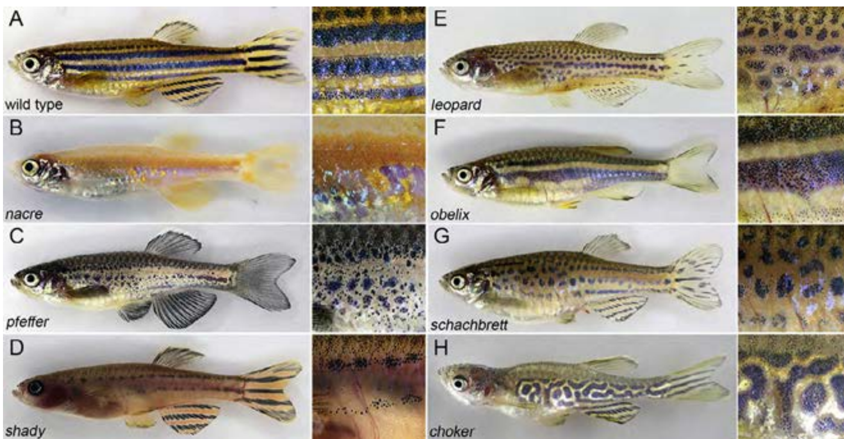

Figure 4 Selected adult-viable pigmentation mutants in zebrafish. (A) Wild-type adult zebrafish male. (B) *nacre* lacks all the neural crest-derived melanophores. (C) *pfeffer* lacks xanthophores, melanophores are reduced in number, and residual dark stripes are discontinuous, displaying a pepper and salt pattern. (D) *shady* mutants lack iridophores and display only two rudimentary melanophore stripes that are broken into spots. Fins are striped. (E) In *leopard*, the stripes break up into spots. (F) *obelix* displays broader and fewer dark stripes and (G) *schachbrett* shows disrupted stripes and spots. (H) In *choker*, which lacks the horizontal myoseptum, a meandering pattern with stripes of normal width is formed.

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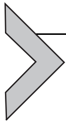
xanthophores and melanophores only (Nakamasu, Takahashi, Kanbe, & Kondo, 2009; Yamaguchi, Yoshimoto, & Kondo, 2007; Yamanaka & Kondo, 2014). This view has been challenged recently by the finding that for the formation of trunk stripes iridophores are absolutely necessary: in mutants lacking iridophores, no dark stripes beyond rudimentary first two stripes are formed in the body, whereas the fins are normally striped (Fig. 4, *shady*) (Frohnhöfer et al., 2013; Haffter et al., 1996; Krauss et al., 2013, 2014; Lopes et al., 2008). In mutants lacking melanophores or xanthophores, residual stripes between iridophores and the remaining cell type are formed in the body, but no stripes are formed in the fins (Fig. 4, *nacre, pfeffer*) (Haffter et al., 1996; Lister et al., 1999; Odenthal et al., 1996). Several mutants display different phenotypes in body and fin stripes. This suggests that models for stripe formation in the body have to be modified substantially to incorporate the recent experimental and genetic data, in particular the dominant role of iridophores (Singh, Fröhnhofer, Irion, & Nüsslein-Volhard, 2015).

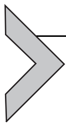
Analyses of mutants missing one single pigment cell type have provided crucial insights into pigment cell interdependency and interactions for their development, survival, and stripe pattern formation. Animals depleted in one pigment cell type, such as melanophores in *nacre*, xanthophores in *pfeffer*, or iridophores in *shady*, *rose*, or *transparent*, initiate stripe morphogenesis but the patterns formed by the two remaining cell types are highly abnormal (Fig. 4). The analysis of chimeric animals obtained by blastomere transplantations indicated a cell-autonomous requirement for all these genes, which means that the abnormal behavior of the remaining two cell types observed in the mutants is indirectly caused by the absence of a given cell type. The analysis of double mutants lacking two types of pigment cells revealed that any single chromatophore alone cannot form a pattern; the residual cells evenly cover the zebrafish trunk (Frohnhöfer et al., 2013). This shows that, on its own, each pigment cell type has a tendency to uniformly cover the skin and that the stable stripe pattern emerges due to restrictions imposed by interactions within and between the three pigment cell types; all three chromatophore types have to communicate and interact to form the final striped pattern. Dense iridophores attract xanthophores, locally suppress melanophore survival but promote it at a distance. Compact xanthophores and melanophores exhibit mutual repulsion at a short range, but they are required for melanophore survival at a distance. These observations point to distinct roles of the three pigment cell types in the process of pattern formation. For a thorough understanding of the

mechanisms, it is required to investigate the origin of the pigment cells and the process of stripe formation during metamorphosis *in vivo*.



4. DEVELOPMENTAL ORIGIN OF THE THREE PIGMENT CELL TYPES

The larval pigment cells develop directly from the neural crest, delaminate during early development, and migrate to the dorsal, lateral, and ventral aspect of the embryo to form a simple striped larval pattern based on morphological landmarks (Eisen & Weston, 1993; Kelsh et al., 1996). The striped pattern of the adult zebrafish arises considerably later during a metamorphic period that begins about 3 weeks postfertilization and lasts for about 1 month. During this time period, the shape of the fish undergoes a substantial reorganization with the appearance of scales and the adult set of fins replacing the larval fin fold. Stripes sequentially appear in the skin, starting with the first light stripe along the horizontal myoseptum. Lineage tracing has revealed that the adult xanthophores descend directly from larval xanthophores, which evenly cover the body and start to proliferate just before the onset of metamorphosis (Mahalwar et al., 2014; McMenamin et al., 2014). In contrast, adult iridophores and melanophores develop from segmentally reiterated, peripheral nerve-associated multipotent stem cells that have been set apart during early development (Fig. 5A–C) (Budi, Patterson, & Parichy, 2011; Dooley, Mongera, et al., 2013; Singh et al., 2014). Sox10-Cre-induced lineage labeling revealed that stem cells may give rise to glia, peripheral neurons, and all three kinds of chromatophores, predominantly iridophores (Singh et al., 2014). Iridophores emerge along the horizontal myoseptum, proliferate, and spread in the skin (Fig. 5C), whereas melanophore progenitors divide while they migrate along peripheral nerves (Fig. 5B). The melanophores hardly proliferate or migrate after they have reached the skin.



5. FORMATION OF A SERIES OF LIGHT AND DARK STRIPES BY PATTERNED AGGREGATION OF IRIDOPHORES

Stripe morphogenesis begins at the onset of metamorphosis. Thyroid hormone acts as a trigger for metamorphosis and also regulates xanthophore proliferation and differentiation (McMenamin et al., 2014). *In vivo* imaging over extended time periods revealed a leading role of iridophores in stripe

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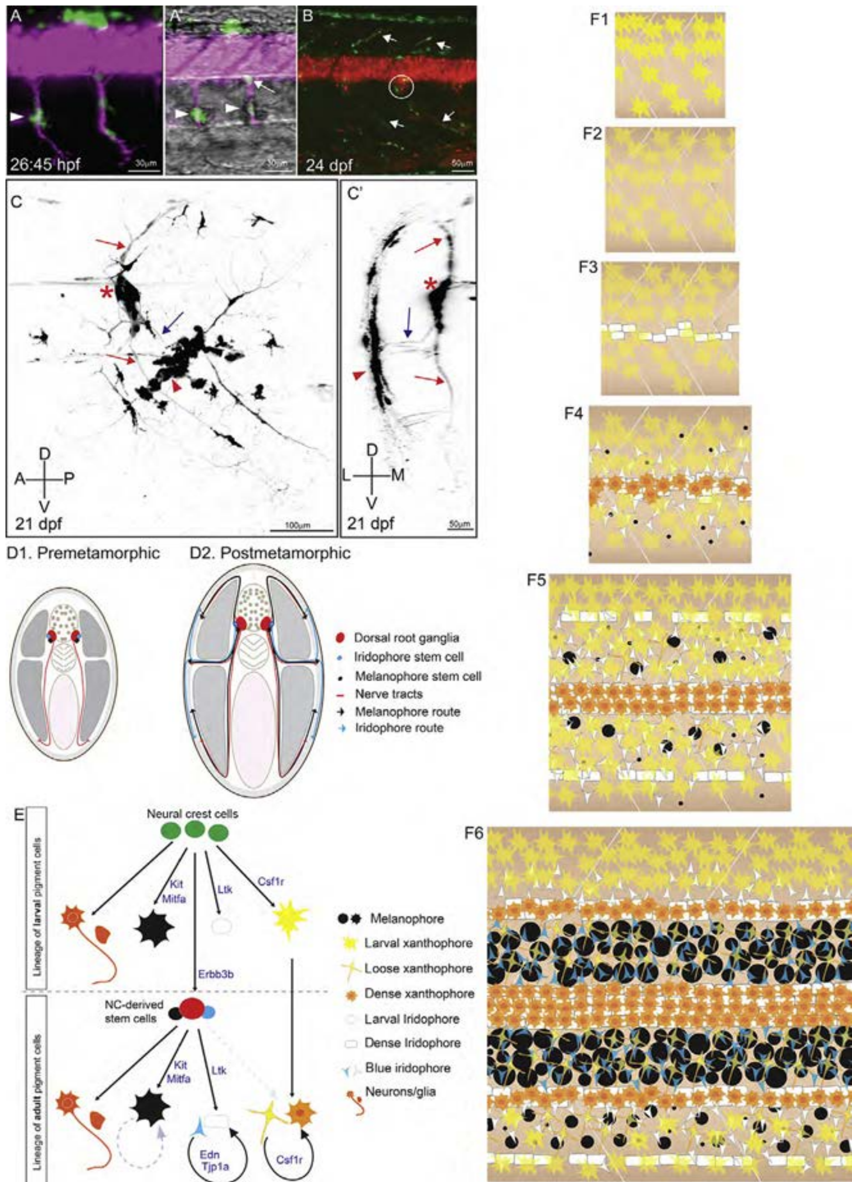

Figure 5 Pigment cell lineage, migration, and stripe pattern formation. (A–A′) During embryogenesis, melanoblasts (Mitfa-positive, green cells) migrate along the primary motor neuron (magenta) and differentiate into larval melanophores (arrowheads in A′). Arrow in (A′) indicates a Mitfa-positive cell that remains at a ventral position of the neural tube, the melanophore stem cell. (B) During metamorphosis, Mitfa-positive (*Continued*)

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formation. Iridophore progenitors migrate along peripheral nerves through the horizontal myoseptum into the skin where they continue to proliferate and spread, leading to the formation of a contiguous sheet of dense iridophores and the formation of the first light stripe (Singh et al., 2014). Interestingly, there is no clonal restriction of iridophores between segments,

Figure 5—Cont'd melanophore progenitor cells are seen at the dorsal root ganglion (encircled) and along the spinal nerves extending dorsally and ventrally to innervate the lateral skin (arrows). (C–C′) A clone of iridophores (arrowhead) associated with the dorsal root ganglion (asterisk). Blue arrows in (C–C′) indicate the neurons that exit through the horizontal myoseptum; red arrows indicate the neuronal tracts associated with the dorsal and ventral myotomes and the myosepta. (C′) The clone in (C) turned by 90° to show a cross section through the trunk region. Skin is to the left. Abbreviations: D: dorsal, V: ventral, A: anterior, P: posterior, L: lateral, M: medial, hpf: hours postfertilization, and dpf: days postfertilization. (D1–D2) Schematic showing the route followed by melanophore progenitors along the peripheral neurons innervating the skin, whereas iridophores migrate through the horizontal myoseptum and disperse dorsoventrally in the skin. (E) Lineage and the postembryonic source of adult pigment cells. Neural crest cells give rise to several larval cell types including neural tissue and develop directly into the three kinds of larval pigment cells in zebrafish. Adult melanophores, iridophores, and neural tissue are derived from postembryonic stem cells, which are located on the segmentally iterated dorsal root ganglia. Larval xanthophores persist and proliferate at the onset of metamorphosis to generate adult xanthophores. Metamorphic iridophores and xanthophores undergo local proliferation in the skin. In rare cases, pigmented melanophores are seen to divide in the skin (faint circular arrow). A fraction of xanthophores may arise from mixed iridophore clones (faint arrow). Genes required for specific lineages are mentioned in blue along the arrows. (F1–F6) Schematics showing stripe pattern morphogenesis during the transition from the larval stage to the adult stage. (F1) Larval xanthophores (5 dpf) (F2) begin to proliferate at the onset of metamorphosis (2 weeks postfertilization; wpf). (F3) Iridophores appear in the skin through the horizontal myoseptum and organize the first light stripe (~3 wpf). (F4) Melanophores develop from the melanoblasts, which enter the skin through the dorsal and the ventral myotomes (~3–4 wpf). Over the dense iridophores, xanthophores become compact and acquire yellow–orange pigment. Loose iridophores appear at the borders of the dense iridophore region and begin to disperse along the dorsoventral axis. (F5) Melanophores grow in size in the stripe region to form contiguous stripes. Loose iridophores aggregate to organize new light stripes at a distance from the first light stripe (~4–5 wpf). (F6) Xanthophores become stellate over the melanophores and compact in the light stripe regions. Loose iridophores on top of melanophores appear bluish and reflective giving blue color to the dark stripes. Light stripes appear golden due to the presence of silvery dense iridophores covered by yellow–orange xanthophores.

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and iridophores derived from a single stem cell distribute over two to four segments along the anterior–posterior axis (Fig. 3C and D) (Singh et al., 2014). After the formation of a first light stripe, iridophores continue to proliferate and spread as blue iridophores bidirectionally in the skin toward the dorsal and ventral aspect of the juvenile fish. They form a loose net of cells covering the flank of the fish, in which additional light stripes appear by patterned aggregation of iridophores into the dense form at a given distance from the first light stripe (Figs. 3C and D and 5F1–F6). By cell division, migration, and cell shape transition, iridophores form new light stripes along the dorsoventral axis, prefiguring the region in which dark stripes are formed after the addition of each new light stripe. Consistent with this, mutants lacking iridophores do not add new light or dark stripes beyond a basic pattern of one light stripe (composed of xanthophores only) and two rudimentary dark stripes broken into spots (Frohnhöfer et al., 2013; Krauss et al., 2013, 2014).

At the cellular level, the process of iridophore shape transition and migration appears qualitatively similar to epithelial-to-mesenchymal transitions (EMT) and mesenchymal-to-epithelial transitions (MET). EMT/MET are important cellular shape changes that underlie several events including migration of neural crest cells in the embryo or cancer progression (Kerosuo & Bronner-Fraser, 2012; Polyak & Weinberg, 2009). While it remains to be seen if there are similarities at the levels of gene regulation and cytoskeletal remodeling between EMT/MET and the iridophore behavior, it is interesting to find parallels in terms of cellular behaviors. The recent cloning and characterization of *schachbrett* (German word for checkerboard), a mutant displaying a spotted phenotype (Fig. 4), provide a first clue for the elements of the cytoskeletal machinery that may underlie cell shape transition of iridophores during pattern formation (Fadeev et al., 2015). The *schachbrett* locus encodes Tight junction protein 1a (Tjp1a), a zebrafish homolog of mammalian ZO-1. Tjp1a is specifically expressed in the densely packed iridophores of the light stripe and its expression is lost in loose migratory iridophores. The presence of Tjp1a allows the cells to undergo shape changes from a dense to a loose form during stripe formation. The failure of this change to occur leads to the invasion of iridophores in their dense shape into the dark stripe regions resulting in a spotted phenotype.

While iridophores lay out the first light stripe, melanoblasts, the unpigmented precursors of melanophores, reach the skin in the presumptive dark stripe regions in close association with the peripheral nerves

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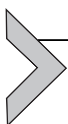
(Fig. 5B and D) (Budi et al., 2011; Dooley, Mongera, et al., 2013; Singh et al., 2014). In contrast to iridophores, melanophores do not proliferate in the skin but dramatically expand in size to form a contiguous sheet of cells, thus forming the dark stripe (Figs. 3E and F and 5F) (Singh et al., 2014). Hence, the dark and light stripes are formed by rather different cellular behaviors. Xanthophores covering the skin as a coherent sheet start to proliferate at the onset of metamorphosis and undergo marked local reorganization acquiring a flat shape with small cytoplasmic protrusions over the dense iridophores of the light stripes, and a stellate shape with long cytoplasmic protrusions and faint pigmentation upon encountering melanophores (Fig. 3G) (Mahalwar et al., 2014). This leads to the exact superposition of the diverse cell types and shapes required for the proper coloration of the pattern. On their own, iridophores cover the entire body uniformly in the dense form (in *nacre; pfeffer* double mutants), indicating that interactions with xanthophores and melanophores are necessary for the appropriate shape changes during stripe formation (Frohnhöfer et al., 2013; Irion, Frohnhöfer, et al., 2014; Maderspacher & Nüsslein-Volhard, 2003; Nakamasu et al., 2009; Patterson & Parichy, 2013; Yamaguchi et al., 2007). Only iridophores exhibit long distance migration in the skin along the dorsoventral axis (Singh et al., 2014), suggesting that these cells in response to the other two pigment cell types may provide the cues to form new light and dark stripes.

Our present view is that the transition between the loose and dense forms of iridophores prefigures the formation of the striped pattern, which is further refined by short- and long-range interactions between chromatophores across the three layers. We propose that pattern variations among closely related species are caused by changes in the spatial parameters regulating the transitions in chromatophore organization.



6. GENETIC AND MOLECULAR REGULATION OF COLOR PATTERN FORMATION

At the cellular level, the process of stripe formation involves several behaviors such as cell type specification, proliferation and spreading, long-/short-range migration of pigment cells, cell shape transition, and acquisition of appropriate pigmentation. Mutant analysis helps to dissect these processes and provides an insight into the molecular basis of cellular interactions (Table 1).

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6.1 Regulation of Pigment Cell Proliferation and Maintenance by Distinct Signaling Systems

The analysis of mutants that specifically lack one type of chromatophore has revealed that different receptor–ligand pairs regulate specification, maintenance, and proliferation of each of the pigment cell types. Most melanophores depend on Kit-signaling; mutations in the receptor (the orthologue of c-Kit, encoded by the gene *sparse*) or the ligand (Kit ligand a, encoded by *sparse-like*) lead to a severe reduction in the number of melanophores (Dooley, Mongera, et al., 2013; Parichy et al., 1999). Leukocyte tyrosine kinase (*shady*) is essential for the specification of iridophores (Lopes et al., 2008). Endothelin signaling is required for dense iridophore development; mutations in endothelin receptor b1 (*rose*) (Parichy, Mellgren, et al., 2000), in endothelin-converting enzyme 2 (*karneol*) (Krauss et al., 2014), or in endothelin 3b (U. I., unpublished) all lead to an absence of dense iridophores in the mutant animals, while loose iridophores are present. Xanthophores depend on colony-stimulating factor signaling; mutations in *pfeffer* (Haffter et al., 1996), which codes for colony-stimulating factor 1 receptor A, lead to the absence of xanthophores (Parichy, Ransom, et al., 2000). Melanophores, in addition, receive unknown cues for maintenance and survival from dense iridophores and xanthophores, whereas the other two cell types develop in a more autonomous manner (Frohnhöfer et al., 2013; Parichy, Ransom, et al., 2000; Walderich, Singh, Mahalwar, & Nüsslein-Volhard, submitted).

Evidence from chimeric animals, where cells of different genetic compositions are juxtaposed, suggests that homotypic cell–cell interactions (interactions between pigment cells of the same kind) regulate pigment cell proliferation and dispersal. Clones of pigment cells that develop in hosts lacking this cell type proliferate more and disperse further compared to similar clones in chimeric animals where the endogenous cells are also present (Walderich et al., submitted). Homotypic competition determines the number, direction of migration, and the individual spacing of pigment cells; it is responsible for the predominant dorsoventral spread of pigment cells of all three kinds during metamorphosis observed in cell lineage experiments (Singh et al., 2014; Walderich et al., submitted). For example, postembryonic iridophore clones generally contribute to all stripes along the dorsoventral axis, but only to about two segments along the anteroposterior axis (Fig. 3C and D).

Pigment cell shape transitions involved in the formation of the striped organization are regulated by more complex heterotypic cell–cell

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interactions (interactions between pigment cells of different kind) (Fadeev et al., 2015; Irion, Frohnhöfer, et al., 2014). To understand the molecular nature of these interactions between the chromatophores, it is helpful to analyze mutants, in which all three cell types are present, but nevertheless stripe formation is not normal. These mutants affect stripe integrity or stripe width.

6.2 Formation of Contiguous Boundaries Between the Light and the Dark Stripes

Stripe integrity with straight boundaries between the dark and light stripes is affected in *leopard, luchs*, *seurat*, and *schachbrett* mutants. All of them are likely to affect contact-dependent cell–cell interactions leading to an expansion of the light stripe regions and an interruption and suppression of the dark stripes. *leopard* and *luchs* code for two different connexins, Cx41.8 and Cx39.4, respectively (Irion, Frohnhöfer, et al., 2014; Watanabe et al., 2006). In both cases, dominant and recessive alleles lead to spotted patterns where light stripe regions invade the dark stripes, whereas the pigment cell composition and layering in the dark and light regions is maintained. Connexins are the subunits of gap junctions, intercellular channels that form by the docking of two hemi-channels from neighboring cells (Kumar & Gilula, 1996; Saez, Berthoud, Branes, Martinez, & Beyer, 2003; Simon & Goodenough, 1998). Both connexins, encoded by *leopard* and *luchs*, are required in melanophores and xanthophores, but not in iridophores (Irion, Frohnhöfer, et al., 2014; Maderspacher & Nüsslein-Volhard, 2003). In double mutants for both connexins, the pattern is completely lost with a reduced number of melanophores appearing as individual cells on a homogeneous background of dense iridophores covered by compact xanthophores. It is suggested that the two connexins form heteromeric gap junctions in and between melanophores and xanthophores resulting in cell–cell communication and ultimately in the instruction of iridophores to undergo a transition from the dense into the loose form characteristic for the dark stripe (Irion, Frohnhöfer, et al., 2014). The mechanism of communication between melanophores and xanthophores on the one hand and iridophores on the other is still unknown. Genetic evidence suggests that in the absence of one of the two connexins homomeric channels can form that still retain some functionality and lead to the formation of spots. Interestingly, in *luchs* the fins are normally striped, whereas in *leopard* no stripes are formed in the fins supporting the notion of a different requirement for fin and body stripes. The short (23 amino acid residues) N-terminal cytoplasmic tail of Cx41.8 is essential for its function. It contains a short motif (ExxxE) predicted to lead

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to polyamine-sensitivity, suggesting that rectification properties of the gap junctions are important for their function (Watanabe, Watanabe, & Kondo, 2012). Several connexins are known to interact with ZO-1 (Giepmans & Moolenaar, 1998; Nielsen et al., 2003), encoded by the gene affected in *schachbrett* mutants (see above). Because *schachbrett* function is specifically required in iridophores, it has been suggested that different, as yet unknown, connexins might be expressed in these cells and involved in the patterning process (Fadeev et al., 2015).

In *seurat* mutants, Igsf11 (immunoglobulin superfamily member 11), a classical cell adhesion molecule of the immune globulin superfamily, is affected (Eom et al., 2012). Igsf11, which is specifically required in melanophores, mediates adhesive interactions between cells and promotes migration and survival of melanophores. It was suggested that differential adhesive properties of the pigment cells might be required for the formation of the pattern.

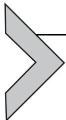
6.3 Determination of the Domain of the Light and the Dark Stripe Regions

In *obelix* (aka *jaguar*) mutants, the number of dark stripes is reduced, they are broader, and xanthophores do not adapt their appearance in the presence of melanophores (Haffter et al., 1996). The function of *obelix*, which codes for an inwardly rectifying potassium channel, Kir7.1/Kcnj13, is exclusively required in melanophores (Iwashita et al., 2006; Maderspacher & Nüsslein-Volhard, 2003). It is assumed that dysfunction of the channel leads to a constant depolarization of the plasma membrane in the mutant melanophores resulting in defects in the repulsive signaling between xanthophores and melanophores (Inaba, Yamanaka, & Kondo, 2012). Experiments with isolated cells *in vitro* suggest that *obelix* mutant melanophores are unable to respond appropriately to contact by xanthophores, which in wild-type cells lead to chase-and-run movements (Yamanaka & Kondo, 2014). The dominant phenotype of *obelix* mutations may be caused by haploinsufficiency for the gene (Maderspacher & Nüsslein-Volhard, 2003). Double mutants of *obelix,* and *leopard* or *luchs* show a strong enhancement of the phenotype with a complete loss of the pattern, arguing for a possible link between the two pathways (Irion, Frohnhöfer, et al., 2014; Maderspacher & Nüsslein-Volhard, 2003).

Stripe width is also affected by manipulations of the Notch signaling pathway; melanophore-specific overexpression of the ligand Delta C or the intracellular domain of Notch 1A results in fewer and wider dark stripes

(Hamada et al., 2014). Delta/Notch signaling promotes melanophore survival and it has been shown that melanophores, expressing the Notch 1A and Notch 2 receptors, extend long projections toward xanthophores, which in turn express the ligands Delta C and Delta-like 4. Our own observations show that a specific point mutation in Notch 2, likely leading to the overactivation of the signaling pathway, is causing the dominant *asterix* phenotype, which is also characterized by fewer and wider dark stripes (Haffter et al., 1996).

These genetic and molecular data suggest that the determination of the stripe width, the formation of the straight boundaries between the light and dark stripes, and the patterned aggregation of iridophores, which defines the stripe areas, are all mediated by direct cell contacts among the chromatophores. This mode of pattern formation, occurring over the three distinct cell layers, is very special and quite different from other patterning processes where mainly secreted extracellular signaling molecules and their membrane-bound receptors are involved. However, not all pigment cells in the zebrafish occupy regions that are striped and it is evident that the tissue environment provides additional cues for the behavior of chromatophores in the distinct body regions.



7. THE ROLE OF TISSUE ENVIRONMENT IN REGULATING COLOR PATTERN FORMATION

Pigment cells are present in several regions of the body including the dorsum, trunk hypodermis, head, fins, intestine, and abdominal wall (Hirata et al., 2005), as well as on the epidermis of the scales. However, stripes are only formed in the trunk region, and in the anal and caudal fins, while in other regions the cell types appear singly or mixed in a more or less random pattern. This suggests a role for the tissue environment in providing a context permissible for stripe morphogenesis. So far, we have no clue on the mechanisms determining the different body regions imposing different interaction rules. Recently, based on expression pattern and *gain-of-function* analysis, it has been suggested that the dorsoventral countershading, a pattern of dark dorsum contrasting with a light ventrum that is observed in many vertebrates including zebrafish, is a process that utilizes agouti signaling and that is largely independent of stripe morphogenesis (Ceinos, Guillot, Kelsh, Cerda-Reverter, & Rotllant, 2015).

Mutations in several genes have been identified that are not required in the pigment cells themselves but in other cells to allow the generation of the

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striped pattern. Some of them encode extracellular ligands for the receptors expressed in the chromatophores, or are responsible for the processing of these ligands (Table 1). These secreted signaling molecules generally affect the respective pigment cells in the entire body, not just the striped regions. Owing to generally low abundances, their distributions are largely unknown.

In other cases, the mechanisms by which the surrounding tissues affect stripe morphogenesis are more complex. In *bonaparte*, the affected gene encodes *basonuclin2*, a nuclear localized zinc finger protein of unknown function (Lang et al., 2009). In the *bonaparte* mutants, the numbers of all the three pigment cell types are highly reduced, and the phenotype is presumably caused by compromised interactions between the chromatophores and the tissue environment (Lang et al., 2009; Patterson & Parichy, 2013). Mutations in the *mau* gene are dominant and cause a spotted pattern with irregular stripe boundaries. *mau* encodes Aquaporin3a, a plasma membrane water channel (Eskova et al., in preparation). Chimeric analyses show that this channel is not required in any of the three pigment cell types but affects an as yet unidentified surrounding tissue. It is not yet clear how a water channel may influence pigment patterning and in which tissue the gene is required. A mutant with fewer dark stripes, but wider light stripes, is *idefix*. The affected gene encodes the enzyme spermidine synthase (U. I., in preparation), which is responsible for the biosynthesis of the polyamine spermidine from putrescine and decarboxylated *S*-adenosyl-methionine. Polyamines, spermidine and its derivative spermine, are important regulators for the gating properties of inwardly rectifying potassium channels (Ficker, Taglialatela, Wible, Henley, & Brown, 1994) and gap junctions (Musa & Veenstra, 2003), underscoring the role of these molecules mediating direct contacts between pigment cells in the patterning process discussed above. We hypothesize that these components modulate the environment such as to allow appropriate function of these channels between pigment cells in all three layers.

An instructive role for the pigment cell environment in determining pigmentation patterns has been demonstrated in mice: dedicated pigment recipient epithelial cells recruit melanocytes to their position in the skin and induce melanin transfer (Weiner et al., 2007). In this context, the mammalian skin has been compared to a coloring book in which the blueprint is created by the pigment recipient cells and the melanocytes fill in the color by transferring melanin (Weiner, Fu, Chirico, & Brissette, 2014). Thus, the tissue environment has an important role in setting up the cues for proper development and organization of the pigment cells in the skin.

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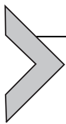
The anatomy clearly has an influence on the final stripe pattern on the trunk as exemplified by the analysis of the zebrafish *choker* mutant that lacks the horizontal myoseptum (Frohnhöfer et al., 2013). In fins, which lack muscles that underlie the hypodermis, the stripes have a different pigment cell composition and arrangement, suggesting the mode of pattern formation differs from that in the body. In the epidermis of the scale margins, all three pigment cells are mixing, and the density decreases from dorsal scales to ventral scales, which are devoid of pigment cells. In conclusion, in different body regions the rules of interaction between pigment cells vary, but the molecular basis determining the local differences is largely unknown.



8. EVOLUTION OF COLOR PATTERNS

The color patterns in closely related *Danio* species are amazingly different (Fig. 2) (McClure, 1999; Parichy, 2006; Parichy & Johnson, 2001). The patterns vary in stripe number, width, and orientation, as well as in stripe integrity, ultimately leading to spotted patterns (Fig. 2). In *Danio albolineatus*, almost no pattern is formed, only a short posterior remnant of the central light stripe persists. Strikingly, only in *D. rerio* the stripes in the body and fins are contiguous and the pattern appears periodic along the dorsoventral axis. Other species display repeated vertical bars along the anterior–posterior axis, a frequent motif in fish patterning (see also Fig. 1). In some species, the set of dorsal, anal, and tail fins displays a common theme, quite distinct from that of the body. This pattern variation offers a great opportunity to investigate the genetic and developmental basis of color pattern evolution in vertebrates, starting from detailed molecular and cellular investigations in one model species, *D. rerio*. Viable hybrids between zebrafish and a number of other *Danio* species have been generated; they all show a striped pattern, demonstrating that the zebrafish pattern forming mechanism is dominant (Fig. 6) (Parichy & Johnson, 2001). The hybrids are sterile, thus precluding most genetic experiments; however, exciting technical developments of the recent years, especially next-generation sequencing technologies and the novel possibilities of genome editing with the CRISPR/Cas9 system (Hwang et al., 2013; Irion, Krauss, & Nüsslein-Volhard, 2014; Jinek et al., 2012), allow for the first time to easily expand from model organisms into other species and directly test the function of genes by targeted knockouts. We have already generated mutants in *D. albolineatus* lacking melanophores, xanthophores, or iridophores, by

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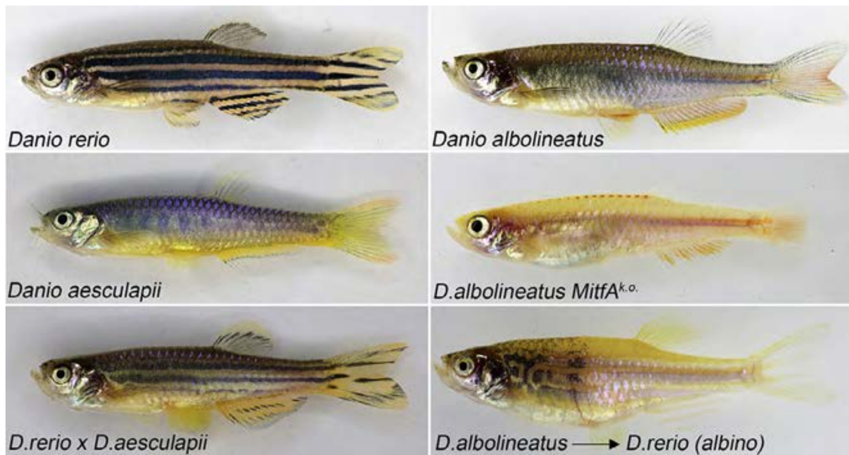

Figure 6 Interspecies hybrid and chimera. Hybrids between *Danio aesculapii* and *Danio rerio* (zebrafish) show a striped pattern very similar to zebrafish; the fins are partially striped. Knockout mutations in *MitfA* in *Danio albolineatus* lead to a loss of melanophores; the patterning, however, is not affected. In chimeric animals obtained by blastomere transplantation, the melanophores derived from *D. albolineatus* can contribute to the stripes of *D. rerio*.

knockouts in the genes coding for MitfA, Csf1r, and Ltk (U. I., unpublished). This demonstrates that the signaling pathways for cell specification, proliferation, and maintenance are conserved between zebrafish and *D. albolineatus*. To identify conserved cellular behaviors, heterologous cell transplantations between the different species can be employed. Transplantations of wild-type blastomeres from *D. albolineatus* into *albino* mutant zebrafish embryos result in chimeras in which the (pigmented) *D. albolineatus* donor melanophores integrate into the (unpigmented) stripes of the host animal (Fig. 6), demonstrating that the melanophores from *D. albolineatus* can correctly respond to the patterning cues present in zebrafish, although they would not produce a striped pattern in their normal environment. The next challenge is to identify genes crucial for the pattering process and exchange alleles of candidates between related species using the CRISPR/Cas9 system.

Understanding color pattern formation in birds and mammals presently rests almost entirely on theoretical considerations because their inclusion into eggs or the womb of a gravid female provides very limited imaging possibilities and makes them hardly accessible to experimental manipulations. Although in mammals there is only one pigment cell type, many

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mammals display patterns composed of two or more colors distributed in vertical stripes or spots. This suggests that there are different types of melanocytes forming the patterns, reminiscent to the most frequent pattern motifs in fish (Watanabe & Kondo, 2012). As fish, amphibians, and reptiles have at least three different pigment cell types, one important question concerns the evolutionary relationship between mammalian melanocytes and the chromatophores of the more basal vertebrates. Iridophores in zebrafish depend on endothelin signaling, as do melanocytes in mammals (Baynash et al., 1994; Frohnhöfer et al., 2013; Krauss et al., 2014; Parichy, Mellgren, et al., 2000). It may not simply have been a case of loss of pigment cell types other than melanophores along the avian/mammalian lineages, but it is conceivable that other pigment cell types acquired the function of melanin production. Stripes in tigers, cats, and zebras may originate from stem cells distributing the melanocytes along the segmentally iterated spinal nerves (Adameyko et al., 2009), possibly explaining their vertical orientation along the dorsoventral body axis, which we also observe in the spreading of the melanophores and iridophores in zebrafish. Although at the level of pigment cells there are profound differences, genes that regulate pigment cell development and color pattern formation are highly conserved between fish, birds, and mammals. Therefore, the studies of zebrafish pigmentation will lay the foundation to understand not only the genetic basis of color pattern variation in fishes but also the evolution of color patterns in other vertebrates.

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