Prospects & Overviews

How fish color their skin: A paradigm for development and evolution of adult patterns

Multipotency, plasticity, and cell competition regulate proliferation and spreading of pigment cells in Zebrafish coloration

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Pigment cells in zebrafish − melanophores, iridophores, and xanthophores − originate from neural crest-derived stem cells associated with the dorsal root ganglia of the peripheral nervous system. Clonal analysis indicates that these progenitors remain multipotent and plastic beyond embryogenesis well into metamorphosis, when the adult color pattern develops. Pigment cells share a lineage with neuronal cells of the peripheral nervous system; progenitors propagate along the spinal nerves. The proliferation of pigment cells is regulated by competitive interactions among cells of the same type. An even spacing involves collective migration and contact inhibition of locomotion of the three cell types distributed in superimposed monolayers in the skin. This mode of coloring the skin is probably common to fish, whereas different patterns emerge by species specific cell interactions among the different pigment cell types. These interactions are mediated by channels involved in direct cell contact between the pigment cells, as well as unknown cues provided by the tissue environment.

Keywords:
- cell competition; coloration; multipotent stem cells; neural crest; pattern formation; peripheral nervous system; zebrafish

Introduction

The striking beauty and diversity of fish color patterns fascinate not only biologists. Despite their importance as targets for both, natural and sexual selection, little is known about the development and evolution of color patterns. Color patterns are traits emerging during post-embryonic development, and their study is therefore difficult due to the long timescale of juvenile growth and the size of adult animals. Colors and color patterns are particularly rich in fishes, which represent ideal models to investigate in vivo many aspects of coloration that are of general interest for biochemistry and cell biology. Examples include the physicochemical basis of biological colors, the cellular interactions involved in the formation of color patterns which serve as signals in social behavior, and the ability of rapidly changing coloration for camouflage, mimicry, and sexual display.

Color patterns serve as signals in social behavior

The primary functions of pigments in animals are to shield against sunlight and hide from predators. Many fish such as herring are cast in a silvery light-reflecting skin, with a dark back and light belly to render them less visible when viewed from top or bottom, respectively. These features are of adaptive value and presumably evolved through natural selection. Other colorations are more difficult to explain by natural selection, because they are costly for the individuals,

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and may render them more conspicuous. Darwin proposed a second mechanism of selection for the evolution of such traits: sexual selection [1]. In these cases it is not the fitness of the individual but the reproductive success that counts. A specific feature of these color patterns is that they serve as triggers for stereotyped, instinctive behaviors [2, 3]. A famous example is the red throat of the male stickleback, which evokes a fighting behavior in other males leading to even spacing of nests within the territory. The red coloration also attracts females to the nest, with a clear correlation between redness and mating success. Astonishing color patterns are also observed in several monogamous coral-reef fish in which the individuals of both sexes aggressively fight fish of the same pattern/ species while completely disregarding fish displaying different patterns in these heavily populated environments [3]. In many instances, color patterns serve as recognition signals in fish living in social groups or shoals such as the zebrafish.

Stripe pattern of Zebrafish as a model to study vertebrate coloration

The zebrafish (*Danio rerio*) has emerged as an important vertebrate model organism as it allows the study of many aspects of animal biology with a rich spectrum of approaches and methods that include genetic manipulations as well as long term imaging [4–6]. Zebrafish live in small streams and ponds in hot climates in India and Southeast Asia. The color pattern of the adult zebrafish is composed of a series of blue and golden horizontal stripes covering the body and the anal- as well as tail fin [7–10]. Closely related *Danio* species with very similar shapes, lifestyle and geographical distribution display strikingly different adult pigment patterns [11, 12]. For example, *Danio albolineatus* is not striped, and *Danio aesculapii*, suggested to be the closest relative of *D. rerio*, displays vertical bars [9, 13, 14]. Other species display light spots on a dark background (*Danio margaritatus*) or dark spots on a light background (*Danio tinwinnie*) (Fig. 1). The adult color pattern in zebrafish is not sexually dimorphic, but males acquire a brighter yellow coloration upon sexual activity. These observations suggest that the striped pattern serves as a recognition signal holding fish flocks and shoals together while sexual attraction occurs via hormonally controlled yellow/orange coloration (Fig. 1).

Three pigment cell types form the pattern in superimposed layers

Fish patterns are composed of several types of differently colored pigment cells: yellow xanthophores absorbing blue light, silvery, or blue iridophores reflecting light and black melanophores absorbing light across the spectrum [7, 15, 16]. The *Danio* larvae are almost completely transparent, displaying rows of individual melanophores and iridophores [17]. Xanthophores are loosely spread dorsally over the head and the flank giving the fish a yellow cast. During metamorphosis the adult pigment cells appear and begin to cover the fish with pigment cells completely [18]. The cells are distributed in three superimposed monolayers under the skin, bounding the underlying tissue: xanthophores on the top layer, iridophores in a middle layer, and melanophores on the bottom layer (Fig. 2) [7]. This is the basic color-forming unit in basal vertebrates, which is modified in the various patterns [15]. Whereas in zebrafish melanophores are only present in the dark stripes, both xanthophores and iridophores are spread over the entire body, albeit in different shapes, and densities in light and dark stripes (Fig. 2). The arrangement of pigment cells differs in different parts of the body [19]: All three pigment cell types are also present on the dorsum, the dorsal, pectoral and pelvic fins, and on the exposed rim of scales; however, in these regions they are intermixed, and formation of stripes is restricted to the flank region, the anal- and tail fin [19, 20].

Pigment cells undergo shape changes when forming the stripes

Interestingly, pigment cell types are quite different not only with regard to the color produced but also with respect to cell shape, density of distribution, size, and origin. *Melanophores* contain the black pigment melanin in melanosomes. Melanophores are dendritic in shape, and extend filopodia in which the pigmented organelles are evenly distributed (Fig. 2D). Melanophores are capable of color change upon physiological stimuli, and by transporting the melanosomes along microtubules toward the cell center, they become pale in appearance. *Xanthophores* undergo conspicuous shape changes depending on their position in the pattern. In the light stripes they appear compact and densely packed, whereas in the dark stripes they are stellate and exhibit long protrusions, keeping contact with one another ([Fig. 2B; [21, 22]]). Interestingly, xanthophore coloration is displayed in a sexually dimorphic manner – males are conspicuously more orange compared to females. This difference in orange coloration becomes particularly prominent during male–female courtship, suggesting that the difference in color is due to the dynamic localization (and amount) of pigment content in xanthophores and not due to the differentiation status of xanthophores. Xanthophores are smaller and more numerous than melanophores. The smallest, but also the most abundant pigment cells are *iridophores*. Their silvery or bluish appearance is due to crystalline guanine platelets that reflect light at different angles. Mature iridophores do not extend filopodia. In the light stripes they form an epithelial-like sheet of dense, whitish cells, whereas a loose net of bluish iridophores are covering the melanophores of the dark stripe (Fig. 2C). A third type, elongated L-iridophores spreads underneath the melanophores. The shiny appearance of the adult fish is enhanced by numerous iridophores situated in the epidermis of the exposed margins of the scales.

Each pigment cell type is regulated by a specific signaling system

Different receptor-ligand pairs regulate specification, proliferation, and maintenance of each pigment cell type. Most melanophores depend on Kit-signaling [23, 24]. Leukocyte
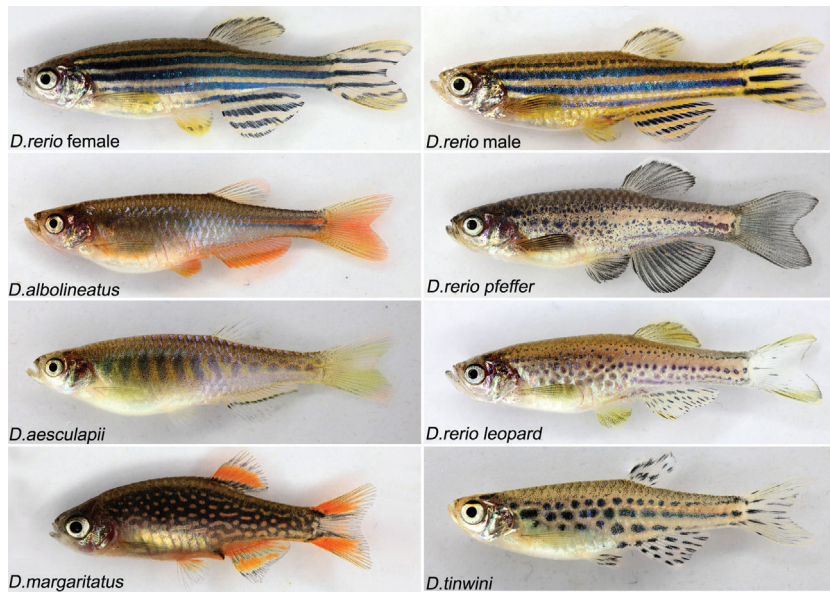
Figure 1. Color pattern diversity in the *Danio* group of fish. Text on the bottom right of each image indicates the name of the species/name of the zebrafish mutant. Figure courtesy Uwe Irion.

tyrosine kinase (Ltk, lacking in *shady* mutants) is essential for the specification of all iridophores [25, 26]. Xanthophores depend on Colony stimulating factor signalling, absent in *pfeffer/csfr1a* (Fig. 1) [27–29]. Melanophore development requires furthermore the transcription factor MitfA [30]; in addition, melanophores receive unknown cues for maintenance and survival from dense iridophores and xanthophores, whereas the other two cell types develop in a more autonomous manner [29, 31–33]. The analysis of mutants that specifically lack one type of chromatophore has revealed that stripe pattern formation depends on a number of long- and short-range interactions between the different pigment cells. Each of the three cell types, in the absence of the other two, is capable to uniformly cover the entire body [31].

Multipotency, plasticity, and cell competition regulate proliferation and spreading of pigment cells in Zebrafish coloration

Neural crest-derived pigment cells populate the skin during metamorphosis

In vertebrates, all color patterns are composed of specialized pigment-producing cells originating from the neural crest [34–36]. This is a transient population of embryonic multipotent cells located at the dorsal neuroectodermal ridge lining the neural plate. The neural crest is an important evolutionary innovation in vertebrates allowing them to become both large and colorful. Neural crest-derived progenitor cells migrate throughout the body and develop into a variety of structures and tissues; they contribute to the

formation of the head with a skull, a jaw and gills that facilitated a predatory life style with a multitude of feeding modes by increasing the surface of the respiratory epithelium [37–42]. Other structures originating from the neural crest are the neurons of the peripheral nervous system and the glia.

Whereas larval pigment cells are directly derived from neural crest cells, which migrate into the skin, the striped pattern of the adult zebrafish arises during metamorphosis, a phase of post-embryonic development that starts about 3 weeks post fertilization and lasts for about 1 month (Fig. 3C). During this period, newly formed pigment cells emerge in the skin to generate the stripes on the flanks and in the anal- and tail fins, which will form the adult pattern. The first light stripe is formed by dense iridophores entering the skin along the horizontal myoseptum, which serves as a morphological prepattern [31]. Iridophores divide and spread in the skin to occupy the available space [5]. Subsequently melanophores appear dorsally and ventrally to form the first dark stripes. The larval xanthophores contribute directly to the adult pattern by giving rise to adult-specific xanthophores; these begin to proliferate at the onset of metamorphosis [21, 43]. The larval melanophores either die or get incorporated in the first two dark stripes; the larval iridophores disappear. The adult melanophores and iridophores emerge in the skin at a time when the neural crest has long disappeared, and their origin has remained obscure until recently when long-term in vivo imaging of fluorescent reporter lines has been developed [5].

Pigment stem cells are located at the ganglia of the peripheral nervous system

The origin of metamorphic melanophores has been traced back to a small number of postembryonic stem cells located at the segmentally reiterated dorsal-root ganglia of the peripheral nervous system [23]. These cells are able to regenerate larval melanophores upon depletion, and contribute to the adult pattern as shown by transplantation experiments. Imaging of *mitfA*:GFP-positive melanoblasts has shown that peripheral nerves serve as niches for melanophore progenitors. Partial absence of the dorsal-root ganglia in the *hypersensitive/picasso/erb3b* mutation, or their deletion by laser ablation or chemical treatment, lead to a defect in the adult striped pattern [23, 44, 45]. These studies have suggested that the dorsal-root ganglia serve as niches for melanophore stem cells. Melanophore progenitors proliferate while migrating along the peripheral nerves and enter the skin as melanoblasts predominantly over the dorsal and ventral myotomes and along the horizontal myoseptum — the structure separating the dorsal and ventral halves of the myotome (Fig. 3A) [46]. Once arrived in the skin, melanoblasts stop dividing and melanize.

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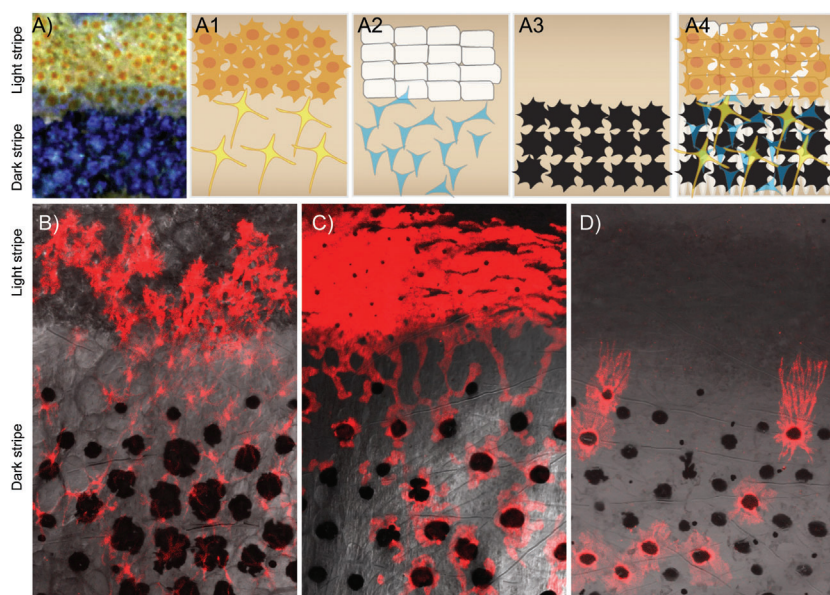
Figure 2. Organization of pigment cells in the trunk skin of adult zebrafish. **A**: Close-up view of the adult skin; **A1–A4**: schematic representation; **A1**: xanthophores; **A2**: iridophores; **A3**: melanophores; **A4**: merge. Fluorescent images (red) showing **B**: xanthophores; **C**: iridophores, and **D**: melanophores.

A more general lineage tracing system that allowed a clonal analysis of all three pigment cell types made use of the neural crest-specific gene *sox10*: All neural crest cells express the transcription factor Sox10, and mutants in *sox10/colorless* lack all pigment cells except the retinal melanophores that are brain-derived [17, 40]. Mongera et al. created a system to genetically label individual neural crest cells such that these targeted cells and their progeny produce red fluorescent protein (RFP) [17, 40]. In this system, an inducible version of the bacterial Cre-recombinase is expressed under the control of the *sox10* promoter. Treatment of fish with a drug induces recombination in a reporter cassette, which will lead to a permanent labeling of recombined cells and all their progeny. In this manner individual clones are produced at specific time points and can be followed through development.

Lineage analysis of clones of larval cells has shown that adult iridophores also develop from segmentally organized stem cells located at the dorsal-root ganglia. Although the majority of cells in postembryonic *sox10*-clones are iridophores, melanophores, and xanthophores, as well as peripheral neurons and glia may be present [5, 46]. In vivo imaging over extended time periods during metamorphosis revealed that iridophores migrate through the horizontal myoseptum where they continue to proliferate and spread after arrival in the skin. Thus, in contrast to melanophores, once in the skin, iridophores proliferate dramatically. After having formed the first light stripe, they change their shape and spread as loose iridophores dorsally and ventrally, to produce the subsequent light stripes by patterned aggregation.

Stem cells remain multipotent at least until metamorphosis

Typically, the *sox10*-clones induced in postembryonic larval stem cells are large and include approximately all iridophores of a hemisegment. Estimates based on results from blastomere transplantations suggest that there may be little more than one stem cell per hemisegment for melanophores [23, 47]. This implies that the adult pattern originates from a small number of stem cells, one or more, for iridophores and melanophores, in every hemisegment along the anterior–posterior axis. However, as different labeling systems were used, it was unclear if already at this stage distinct stem cells were committed for melanophores or iridophores, or whether multipotent pigment stem cells give rise to both pigment cell types. Our present view is that there is only one type of stem cell, which however, can contribute to a large spectrum of clones with highly variable sizes and composition (Fig. 4) [46].

The multipotency of the stem cells at the dorsal-root ganglia came as a surprise, as the neural crest cells had already delaminated and migrated away from the dorsal crest, and commitment to distinct cell fates could have occurred before delamination. Therefore, it was important to investigate at which time, if at all, the progenitors were firmly committed to only one or a small subset of cell fates. To this end, pigment cell clones were induced at four successive time-points from embryogenesis (16 hours after fertilization) to early metamorphosis (21 days), when the adult color pattern begins to develop. Clone size and composition was analyzed in fully pigmented 2–3-month old fish [46]. Surprisingly, at all time points a large fraction of the clones included all three pigment cell types, as well as neuronal tissue (Fig. 3B and D). This shows that pigment cell progenitors remain multipotent at least until metamorphosis. Clones are of highly variable composition suggesting the absence of highly regulated, stereotyped commitments, and that no distinct stem cells for one particular cell type exist at any time point.

A dual origin of xanthophores

At all stages, a substantial fraction of clones are fate restricted to xanthophores (Fig. 3D). This was expected as it had been shown that adult xanthophores arise from larval xanthophores. These clones presumably have not been induced in the stem cells but in one of the six to eight larval xanthophores per segment [21, 43]. Xanthophores also appear in the mixed clones derived from multipotent progenitors [5, 46], and a dual origin of xanthophores had also been deduced from the fact that upon complete depletion they can fully regenerate [43, 47]. Mixed clones including all three cell types as well as neuronal cells are the most frequent clone type, while the number of iridophore-only clones increases at later times of induction (Fig. 4) [46]. Clone sizes are much larger in mixed
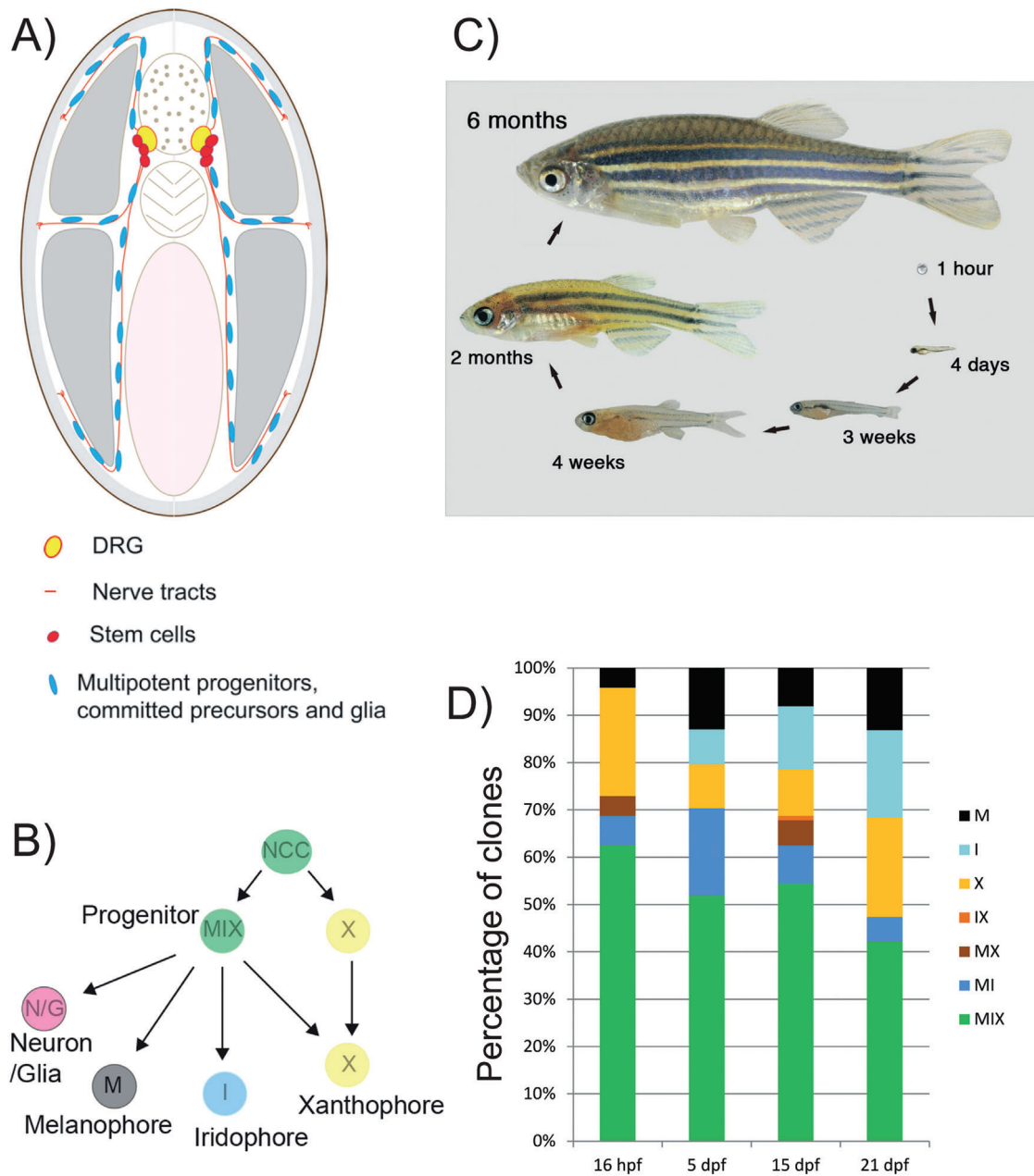
Figure 3. Pigment cell progenitors remain multipotent beyond embryogenesis through metamorphosis. **A**: Schematic representation of the location of pigment cell progenitors in metamorphic fish; cross-sectional view through the body. **B**: The relationship between the neural crest cells and adult pigment cells. **C**: Life-cycle of zebrafish. **D**: Percentage of pigment cell clones obtained by Cre induction at different time-points until metamorphosis. hpf, hours post fertilization; dpf, days post fertilization. (**A**, and **D**: Data from [46], **C** figure courtesy Nicolas Rohner).

clones derived from multipotent progenitors suggesting that the rate of proliferation decreases with fate restriction as the progenitors migrate into the skin. However, during the 3 weeks until metamorphosis only little fate restriction seems to occur. Clones sharing two cell types are relatively rare, and restricted to melanophores with either of the other two cell types (the single iridophore-xanthophore clone observed in a total of more than 200 clones may have been due to a double induction event) (Fig. 3D). Fate restricted xanthophore- and iridophore-only clones indicate that these cell types are

capable to divide and spread in the skin as differentiated cells, in contrast to melanophores, which produce only one or two cells in fate-restricted clones (Fig. 4, top row). This observation supports the notion that melanophore number increases due to proliferation of progenitor cells and not after differentiation has occurred. This means that melanoblasts are not fate restricted and give rise to pigment cells other than melanophores. It further underlines the importance of the association with the peripheral nerves in guiding pigment cells of all types to the skin. The progenitors associated with

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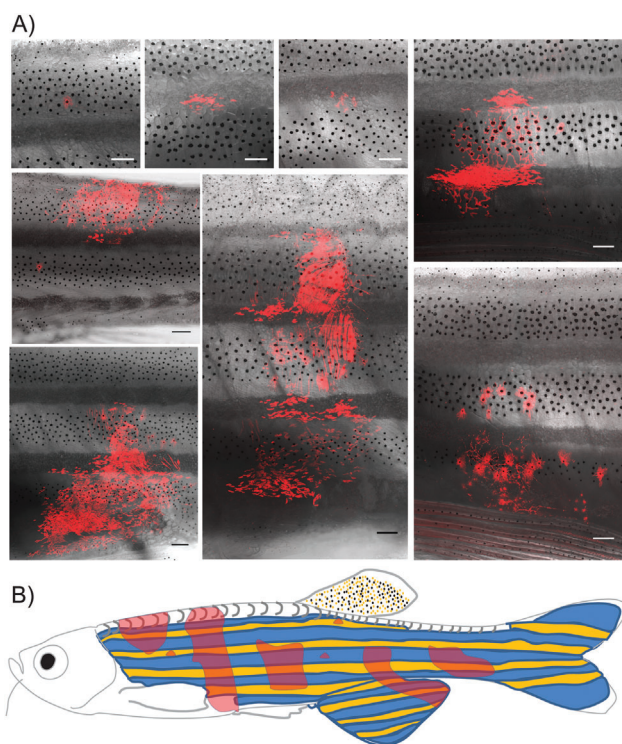
Figure 4. Pigment cell clones obtained using inducible *sox10-Cre*.
A: Examples of pigment cell clones. Scale bars = 250 μm.
B: Schematic overlay of several such clones on the zebrafish skin.
For details see [46].

the dorsal-root ganglia remain multipotent and are the adult stem cells giving rise to pigment cells and neurons during metamorphosis. Neurons are continuously added to the fish dorsal-root ganglion – the larval dorsal-root ganglion contains five to six neurons on 5 dpf, and this number can increase up to 100 neurons by 28 dpf [48, 49]. The new neurons are added by proliferation of *sox10*-positive cells that reside within the dorsal-root ganglion [49]. As mentioned, dorsal-root ganglion-associated cells regenerate new melanophores upon depletion of existing melanophores [23]. This supports the notion that some, if not all the *sox10*-positive multipotent progenitors at the dorsal-root ganglion behave like stem cells.

The distribution of pigment cells is not restricted by morphological structures

Clone shapes indicate that pigment cells tend to spread along the dorso-ventral axis, and clones may contribute to all light and dark stripes along the entire dorso-ventral pattern when induced early in development. Most mixed clones include either the ventral-most or dorsal-most parts of the pattern, whereas fate restricted clones appear predominantly close to the horizontal myoseptum, often including it (Fig. 4). This confirms the paths taken by the progenitors along the dorsal or ventral spinal nerves, the dorsal, and ventral rout, respectively (Fig. 3A). Significantly, no restrictions imposed by morphological structures could be observed, and the

striped pattern does not play a role for the spreading of pigment cells. Clones may include both skin and scale pigmentation and extend into the unstriped dorsum as well as the fins. Within each clone, iridophores and xanthophores acquire the shapes depending on the position in the striped pattern: dense and compact packing in the light stripes, and loose nets in the dark stripes.

The dorso-ventral orientation of the clones reflect their segmental origin from the dorsal-root ganglia, as pigment cells emerging from neighbouring segments prevent expansion along the anterior–posterior axis. However, cells from adjacent segments intermingle and no clonal restriction between segments is observed. This is in contrast to the mode of epidermal development and patterning in insects such as *Drosophila*, where clonal restrictions define specific compartments along the antero–posterior and dorso-ventral axes.

Pigment cell progenitors remain plastic and show variable rates of proliferation

The variability in clone size and the lack of commitment of the progenitors suggest that pigment cells populating the skin constantly communicate with their neighbors in order to regulate their clonal expansion. Each cell type is dependent on a different signalling system and the analysis of clones induced in mutants lacking iridophores (*shady*) or xanthophores (*pfeffer*) indicates that the lack of one cell type does not impair the spreading of the other [47]. Indeed, an even and dense spreading of xanthophores or iridophores alone occurs in mutants lacking the other cell types. Melanophores, on the other hand, seem to need support from both iridophores and xanthophores, as their density is low in mutants lacking these cell types [31].

Proliferation and migration are influenced by the density of cells of the same kind of pigment cells. It is possible that ligand availability for the respective signalling systems regulates the differentiation and proliferation of each cell type. Plasticity in adjusting the rate of cell division and differentiation may allow a regular and even spacing of cells within each cell layer. Such a mode of origin enables the adjustment to variations of sizes of segments and shape of the body.

Homotypic competition regulates proliferation and spreading of pigment cells

To test the role of interactions between pigment cells of the same kind within the cell layers, the proliferation and spreading of pigment cells in the absence of competing cells of the same type was analyzed for each of the three cell types [47]. Clusters of labeled xanthophore progenitors were obtained by blastomere transplantations into blastulae of the mutant *pfeffer* lacking xanthophores and the chimeric animals were analyzed throughout development. In control experiments cells were transplanted into animals with all three cell types present (Fig. 5, top panel). In *pfeffer* mutants, donor-derived clusters of labeled xanthophores proliferate at a faster rate than in their normal surrounding and may eventually cover large regions of the skin. Clones are no longer
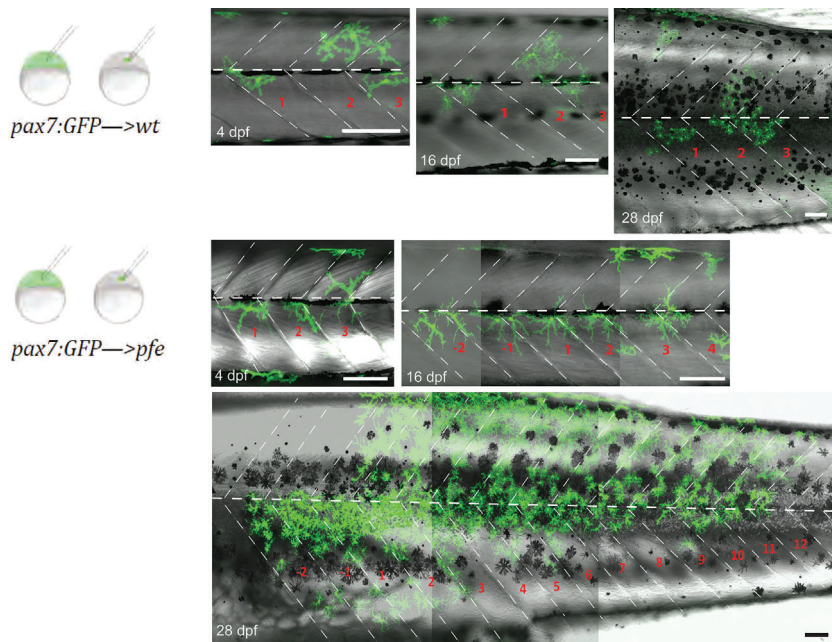
Figure 5. Xanthophores cover the skin by homotypic interactions. In the absence of xanthophores in the neighbourhood, wild-type xanthophores (green) disperse into several more segments (bottom panel) than when neighbouring xanthophores are present (top panel). Scale bars: 100 µm (Data from [47]).

oriented dorso-ventrally but may span many segments along the antero–posterior axis (Fig. 5, bottom panel). If given enough time, the entire pattern within each segment can be covered by donor-derived xanthophores. Interestingly, in these chimeras xanthophores start to proliferate already during larval stages (16 dpf in Fig. 5), whereas in the wild-type environment xanthophore proliferation commences only with the beginning of metamorphosis (Fig. 5). This indicates that it is not a hormonal trigger that induces xanthophore proliferation but the increase in distance between individual xanthophores due to growth.

A few clusters of iridophores introduced into *shady* hosts suggested a similar behavior for iridophores which was corroborated by regeneration experiments: upon depletion of the dorsal-root ganglia with a drug inhibiting ErbB-signaling, animals lacking large regions of dorsal-root ganglia-derived melanophores and iridophores can be obtained. These regenerate the light stripe region due to proliferation and dispersal of iridophores from the neighboring segments into the gap devoid of iridophores. In this manner, gaps of more than ten segments can be covered, albeit in a wavy pattern [47]. In another study, *moonstone*, a mutant with hyperactive Ltk signaling was investigated [26]. Mutants display ectopic iridophores in larvae, and in the adults excessive iridophores in the scale margins lend them a conspicuous green shine. However, the striped pattern is not affected and both iridophores and melanophores display a normal appearance. This indicates that over-proliferation only occurs in regions bounded by areas devoid of iridophores whereas in the striped

region, where all cells are poised to produce more iridophores through hyper-active Ltk signalling, they keep each other in check. *moonstone* mutant iridophores may display dramatic over-proliferation in the striped pattern and produce multilayered clusters when put in contact with wild-type cells having normal Ltk levels in chimeric animals. In this situation, cells with different Ltk levels are juxtaposed, giving the over-active cells a growth advantage [26].

These cellular behaviors show that competition between cells of the same type limits their proliferation and spreading, and suggest a mechanism that explains the dorso-ventral orientation of clones observed in normal development. In the absence of competing cells within a layer in the skin, donor-derived clusters of xanthophores and iridophores proliferate at a faster rate than in their normal surrounding and spread also laterally to cover larger regions of the skin.

Interestingly, in the case of melano-phores the influence of homotypic competition is less pronounced: while clusters are significantly larger when introduced into *nacre* mutants lacking melanophores, they never cover extensive regions along the antero–posterior axis and remain restricted to a maximum of about six meta-mers [47]. In the regeneration experiment mentioned above, where gaps are induced in the pattern by chemical ablation of dorsal-root ganglia, melanophores have a more limited ability to fill the region devoid of pigment cells. This is in agreement with the close association of proliferating melanoblasts and the segmental peripheral nerves, as well as the lack of ability of melanophores to divide once in the skin.
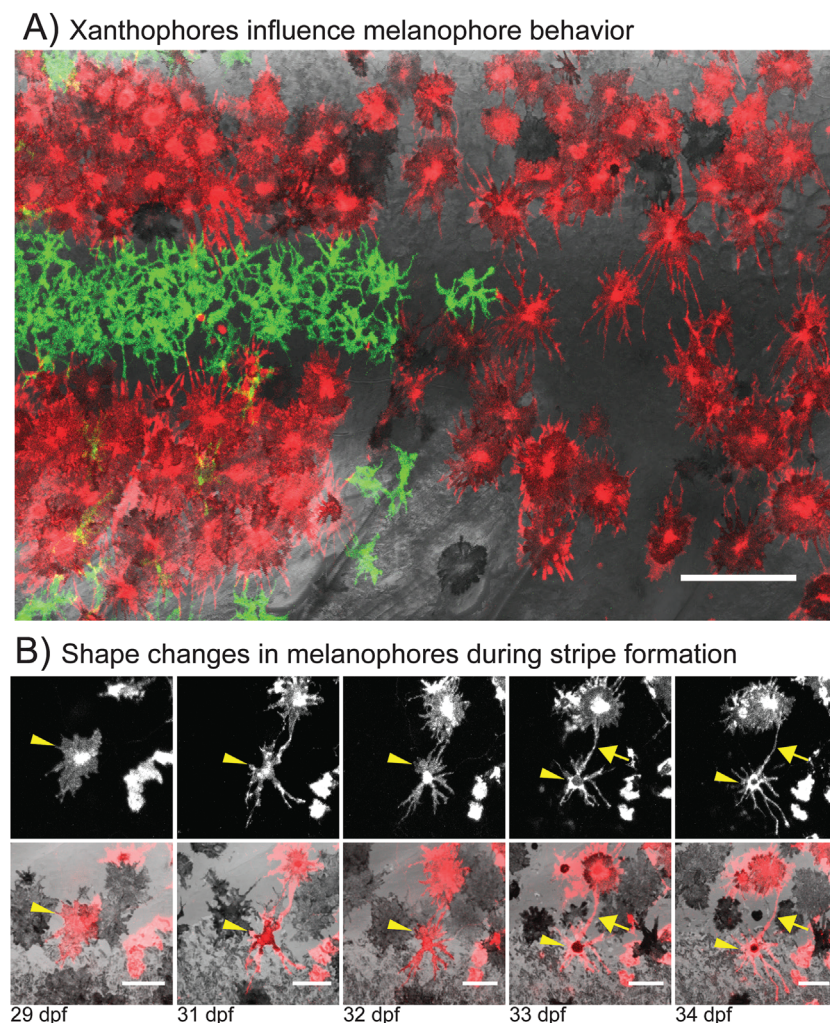
Taken together, these data indicate that in normal development the striped pattern is stitched together by adjacent clones derived from stem cells at the dorsal-root ganglia of neighbouring segments [46]. The lateral extension of the clones is restricted by homotypic competition [47]. Thus, the dorso-ventral orientation of the pigment cell clones observed in normal development is the result of competitive interactions between cells from neighbouring segments and not the product of a hypothetical, morphogen-based directed migration.

Toward a general mechanism of pigmentation for all fish: Problems and paradigms

Contact dependent interactions self-organize color patterns

Mutant analysis has revealed that stripe width and composition depend on self-organization, whereas the orientation along the antero–posterior axis is determined by a larval prepattern, which is set by the horizontal myoseptum [31]. Experimental and theoretical analyses of zebrafish color pattern formation over the past decade have

A) Xanthophores influence melanophore behavior

B) Shape changes in melanophores during stripe formation

29 dpf   31 dpf   32 dpf   33 dpf   34 dpf

Figure 6. Heterotypic interactions between melanophores and xanthophores. **A**: Xanthophores when introduced by transplantation into the *pfeffer* mutant (see Fig. 5) cause the host melanophores (red) to arrange in a coherent organization, and their disappearance from the region occupied by xanthophores. Scale bar = 200 μm. **B**: Melanophores, when trapped in an expanding region of dense iridophores forming the light stripe, gradually change their shape to eventually disappear. Scale bars = 50 μm. Wild-type xanthophores (green), melanophores (red); (**A**) data courtesy: Prateek Mahalwar and Brigitte Walderich. See also: [47]. (**B**) for detail see [5].

confirmed and substantiated the role of cell−cell interactions between different pigment cell types during stripe pattern formation [28, 29, 31–33, 50–52]. This has recently been extensively reviewed [9, 10, 53, 54]. Key cellular outcomes of such heterotypic interactions are the regulation of cell survival, cell migration and cell shape transition. For example, xanthophores and melanophores mutually repel each other (Fig. 6A), and sharpening of the stripe boundary cause shape changes in melanophores by interaction with dense iridophores (Fig. 6B).

Direct cell−cell contact involving potassium channels and gap junctions are thought to be the primary mode of communication among different pigment cell types. Genetic

screens have identified several components of this communication system. These components directly participate in channel/ junction formation, such as Kcnj13, Connexin 41.8, and Connexin 39.4 [55–58], or modulate their function, such as spermidine [59]. Tight junction protein 1a acts potentially downstream of gap junction-derived signals to mediate iridophore behaviour during pattern formation [60]. Although several genes have been identified that are involved in zebrafish color pattern formation [8–10, 61], the actual underlying biochemical and molecular events remain highly conjectural. For instance, although it is likely that the gap junction-dependent communications are important for pattern formation, in vivo data for the expression patterns of the components of gap junctions is still lacking, and it is not known if indeed there are gap junctions formed between pigment cells during the relevant stages of development. The challenge is not easy to tackle − patterning processes take weeks and months to accomplish and during these developmental stages the zebrafish skin is neither very amenable to conventional techniques of expression pattern analysis nor approaches to test intercellular communications. Only in one instance- *schachbrett*/Tjp-1- has expression in a specific cell type, iridophores, been documented by immunostaining [60]. Development of CRISPR/Cas9-based knock-in tools for analysis of endogenous expression patterns of patterning molecules will be a crucial step forward in determining the specific aspects of cell−cell interactions that are regulated by gap junctions (and other proteins) in vivo.

How do interactions among pigment cells depend on the tissue environment?

Having accumulated insightful information on the cellular and molecular basis of color pattern formation, how shall we proceed to derive the essential developmental principles underlying diverse color patterns that are seen in fishes of the world? A common feature of color patterns seems to be the layered arrangement of the pigment cells in the skin: xanthophores are typically present on the outermost layer, iridophores in the middle and melanophores in the inner layer [15, 62]. We propose that the lineage and dispersal, as revealed in these analyses, is probably a general mechanism common to all fish. However, our knowledge of the anatomy and development of the layered organization of pigment cells is poor even in zebrafish. Which cell types or extracellular matrix components compose the substratum on which pigment cells proliferate and migrate? Are extracellular matrix structures involved? Genetic evidence has pointed to a role of non-pigment cells in shaping the pattern but the responsible tissues have not been identified [32]. The mode of

interactions among pigment cells depends significantly on their position in the body. Despite a common origin, in the dorsum, in some fins and on the scales the three cell types mix, and pattern formation in the body is restricted to the flank region in zebrafish (striped) as well as in the other *Danio* species (diverse patterns). We have no cues how this patterned region is spatially demarcated. In zebrafish, also the anal- and tail fins are striped, and genetic evidence suggests that the mechanism of stripe formation differs from that of the body [53]. This notion is supported by the fact that in other *Danio* species the fins display pattern motifs very different from the body, and the congruent striping observed in zebrafish appears to be a rather special, derived feature (Fig. 1). We do not know the mechanisms causing the various fins to adopt different patterning modes.

An important environmental cue in setting up the color pattern is the horizontal myoseptum. As mentioned, the horizontal myoseptum plays an important role in orienting the stripes along the anterior–posterior axis, as the iridophores forming the first stripe emerge along this site. Horizontal bands of pigment cells parallel to this anatomical landmark are seen in many fishes. However it is not clear whether the horizontal myoseptum serves just as a morphological exit point for the emerging iridophores or instead molecular cues are located at this structure guiding the formation of the first light stripe. Vertical bars, a frequent pattern motif, remind on the dorso-ventral orientation of clones originating from the segmentally reiterated stem cells. It is conceivable that in other fish species other anatomical landmarks are triggers of patterning such as the dorsal midline or the scale margins. Interestingly, morphogen gradients that pattern cuticular structures in insects do not seem to play a role at all in fish pigment pattern formation. As none of the other *Danio* patterns involves parallel stripes, the seeming periodicity of the zebrafish pattern may also be a rather special feature.

What is the genetic basis of pattern variation and evolution?

Recent advances in whole genome sequencing techniques and genome editing have made this fundamental question accessible to experimental analyses. In particular, *Danio* species and cichlids offer a valuable resource to discover common principles underlying diversification of color patterns, and lay a platform for genetic analysis of pattern evolution [13, 63, 64]. Comparative genetic analyses between diverse *Danio* species, particularly between zebrafish (*D. rerio*) and pearl danio (*D. albolineatus*), have provided insights into potential cellular and genetic mechanisms underlying pattern diversification between the two species [65, 66]. Precocious differentiation of xanthophores over a wide region in the skin of *D. albolineatus* has been suggested to have led to loss of stripe pattern in this species [67].

Which cellular behaviours occur during proliferation and dispersal of pigment cells?

Genetic and theoretical studies have identified short-range and long-range communication between pigment cells [29, 31, 33, 51].

The short-range communication is thought to involve direct cell–cell contacts. Recent studies have implicated long cellular projections as mediators of long-range cell–cell interactions involving Notch signalling [68, 69]. How these multiple known and unknown molecules co-operate during color pattern formation remains a challenging question. Aspects of pigment cell behaviour bear resemblance to their origin – the neural crest cells. Iridophores display epithelial-to-mesenchymal-like transition during formation of light and dark stripe regions [5, 60]. Homotypic interaction-dependent coverage of the skin by pigment cells resemble that of neural crest cells, which undergo collective cell migration and display co-attraction despite contact inhibition of locomotion [47]. Comparisons with other systems of cell migration provide a context for identifying the underlying cellular and molecular mechanisms. It will be interesting to explore the role of known regulators of epithelial-to-mesenchymal transition and collective migration in the context of color pattern formation.

Conclusion

It has been long known that all pigment cells in vertebrates are neural crest-derived, however, their lineage commitment and mode of distribution in the skin has been poorly understood. Recently the cell lineage of all three pigment cell types has been analyzed in detail [5, 21, 23, 45, 46]. Lineage tracing of Cre-lox-induced clones in situ revealed that, surprisingly, progenitors of pigment cells remain multipotent and share the lineage with neurons and glia of the peripheral nervous system well into metamorphosis [46]. They remain plastic and their composition and growth rate is highly variable. Despite the common origin and the shared lineage, each cell type behaves differently. Xanthophores also originate from larval cells. Iridophores and xanthophores are capable of proliferating and spreading in the skin as differentiated pigmented cells, whereas pigmented melanophores hardly ever divide or migrate. The rate of proliferation depends on the presence or absence of cells of the same type (homotypic competition), such that clones of xanthophores and iridophores can cover large regions in the skin when introduced by transplantation into individuals lacking the respective cell type [47]. Collective migration and competition between cells of the same type lead to an even spacing of pigment cells in the skin. We propose that pigment cell lineage and their dispersal, as revealed in these analyses, are common to all fish, whereas the shape changes that occur in iridophores and xanthophores between light and dark stripes in zebrafish are determined by species-specific interactions between the different cell types (heterotypic interactions). Color patterns provide a novel paradigm for morphogenesis and pattern formation by contact-dependent cell–cell interactions among pigment cells across different layers; novel color patterns may arise by modifying this layered organization of pigment cells. It is a nascent field holding great promise for evolutionary developmental biology.

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The authors have declared no conflict of interest.

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