The evolution of morphological complexity in zebrafish stripes

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The zebrafish pigment stripe pattern is a complex tissue containing iridophores, xanthophores and multiple melanocyte types. Mutational analysis reveals that both ancient and recent gene duplications are involved in the generation or maintenance of the pattern complexity. Receptor tyrosine kinases kit and fms, products of an ancient gene duplication, are required in distinct types of melanocytes and xanthophores. Transcription factors mitfa and mitfb, results of a teleost-specific duplication, partition gene expression and function between different sets of melanocytes. Understanding the roles of these duplicated genes in zebrafish allows us to predict roles for their precursors in ancestral vertebrates.

A general role for gene duplication in the evolution of morphological complexity was first suggested by Ohno [1]. Ohno proposed that natural selection tends to prevent mutations at single gene loci that might lead to new functions. When the gene is duplicated, the two copies are functionally redundant. Thus, selection on each is relaxed and formerly forbidden mutations can arise. These could either completely inactivate one copy of the duplicated gene, or generate loci with new or different functions. By these means, new gene functions can lead to additional morphological complexity, be it the complex brain of humans or the radiant pigment stripes of zebrafish.

Genes can duplicate by a variety of mechanisms, including tandem gene duplication, segmental duplication and whole-genome duplication. The concomitant increase in gene number might then fuel morphological innovations. Two large-scale gene duplications or whole-genome duplications were first posited by Ohno [1] and later by Holland [2] to correspond to significant stages in the morphological evolution of the vertebrates. This was supported by the finding of one *Hox* cluster in amphioxus [3], compared with four *Hox* clusters in mammals [4], as well as the recognition that *Hox* genes are found in conserved groupings in mice and humans [5–7]. These events, thought to have occurred after the divergence of amphioxus from the vertebrate lineage [2], temporally correspond to the evolution of many of the major innovations of vertebrates, including the NEURAL CREST (see Glossary) and placodes, cartilage, bones and teeth [8] (Fig. 1). Presumably, the emergence of these innovations was facilitated by the presence of extra genes, and the relaxed selective pressure following genome duplication. Evidence that segmental duplications continue to shape the human genome is emerging with the completion of the Human Genome Project. Relatively recent segmental duplications (90–98% sequence identity) could make up as much as 5% of the human genome [9]. That these duplications are often associated with chromosomal rearrangements and deletions that lead to clinical syndromes [10] further illustrates the ebb and flow of genes in the genome.

Identification of seven *Hox* clusters in zebrafish [11], and two copies of many zebrafish genes where only one has been found for human or mouse, has fueled speculation of either recent whole-genome duplication in teleosts [11], or continuous duplication of chromosomal segments [12]. When duplicated copies of zebrafish genes are preserved, they often have complementary expression patterns that together are

Glossary

Early stripe melanocytes (ESMs): Melanocytes (see below) that develop during the first phase (2–3 weeks of development) of the larva-to-adult transition (2–4 weeks of development).
Embryonic melanocytes: Neural-crest-derived melanocytes (see below) that develop during the first 24–36 hours of development. They are not thought to contribute to the final adult melanocyte stripe pattern.
Epistasis group: A group of genes for which double mutants cause the same or similar phenotype as either single mutant. By extension, double mutants between epistasis groups show additive phenotypes.
ESM epistasis group: Mutant (*kit*) that affects development of early stripe melanocytes but not late stripe melanocytes, indicating that the *kit* gene is required only in a subset of melanocytes.
Iridophores: Iridescent or reflective pigment cells derived from neural crest. These cells contain guanidine-based reflective platelets responsible for their silver or gold appearance in zebrafish.
Late stripe melanocytes (LSMs): Melanocytes that develop at late stages (3–4 weeks of development) of the larva-to-adult transition.
LSM epistasis group: Mutants (*ednrb, fms, leopard, primrose*) that affect the development of late stripe melanocytes but not early stripe melanocytes, indicating that the corresponding genes are only required in a subset of adult melanocytes.
Melanocyte: Pigment cells derived from neural crest or are part of the RPE that have melanin containing structures termed melanosomes, giving them a black or brown appearance.
Neural crest: A transient organ of embryogenesis. In zebrafish, neural crest cells arise by delaminating from the neuroepithelium of the developing neural tube. They then migrate from their initial location dorsal to the neural tube on one of two pathways to target sites (Box 1). Neural crest cells give rise to many different cell types such as cartilage, connective tissue, sympathetic and sensory neurons, Schwann cells, glia and pigment cells.
Retinal pigmented epithelium (RPE): A sheet of melanin-containing cells that form the margin of the eye. These cells arise from the optic vesicle rather than the neural crest.
Xanthophore: Pigment cells derived from neural crest that contain pteridine and/or carotenoid pigments and are yellow or orange in appearance.

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Box 1. Pigment cell development from neural crest

Most vertebrate pigment cells develop from the neural crest. In zebrafish, neural crest cells delaminate from the dorsal neural tube and migrate between the somites and the neural tube, or between the ectoderm and the somites (Fig. I; red arrow) to target locations [a]. As they migrate, neural crest cells differentiate into a variety of cell types including peripheral neurons, cartilage, connective tissue, Schwann cells, glia and pigment cells [b]. Zebrafish trunk neural crest cells are thought to be specified before migration [c]. This figure, showing tangential sections through the trunk of a zebrafish embryo at three progressive stages, specifically depicts melanocyte development from neural crest. Xanthophores and iridophores arise in a similar fashion but require different genes for their specification and migration. In zebrafish, neural crest derived embryonic melanocyte precursors first require the *microphthalmia transcription factor* gene (*mitf*) for melanocyte fate specification, and then require the *kit* growth factor receptor tyrosine kinase gene for migration to target sites and subsequent survival [d,e]. The role for *endothelin receptor B* (*ednrb*) in zebrafish embryonic melanocyte development is less clear [f], however, work from mouse suggests *ednrb* acts before *kit* [g] and may be required for lineage expansion or specification [g,h]. NC, neural crest; S, somite; N, notochord; NT, neural tube.

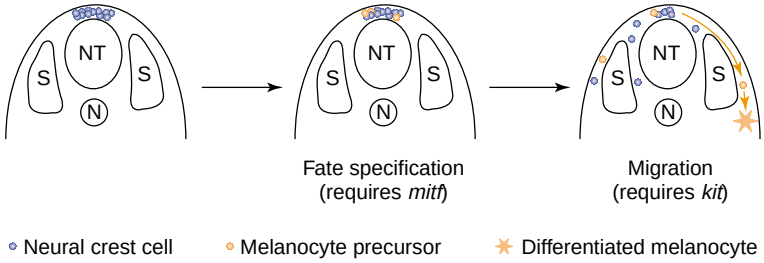

Fig. I

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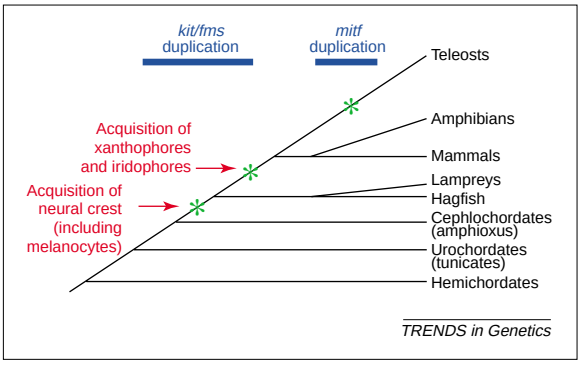

Fig. 1. Association of neural crest and pigment cell innovations with gene duplications during vertebrate and teleost evolution. This probable phylogeny of vertebrate evolution is adapted from Ref. [2]. Predicted genome duplications or large-scale gene duplications are indicated by green asterisks. Other independent partial or whole genome duplications are thought to have occurred in individual species in certain lineages such as in *Xenopus laevis*. The lower red arrow indicates the predicted time when vertebrate specific features, such as neural crest (including melanocytes), placodes, cartilage, bones and teeth, originated [8]. The upper arrow indicates when the other neural-crest-derived pigment cell types (xanthophores and iridophores) presumably originated. This is based on the observation that amphibians and teleosts have melanocytes, xanthophores, and iridophores whereas lampreys appear to only have melanocytes (D. McCauley and J. Langeland, pers. commun.). Xanthophores and iridophores are thought to be lost in the mammalian lineage. We suggest that the duplication of a *kit/fms* ancestor producing the *kit* and *fms* genes occurred about the same time as ancestral duplications that preceded the divergence of fish and mammals. Whether or not the *kit/fms* duplication preceded the emergence of xanthophores and iridophores, possibly facilitating their evolution, or followed the emergence of these pigment cell types is unknown. The duplication event that produced zebrafish *mitfa* and *mitfb* appears to coincide with a predicted genome duplication or large-scale gene duplication in the teleost lineage.

equivalent to the expression pattern of the single gene in mouse. For instance, in mouse, the *Engrailed1* (*En1*) gene is expressed in a subset of central nervous system (CNS) neurons and limb buds [13]. This expression pattern is partitioned between two *En1* co-orthologs in zebrafish. The first, *eng1*, is expressed in zebrafish pectoral fin buds, and the second, *eng1b*, is expressed in CNS neurons [14]. Together, this suggests that the mouse expression pattern reflects the expression pattern of the ancestral vertebrate *En1*. Duplication of the *En1* locus in the teleost lineage might have relaxed selective pressure on these genes, leading to degeneration of tissue-specific enhancers in each of the duplicated genes, resulting in their expression in complementary patterns in the zebrafish embryo. Force and colleagues have proposed this model (duplication–degeneration–complementation) as one means by which duplicated copies of genes are preserved [14].

An opportunity for studying how the function and expression of duplicated genes can be partitioned between different but related cell types is provided by the multiple pigment-cell types of the zebrafish pigment pattern. Identification of genes affected by zebrafish pigment pattern mutations reveals several such cases.

Complexity in the zebrafish pigment pattern

Identifying the pleiotropic roles of a gene that acts at multiple stages of development or in multiple cell types is hindered if mutants for this gene die at the stage when the gene is first required or if resulting pathologies obscure the role of the gene in other cells. Because the pigment pattern in fish is dispensable under laboratory conditions, many mutations can be identified that are viable (and indeed, beautiful; Fig. 2a) with no associated pathologies. Viability of the

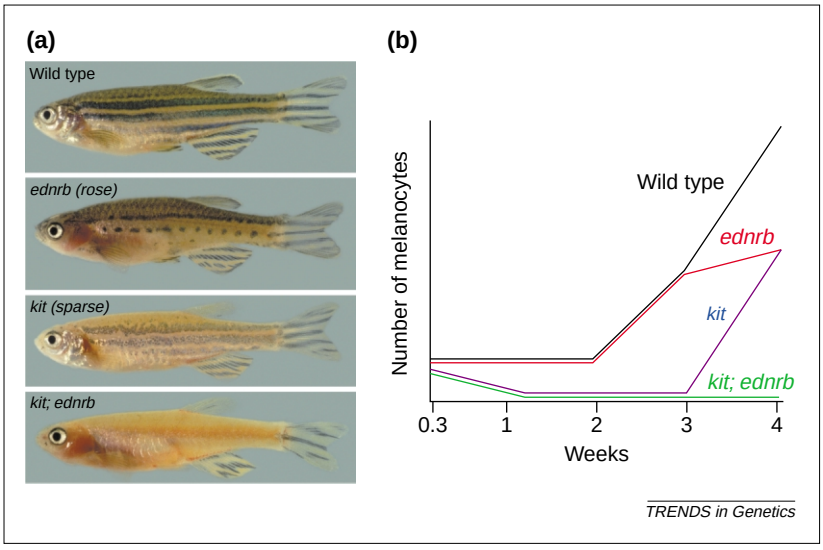

Fig. 2. Mutations in *kit* and *ednrb* reveal that three distinct populations of melanocytes contribute to the adult stripes of the zebrafish. (a) *ednrb* (*rose*) and *kit* (*sparse*) mutants each have approximately half the number of melanocytes present in wild-type body stripes [17]. *ednrb* mutants also lack adult iridophores [27]. The double mutant (*kit; ednrb*) lacks all body stripe melanocytes [17], and residual melanocytes reveal a third population (secondary fin melanocytes) [43] in the caudal and anal fins of the zebrafish. (b) Quantitative analysis reveals that melanocytes in *kit* and *endrb* mutants develop at different stages during the larval-to-adult transition. *ednrb* mutants lack late stripe melanocytes (LSMs) that develop between three and four weeks, and *kit* mutants lack melanocytes that develop early (ESMs) between two and three weeks. An LSM epistasis group is revealed by similar quantitative analysis of *primrose*, *leopard* and *panther(fms)* mutants, that also have approximately half the normal complement of body stripe melanocytes, also lack virtually all body stripe melanocytes when doubly mutant with *kit*, and show no significant additional melanocyte deficit when doubly mutant with each other [17,31].

mutant then allows the potential for all pleiotropic requirements of the gene to be revealed. The dispensability of zebrafish pigment cells has allowed the identification of numerous mutations that affect embryonic and adult pigment pattern development (reviewed in Ref. [15]). Despite being dispensable in the lab, pigment patterns are presumably intensely acted upon by evolutionary selection in the wild [16], and analysis of the genes that affect pigment pattern could help reveal the evolutionary mechanisms, such as gene duplication, involved in generating morphological complexity.

Zebrafish pigment pattern contains a complexity of cell types beyond that of the coat colors of mammals. A first level of complexity is the overtly different types of pigment cells in zebrafish. In addition to MELANOCYTES (shared with mammals), zebrafish have yellow XANTHOPHORES, and gold and silver IRIDOPHORES that together make the familiar stripe pattern [17].

More-covert complexity is revealed by genetic analysis that places most mutations that affect adult zebrafish melanocyte development in one of two EPISTASIS GROUPS. One epistasis group affects LATE STRIPE MELANOCYTES (LSMs), and includes mutants for *rose*, *primrose*, *leopard* and *panther*, and a second epistasis group affects EARLY STRIPE MELANOCYTES (ESMs) and consists of mutations at a single locus, *sparse* (Fig. 2). Double mutants for *sparse* and any one of the genes in the LSM EPISTASIS GROUP lack most or all body stripe melanocytes. We have interpreted this epistasis analysis to indicate that ESMs and LSMs are distinct populations of melanocytes that develop under different genetic control [17]. No similar complexity of mammalian melanocyte type has been observed.

Despite the differences in complexity of the mouse and zebrafish pigment patterns, mouse coat-color genetics provides a foundation for studies of zebrafish pigment-pattern mutations. In mouse, a handful of coat-color mutations have been identified that ablate all neural-crest-derived melanocytes. These include *micropthalmia* (*Mitf*, mutant for the Mitf transcription factor [18]), *piebald-lethal* (s^l; mutant for endothelin receptor B [Ednrb] [19]) and *dominant spotting* (*W*; mutant for Kit, a receptor tyrosine kinase [RTK] [20]). Mutants at these loci are thought to cause defects in specification of the melanocyte lineage (*Mitf*) [21,22], failure to expand melanocyte precursors before migration from the neural crest (*Ednrb*) [23,24], or failure to migrate from neural crest and survive (*Kit*) [25]. *Mitf* mutants also lack the RETINAL PIGMENTED EPITHELIA (RPE) [18]. One prediction is that mutations in each of these genes would ablate all melanocytes or all pigment cells in zebrafish.

Zebrafish orthologs for *kit* [26], *ednrb* [27], and *mitf* [28] have been identified. Surprisingly, defects in these genes correspond to some of the pigment pattern mutations discussed above. Thus, *sparse* mutants (which lack ESMs), are defective for a zebrafish *kit* ortholog, and *rose* mutants (which lack LSMs) are defective for a zebrafish ortholog of *ednrb*, and each mutant retains the complementary subset of melanocytes. By contrast, all mouse neural-crest-derived melanocytes require both *Kit* and *Ednrb* for their development (as discussed above). Additionally, mutants for *nacre*, which lack all neural-crest-derived melanocytes, but have normal RPE, are defective for a zebrafish ortholog of *mitf* [28]. This differs from the mouse *Mitf* mutant discussed above that has defects in both RPE and neural-crest-derived melanocytes. In each case, the partial pigment pattern or melanocyte deficit described above results from complete inactivation of the zebrafish gene, confirming that the partial loss of pigment cells was not the result of leaky gene function for these mutations. In light of a possible recent genome duplication in the zebrafish lineage [11], the differences between the mouse and zebrafish phenotypes for mutations in these closely related genes presents several opportunities for exploring the role of gene duplications in morphological diversification.

Are recent or ancient gene duplications involved in zebrafish pigment pattern?

An ancient duplication of an RTK used for melanocyte development

One inconsistency between mouse and zebrafish pigment-pattern genetics is that mutants with null or severe loss-of-function alleles for *Kit* and *Ednrb* in mouse lack all neural-crest-derived melanocytes [19,20], whereas zebrafish mutants for orthologs of these genes lack complementary populations of

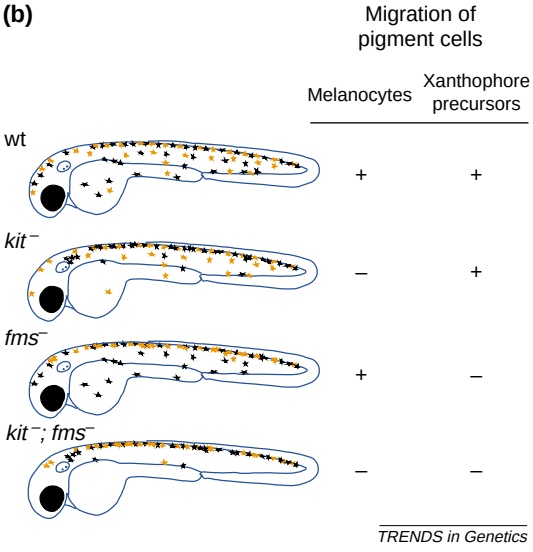

Fig. 3. Requirement of different pigment cell types on paralogous receptor tyrosine kinases kit and fms in adult and embryonic zebrafish. (a) Adult fish singly or doubly mutant for *kit* or *fms*. The finding that double mutants lack almost all adult melanocytes suggests that *fms* is required for development of LSMs [31]. Absence of yellow pigmentation in the *fms* mutants indicates *fms* is also required for development of all xanthophores. Note iridophores and secondary fin melanocytes are unaffected in *kit; fms* double mutants. (b) Cartoon of *kit* and *fms* mutant embryos [26,31]. Melanocytes in *kit* mutants (black) and xanthophore precursors (red) in *fms* mutants fail to migrate from the embryonic neural crest, indicating similar mechanistic role for these paralogous RTKs in different pigment cell types.

melanocytes [17]. One possible explanation for this difference is that each population of zebrafish melanocytes uses a different type of genetic mechanism for its development (e.g. one population requires an RTK, such as kit, and the other population requires a G-protein-coupled receptor, such as the ednrb receptor, for growth factor receptor functions). Alternatively, the same type of genetic mechanism might be employed for development of each type of melanocyte (e.g. both populations require an RTK and a G-protein-coupled receptor). Given the high frequency of duplicated genes reported in zebrafish [29,30], one prediction of this second model is that a recently duplicated copy of *kit* could promote similar functions in LSMs as *kit* promotes in ESMs. The identification of a *kit* paralog as the mutated gene among the LSM epistasis group would tend to support this second model.

Evidence for the second model above comes from the discovery that mutations in the zebrafish *fms* (or *csf1r*) gene encoding a type III RTK are responsible for the *panther* LSM deficit [31] (Fig. 3a). With this result, we have identified a type III RTK responsible for development of both ESMs (*kit*) and LSMs (*fms*). Our second model predicted that the RTK responsible for LSM development would be the result of a recent duplication of the *kit* locus specific to the teleosts [11]. Instead, we found an ancient duplication event [32] that preceded the divergence of mammals and fish was responsible for generating the two RTKs that control development of these different populations of melanocytes (Fig. 4a). Mouse mutants for *Fms* have yet to be described; however, mutants for the *Fms* ligand, M-CSF (mutated in the *op* mouse) have no deficit in melanocyte development [33], suggesting that loss-of-function mutants for *Fms* will likewise have no melanocyte deficit. Whether this suggests that mouse *Fms* gene has lost its role in melanocyte development or zebrafish *fms* has gained this role is not yet clear, although analysis in amphibians could help resolve this issue.

Zebrafish studies have revealed another role for *fms* – promotion of the development of the yellow xanthophore pigment cells. This role provides an additional way to demonstrate the parallel requirements for *kit* and *fms* in development of different pigment cells types (Fig. 3b). In embryos mutant for *kit*, melanocytes mostly fail to migrate from the neural crest, but xanthophores or their precursors migrate normally [26]. By contrast, xanthophore precursors fail to migrate from the neural crest in *fms* mutants [31]. Notably, where the mechanistic basis of the deficits in ESM and LSM in larval or adult development caused by *kit* and *fms* mutants is still unknown (but see Ref. [34]), the phenotype of these mutants in embryos show parallel mechanistic roles in promoting migration of pigment cells from the neural crest.

Which roles for *fms* are ancestral? One ancestral role for *fms* could be in promoting development of osteoclasts and macrophages. These cells are not derived from the neural crest, but they also require *fms* and *fms* ligand for development in both zebrafish and mouse [31,35–37]. Because *fms* presumably does not have a role in mammalian melanocytes, and mammals lack xanthophores, analysis of additional vertebrate groups will be necessary to determine whether *fms* was required in these cells in the ancestral vertebrate. One opportunity to address this question could come from analysis of *fms* in other tetrapods, such as urodeles (salamanders) and anurans (frogs), which, similar to zebrafish, have xanthophores. Finding that *fms* is expressed in xanthophores in these animals or that mutants for their *fms* ortholog lack xanthophores would tend to suggest that the role observed in zebrafish xanthophores is ancestral.

A recent duplication of mitf

Our analysis of *kit* and *fms* showed duplications that occurred in vertebrates ancestral to fish and mammals are important in the complexity of pigment pattern in zebrafish. Given the frequency of duplicated gene pairs

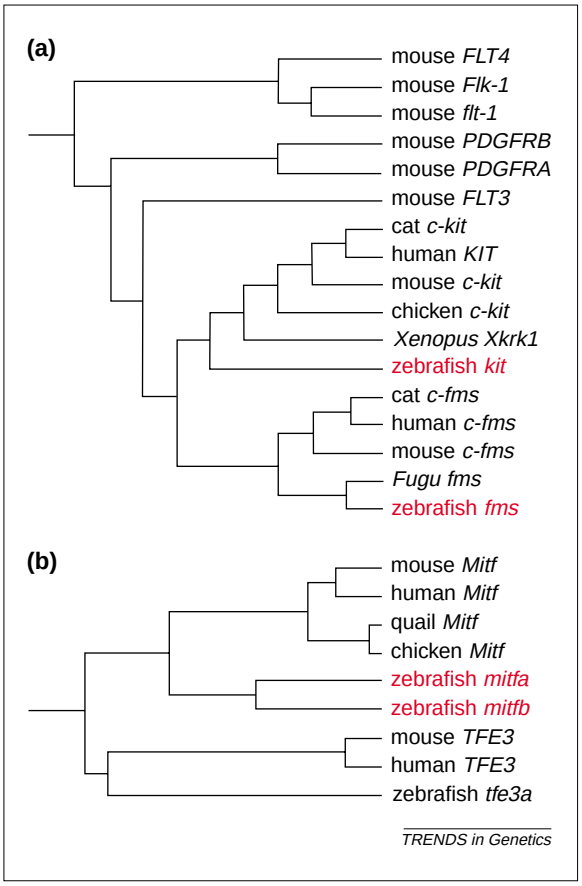

Fig. 4. Phylogenic analysis of zebrafish pigment pattern genes. (a) Zebrafish *kit* and *fms* result from an ancient duplication that occurred before the divergence of zebrafish and tetrapods. Additional gene duplications resulted in PDGFRA, PDGFRB, FLK1, FLT3, FLT1 and FLT4 receptor tyrosine kinases (RTKs), that could provide the analogous RTK function for iridophores that *kit* and *fms* provide melanocytes and xanthophores (see text). (b) Zebrafish *mitfa* and *mitfb* result from a recent duplication that occurred following the divergence of zebrafish and tetrapods. Phylogenetic trees shown are adapted from Refs [31,38].

in zebrafish (where there is a single gene for mammals) [29], it seems probable that recent duplications might also contribute to the increase in pigment-pattern complexity in zebrafish when compared with mammals. Analysis of the *mitf* genes provides one example. Similar to the unexpected discrepancy of phenotypes of zebrafish and mouse *kit* and *ednrb* mutants, zebrafish *mitfa* mutants lack all neural-crest-derived melanocytes but show no defects in RPE [28], whereas in mouse, strong loss-of-function *Mitf* mutants also lack RPE [18]. RPE in zebrafish express *mitfa*, indicating that the role in RPE development for *mitfa* might be conserved in zebrafish but be masked by a partially redundant copy [28]. A second *mitf* (*mitfb*) has recently been identified. *mitfb* resulted from a duplication of the *mitf* locus after the divergence of fish and mammals (Fig. 4b), and it is also expressed in RPE but not neural-crest-derived melanocytes [38].

The recent duplication of *mitf* in the zebrafish lineage provides an instructive example of the mechanisms involved in partitioning gene function following duplication. The mouse *Mitf* locus is organizationally complex, with different transcripts expressed in RPE and neural-crest-derived melanocytes [39]. If this reflects the organization of the ancestral *mitf* gene, perhaps gene function and expression domains were partitioned between the duplicated genes by retention or loss of specific enhancers, promoters or transcription initiation sites. Some evidence for this is provided by the observation that the *mitfa* cDNA is similar to the mouse *Mitf* M transcript isoform (having expression specific to melanocytes), and the *mitfb* cDNA is most similar to the *Mitf* A transcript isoform (having expression enriched in the RPE) [38]. Possibly zebrafish *mitfb* has lost a neural-crest-specific enhancer or transcription initiation site [38], although in this case, both genes retain the RPE enhancer or initiation site.

Table 1. Requirement of pigment pattern genes (identified and predicted) in different pigment cell populations[a]

	RTK[b] requirement			*ednrb* requirement	
Pigment cell population	***kit* [26]**	***fms* [31]**	**Predicted additional RTK[b]**	***ednrb* [27]**	**Predicted additional *ednrb***
Melanocytes					
Embryonic	+	–	–	–	+
Early stripe (ESMs)	+	–	–	–	+
Late stripe (LSMs)	–	+	–	+	–
Primary fin	+	–	–	–	+
Secondary fin [43]	–	–	+	–	–
Iridophores					
Adult	–	–	+	+	–
Embryonic	–	–	+	–	+
Xanthophores	–	+	–	–	+

[a]Requirement (+) or lack of requirement (–) of genes involved in pigment pattern development for different pigment cell populations.
[b]RTK, receptor tyrosine kinase.

The search for missing links

Our analyses suggest that pigment cells in zebrafish require homologs of many of the genes required for mouse melanocyte development. When mutants for zebrafish homologs of mouse coat-color genes retain one or more classes of pigment cells, our experience indicates it might point the way to additional members of the gene family acting in these residual pigment cells. Several additional homologs of RTKs and endothelin receptors are predicted (Table 1) on the basis of the mutant phenotypes of genes described above or their expression patterns.

The finding that the ancient paralogs *kit* and *fms* control parallel development of ESMs and LSMs raises the possibility that a paralog of *ednrb* (which is required for development of LSMs), could control development of ESMs. Similarly, the fact that *ednrb* is required for development of adult iridophores (Fig. 2a) raises the possibility that endothelin receptor function is generally required for development of all neural-crest-derived pigment cells. Each of the embryonic pigment cells (melanocytes, xanthophores and iridophores) express *ednrb* but show no deficit in *ednrb* mutants [27] (Table 1). This suggests that one or more redundant endothelin-receptor-like genes that have yet to be identified are acting to promote the development of these pigment cells. To date, only one *ednrb* gene has been identified in zebrafish, and our searches of zebrafish expressed sequence tag (EST) database [30] and whole-genome shotgun sequence database (2× coverage; The Sanger Centre, http://trace.ensembl.org) show no

evidence for a closely related paralog (>75% sequence identity) from a recent duplication. One possibility is that the product of a more-ancestral duplication provides the redundant function that enables development of pigment cells not affected in *ednrb* mutants. Likewise, that zebrafish *ednrb* mutants have no apparent deficit in enteric neurons [27] (which are severely affected in the mouse mutants [19]) suggests that additional endothelin-receptor-like genes might promote their development as well.

Similar logic can now be applied to suggest that pigment cells that are not affected in *kit* and *fms* mutants could use another RTK to promote their development. Fish doubly mutant for *kit* and *fms* retain iridopohores and secondary fin melanocytes (Fig. 3a), suggesting that at least one additional RTK that affects these cells remains to be identified. Perhaps mutations that ablate iridophores, such as *transparent* (C. Walker and S.L. Johnson, unpublished) or *shady* [40] are defective for such an RTK. As with *ednrb* analysis, we find no evidence that paralogs of *fms* or *kit* from a recent duplication persist in the zebrafish genome. One attractive possibility is that RTKs related to *kit* or *fms* by more-ancestral gene duplications (such as PDGFRs or FLTs [29]) might provide the RTK function for these pigment cells. Similarly, *Kit* is required for germ-cell and blood development in mouse [41,42] but not in zebrafish [26], and one or more of these paralogs from more ancient duplications could be providing the Kit-like RTK role for these cells.

Conclusions and speculation

We have discussed two examples of gene duplications in the evolution of vertebrates or teleosts that had significant roles in generating or maintaining complexity of fish pigment patterns. We have described examples of ancient (*kit* and *fms*) [26,31] and recent (*mitfa* and *mitfb*) [28,38] gene duplications that segregate gene function between different types of pigment cells. We can now use these examples to make predictions about the types of pigment cells that might have existed in vertebrate ancestors and the requirements for the ancestral genes in those pigment cells.

It seems probable that the immediate ancestor of fish and mammals had melanocytes, xanthophores and iridophores. One major reason for drawing this conclusion is that xanthophores and iridophores are found not only in fish, but also in anurans (frogs), urodeles (salamanders) and reptiles, which branched from the mammalian lineage more recently than fish (Fig. 1). That lampreys seem to lack xanthophores and iridophores (D. McCauley and J. Langeland, pers. commun.) raises the possibility that these pigment cell types were acquired after the divergence of lampreys, and before the divergence of tetrapods (such as amphibians and mammals) from the teleost lineage. Presumably, neural-crest-derived xanthophores and iridophores have been lost subsequently from the mammals.

Did the common ancestor to fish and mice have two populations of melanocytes or one? It is possible that the zebrafish lineage gained a second melanocyte population following the divergence of fish and mice, or that the mouse lineage might have lost the *fms*-dependent population of melanocytes since this divergence. Finding that *fms* orthologs are expressed in a subpopulation of developing melanocytes in other mammals, anurans, urodels or other tetrapods might suggest that the mouse lineage has lost *fms*-dependent melanocytes. Alternatively, expression studies showing that only teleosts express *fms* in melanocytes might indicate that the *fms*-dependent population is a derived feature of the teleosts.

A more difficult problem is what type of pigment cells existed in the chordate or vertebrate ancestor before the *kit*/*fms* duplication. Although it seems probable that most or all pigment cells (except RPE and possibly iridophores) required the ancestral *kit*/*fms* gene, it is not clear whether the different pigment cell types of melanocytes, xanthophores and iridophores had evolved by this stage of vertebrate evolution. One possibility is that this ancestor had a single type of pigment cell, and that gene duplications, such as the *kit* and *fms* duplication, led to the diversification of different pigment cell types. Alternatively, this ancestor might already have had multiple types of pigment cells, and RTK gene duplication and subsequent partitioning of expression and function between cell types could have allowed for more exquisite control in generating pattern and morphology. Identification of *kit* and *fms* orthologs, and analysis of their expression in all pigment cells, in amphibians or in deeper branches of vertebrate evolution (such as lamprey, hagfish and amphioxus) could help elucidate the role of the *kit*/*fms* duplication in generating different pigment cells and their ability to generate morphological complexity.

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Establishment of polarity in angiosperm lateral organs

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In seed plants, lateral organs such as leaves and floral organs are formed from the flanks of apical meristems. Therefore, they have an inherent positional relationship: organ primordia have an adaxial side next to the meristem, and an abaxial one away from the meristem. Surgical and genetic studies suggest that a morphogenetic gradient, which originates in the meristem, converts the inherent polarity into a functional one. Once an adaxial–abaxial axis of polarity is established within organ primordia, it provides cues for proper lamina growth and asymmetrical development. Several key participants in this process have been identified, and analyses of these genes support and refine our views of axis formation in plants. The complex relationships between and within various members of these plant-specific gene families (class III HD-ZIPs, YABBYs and KANADIs) might account for a substantial part of the morphological variation in lateral organs of seed plants.

The bodies of SEED PLANTS (see Glossary) are generally composed of two distinct classes of organ: roots and stems have overall radial symmetry, whereas LATERAL ORGANS, such as leaves and floral organs, display lateral growth and distinct asymmetrical development. The inherent relationship between the lateral organs and the apical meristem from which they develop is the basis for the definition of three primordial axes (Fig. 1). First, primordia have a proximal–distal axis that is clearly defined by their attached (proximal) and free (distal) ends. Asymmetry along the proximal–distal axis is inherent and can be seen in many leaves that have proximal petiole and distal blade. Second, primordia also have a left–right axis whose positional relationship to the meristem is unclear, but could be associated with PHYLLOTAXY. In some cases, for example the leaves of most *Eucalyptus* species, asymmetries are evident in the left–right axis. Third, primordia have an adaxial–abaxial axis (Fig. 1), with one side forming next to (*ad*, close to) and the other side away from (*ab*, away from) the meristem. In most angiosperm leaves, polarity in the adaxial–abaxial

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