Primer

Pigment patterns: fish in stripes and spots

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From tiger stripes to the spots on a butterfly wing, we all grow up learning about the beauty and diversity of animal pigment patterns. Such patterns are one of the most obvious traits of animals and serve a variety of functions. Some provide camouflage (zebra stripes) or warnings (the colors of poison arrow frogs). Other patterns have roles in social aggregation and mate choice (guppies), and have important roles in adaptive radiations and speciation (African cichlid fishes).

Given their prominence and ecological functions, pigment patterns often are targets of natural selection and thus of particular interest to evolutionary biologists. They have been of long-standing interest to developmental and cell biologists as well: their accessibility to observation and manipulation has made them a classic and enduring system for studying basic genetic and cellular mechanisms.

Recent years have seen the emergence of pigment patterns specifically as a model for post-embryonic development. Discovering why adult organisms look the way they do is critical to understanding the evolution of morphology. Despite great strides in understanding embryogenesis, however, we still know little about the generation of adult form. In this regard, pigment patterns are an especially tractable system for identifying mechanisms of pattern formation and morphogenesis that make an adult. They also offer the prospect of truly integrative research spanning several levels of biological organization, from molecules to cells and phenotypes, and from individuals to populations and species. One prime model organism for dissecting mechanisms of pigment pattern formation and evolution is the zebrafish, *Danio rerio*.

Danio pigment patterns and the diversity of neural crest-derivatives

Vertebrates exhibit a stunning array of pigment patterns, which are highly varied especially in teleost fishes. The *Danio* genus captures some of this diversity among its many species (Figure 1). The patterns in danios and other vertebrates depend on pigment cells, which have their origin in the neural crest, a transient population of cells that arises during neurulation along the dorsal neural tube. Neural crest cells then disperse throughout the embryo in one of the most dramatic examples of cell migration known. Besides pigment cells, neural crest cells also contribute to a diverse array of other cell types and organ systems. Indeed, many of the shared, derived traits of vertebrates have their origin at least in part within the neural crest. Thus, an understanding of how these remarkable cells are patterned — and how these patterning mechanisms evolve — should provide considerable insight into the evolution of vertebrate form.

What's in a stripe?

The adult stripes of zebrafish result from three classes of pigment cells, or chromatophores: black melanophores (containing melanin); yellow or orange xanthophores (containing pteridines and carotenoids); and silvery iridophores (containing guanine-rich reflecting platelets). Dark stripes include melanophores and iridophores, with only a few xanthophores, whereas light stripes include xanthophores and iridophores, with few if any melanophores (Figure 2).

The diversity of ectotherm chromatophores differs from amniotes, which exhibit just a single type of skin pigment cell: the melanocyte. However, molecular analyses are revealing a conservation between teleosts and mammals for many aspects of pigment cell development, e.g. a microphthalmia-associated transcription factor (mitf) is essential for specifying both melanocytes in mouse and melanophores in zebrafish.

Metamorphosis: a very special episode in the life of a fish

Many teleosts undergo a metamorphosis that is reminiscent of amphibian metamorphosis. In the zebrafish, this transformation involves development of scales as well as adult fins, changes to the gut, skeleton and sensory systems, as well as resorption of the larval fin fold. The color pattern changes dramatically during metamorphosis as well (Figure 2,3). Unlike adults, larvae exhibit a simple series of melanophore stripes (containing a few iridophores) and xanthophores scattered widely over the flank. This pattern develops during embryogenesis and remains largely unchanged for the first two weeks. At the onset of metamorphosis, melanophores appear outside the larval stripes and during the next two weeks a new pattern forms, consisting initially of two adult "primary" dark stripes dorsal and ventral to a

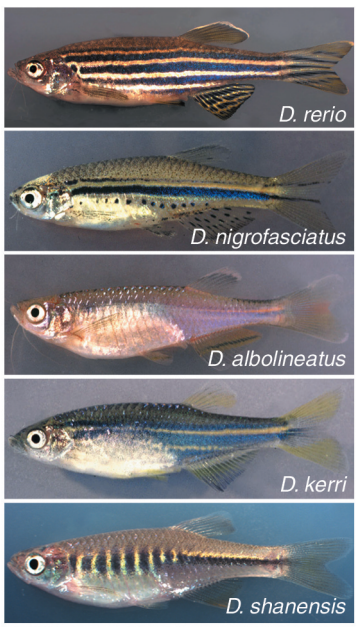

Figure 1. Diversity of *Danio* adult pigment patterns.
Danios exhibit stripes, spots, bars, uniform patterns, and more. These fishes are native to central and southeast Asia as well as China.

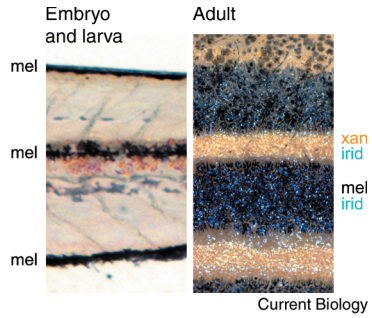

Figure 2. Pigment patterns at different stages.
The early larva at the start of metamorphosis (left) has melanophore stripes dorsally, ventrally, and in the middle of the flank and clusters of xanthophores and iridophores beneath the middle stripe. The adult (right) has alternating light and dark stripes containing xanthophores and melanophores, respectively.

primary light stripe. As the fish grows, new "secondary" stripes are added until 5–6 melanophore stripes are present. There is no obvious relationship between the location of larval stripes and those of the adult. So where do the adult stripes come from?

Dual origins of adult melanophores

Two extreme models might be suggested for the source of adult pigment cells: They could arise entirely from stem cells that are recruited during metamorphosis; alternatively, chromatophores that differentiated at embryonic stages could be responsible for making the adult pattern via differential proliferation, death, and migration. Cellular and genetic analyses support an intermediate model.

An important role for stem cells is revealed by mutants such as *puma*, which develop normal embryonic — but not adult — pigment patterns. Whereas in wild-type new melanophores differentiate over the flank during metamorphosis, very few differentiate in *puma* mutants (Figure 3). Instead, remnants of the embryonic pigment pattern persist into the adult and only much later irregular adult stripes form (Figure 4; see movie on line at: http://www.biosci.utexas.edu/ib/faculty/parichy/movies.html). A variety of analyses further suggests that the *puma* gene is essential for recruiting neural crest-derived stem cells into chromatophore and glial fates, specifically during metamorphosis.

What is the fate of the embryonic melanophores during normal development? Some are lost, but others are incorporated into the adult pigment pattern. So, the adult pigment pattern actually comprises distinct classes of melanophores: a major contribution from metamorphic melanophores, and a lesser contribution from persisting embryonic melanophores.

Subpopulations of metamorphic melanophores

The first insights into the genetic requirements for metamorphic melanophores came from studies on the timing of melanophore appearance in wild-type zebrafish, as well as in *sparse* and *rose* mutants (Figure 4). In wild-type, melanophore numbers increase steadily during metamorphosis. *sparse* mutants, however, develop fewer embryonic melanophores, lose all of these cells by the onset of metamorphosis, and develop many new melanophores during late metamorphosis, illustrating the ability of stem cells to produce an adult pigment pattern. By contrast, *rose* mutants have normal melanophore numbers through early metamorphosis, but fail to produce as many new melanophores during late metamorphosis.

This complementary timing of melanophore appearance suggests that metamorphic melanophores are separable into an early appearing population dependent on *sparse* and a later appearing population dependent on *rose*. In support of this idea, fish doubly mutant for *sparse* and *rose* lack all body melanophores (Figure 4).

sparse and *rose* encode orthologs of genes long known for their roles in amniote melanocyte development. *sparse* encodes an ortholog of *kit*, a type III receptor tyrosine kinase, whereas *rose* encodes an ortholog of *endothelin receptor b1* (*ednrb1*), a heptahelical G protein-coupled receptor. Both are expressed by

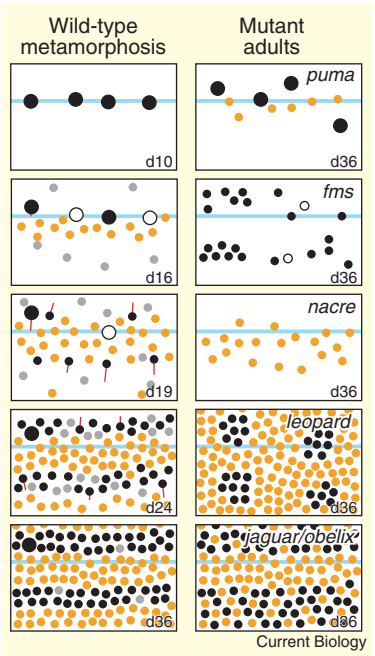

Figure 3. Chromatophore arrangements
In wild-type (left), larval melanophores (large black circles) are present along the horizontal myoseptum (blue line). Some of these cells die (open circles), others move into the developing adult stripe (red tracks). Simultaneously, xanthophores (orange) begin to appear in the middle of the flank and metamorphic melanophores (gray) appear over the flank. Some metamorphic melanophores migrate into the position of adult stripes; others differentiate already at the site of stripe formation. After metamorphosis, the pigment pattern has little resemblance to that of the embryo. In pigment pattern mutants (right), chromatophore numbers and arrangements differ dramatically from wild-type. A supplementary movie showing pigment pattern metamorphosis in wild-type zebrafish and its failure in *puma* mutants is available on line at : http://www.biosci.utexas.edu/ib/faculty/parichy/movies.html.

pigment cells and their precursors and null alleles of mouse *Kit* and *Ednrb* completely lack melanocytes. Thus, the many residual melanophores in zebrafish mutants are somewhat surprising. The zebrafish mutants also lack severe pleiotropic effects seen in the mouse. Perhaps additional copies of *kit* and *ednrb* are present in zebrafish and provide some functional redundancy.

Double mutant analyses have associated several additional genes with late metamorphic melanophores. For example, fish doubly mutant for *panther* and

sparse (*kit*) lack almost all body melanophores, similar to *sparse; rose* double mutants. Yet, fish doubly mutant for *panther* and *rose* (*ednrb1*) have as many melanophores as single mutants (though the cells are uniformly dispersed; Figure 4). These results indicate that *panther* is required by late, but not early metamorphic melanophores. Intriguingly, *panther* corresponds to a zebrafish ortholog of *fms* (*Csf1r*), the closest known homolog of *kit*. Thus two essentially paralogous genes, *kit* and *fms*, promote the development of parallel populations of metamorphic melanophores.

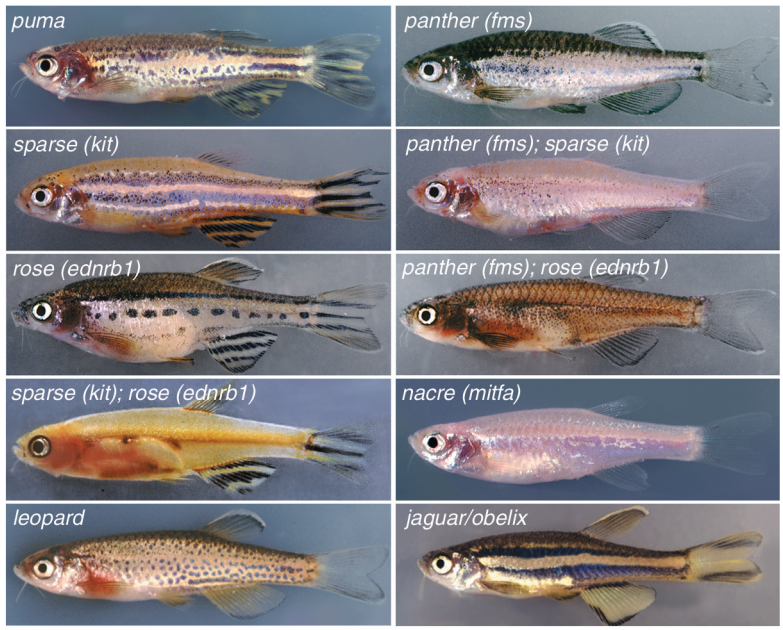

Figure 4. Zebrafish pigment pattern mutants.
Just a few of the many adult pigment pattern mutants. Analyses of residual chromatophore patterns in single and double mutants have helped to outline the basic mechanisms of pigment pattern metamorphosis.

Stripe organization: more than just melanophores

During pigment pattern metamorphosis, new melanophores initially are scattered over the flank. How are these cells organized into stripes? A clue came from comparing wild-type and *fms* mutant zebrafish. In wild-type, dispersed metamorphic melanophores actively migrate into stripes. In *fms* mutants, however, these movements are uncoordinated and only a rudimentary stripe pattern forms (Figure 3,4). However, *fms* is not actually expressed by melanophores. Rather, *fms* is expressed by xanthophores and their precursors, and in *fms* mutants xanthophores are absent. This raised the possibility that *fms*-dependent xanthophores might normally be required for organizing melanophores.

Such a role for xanthophores has been confirmed by studies that examined chimeras derived from *fms* mutants or wild-type fish (Figure 5), as well as *nacre* mutants. *nacre* mutants lack melanophores owing to a lesion in *mitfa* (Figure 3,4). Thus, *nacre* mutants are unable to make their own melanophores and can supply only the xanthophores to any resulting pigment pattern.

The requirement for xanthophores in organizing melanophore stripes is demonstrated by transplanting wild-type or *nacre* mutant cells into *fms* mutant hosts: in contrast to the disrupted stripes of the typical *fms* mutant, chimeras with donor xanthophores form well-organized stripes of melanophores (Figure 5). Additional analyses show the interaction is mutual, as xanthophore organization in light stripes is dramatically improved in the presence of melanophores.

Thus, introducing one chromatophore class into a mutant possessing only the other class is sufficient to make melanophore stripes and to organize xanthophores within light stripes. Interactions between melanophore and xanthophore lineages seem to be short-range, as only chromatophores in close proximity to cells of the "other" class participate in stripe formation.

When are melanophore–xanthophore interactions needed? Analysis of a temperature-sensitive *fms* allele shows that curtailing Fms activity at any point from embryo to adult causes the loss of xanthophores and the degeneration of melanophore stripes; conversely, activating Fms at any stage allows xanthophore development and the assembly of melanophore stripes. Thus, melanophore–xanthophore interactions are needed first to establish and then to maintain adult stripes.

Making the first stripes: cues for pattern formation?

While interactions between melanophores and xanthophores are needed to make stripes, they probably do not determine stripe orientation. Evidence comes from the caudal fins of temperature-sensitive *fms* mutants: when xanthophore development is permitted only in the adult, stripes form but their orientation is haphazard. Cues that orient fin stripes during normal development are presumably no longer present at later stages.

What are these cues? Despite tantalizing hints, this problem has yet to be solved. For example, xanthophores first appear at metamorphosis over the middle of the myotomes where the primary light stripe will form (Figure 2,3). This might serve as a prepattern upon which the adult stripes are built. What establishes it remains unclear. The lateral line sensory system might be involved, as the lateral lines initiate a stripe pattern in salamander larvae, and zebrafish mutants such as *puma*

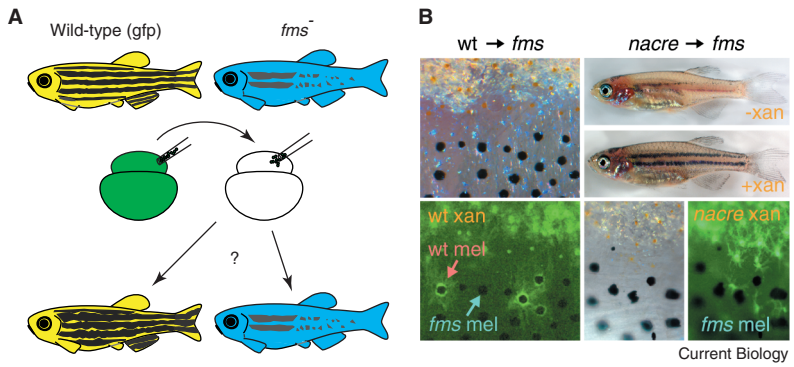

Figure 5. Interactions between melanophores and xanthophores.
(A) Genetic mosaics can be made by transplanting GFP-expressing cells between embryos. (B) Transplantation of wild-type (wt) cells to *fms* mutants allows stripe recovery. Xanthophores originate from the wild-type donor, whereas melanophores in stripes are both donor and host derived. Similarly, recombining xanthophores and melanophores by transplanting cells from *nacre* mutants also allows stripes to form (B). When donor cells fail to differentiate as xanthophores, a typical *fms* mutant pattern develops (top). When donor cells form xanthophores, however, these can spread widely over the flank and organize a melanophore pattern (lower panels).

exhibit defects both in adult stripe formation and lateral line metamorphosis.

Stripes and spots: establishing some boundaries

Whereas studies of *fms* mutants identified a role for cellular interactions during stripe formation, the two mutants *leopard* and *jaguar/obelix* provide insights into the nature of the interactions themselves. *leopard* mutants often have spots, but an allelic series ranges from wavy stripes broken only intermittently, to melanophores being scattered uniformly over the flank and extensively intermingled with xanthophores. *jaguar/obelix* mutants exhibit broader stripes than wild-type fish and fail to develop secondary adult stripes as they grow (Figure 4). Similar to severe *leopard* alleles, *jaguar/obelix* mutants also have numerous xanthophores intermingled with melanophores (Figure 3). Thus, *leopard* and *jaguar/obelix* mutants fail to produce normal boundaries between chromatophore classes.

Cell transplantations and genetic analyses show that both genes, *jaguar/obelix* and *leopard,* act within the melanophores to promote their aggregation, whether or not xanthophores are present. Genetic analyses further suggest a similar role for *leopard* within xanthophores, restricting these cells to regions where light stripes should form. It will be especially interesting to identify the molecular nature of these genes and whether they exert their effects by mediating cell–cell adhesion, or the response of chromatophores to cues in their extracellular environment. Remarkably, the range of *leopard* mutant phenotypes can be closely modeled by reaction–diffusion simulations, suggesting that a relatively simple mechanism could explain the diversity of spotted and striped patterns of zebrafish and other species.

Pigment patterns as a model for evolution and behavior

The analysis of zebrafish pigment pattern development suggests a model for the genes and cell populations required to make an adult pigment pattern and provides a useful starting point for identifying the mechanisms underlying pigment pattern evolution across species.

A recent approach has used interspecific hybrids. When zebrafish are crossed to other danios (Figure 1), the resulting pigment patterns typically resemble that of zebrafish more than the other species, suggesting that pigment pattern genes in other species often are recessive to those of zebrafish. This has allowed for interspecific complementation tests to determine whether genes identified as zebrafish pigment pattern mutants might contribute to differences between taxa. For example, hybrids between wild-type zebrafish and *D. albolineatus* form stripes like zebrafish, whereas hybrids between *fms* mutant zebrafish and *D. albolineatus* lack stripes, like *D. albolineatus*, identifying *fms* as a candidate gene for stripe elaboration in zebrafish, or stripe loss in *D. albolineatus*.

Danio pigment patterns offer an outstanding opportunity for integrative studies that link genetic and cellular mechanisms to behavior, ecology, and natural selection in the wild. In the future, identification of additional genes and cell behaviors required for pigment pattern formation in zebrafish will provide valuable resources and testable hypotheses for dissecting the evolution of neural crest derivatives in danios and vertebrates more generally.

Further reading

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