

Pigment Pattern Formation in the Guppy, *Poecilia reticulata*, Involves the Kita and *Csf1ra* Receptor Tyrosine Kinases

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ABSTRACT Males of the guppy (*Poecilia reticulata*) vary tremendously in their ornamental patterns, which are thought to have evolved in response to a complex interplay between natural and sexual selection. Although the selection pressures acting on the color patterns of the guppy have been extensively studied, little is known about the genes that control their ontogeny. Over 50 years ago, two autosomal color loci, *blue* and *golden*, were described, both of which play a decisive role in the formation of the guppy color pattern. Orange pigmentation is absent in the skin of guppies with a lesion in *blue*, suggesting a defect in xanthophore development. In *golden* mutants, the development of the melanophore pattern during embryogenesis and after birth is affected. Here, we show that *blue* and *golden* correspond to guppy orthologs of *colony-stimulating factor 1 receptor a* (*csf1ra*; previously called *fms*) and *kita*. Most excitingly, we found that both genes are required for the development of the black ornaments of guppy males, which in the case of *csf1ra* might be mediated by xanthophore–melanophore interactions. Furthermore, we provide evidence that two temporally and genetically distinct melanophore populations contribute to the adult camouflage pattern expressed in both sexes: one early appearing and *kita*-dependent and the other late-developing and *kita*-independent. The identification of *csf1ra* and *kita* mutants provides the first molecular insights into pigment pattern formation in this important model species for ecological and evolutionary genetics.

THE guppy (*Poecilia reticulata*) is thought to be among the most color-polymorphic vertebrates (Endler 1983). Male guppies have an outstanding degree of variation in their ornamental patterns, which are shaped by a complex interplay between natural and sexual selection in wild populations. Along with introduction experiments, studies on guppy life-history traits, mate choice behavior, and predator–guppy as well as guppy–environment interactions have demonstrated that guppy populations can adapt rapidly to new environments (for an overview, see Magurran 2005).

The guppy is therefore a prime model organism for the study of “evolution in action.”

Despite our wealth of knowledge about the ecological importance of coloration, the genes and developmental pathways underlying guppy pigment pattern formation are unknown. Both forward and reverse genetic studies are hampered by the fact that guppies are livebearers with internal fertilization, an average gestation period of 3–4 weeks, and a relatively small brood size (Houde 1997). The genetic basis of sex determination is highly variable within the Poeciliid family, to which the guppy belongs. The guppy itself has incipient X and Y chromosomes that include a non-recombining part (Traut and Winking 2001). Only males develop highly polymorphic ornaments during puberty, which are under hormonal control (Houde 1997). The genetic analysis of male guppy ornaments first attracted attention >80 years ago, when Winge described a total of 18 putative ornamental loci, of which 17 showed sex-linked inheritance and 9 were strictly Y-linked (Winge 1922, 1927). Many more pigment pattern loci, which can be Y-linked, X-linked, XY-linked, or autosomal, have since been described (Lindholm and Breden 2002). Ornamental traits linked to the sex

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Data for File S1 and File S2 are available at ftp://ftp.tuebingen.mpg.de/ebio/csf1ra_kita_mutants

Sequence data from this article have been deposited under GenBank accession nos. KC143122 (*csf1ra*); KC143123 (*csf1ra blue* allele); KC143124 (*kita*); KC143125 (*kita*); and KC143126

(part of *kita* genomic locus in *golden* mutants).

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chromosomes are typically expressed only in males, but females can develop some male color patterns when treated with testosterone (Clemens *et al.* 1966; Lindholm and Breden 2002). An analysis of quantitative trait loci (QTL) has confirmed that most male color traits are controlled by multiple genes, including genes on autosomes (Tripathi *et al.* 2009b). In contrast to the sex-specific genes, several autosomal color factors behave as ordinary Mendelian recessive genes and are expressed in both sexes (Goodrich *et al.* 1944; Dzwillo 1959; Lindholm and Breden 2002).

The pigment pattern of the guppy consists of three to four different types of neural crest-derived chromatophores: black melanophores, yellow/orange to reddish xanthophores, blue iridescent iridophores, and, possibly, white leukophores (Takeuchi 1976; Tripathi *et al.* 2008). Guppy embryos are staged according to the differentiation of their eyes. In the middle-eyed stage, the retina is fully pigmented and the first melanophores differentiate on the head above the midbrain (Martyn *et al.* 2006). More melanophores appear during the late-eyed stage and form dark stripes along the lateral midline, on the back, and on the belly (Martyn *et al.* 2006). In the very late-eyed stage shortly before birth, a rhombic reticulate pattern consisting of melanophores emerges on the trunk (Martyn *et al.* 2006). It has also been referred to as a ground, diamond, or camouflage pattern (Goodrich *et al.* 1944; Martyn *et al.* 2006; Tripathi *et al.* 2008). All pigment cell types are present in wild-type embryos at birth and contribute to the newborn pattern (Figure 1A); *e.g.*, the yolk is partially covered by iridophores and melanophores, while xanthophores are widely dispersed (Martyn *et al.* 2006).

The reticulate pattern of very late-eyed embryos and newborn guppies appears to persist into adulthood (Tripathi *et al.* 2008). This pattern is expressed in both sexes, but becomes overlain in males by male-specific ornaments (Figure 1, B–E). Two different melanophore types occur in adult wild-type guppies: large, roundish corolla and more heterogeneously shaped dendritic melanophores (Goodrich *et al.* 1944). The reticulate pattern is composed of corolla melanophores in deep skin layers, which are additionally arranged irregularly over the whole body, whereas dendritic melanophores are distributed superficially and are associated with the scales (Figure 1E) (Goodrich *et al.* 1944; Winge and Ditlevsen 1947).

Two autosomal color loci that are expressed in male and female guppies are *blue* and *golden* [also known as *fredlini* (Haskins and Druzba 1938; Winge and Ditlevsen 1947) and not to be confused with the zebrafish (*Danio rerio*) *golden* locus (Lamason *et al.* 2005)]. The mutations in both genes occurred spontaneously and act recessively (Goodrich *et al.* 1944; Dzwillo 1959). *Blue* mutants lack orange pigmentation in their skin, indicating a xanthophore defect (Figure 1) (Dzwillo 1959).

In *golden* mutants, the development of the melanophore pattern during embryogenesis and after birth is affected (Haskins and Druzba 1938; Goodrich *et al.* 1944; Winge and Ditlevsen 1947). Golden guppies of both sexes lack

melanophores in the skin at birth and have only a few peritoneal melanophores above the swim bladder (Figure 1A), but eventually develop an incomplete reticulate pattern, which gives them a “coarsely mottled appearance” (Goodrich *et al.* 1944; Winge and Ditlevsen 1947). Corolla melanophores are restricted to the reticulate pattern in *golden* mutants, which also have only a very few dendritic melanophores (Figure 1E) (Haskins and Druzba 1938; Goodrich *et al.* 1944; Winge and Ditlevsen 1947). *golden* mutant females have only half of the normal number of melanophores in total (Goodrich *et al.* 1944). *golden* mutant males develop male-specific ornaments (Figure 1, B and C) (Haskins and Druzba 1938).

Among teleost fish, pigment pattern formation has been most extensively studied in zebrafish. Zebrafish undergo a complex pigment pattern metamorphosis during the transition from the embryonic/early larval to the juvenile/early adult stage (Johnson *et al.* 1995; Parichy and Turner 2003a,b; Kelsh *et al.* 2009; Parichy *et al.* 2009; Budi *et al.* 2011). The two type III receptor tyrosine kinases encoded by *kita* and its ancient paralog *colony-stimulating factor 1 receptor a* (*csf1ra*; previously called *fms*) (Braasch *et al.* 2006) play key roles during the establishment of the adult zebrafish pigment pattern: early metamorphic melanophores require *kita* for their development, while late metamorphic melanophores depend on *csf1ra* and *endothelin receptor b1a* (*ednrb1a*) (Parichy *et al.* 1999, 2000a,b; Parichy and Turner 2003b). *Csf1ra* is also crucial for xanthophore development (Parichy *et al.* 2000a; Parichy and Turner 2003b). In other teleost species, the functions of *kita* and *csf1ra* are less well understood; comparative studies including interspecies hybrids suggest some functional diversification of both receptor tyrosine kinases even within the *Danio* genus (Quigley *et al.* 2005; Parichy 2006; Mills *et al.* 2007).

Here, we present evidence that *golden* and *blue* correspond to guppy orthologs of *kita* and *csf1ra*. Both an early *kita*-dependent and a later-appearing *kita*-independent melanophore population contribute to the adult reticulate pattern in this species. In contrast to zebrafish, *csf1ra* is not required for the development of the late-appearing *kita*-independent melanophores. *Csf1ra*, however, is essential for xanthophore development and the formation of the male-specific melanophore pattern, which also requires *kita*.

Materials and Methods

Fish husbandry

All fish were maintained at 25° in a 12-hr light and dark cycle and fed 6 days a week with *Artemia*. No more than eight fish were kept per 1.5-liter tank. We used virgins for crosses, as guppy females are capable of storing sperm.

Strains

Guppies of the following inbred strains were used in this study; available phenotypes other than wild-type are shown in parentheses: Maculatus (MAC) (*golden*) (Tripathi *et al.*

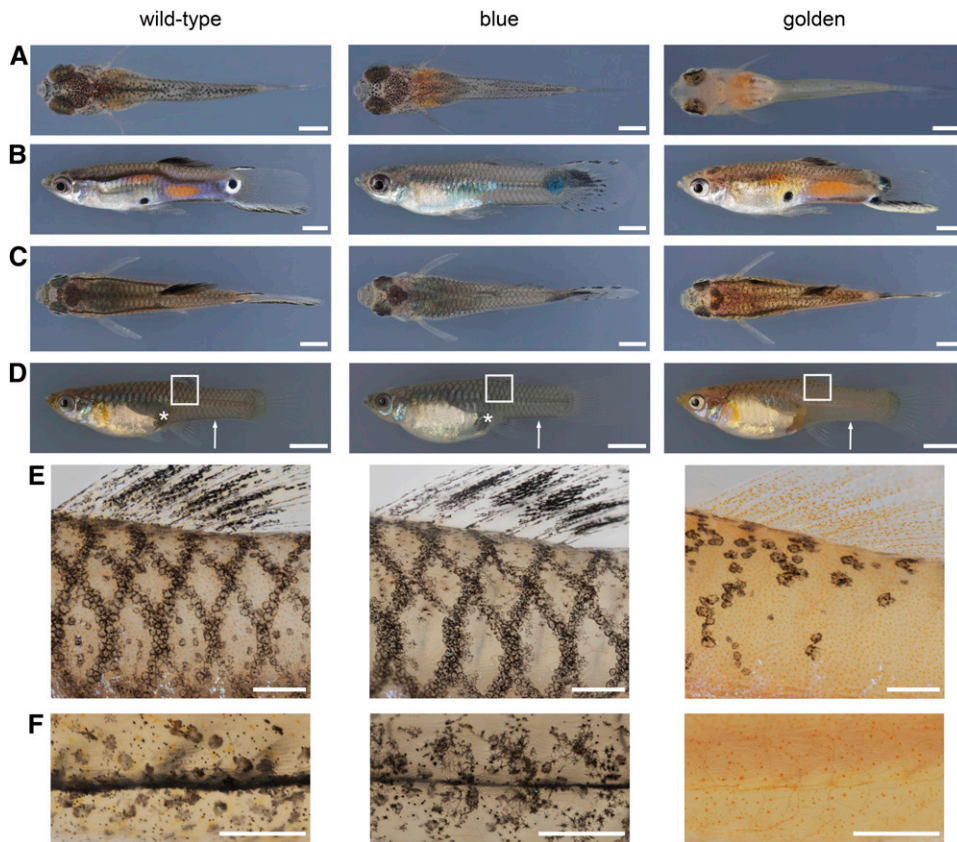


Figure 1 Blue and golden phenotypes. (A) Dorsal aspect of newborns. (B) Lateral aspect of adult males. (C) Dorsal aspect of adult males. (D) Lateral aspect of adult females. (E) Details of areas boxed in D showing the reticulate pattern. (F) Ventral view of the caudal peduncle of females (indicated by white arrows in D). *golden* mutants of both sexes lack a ventral black stripe and have only a few melanophores on the anterior head, including the choroid of the eyes. Golden females lack the female pigment spot above the anal fin (white asterisks in D). Individuals shown are from the BDZW1 (wild type, golden) and BDZW2 (blue) background. Bars: (A) 1 mm; (B and C) 2 mm; (D) 5 mm; (E and F) 0.5 mm.

2008); BDZW1 (golden, golden blue) and BDZW2 (blue only) (Dzwillo 1959); Armatus (golden) (Winge 1927); Guanapo and Quare6 (Reznick and Endler 1982); Quare6 family II 215-3 (Tripathi *et al.* 2008); and Cumaná (Alexander and Breden 2004). Maculatus, BDZW1, BDZW2, and Armatus are domesticated strains that have been bred in captivity for >50 years [in our laboratory since 2003 (Maculatus, BDZW1) and 2011 (Armatus, BDZW2)]; the others are derived from wild individuals caught in Trinidad (Guanapo and Quare rivers) and Venezuela (Cumaná region). Quare and Cumaná guppies were obtained in 2003 and Lower Guanapo (Twin Bridge) fish in 2009. All guppy strains are kept separately in small groups, usually consisting of two to three females and four to five males per tank and are allowed to reproduce freely, except for the Guanapo fish that are inbred by brother–sister mating.

blue was found in two strains that likely both were derived from the original population of *blue* mutants described by Dzwillo in 1959. We renamed them BDZW1 and BDZW2 for clarity. BDZW1 was obtained under the name “Blau” and comprised individuals heterozygous for *golden* and *blue*; BDZW2 (“Dzwillo 1959 Blau”) fish were all homozygous for *blue*.

Phylogenetic analyses

Only unambiguously annotated sequences were used for the analyses (ORF begins with a start codon; number of exons are as predicted for other species; and introns begin and end

with splice sites). Sequences were aligned with Clustal Omega (v1.1.0) (Goujon *et al.* 2010; Sievers *et al.* 2011). Maximum-parsimony and maximum-likelihood phylogenetic trees were constructed by the PhylipParsimony algorithm (Felsenstein 1989) via the SplitsTree4 (v4.12.8) interface (Huson and Bryant 2006) and by PhyML (Guindon *et al.* 2010), respectively. The topologies of the maximum-likelihood trees were not fully resolved (therefore not shown), but, as the maximum-parsimony trees, they suggest that the sequenced guppy ORFs are most similar to *kita*, *kit ligand a* (*kitla*), and *csf1ra* of other teleost species.

Complementary DNA analyses

We used polymerase chain reaction (PCR) to amplify genes of interest from first-strand complementary DNA (cDNA). Total RNA was extracted from 10 to 15 pooled early to very-late eyed embryos with TRIzol (Invitrogen) according to the manufacturer’s instructions. Total RNA was then directly used for first-strand cDNA synthesis using PrimeScript Reverse Transcriptase (TaKaRa) and the oligo(dT) primer 5’-ATTCTAGAGGCCGAGGCGGCCGACATGT(18)VN-3’. We added 1 U/μl SUPERaseIn RNase Inhibitor (Ambion) to each reaction. First-strand cDNA was used as template for PCR, which was carried out with Advantage 2 Polymerase Mix (Clontech) according to the manufacturer’s instructions. PCR program was 10 cycles touchdown [95° for 15 sec, T_m (melting temperature) of primers (lower one) + 5° –0.5°/cycle for 30 sec, 68° for 2–5 min] followed by 27 cycles

without touchdown (95° for 15 sec, Tm for 30 sec, 68° for 2–5 min]. Elongation time was adapted to the length of the expected product (~1 min/kb). Primer sequences and methods used for full-ORF amplification of candidate genes are listed in [Supporting Information, Table S1](#). PCR products were analyzed by gel electrophoresis, purified with MinElute Gel Extraction Kit (QIAGEN), and subsequently cloned into pGEM-T Easy vector (Promega) following the manufacturer's instructions. Plasmid DNA was purified with Wizard Plus SV Minipreps DNA Purification System (Promega) according to the instruction manual and sequenced.

To investigate whether the *kita* transcripts V1–V6 cosegregate with the golden phenotype, we prepared first-strand cDNA from adult individuals as described before. Total RNA for cDNA synthesis was extracted separately from liver tissue of the parental male and the parental female and from pooled liver tissue of seven wild-type N2 fish and 11 golden N2 fish. Liver tissue was used as total RNA isolated from liver usually is of very high quality (personal observation). We used primers in exon 5 (forward: 5'-GATGCTGGGAGT TACAAATGCGTAG-3') and exon 9 (reverse: 5'-AACAGT ATGTAGGCTTGCTCTCC-3') for PCR amplification with Advantage 2 Polymerase Mix (Clontech) and cloned the products into pGEM-T Easy vector (Promega). We sequenced the purified plasmid DNA of 24 colonies per parent and N2 pool to identify wild-type and golden mutant *kita* transcripts.

Real-time quantitative PCR

Total RNA for real-time quantitative PCR was prepared as described above from skin of adult wild-type, golden, and blue females that were 6–9 months old. Following DNaseI treatment, first-strand cDNA was prepared from 830 ng of total RNA primed with oligo(dT)₁₈ using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. First-strand cDNA was diluted 10-fold for real-time quantitative PCR that was conducted with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) according to the instruction manuals provided by the manufacturers. PCR program was 95° for 3 min, 40 times (95° for 10 sec, 60° for 10 sec, 72° for 5 sec), and 95° for 10 sec. Expression of *csf1ra*, *csf1rb*, *kita*, and *kitb* was determined by using three biological replicates with three technical repetitions each. Expression of the target genes was normalized to *glyceraldehyde-3-phosphate dehydrogenase* expression. Standard deviation and normalized expressions ($\Delta\Delta C_q$) were calculated with CFX Manager software. Primer sequences and efficiencies (Pfaffl 2001; Vandesompele *et al.* 2002) are given in [Table S2](#).

Genomic DNA analyses

Genomic DNA was prepared with DNeasy Blood and Tissue Kit (Qiagen) from trunk tissue of adult guppies. We used 100 ng of DNA per PCR reaction. If not mentioned otherwise, Advantage 2 Polymerase Mix (Clontech) was used to carry out the PCRs. *Kita*^{insert} was first amplified using

a forward primer in exon 6 (5'-TGTCTCTGAACGTTAG CATGGAG-3') and a reverse primer in exon 7 (5'-ACACG GAGAAGTTCTGCTTTACC-3') of *kita* (elongation time: 5 min). To test whether *kita*^{insert} and the golden phenotype are associated, we designed PCR assays for *kita*^{insert} and *kita*^{wt} using Phusion High-Fidelity DNA Polymerase (New England Biolabs) according to the manufacturer's instructions. Details can be found in [Table S3](#). PCR products were analyzed by gel electrophoresis. PCR products of *csf1ra*^{wt} and *csf1ra*^{indel} were purified with 0.2 U/ μ l FastAP Thermosensitive Alkaline Phosphatase (Fermentas) and 2 U/ μ l exonuclease I (Fermentas) (37° for 15 min, 85° for 15 min) and subsequently sequenced. Primer sequences are given in [Table S3](#).

Sequence analysis

Purified plasmid DNA and PCR products were sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a DNA Analyzer ABI Prism 3730XL (Applied Biosystems). Sequences were analyzed using the Staden package (Pregap4 v1.5 and Gap4 v4.10; <http://staden.sourceforge.net/>) and inspected manually. Exon–intron structures were predicted according to the gene structure of *kitla*, *kita*, and *csf1ra* of other teleost species (for species, see [Table S1](#); exon–intron structure was inferred from <http://www.ensembl.org>).

Imaging

Photos of whole embryos or details of adult fish were taken with a Leica MZFLIII dissecting microscope connected to a Zeiss AxioCam HRc color camera and processed with AxioVision Software Release 4.7.2. Photos of fish after birth and adult fish were taken with a Canon EOS 10D digital camera using a Canon Macro Photo Lens MP-E 65 mm or Canon Macro Lens EF 100 mm. Adult males were at least 4 months old to ensure that their pigment pattern was fully developed. All photos were taken under incident light conditions. Fish were anesthetized in 0.1% (w/v) tricaine (ethyl 3-aminobenzoate methanesulfonate salt) solution (pH 7) before imaging. The background of most images was equalized with Adobe Photoshop software version 12.1; all original images are available upon request.

Analysis of melanophores

To analyze melanophore pattern development, photos of the same fish were taken every 3 days (day 1–40 after birth). Each fish was kept separately in a 1.5-liter tank. Fish were briefly anesthetized in 0.04% tricaine solution before imaging. Their siblings were kept as control; none of the imaged fish showed retarded development. Newly arising melanophores were detected by comparing consecutive images to each other.

To analyze the number of melanophores, fish were immersed in standard E2 solution (Nusslein-Volhard and Dahm 2002) containing 2.4 mg/ml epinephrine for ~5 min to contract melanosomes. Afterward, fish were anesthetized, and the right side under the dorsal fin, as well as the complete

fish, were imaged. Melanophores were counted manually utilizing Adobe Photoshop software version 12.1. The number of melanophores was plotted against the area in square millimeters using Microsoft Excel for Mac 2011 version 14.2.3.

Results

Identification of golden as kita:

We used a candidate gene approach to identify *golden* at the molecular level. Based on a comparison with zebrafish pigment pattern mutants, we investigated two potential candidates for *golden*, *kita*, and *kitla* (also called *stem cell/steel factor*). *Kitla* is the ligand for the Kita receptor, and together the two are required for melanophore migration and survival in zebrafish (Hultman *et al.* 2007). While only one copy of *kit* and *kitl* exists in mouse and humans, two copies of each gene are present within the Teleostei. These copies can be considered as “ohnologs” since they are derived from ancestral *kit* and *kitl* genes that were duplicated during the teleost-specific whole-genome duplication event (Mellgren and Johnson 2005; Braasch *et al.* 2006; Hultman *et al.* 2007). Mutations in zebrafish *kita*, as in *golden*, greatly reduce melanophore number (Parichy *et al.* 1999). *Kita* is located on guppy autosomal linkage group 4 (Tripathi *et al.* 2008). *Kitla* has not been mapped so far.

To amplify the guppy orthologs of *kita* and *kitla*, we used rapid amplification of cDNA ends (Table S1). Phylogenetic analyses demonstrated that the obtained full-ORF sequences are orthologous to *kita* (GenBank accession no. KC143124) and *kitla* (GenBank accession no. KC143125) of other teleost species (Figure 2A and Figure S1). We also identified a potential guppy *kitb* ortholog by performing BLAST searches of zebrafish *kitb* against a preliminary genome and transcriptome assembly of the guppy (E. Sharma, A. Künstner, B. A. Fraser, M. Hoffmann, V. A. Kottler, G. Zipprich, D. Weigel, and C. Dreyer, unpublished data) (Figure 2A) (Altschul *et al.* 1990). We could not determine whether a guppy *kitlb* ortholog exists, as BLAST yielded no significant results.

Golden mutant guppies did not have any obvious polymorphisms in the *kitla* ORF. In contrast, among cDNAs from golden fish of the MAC and BDZW1 backgrounds, we found a total of six different *kita* splice variants, none of which was wild type. Most of these variants contained inserts with a length of 17, 36, or 53 bp and/or lacked parts of or the complete exon 6 (Figure 2B and Table S4). Exons 7 and 8 were additionally absent in two variants (Figure 2B and Table S4). In all cases, these alterations cause frameshifts and truncate the ORF. These *kita* variants are likely non-functional since the encoded proteins would lack the transmembrane, juxtamembrane, and split kinase domains, all of which are required for normal protein function (Mol *et al.* 2003).

Analysis of genomic DNA indicated that *kita* exon 6 of *golden* mutants has an insertion of 1678 bp. This insertion consists of two potential short exons of 17- and 36-bp length that surround a 1625-bp intron delineated by 5' GT-AG 3'

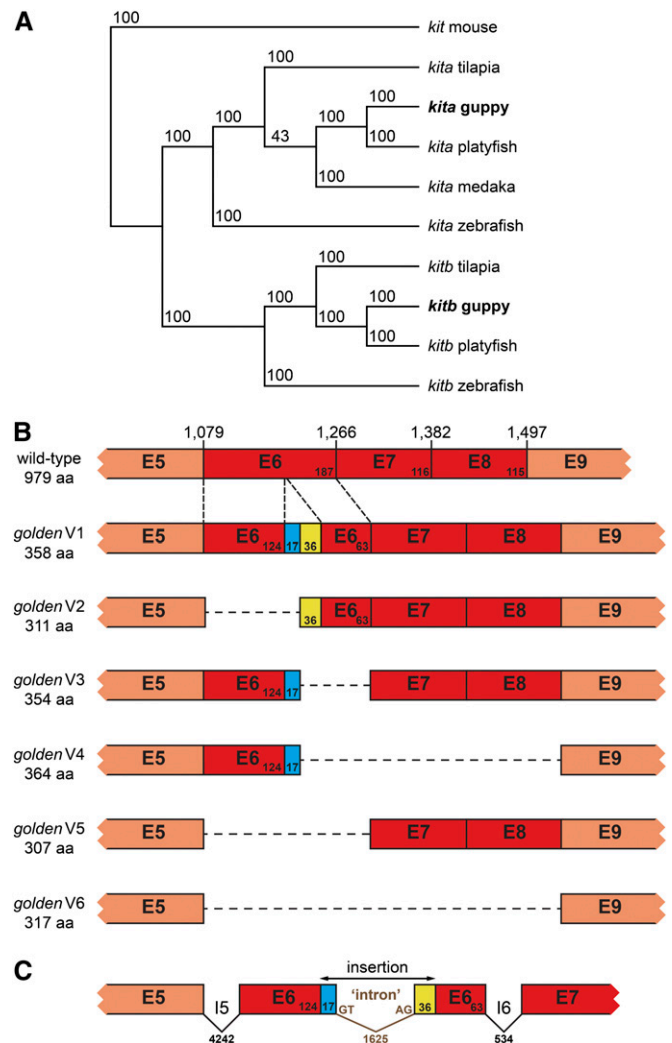


Figure 2 *golden* is an ortholog of *kita*. (A) Maximum-parsimony phylogenetic tree of *kita* and *kitb* ORF sequences. Mouse *kit* was used as an outgroup. Bootstrap support values from 100 replicates are shown. Accession numbers of sequences are the following: guppy *kita*, KC143124; medaka (*Oryzias latipes*) *kita*, ENSORLT00000000707; mouse (*Mus musculus*) *kit*, NM_021099; platyfish (*Xiphophorus maculatus*) *kita*, ENSXMAT00000013579; platyfish *kitb*, ENSXMAT00000017731; tilapia (*Oreochromis niloticus*) *kita*, ENSONIT00000003735; tilapia *kitb*, XM_003452144; zebrafish *kita*, NM_131053; and zebrafish *kitb*, NM_001143918. Guppy *kitb* sequence is available upon request. (B) Exons 5–9 of *kita* cDNAs sequenced from wild-type and *golden* mutant [variants (V) 1–6] backgrounds; exons affected by splicing defects are shown in dark red. Length in amino acids (aa) of the respective predicted protein is shown on the left. Numbers above wild type refer to the wild-type *kita* cDNA sequence deposited at GenBank (KC143124) and demarcate the last nucleotide of each exon. (C) Part of *kita* genomic locus in *golden* mutants (*kita^{insert}*) (GenBank accession no. KC143126). Numbers refer to base pairs. Exons (E), introns (I), and inserted sequence are not drawn to scale.

canonical splice sites (Figure 2C). The splice sites within the novel intron seem to be recognized by the spliceosome and lead to mis-splicing of the *kita* pre-messenger RNAs (mRNAs) in the *golden* mutants. Depending on which splice sites are used, this leads to short insertions or removal of parts of exon

6 or of exons 6–8 from the mature transcript. Comparison with a preliminary genome assembly of the guppy (A. Künstner, E. Sharma, B. A. Fraser, M. Hoffmann, V. A. Kottler, D. Weigel, and C. Dreyer, unpublished data) suggests that >50 copies similar to the 1678-bp insertion, which all include a long terminal repeat of ~300 bp, exist (data not shown).

To confirm that *kita*^{insert} corresponds to *golden*, we tested for genetic linkage. Forty *golden* mutant fish from the MAC, BDZW1, and Armatus (ARM) backgrounds were homozygous for *kita*^{insert} and complementation test crosses between the strains demonstrated allelism (Figure S2B and S1C). In 28 wild-type MAC, BDZW1, and ARM individuals, only *kita*^{wt} could be detected. This indicates that *kita*^{insert} was likely introduced into several guppy strains by breeders because of the golden coloration. Next, we investigated whether *kita*^{insert} is also linked to the golden phenotype in a segregating backcross N2 population: we crossed a *golden blue* double-mutant female of the BDZW1 strain to a wild-type male from the Guanapo river in Trinidad; F₁ males were then backcrossed to produce X^{BDZW1}X^{BDZW1}/X^{BDZW1}Y^{GU} N2 individuals. Forty-six golden and golden blue N2 fish were homozygous for *kita*^{insert}, while 12 wild-type and blue fish were heterozygous *kita*^{insert}/*kita*^{wt}. In addition, we could amplify only *kita*^{insert} variants from pooled cDNA of *golden* mutant N2 offspring. Taken together, all of these results make it very likely that guppy *golden* is allelic to *kita*.

Identification of *blue* as *csf1ra*:

We hypothesized *csf1ra* to be a candidate for *blue*, since *blue* mutants lack xanthophores as do *csf1ra* mutants of zebrafish (Dzwillo 1959; Parichy *et al.* 2000a). *Csf1ra* and *csf1rb* ohnologs have been identified in several teleost species (Braasch *et al.* 2006). *csf1ra* has previously been mapped to guppy autosomal linkage group 10 (Tripathi *et al.* 2008).

Rapid amplification of cDNA ends was used to amplify the guppy *csf1ra* ortholog (Table S1). Phylogenetic analyses showed that the sequenced cDNA is closely related to *csf1ra* of other teleost species (Figure 3A). We also identified a presumptive guppy *csf1rb* ortholog within a preliminary genome and transcriptome assembly of the guppy (E. Sharma, A. Künstner, B. A. Fraser, M. Hoffmann, V. A. Kottler, G. Zipprich, D. Weigel, and C. Dreyer, unpublished data) (Figure 3A).

Blue mutant guppies carry a complex change in exon 17 of *csf1ra*, with a deletion of 5 bp and an insertion of 7 bp (Figure 3B). This indel causes a frameshift and truncates the ORF. The predicted protein lacks part of the second half of the split kinase domain, which is required for the activity of type III receptor tyrosine kinases (Yarden and Ullrich 1988; Mol *et al.* 2003). A similar mutation in zebrafish, *fms*^{j4blue}, inactivates *csf1ra* and leads to loss of xanthophores (Parichy *et al.* 2000a).

We used the same cross as above to test for linkage of *csf1ra* and *blue*. Forty-six blue and golden blue N2 fish were homozygous for *csf1ra*^{indel}, while 12 wild-type and golden N2 individuals tested were heterozygous *csf1ra*^{indel}/*csf1ra*^{wt}. Consistent with this, 24 *golden blue* mutant guppies of the

BDZW1 strain were homozygous for *csf1ra*^{indel}, whereas only *csf1ra*^{wt} could be identified in 12 wild-type and golden fish of the same population. Another *blue* strain obtained from a hobby breeder, BDZW2, was also homozygous for the *csf1ra*^{indel} allele, and complementation test crosses to BDZW1 confirmed allelism (Figure S2A). *blue* is therefore likely to be the guppy ortholog of *csf1ra*, with the same *csf1ra*^{indel} mutation present in both strains tested.

In zebrafish, *csf1ra* promotes the migration of the xanthophore precursors from the neural crest (Parichy *et al.* 2000a), and both embryonic and metamorphic xanthophore populations require *csf1ra* activity (Parichy and Turner 2003b). To investigate whether *csf1ra* mutant guppies entirely lack xanthophores, we thoroughly inspected 7-month-old golden blue and blue fish that shared the same grandparents. In contrast to a previous study (Dzwillo 1959), we found a small number of xanthophores in 19 of 20 *golden blue* mutant individuals and in 5 of 17 *blue* mutants (Figure 3, C and C'). Most of the xanthophores were arranged on scale margins close to the dorsal fin. We could not detect any xanthophores on the lateral or ventral side of adult golden blue or blue fish or in blue embryos and newborns (we here refer to guppies as newborns 1–3 days after birth). A small number of xanthophores can also be found in zebrafish larvae, but not in adults, which are homozygous for *salz*^{tl41a} or *pfeffer*^{tm36b}, two alleles that fail to complement *fms*^{j4blue} (Maderspacher and Nusslein-Volhard 2003).

Expression of *kita*, *csf1ra*, and their ohnologs in female skin

We could detect *kita*, *kitb*, *csf1ra*, and *csf1rb* expression in the skin of adult wild-type, golden, and blue females by Reverse Transcriptase-PCR (data not shown). To assess whether *Kitb* and *Csf1rb* might be upregulated to compensate for the loss of *Kita* and *Csf1ra* function in the *golden* and *blue* mutants, respectively, we investigated the expression levels of these genes and their a-paralogons by real-time quantitative PCR. We found that *csf1ra* expression is downregulated in the skin of *blue* mutant females compared with the wild type, while there was no significant difference in the expression levels of *csf1rb* (Figure S3). In zebrafish, *csf1ra* is expressed in the xanthophore, macrophage, and osteoclast lineages (Parichy *et al.* 2000a). Our data suggest that the guppy ortholog of *csf1ra* is expressed in guppy xanthophores, as the almost complete absence of these cells in blue skin coincides with a low *csf1ra* expression level. Additionally, the blue transcript of *csf1ra* might undergo nonsense-mediated mRNA decay triggered by the premature termination stop codon. In contrast, the expression levels of *kita* and *kitb* were not significantly different in golden skin compared with wild type, respectively (Figure S3), which suggests that the golden *kita* transcripts are not subjected to nonsense-mediated decay. In conclusion, no significantly elevated expression of the *kit* and *csf1r* b-paralogons could be detected by real-time quantitative PCR in the skin of *golden* and *blue* mutants (Figure S3). Yet we cannot

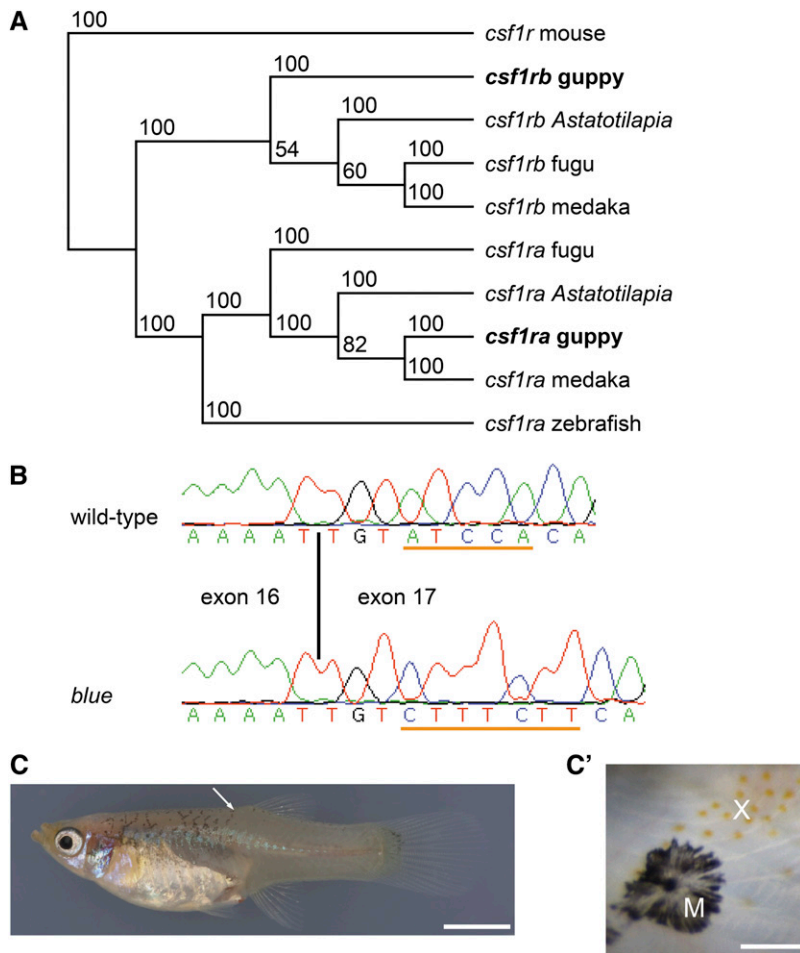


Figure 3 *blue* is an ortholog of *csf1ra*. (A) Maximum-parsimony phylogenetic tree of *csf1ra* and *csf1rb* ORF sequences. Mouse *csf1r* was used as an outgroup. Bootstrap support values from 100 replicates are shown. Accession numbers of sequences are the following: *Astatotilapia burtoni csf1ra*, DQ386648; *A. burtoni csf1rb*, DQ386647; fugu (*Takifugu rubripes*) *csf1ra*, U63926; fugu *csf1rb*, AF411927; guppy *csf1ra*, KC143122; medaka *csf1ra*, ENSORLT00000006111; medaka *csf1rb*, XM_004076731; mouse *csf1r*, NM_001037859; and zebrafish *csf1ra*, AF240639. Guppy *csf1rb* sequence is available upon request. (B) Partial sequence electropherograms of cDNAs from wild-type and *blue* mutant fish, which carry a deletion of 5 nt in exon 17 (underlined in wild-type sequence) that simultaneously contains a 7-nt insertion (underlined in *blue* sequence). The length of the predicted protein produced by *blue* mutants is 794 aa, with the last 12 new. The wild-type protein is 978 aa long. The first nucleotide of exon 17 corresponds to nucleotide 2392 in the 3084-bp wild-type *csf1ra* sequence (GenBank accession numbers of cDNAs: wild-type, KC143122; *blue*, KC143123). (C) *golden blue* mutant female; white arrow points to detail shown in C'. (C') Small patch of xanthophores (X) and melanophore (M) on the back close to the dorsal fin of the female. Variation in the number of xanthophores was high in *blue* and *golden blue* mutant fish. Xanthophores were abundant and evenly distributed in wild-type and *golden* mutant females (Figure 1, E and F). Bars: (C) 5 mm; (C') 50 μ m.

exclude that less efficient salvage pathways mediated by *Kitb* and *Csf1rb* compensate for the loss of *Kita* and *Csf1ra* function in the mutants based on these findings.

Contribution of distinct melanophore populations to the adult reticulate pattern

Kita-dependent and -independent metamorphic melanophores contribute to the adult pigment pattern in zebrafish (Parichy *et al.* 1999; Parichy and Turner 2003b). Additionally, *Kita* activity is required for the dispersal of melanoblasts from the neural crest in zebrafish embryos (Parichy *et al.* 1999). To investigate whether temporally and genetically distinct melanophore populations exist in the guppy, we examined *golden* mutant guppies at different developmental stages.

First, we explanted wild-type and *golden* embryos 12 days after last parturition, which corresponds to the middle-eyed stage, at which the first melanophores differentiate in the skin above the midbrain in wild-type embryos (Martyn *et al.* 2006). *golden* mutant embryos lacked melanophores on the head and the trunk at this stage (Figure 4A). Second, we investigated the development of the pigment pattern between the 1st and the 40th day after birth by taking photographs of the same individuals every 3 days.

Immediately after birth, *golden* mutants had few patches of peritoneal melanophores, as described previously (Goodrich

et al. 1944; Winge and Ditlevsen 1947). Additionally, we found some melanophores in their skin and close to the neural tube (Figure 4B). Four and 7 days after birth, few scattered melanophores were present close to the dorsal midline and on the head of *golden* mutants (Figure 4C and File S1). After 10 days, melanophores had become more abundant on the dorsal part of *golden* mutant fish and formed an incipient reticulate pattern, which became quite prominent after 16 days (Figure 4, D and E). The formation of the reticulate pattern seemed to be completed \sim 22 days after birth in the mutants (Figure 4, F and G), although we detected a few newly arising melanophores even in 40-day-old *golden* individuals (File S1). This suggests that two melanophore populations that are temporally and genetically distinct contribute to the adult reticulate pattern: one that develops early and depends on *Kita* and one that appears late and is independent of *Kita*. In wild-type fish, melanophores were abundant and distributed over the whole body from the day of birth onward (Figure 4, B–G).

Adult *golden* mutant females have about half the number of skin melanophores of wild-type females (Goodrich *et al.* 1944) and lack the pigment spot by the anal fin (Figure 1, D and E). Additionally, the amount of superficial dendritic melanophores associated with the scales is strongly reduced (Haskins and Druzba 1938; Goodrich *et al.* 1944; Winge and

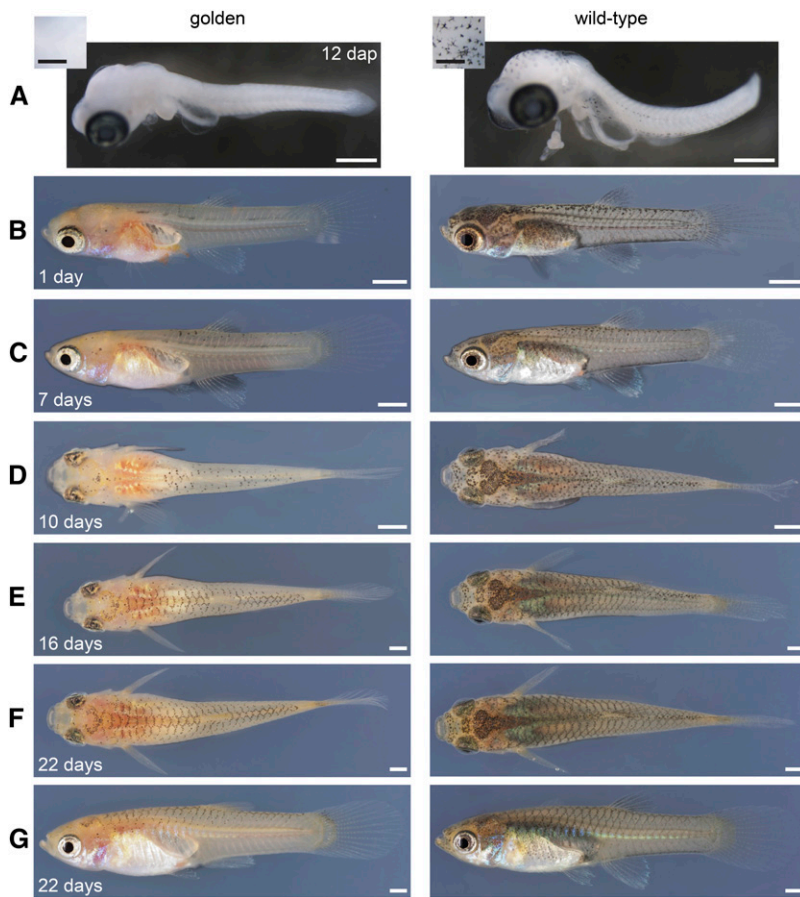


Figure 4 Melanophore pattern development in BDZW1 wild-type and *golden* mutant fish. (A) Embryos explanted 12 days after last parturition (dap). Yolk sacs were removed and embryos were fixed overnight at 5° in 4% paraformaldehyde and 1% dimethyl sulfoxide. Insets: Dorsal aspect of the midbrain region with individual melanophores apparent in wild type. (B, C, and G) Lateral, (D–F) dorsal aspects of the same females over a 3-week time course (days are after birth). All images taken, including the ones of males, can be found in File S1. Bars: (A) 500 μ m, (insets) 250 μ m; (B–G) 1 mm.

Ditlevsen 1947), and black pigment is scarce on the choroid of the eyes as well as on the anterior head in golden fish, indicating that these pigmentation traits depend on Kita (Figure 1, B–E). The arrangement of the melanophores along the scales seems to be independent of Kita, as the size of the reticulate pattern (diameter of rhombi) was similar in golden and wild-type fish (data not shown). Onset of puberty in golden and wild-type males was observed between day 19 and 28 (File S1); the development of the reticulate pattern in golden males was similar to that of golden females (File S1).

csf1ra-independent melanophore populations

In zebrafish, early *kita*-dependent metamorphic melanophores are independent of *Csf1ra*, whereas late-differentiating *kita*-independent melanophores require *Csf1ra* and *Ednrb1a* activity for their differentiation (Parichy *et al.* 2000a,b; Parichy and Turner 2003b). *kita csf1ra* double-mutant zebrafish lack almost all melanophores because of the strong additive effect of mutations in these two genes (Parichy *et al.* 2000a). Since we identified a *kita*-dependent and -independent melanophore population in the guppy, we asked whether any of them requires *csf1ra*.

Inspection of embryos and newborns revealed no major differences between the *blue* mutant and wild-type melanophore patterns (Figure S4A), although we cannot exclude that a small subset of the early appearing melanophores

depends on *Csf1ra* signaling since we could not count these early cells (see Figure S4 legend). To investigate whether the late-differentiating melanophores depend on *csf1ra*, we compared an area below the dorsal fin in golden and golden blue adult females (Figure S4, E and E'). We found that both single- and double-mutant fish have similar numbers of melanophores per area (Figure S4F). Therefore, unlike in zebrafish, mutations in *kita* and *csf1ra* have no additive effect on the reticulate pattern of the guppy, which is further supported by the observation that the reticulate pattern of *blue* mutant guppies develops as in the wild type until at least day 40 after birth (Figure S4, A–D; File S1). Consequently, the late-appearing melanophores of the guppy reticulate pattern are independent of both Kita and *Csf1ra* signaling.

Requirement of Kita and *Csf1ra* signaling for male-specific ornaments

Guppy male-specific pigmentation patterns vary tremendously within and among populations, yet the within-population variance decreases considerably with inbreeding (personal observation). To investigate the roles of Kita and *Csf1ra* in male color pattern formation, we compared the ornamental patterns of related wild-type and mutant males. We crossed *golden blue* mutant BDZW1 females with wild-type males from the inbred wild-derived Cumaná (CUM), Guanapo (GU), Quare6 family II 215-3 (QUII), and Quare6

(QU) strains. The phenotypically wild-type F_1 males were backcrossed to *golden blue* mutant BDZW1 females to produce a N2 generation (Figure 5). As a result of the backcross, the grandfather's Y chromosome was combined with an X chromosome of the BDZW1 strain in all N2 males. Since recombination frequency of sex chromosomes is comparatively low in male meiosis (Tripathi *et al.* 2009a), this experimental design minimized the amount of pattern variation caused by X chromosomes derived from different strains, thereby allowing us to study the influence of the mutant autosomal genes on the pattern directed by a given Y chromosome. The number of male offspring derived from each cross is given in Table 1, and all images of the backcrosses can be found in File S2.

For our analysis, we focused on the most prevalent characteristic traits of each cross, as seen on the grandfather and its wild-type male offspring (Figure 6, Figure 7, Figure 8 and Figure 9; Figure S5). Furthermore, we tried to homologize the male ornaments of the different strains based on their color, shape, and approximate positions. A summary of all traits and generalization of our results is shown in Figure 9, which should facilitate tracking of single traits within the complex male patterns. We are, however, aware of the fact that superficially similar-looking traits need neither be directed by the same developmental pathways nor be derived from the same putative cell precursor pool.

Regardless of the origin of the Y chromosome, *blue* mutant N2 males lacked all orange color traits, indicating that *Csf1ra* activity is also required for the dispersal or differentiation of male-specific xanthophores (Figure 6, Figure 7, and Figure 9). In addition, the location and shape of the black male-specific ornaments were modified in the mutants.

Previous studies of the male-specific color pattern of the Cumaná guppy have shown that the blue iridescent spot on the trunk close to the dorsal fin, the combination of black and orange on the dorsal fin, and the ventral black lining of the caudal peduncle constitute strictly Y-linked traits (Figure 6A and Figure 9) (Tripathi *et al.* 2008, 2009a,b). The male-specific ventral black lining is more pronounced than the ventral black stripe of both sexes described in Figure 1F. Typically, two thicker black horizontal stripes, an anterior and a posterior one, are present on the trunk of Cumaná males (Figure 6A and Figure 9). The orange-black lining of the tail fin, which is often more pronounced on the ventral margin, is another male-specific Cumaná trait (Figure 6A and Figure 9). The prominent ventral black spot on the tail fin of many Cumaná males (Figure 6A and Figure 9; Figure S5) is very likely directed by one or more dominant factors located in the pseudo-autosomal region of the Cumaná sex chromosomes (Tripathi *et al.* 2008).

Most Cumaná male-specific traits were fully developed in the grandfather, in all F_1 , and in all wild-type N2 males (Figure 6, A–C; for the ventral black spot on the tail fin, see Figure S5). Of the *golden* mutant N2 males, all but one showed all of these traits as well (Figure 6D and Figure 9; the pattern of the one exceptional individual might be the

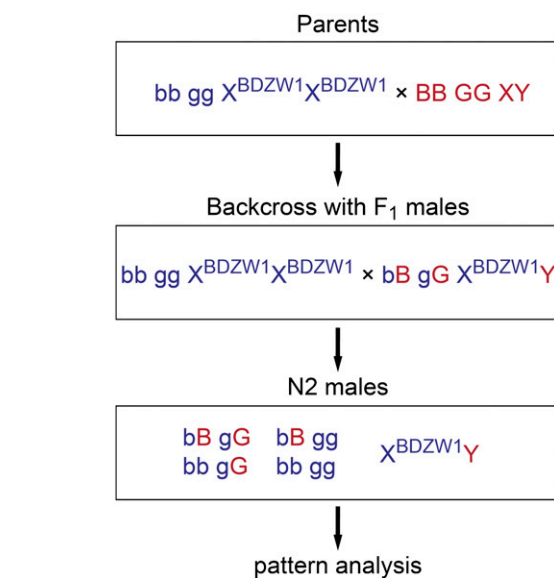


Figure 5 Crossing scheme that was used in the case of the CUM, GU, QUIL, and QU strains. The color patterns of the wild-type, golden, blue, and golden blue N2 males were analyzed. *blue* (b) and *golden* (g) are located on different autosomes and can therefore recombine freely. “B” and “G” indicate wild-type alleles. BDZW1 and MAC males were crossed with females of the same strain (for details see Figure 8).

outcome of a rare recombination event). Comparisons between wild-type and golden males revealed that (i) *golden* mutants had less black on the dorsal fin; (ii) the ventral black lining of the caudal peduncle was shifted upward and was often discontinuous in golden fish; (iii) the posterior black stripe of *golden* mutants was shifted downward and was often discontinuous (Figure 6D and Figure 9). In addition, a concise posterior orange spot on the caudal peduncle was absent in 94% of the *golden* mutants (Figure 6D). The anterior black stripe, the anterior orange spot, the blue iridescent spot, the orange-black lining, and the ventral black spot on the tail fin were not obviously changed by the mutation in *kita* (Figure 6D and Figure 9). The *blue* mutant N2 males not only lacked all orange traits, but also lost the black components of the orange-black lining of the tail fin, whereas the ventral black spot persisted (Figure 6E and Figure 9). Other defects of the *blue* mutant fish were the following: (i) the anterior black stripe was absent or very diffuse; (ii) the posterior black stripe was shifted downward and seemed more diffuse, and small incoherent patches of melanophores were often found on the dorsal trunk; (iii) in only 43% of the blue males a comparatively small blue iridescent spot was visible close to the dorsal fin (Figure 6E and Figure 9). The phenotype of the *golden blue* mutant N2 males resembled the *blue* mutant fish, but they showed fewer melanophores on the dorsal fin and trunk (Figure 6F).

The pattern of the Guanapo grandfather was characterized by several black crescents forming a labyrinthine pattern in proximity to the gonopodium (Figure 6G and Figure 9). The melanophores on the proximal and distal parts of the tail fin were concentrated on the ventral half of the fin, and an

Table 1 Male offspring obtained from each cross

Male parent	F ₁	N2			
		Wild type	Golden	Blue	Golden blue
Cumaná (CUM)	55	39	33 ^a	52	53
Guanapo (GU)	30	24	25	18	21
Quare6 family II 215-3 (QUII)	34	16	16	11	10
Quare6 (QU)	43	17	18	25	14
		F ₂			
		Wild type			Golden
BDZW1	24	56			18
Maculatus (MAC)	12	30			13

^a Actual number was 34, but one male had a BDZW1 instead of a CUM-like color pattern (see text).

orange spot was located close by (Figure 6G and Figure 9). The anterior and posterior black horizontal stripes, the ventral black lining of the caudal peduncle, and the anterior and posterior orange spots on the trunk appeared weaker compared with the Cumaná male (Figure 6G). The posterior black stripe approximately demarcated the lateral midline in the Guanapo grandfather.

All of the F₁ and wild-type N2 males showed the Guanapo traits described above, but only 41% of the wild-type males had an anterior orange spot (Figure 6, H and I). Furthermore, the F₁ and wild-type N2 males tended to have more complex black labyrinthine ornaments close to the gonopodium than the Guanapo grandfather, suggesting that cofactors derived from the autosomes or X chromosome of the BDZW1 strain modulate this trait (Figure 6, H and I). The black pigment pattern of the *golden* mutant N2 males was substantially changed in the following ways: (i) black pigment in the dorsal fin was reduced; (ii) the labyrinthine black ornaments close to the gonopodium were mostly lost; (iii) black pigment in the proximal part of the tail fin was concentrated dorsally; (iv) anterior and posterior black stripes were often discontinuous; and (v) the ventral black lining was shifted upward (Figure 6J and Figure 9). The orange ornaments persisted, and an anterior orange spot was found in 32% of the golden N2 males (Figure 6J and Figure 9). The *blue* mutant N2 males had an even more severe phenotype: (i) only one to two round black spots were present on the trunk that were in most individuals not located near the gonopodium; (ii) as in golden N2, the black in the proximal part of the tail fin was located dorsally, and several males had black tail-fin margins; and (iii) the anterior black stripe was absent or diffuse (Figure 6K and Figure 9). The *golden blue* mutant N2 males had fewer melanophores on the fins and trunk than the *blue* mutants (Figure 6L).

The pigmentation traits of the Quare6 family II 215-3 grandfather are shown in Figure 7A. Compared with the Cumaná grandfather, the black pigment on the dorsal fin and the ventral black lining of the caudal peduncle of the Quare6 family II 215-3 grandfather appeared weak. It had a black spot associated with its anterior black horizontal stripe, which we found in 83% of its wild-type and in 56% of its golden N2 male offspring (Figure 7, A–D). We found

that *golden* mutant N2 males of this backcross had (i) less black on the dorsal fin, (ii) a more diffuse posterior black horizontal stripe, (iii) an upwardly shifted or no ventral black lining of the caudal peduncle, and (iv) a slightly upwardly shifted central black spot (Figure 7D and Figure 9). In *blue* mutant N2 males, black ornaments on the trunk were mostly reduced to a few spots, and in some individuals a spot appeared at an unusual position just behind the operculum (Figure 7E).

Fewer black ornaments on the dorsal fin were also observed in *golden* mutant males of the highly inbred BDZW1 and Maculatus strains (Figure 8). The black spot in the dorsal fin of the Maculatus strain is considered to be a strictly Y-linked trait (Winge 1922), which illustrates that the expression of such traits nevertheless depends on autosomal cofactors. Wild-type males of the BDZW1 strain have a creamy-black margin of the tail fin and a prominent black-and-white eye spot on the caudal peduncle (Figure 8, A–C, and Figure 9). The colors of the creamy-black tail-fin margin were intermingled or switched in *golden* mutant BDZW1 males, and, instead of the eye spot, they had black spots at a more dorsal position on the trunk or ventral position on the tail fin (Figure 8, D–F, and Figure 9; occasionally two spots were present). Hence, the position of the eye spot is more variable in *golden* mutant BDZW1 males compared with the wild type. The posterior black horizontal stripe of golden BDZW1 males was often diffuse (Figure 8, D–F, and Figure 9), while it was curved, absent, or diffuse in golden Maculatus males (Figure 8, G, K, and L, respectively, and Figure 9). The ventral black lining of the caudal peduncle was shifted upward (BDZW1, Figure 8, D–F, and Figure 9) or absent (MAC, Figure 8, G, K, and L, and Figure 9). *golden* mutant Maculatus males showed dorsal or ventral black spots on the tail fin as well, which were only rarely seen in wild-type males (Figure 8, G–L).

Discussion

How the extreme variation in colorful ornaments satisfies the conflicting demands of predator evasion and mate attraction in male guppies has fascinated scientists for almost a century (Winge 1922, 1927; Lindholm and Breden 2002; Magurran 2005). Importantly, despite the extreme interindividual differences, many color traits are highly heritable, and sons greatly resemble their fathers (Winge 1922, 1927; Dzwillo 1959; Lindholm and Breden 2002). A better understanding of these issues requires better knowledge of the ontogeny of guppy pigmentation. This has unfortunately been challenging, due to the intrinsic difficulties of working with a livebearer for which many standard techniques that can be utilized in model organisms are not available. Here, we have exploited the power of forward genetics to advance the understanding of pigmentation in guppies. We have discovered that mutations in the two type III receptor tyrosine kinase genes *kita* and *csf1ra* underlie the guppy *golden* and *blue* phenotype, respectively, and have studied the effects of the mutations on both the reticulate pattern shared by females and males

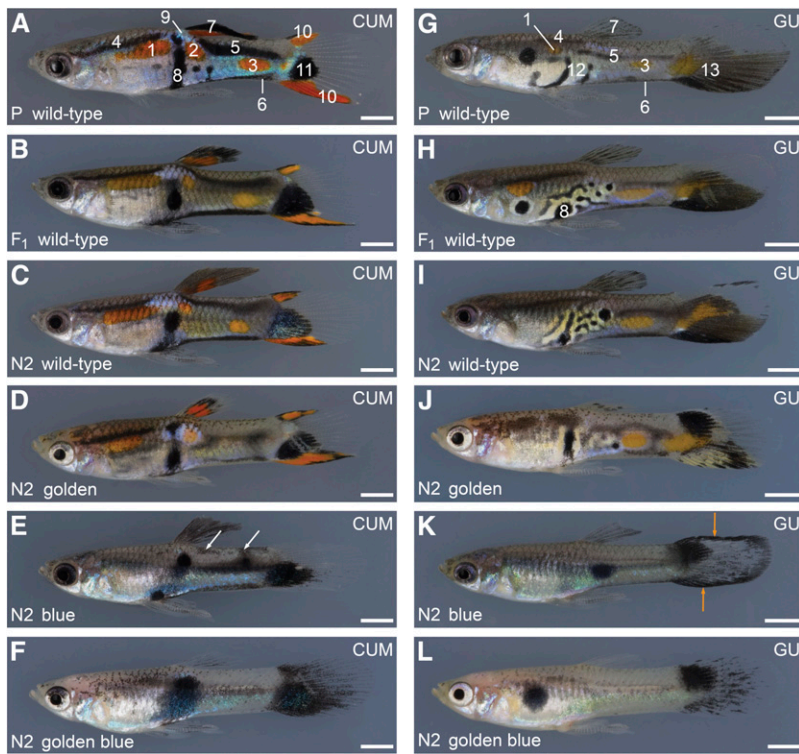


Figure 6 Ornaments in *golden* and *blue* mutant males from the Cumaná and Guanapo backgrounds. (A–F) Cross between a *golden blue* mutant BDZW1 female and a wild-type Cumaná male (A). Representative F₁ (B) and N₂ males with different phenotypes (C–F) are shown. Cumaná traits are highlighted in A. White arrows in E show incoherent patches of melanophores described in the text. (G–L) Cross between a *golden blue* mutant BDZW1 female and a wild-type Guanapo male from a F₅ Guanapo laboratory inbreeding population (G). Representative F₁ (H) and N₂ males with different phenotypes (I–L) are shown. Guanapo traits are highlighted in G and H. I–L lack the anterior orange spot. Orange arrows in K show the black margins of the tail fin described in the text. Traits are labeled with numbers that correspond with numbers used in Figure 9: 1, 2, and 3: anterior, central, and posterior orange spot; 4 and 5: anterior and posterior black horizontal stripes; 6: ventral black lining of caudal peduncle; 7: black pigment on dorsal fin (in CUM in combination with orange); 8: central black spot near gonopodium; 9: blue iridescent spot; 10: orange-black lining of tail fin; 11: ventral black spot on tail fin; 12: black crescents forming a labyrinthine pattern close to the gonopodium; and 13: orange spot and ventrally concentrated melanophores on tail fin. N₂ males shown for each backcross are siblings or cousins. All photos taken from the backcrosses can be found in File S1. Bottom left in each panel: generation (P, grandfather; F₁; N₂) and phenotype. Top right in each panel: Y chromosome origin. Bars: 2 mm.

and the male-specific ornaments. We found that the mutations in *kita* and *csf1ra* have strong effects on the expression of male-specific color patterns. In general, the mutation in *kita* made none or only minor changes in orange ornaments and affected the male melanophore pattern more subtly and in a more reproducible manner than the mutation in *csf1ra*. The salient feature of the *csf1ra* mutant males was the absence of all orange traits, with concomitant severe changes in black ornaments.

In many teleost species, including zebrafish, medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), and Japanese flounder (*Paralichthys olivaceus*), larvae and adults differ in their pigmentation patterns (Johnson *et al.* 1995; Parichy and Turner 2003a,b; Lynn Lamoreux *et al.* 2005; Kelsh *et al.* 2009; Yamada *et al.* 2010; Budi *et al.* 2011; Greenwood *et al.* 2012). Our study shows that the camouflage reticulate pattern of newborn guppies is not yet completely developed and that it is fully elaborated only during the first month after birth. Absence of most melanophores in embryonic and newborn *golden* mutants suggests that *Kita* is essential for the differentiation of an early melanophore population. A second melanophore population arises after birth and remains restricted to the dorsal half of the body in *golden* mutant fish, indicating that *Kita* signaling is not required for the differentiation of this melanophore subpopulation, yet that it might be essential for its proper migration. Alternatively, the migratory behavior of this subpopulation might be normal in *golden* mutants, with the later-differentiating melanophores enhancing the camouflage in wild-type fish on the dorsal side only. This could be investigated in

the future by selective labeling and tracing of the *kita*-independent population or by finding another mutation that eliminates this population. Independently of these details, we conclude that the guppy has an early-appearing *kita*-dependent and a later-developing partially or fully *kita*-independent melanophore population and that both populations are required to form the non-sex-specific reticulate pattern. Whole-mount *in situ* hybridization experiments turned out to be extremely difficult in guppy embryos due to their size and very low permeability (U. Martyn, and C. Dreyer, unpublished data), but could potentially help to determine the developmental time point at which the first melanophores differentiate in guppy embryos, especially as the melanization of these melanophores might be delayed in a livebearing fish like the guppy. The tracking of putative pigment cell precursors for adult ornaments, however, would require analysis of serial sections of specimens from early embryogenesis to puberty.

Our study suggests that *Kita* functions have at least partially been conserved across teleosts. The last common ancestor of zebrafish and guppies lived probably >300 million years ago (Kasahara *et al.* 2007); nevertheless, the adult pigment pattern of both species depends on an early *kita*-dependent and a late *kita*-independent melanophore population, and loss-of-function mutations in *kita* decrease the amount of melanophores, including scale melanophores, in both species (Haskins and Druzba 1938; Goodrich *et al.* 1944; Winge and Ditlevsen 1947; Parichy *et al.* 1999; Parichy *et al.* 2000a). Yet the teleost-specific whole-genome duplication likely also facilitated subfunctionalization and

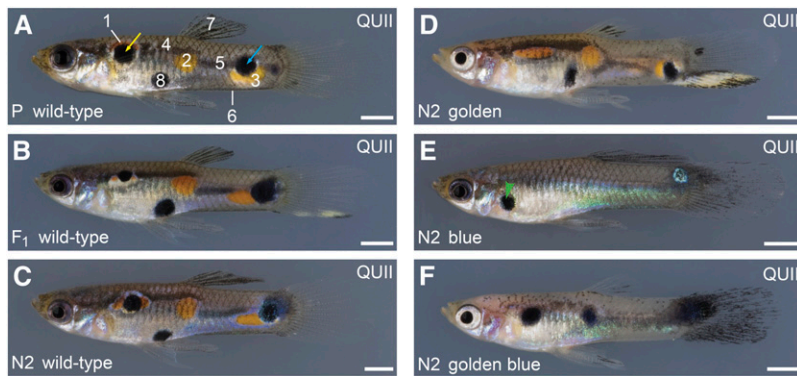


Figure 7 Ornaments in *golden* and *blue* mutant males from the Quare6 family II 215-3 background. Cross between a *golden blue* mutant BDZW1 female and a wild-type Quare6 family II 215-3 male (A). Representative F₁ (B) and N2 males with different phenotypes (C–F) are shown. Quare traits are highlighted in A. Yellow arrow points to black spot associated with anterior black horizontal stripe. Blue arrow points to black spot close to the tail fin that was not included in the analysis as its location was highly variable in wild-type fish. The green arrowhead in E shows a black spot close to the operculum described in the text. Traits are labeled with numbers corresponding with numbers used in Figure 6 and Figure 9. N2 males shown are cousins. All photos from this backcross and from the Quare6 backcross that was very similar regarding the male patterns can be found in File S2. Bottom left: generation (P, grandfather; F₁; N2) and phenotype. Bars: 2 mm.

phenotypic diversification, as revealed in different *Danio* species (Braasch *et al.* 2006; Mills *et al.* 2007).

An early xanthophore population contributes as regularly spaced cells to the reticulate pattern of guppy juveniles and adults (Figure 1, E and F). Our study shows that these non-sex-specific xanthophores depend on *Csf1ra* signaling. The presence of isolated dorsal clusters of xanthophores in *blue* mutant fish suggests that *Csf1ra* activity might not be absolutely required for differentiation, but for proliferation and dispersal of guppy xanthoblasts. We do not know yet when during ontogeny *csf1ra* acts and whether different xanthophore populations exist, but we showed that *Csf1ra* is not required for the formation of the non-sex-specific black reticulate pattern of the guppy. In contrast, *csf1ra* mutant zebrafish cannot form a complete non-sex-specific stripe pattern (Parichy *et al.* 2000a; Parichy and Turner 2003b).

Guppies are conspicuously sexually dimorphic in their pigmentation, and mate-choice experiments have demonstrated that females prefer males with pronounced orange and blue iridescent ornamentation (Endler 1983; Kodric-Brown 1985). Males are also able to intensify their black ornaments while courting (Endler 1983). Crosses between *golden blue* mutant females with male guppies of geographically and genetically diverse origins (Willing *et al.* 2010) gave us the opportunity to study the influence of Kita and *Csf1ra* loss-of-function on the diverse male-specific patterns of the guppy. The males originated from West Trinidad (Guanapo), East Trinidad (Quare), and Venezuela (Cumaná). The latter two strains had previously been used for genetic mapping and QTL analysis (Tripathi *et al.* 2009b). Our study demonstrates that the male-specific xanthophores of the guppy, whose development is induced during puberty like the one of male-specific melanophores and iridophores, also depend on *csf1ra* and that loss of *Csf1ra* and *Kita* function substantially changes the formation of male ornaments.

Comparison between wild-type and golden males showed that black stripes and spots appeared ectopically in *golden* mutants, although in a manner that varied between populations and individuals; *e.g.*, the ventral melanophores on the Guanapo tail fin were shifted to a more dorsal position, and

golden mutant *Maculatus* males had novel black spots on their tail fins. In the BDZW1 strain, the arrangement of the creamy-black tail-fin margin, which might involve iridophores (personal observation), appeared reversed. In contrast, loss of *Kita* function did not change the dorso-ventral arrangement of the orange-black lining of the tail fin of golden N2 from the Cumaná cross. Interestingly, the marginal black components of the Cumaná tail-fin ornaments were lost together with the orange in *blue* mutants, whereas the major black spot on the tail fin persisted. In zebrafish, *kita* is expressed in melanoblasts, while *csf1ra* acts nonautonomously via short- and long-range xanthophore–melanophore interactions to promote melanophore migration and death during adult stripe formation (Parichy *et al.* 1999; Parichy and Turner 2003b; Nakamasu *et al.* 2009; Inaba *et al.* 2012). As transplantation experiments are not yet possible in the guppy, we could not determine whether *Kita* and *Csf1ra* act cell-autonomously or non-cell-autonomously during male pattern formation. However, as downregulation of *csf1ra* expression coincides with the absence of almost all xanthophores in the skin of blue fish, it is likely that *csf1ra* acts cell-autonomously within guppy xanthophores. *Kita* is an early melanoblast marker not only in zebrafish, but also in mice, and therefore most likely is expressed in guppy melanophores as well (Kelsh *et al.* 2009). Our observations suggest that some pattern elements in guppy males depend on coordinate expression of different cell types and that the formation of some of these pattern elements requires interactions between, or joint contribution from, different cell types. For example, xanthophore–melanophore interactions might underlie the formation of the orange-black lining of the tail fin of Cumaná males, but not the development of the black spot on the tail fin. *Kita* might directly affect the migration and/or survival of a subset of male melanophores. Terminal deoxynucleotidyl transferase-dUTP Nick End Labeling (TUNEL) assays could reveal whether cell apoptosis plays a role during male pattern formation in the guppy.

Comparisons between wild-type and blue offspring of the backcrosses suggest that compact black spots can form in absence of *Csf1ra*, most probably without interactions between

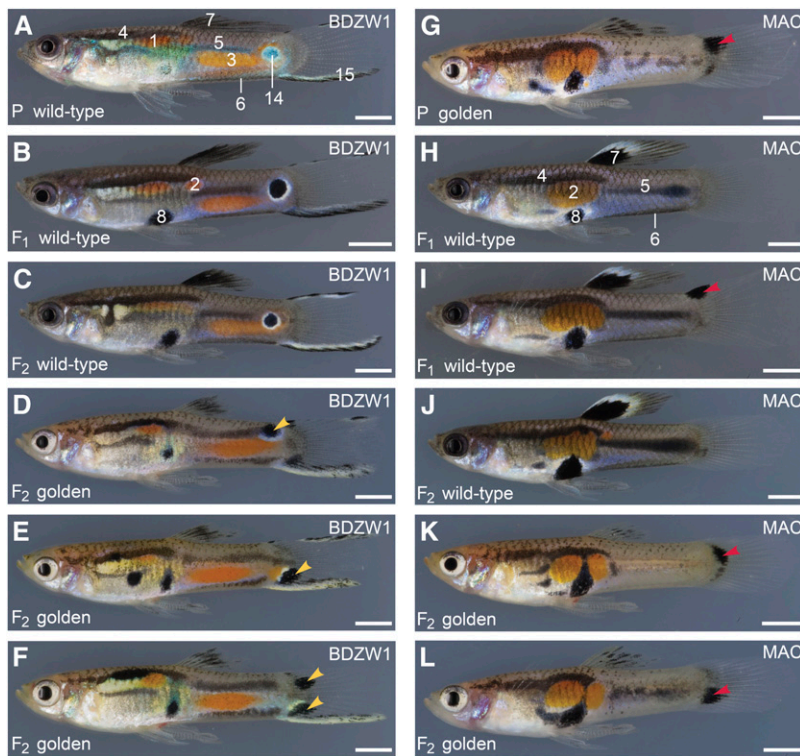


Figure 8 Ornaments in *golden* mutant males from the BDZW1 and Maculatus backgrounds. (A–F) Cross between a *golden* mutant BDZW1 female and a wild-type BDZW1 male (A). F₁ siblings were crossed to produce a F₂ generation. Representative F₁ (B) and F₂ males (C–F) are shown. BDZW1 traits are highlighted in A and B. All wild-type F₂ and 92% of the wild-type F₁ males had a central black-and-white eye spot on the caudal peduncle. Instead of this eye spot, one-half of the *golden* mutant F₂ males had a spot located more dorsally on the trunk, while the other half had a spot at a more ventral position, mostly on the tail fin (yellow arrowheads in D and E). Three males had two spots (yellow arrowheads in F). (G–L) Cross between a wild-type Maculatus female and a *golden* mutant Maculatus male (G). F₁ siblings were crossed to produce a F₂ generation. Representative F₁ (H and I) and F₂ males with different phenotypes (J–L) are shown. Maculatus traits are highlighted in H. Red arrowheads in (G, I, K, and L) indicate untypical black spots on the tail fin. Only one of the F₁ males showed such a black spot (I). It was not seen in any of the wild-type F₂ males, but it was present in 85% of the *golden* mutant F₂ males (K and L). Traits are labeled with numbers corresponding with those shown in Figure 6 and Figure 9. Additional traits to those shown in Figure 6: 14, black-and-white eye spot; 15, creamy-black margin of tail fin. F₂ males shown for each cross are siblings or cousins. Bottom left: generation (P, grandfather; F₁; F₂) and phenotype. Top right: Y chromosome origin. Bars: 2 mm.

xanthophores and melanophores, yet the final positions of these spots appear unpredictable. The labyrinthine ornaments close to the gonopodium in Guanapo males provide an example of interacting genetic cofactors in that backcross and potentially also of interactions between different cell types. Compared with wild type, these ornaments were greatly reduced in complexity in *golden* mutant N2 and lost in *blue* and *golden blue* mutant N2. Since only faint yellow pigment is seen in this area in wild-type fish, the contribution of xanthophores and their interactions is here hard to assess. The loss of the labyrinthine ornaments in *golden* mutant N2 might be explained by a reduced migratory potential of the *kita*-independent melanophores or their precursors in golden N2. We can, however, not exclude that a *kita*-dependent subpopulation of late melanophores contributes to this complex trait in wild-type fish.

golden blue double-mutant guppies from four different backcrosses always had less total black than *blue* mutants, but much more than zebrafish *kita csf1ra* double mutants (Parichy 2006). This might reflect different requirements for *Kita* and *Csf1ra* signaling: while xanthophores enhance the survival of adult stripe melanophores in zebrafish (Parichy and Turner 2003b), the survival of male hormone-induced melanophores in the guppy might not require xanthophores; yet the ability of these melanophores to form some of the complex traits might depend on interactions with these cells.

Taken together, we conclude that at least three temporally and genetically distinct melanophore populations occur in the guppy: first, a *kita*-dependent population differentiat-

ing during embryogenesis; second, a partially or fully *kita*-independent population mostly differentiating after birth; and third, a male-specific melanophore population whose differentiation, migration, and proliferation might be induced by testosterone during puberty. It remains to be resolved where the precursors of these male-specific pigment cells reside and whether they might be derived from the same pool as the late non-sex-specific *kita*-independent melanophores. Only a few recent publications have addressed the routes and fates of pigment cell precursor pools destined for delayed differentiation in other vertebrates (Watanabe *et al.* 2008; Adameyko *et al.* 2009; Yamada *et al.* 2010; Budi *et al.* 2011). While the embryonic and early larval pigment pattern of zebrafish is formed by melanoblasts that are derived directly from the neural crest, later-appearing metamorphic melanophores of zebrafish develop from post-embryonic extrahypodermal pigment cell precursors, which migrate to the hypodermis during pigment pattern metamorphosis (Budi *et al.* 2011). These precursors are associated with nerves and depend on *ErbB3b* and *Tubulin α 8-like 3a* signaling (Budi *et al.* 2011). Improvement of *in vitro* culture methods of guppy embryos (Martyn *et al.* 2006) may facilitate the ability to treat, and to subsequently raise, explanted guppy embryos with an *ErbB* inhibitor, which might reveal whether *ErbB* signaling is required to establish melanophore stem cells in the guppy as well.

In stickleback, regulatory mutations in *kitla* are associated with the light coloration of gills and ventral skin in several freshwater populations (Miller *et al.* 2007). This indicates that differential distribution of *Kitla* can lead to

golden

blue

General traits

Orange traits

1 Anterior orange spot	present ^a	absent
2 Central orange spot		
3 Posterior orange spot		

Black traits

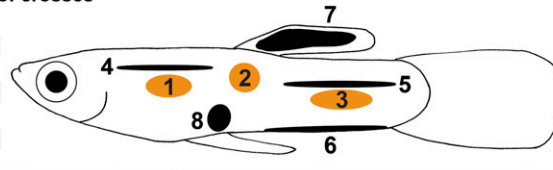
Complexity of black ornaments on body

reduced

4 Anterior black horizontal stripe	present ^b	diffuse/absent
5 Posterior black horizontal stripe	shifted/diffuse/absent	shifted/diffuse/absent
6 Ventral black lining of caudal peduncle	shifted/diffuse/absent	present
7 Black pigment on dorsal fin	reduced	present
8 Central black spot near gonopodium	present at same position/slightly shifted	shifted/absent

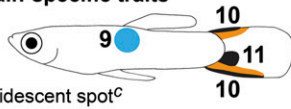
Presence of traits in wild-type males of crosses

CUM	1	2	3	4	5	6 ^c	7 ^c	8
GU	(1)		3	4	5	6	7	(8)
QUII	1	2	3	4 ^d	5	6	7	8
BDZW1	1	(2)	3	4	5	6	7	8
MAC		2 ^c	4	5	6	7 ^c	8 ^c	



Additional strain-specific traits

Cumaná



9 Blue iridescent spot ^c	present	small spot visible in 43% of males
10 Orange-black lining of tail fin	present	absent
11 Ventral black spot on tail fin	present	present

Guanapo



12 Labyrinthine pattern close to gonopodium	mostly absent	absent
13 Orange spot and ventrally concentrated melanophores (m) on tail fin	m concentrated dorsally	orange absent; m concentrated dorsally

BDZW1



14 Black and white eye spot	shifted	n/a
15 Creamy-black margin of tail fin	colors intermingled/switched	n/a

Figure 9 Summary and generalization of the results regarding the male-specific pattern. Both *Csf1ra* and *Kita* are required for the formation of male ornaments of the guppy. The black pigment on the dorsal fin can be a distinct patch or diffuse. Numbers in parentheses refer to traits that were not present in all wild-type males of the specific strain. Traits that were too variable in wild-type males were not included into the analysis. More details are described in the text and shown in Figure 6, Figure 7, and Figure 8 and in Figure S5. ^aException: a concise posterior orange spot was absent in 94% of the golden N2 males with a Y^{CUM} background. ^bException: often discontinuous in golden N2 males with a Y^{GU} background. ^cY-linked in respective strain. ^dAssociated with a black spot in 83% of the wild-type males with a Y^{QUII} background.

distinct pigmentation patterns in natural populations. In contrast to the receptor *Kita*, the functions of *Csf1ra* seem to be less conserved even among species in the genus *Danio*, as indicated by limited complementation of *csf1ra* loss of function in interspecies hybrids (Quigley *et al.* 2005). Furthermore, a study with haplochromine cichlids has suggested that positive selection has acted on *csf1ra*, which is expressed in the yellow egg spots of these fish (Salzburger *et al.* 2007). QTL mapping with higher marker density may reveal whether or not *Kita* and *Csf1ra*, and/or their ligands, also affect natural variation of guppy ornaments. For this purpose, we are generating a denser genetic map of the guppy based on Restriction-site Associated DNA (RAD) markers. Combined with the ongoing whole-genome sequencing, these

experiments will further enhance our efforts to unravel the network of genetic factors that cooperatively maintain the highly complex male ornaments of the guppy.

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We thank Harald Auer for the donation of the ARM and BDZW2 guppy strains; Axel Meyer for the BDZW1 guppy strain; David Reznick for guppies from the Quare and Guanapo rivers; Christopher Dooley for general advice on zebrafish pigment pattern mutants; Tobias Langenecker for assistance with real-time quantitative PCR; Gertrud Scheer, Alexandra Schnell, and Philipp Vollmer for guppy images; Richard Neher for help with data analysis; Joffrey Fitz for

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GENETICS

Supporting Information

<http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.151738/-/DC1>

Pigment Pattern Formation in the Guppy, *Poecilia reticulata*, Involves the Kita and Csf1ra Receptor Tyrosine Kinases

Verena A. Kottler, Andrey Fadeev, Detlef Weigel, and Christine Dreyer

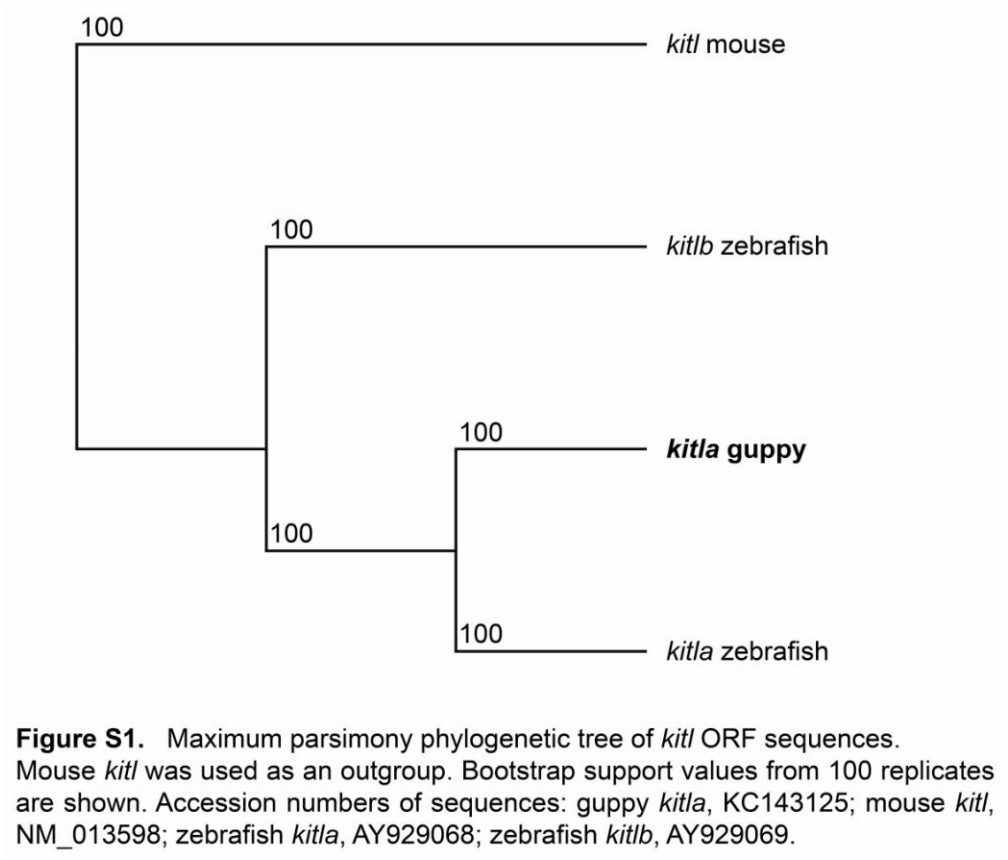




Figure S2. Complementation analyses.

(A) Non-complementation of the blue phenotype. All F_1 from a cross between a golden blue BDZW1 female and a blue BDZW2 male were blue. (B,C) Non-complementation of the golden phenotype. All F_1 from crosses of golden BDZW1 females to golden ARM and MAC males were golden. Parents (P) and representative F_1 male are shown for each cross. Scale bars: males 2 mm; females 5 mm.

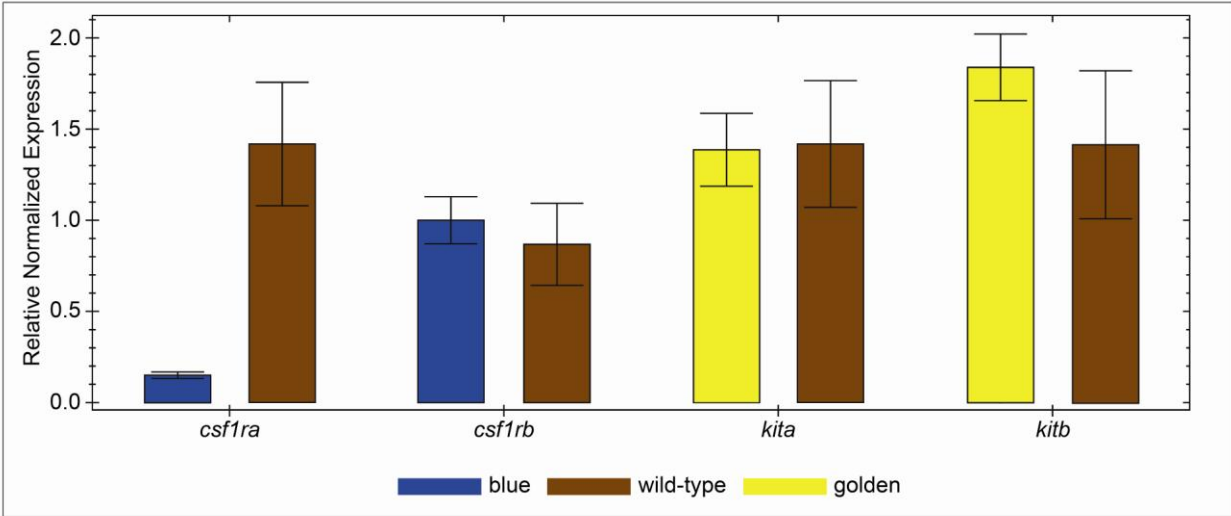


Figure S3. Expression levels of *csf1ra*, *csf1rb*, *kita*, and *kitb* in adult female skin. Normalized expressions ($\Delta\Delta C_Q$) of the genes in wild-type, golden, and blue female skin are shown. Expression levels were determined by real-time quantitative PCR using three biological replicates (one replicate refers to skin of one female) with three technical repetitions each. Expression was normalized to *gapdh* expression. Primer efficiencies were: *gapdh*, 85.3%; *csf1ra*, 96.4%; *csf1rb*, 97.6%; *kita*, 98.2%; *kitb*, 95%.

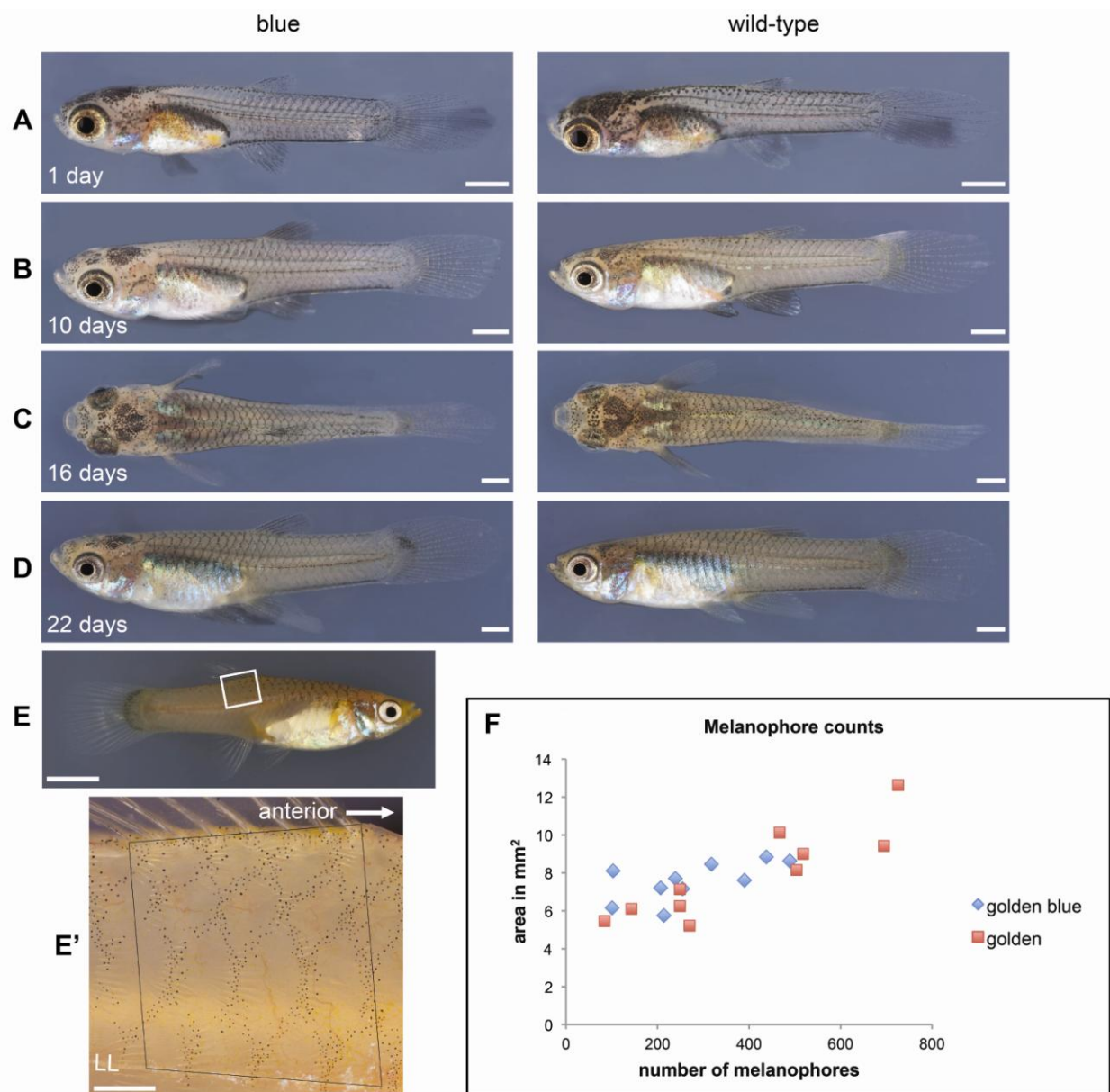


Figure S4. Melanophore pattern development in wild-type and *blue* mutant fish.

(A-D) Photos of the same males over a three-week time course (days are after birth). The blue fish has a BDZW2, the wild-type fish a BDZW1 background. The amount of melanophores of *blue* mutant and wild-type newborns was not compared, as variation in melanophore number is high between guppy strains (unpublished observation). (E') Detail of area boxed in (E); black rectangle indicates area below dorsal fin in which melanophores were counted. (F) Melanophore counts in 10 *golden* and 10 *golden blue* mutant females. The number of melanophores is strongly correlated with the size of the area. All fish shared the same grandparents and were approximately one year old. LL: Lateral line. Scale bars: (A-D) 1 mm; (E) 5 mm; (E') 1 mm.

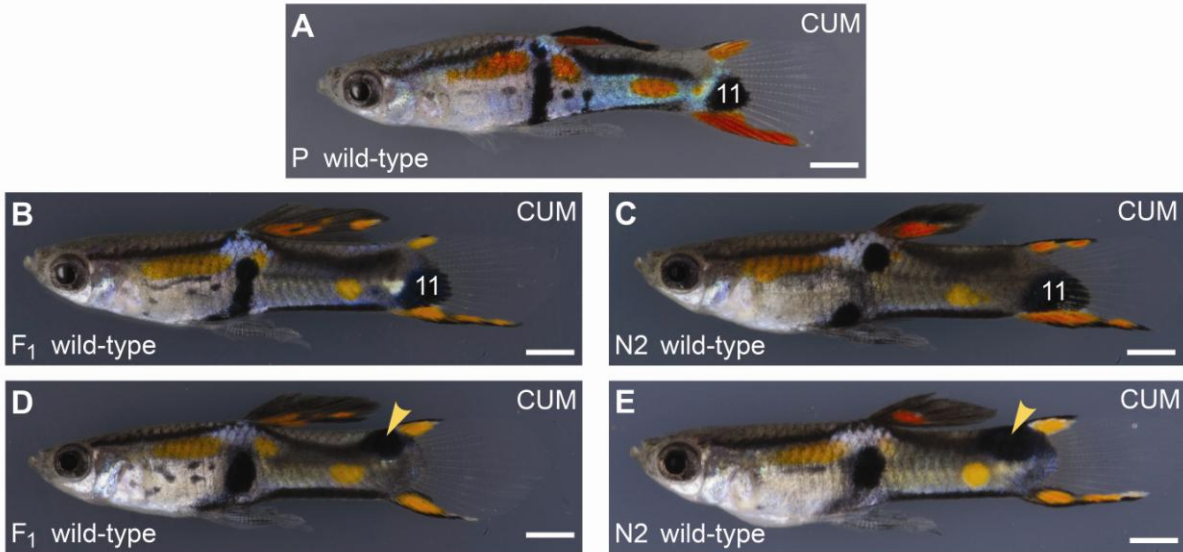


Figure S5. Inheritance of the ventral black spot on the tail fin of the Cumaná strain. (A) Grandfather, (B) F_1 , and (C) N_2 males with a ventral black spot on the tail fin (11). (D) F_1 , and (E) N_2 males with a dorsal black spot on the caudal peduncle (yellow arrowheads). The black spot on the tail fin was present in 33 of 55 F_1 males; all other males had a black spot on the caudal peduncle. This suggests that cofactors exist that modulate this trait, which might be derived from autosomes or the X chromosome of the BDZW1 strain in this case. All N_2 males had the spot at the same position as their father. (C) and (E) are the sons of (B) and (D), respectively. P, grandfather. Scale bars: 2 mm.

File S1

Photos tracking the development of the melanophore pattern after birth.

Image names include image number and days after birth at which the image was taken. Additional pictures with higher magnification of some of the fish are available upon request. Note that the gestation time in the guppy varies; although all fish were imaged at identical time points after birth, they might not be in the exact same developmental stage. We did not take any photos from the dorsal aspect during the first week after birth, as most fish did not survive this at this age. Dataset was deposited at: ftp://ftp.tuebingen.mpg.de/ebio/csf1ra_kita_mutants/

File S2

Photos of males taken from backcrosses to investigate the influence of the golden and blue mutations on the male-specific ornaments.

Cross20_BDZW1_CUM: cross between a golden blue BDZW1 female and a wild-type Cumaná male. Seven F1 males were backcrossed to golden blue BDZW1 females (BC1 to 7 pairs) to produce a N2 generation. The color pattern of one of the golden males (cross20_F1fBC6_whitem_0488) resembled the one of the BDZW1 strain rather than the one of the Cumaná strain, which might be the outcome of a rare recombination event. Cross21_BDZW1_GU: cross between a golden blue BDZW1 female and a wild-type Guanapo male. Four F1 males were backcrossed to golden blue BDZW1 females (BC1 to 4 pairs) to produce a N2 generation. Cross23_BDZW1_QU: cross between a golden blue BDZW1 female and a wild-type Quare6 male. Four F1 males were backcrossed to golden blue BDZW1 females (BC1 to 4 pairs) to produce a N2 generation. Cross24_BDZW1_QUII: cross between a golden blue BDZW1 female and a wild-type Quare6 family II 215-3 male. Four F1 males were backcrossed to golden blue BDZW1 females (BC1 to 4 pairs) to produce a N2 generation. Image names include cross number, generation, phenotype, sex, and identification number. P, grandparents; BCs, backcrosses; m, male; f, female; F1fBC1, F1 offspring (here called N2 generation) from BC1 pair; wtm, wild-type male; whitem, golden male; bluem, blue male; ghostm, golden blue male. Dataset was deposited at: ftp://ftp.tuebingen.mpg.de/ebio/csf1ra_kita_mutants/

Table S1 Methods used for full-ORF amplification of pattern formation candidate genes

Gene	Origin of partial sequences	Primers for RACE PCR	Primers used for full-ORF amplification by PCR	Primers used for internal sequencing of full-ORF clones
Kitla	Consensus primers:	3'-RACE PCR primers (forward):		
	Forward: 5'-AAGTT GGATACGCGTCTG TGTCC-3'	5'-CTCATCAGCTTCGTTGCCAA GTC-3' (reaction 1)	5' UTR forward: 5'-ATTGGATAT GTGCACACAGGATGATGAC-3'	
	Reverse: 5'-CTTCC ACACMAGCAGGA AWA- 3'	5'-CACTGCTGGCCCTAATATTC CTG-3' (reaction 2)	3' UTR reverse: 5'-GTTTGTCTT TCACCTTTGAACACCAACAG-3'	
<p>We amplified a short piece of the guppy ortholog of <i>kitla</i> by using consensus primers that were chosen based on an alignment of <i>kitla</i> of other teleost species^a. We then designed guppy-specific <i>kitla</i> primers for RACE PCR; however, the 5'-RACE PCR failed. To obtain a part of the guppy <i>kitla</i> 5' UTR, we ordered forward consensus primers in the 5' UTR of <i>kitla</i> based on an alignment of <i>kitla</i> sequences of other teleost species^b. 5'-ATTGGATATGTGCACACAGGATGATGAC-3' was used successfully in combination with guppy-specific <i>kitla</i> reverse primers. Subsequently, we used this primer in combination with a reverse primer in the 3' UTR for full-ORF amplification.</p>				
Kita	<i>Kita</i> marker sequence (Tripathi et al., 2008)	3'-RACE PCR primers (forward):		5'-GACAGTAAAGAAAGTCC CTTTAGGTCC-3' (forward)
		5'-CAGAGCTGAAGGTCTCAGTT ACTTGG-3' (reaction 1)	5' UTR forward: 5'-AGAGCT ACACCAGCTTGACCAC-3'	5'-GGAACTCCCACTGGTGAT TGTAG-3' (reverse)
		5'-TCAGCACCTGTGAGCTAAAGG AGAAC-3' (reaction 2)	3' UTR reverse: 5'-CGTAAC ATCACAGGCACTTAGAGG-3'	5'-GACAAGTCGTCCTCATCG AACATCTC- 3' (reverse)
<p>We used a partial <i>kita</i> sequence to design primers for RACE PCR. The 5'-RACE PCR failed; to obtain a part of the guppy <i>kita</i> 5' UTR, we ordered forward consensus primers in the 5' UTR of <i>kita</i> based on an alignment of <i>kita</i> sequences of other teleost species^c. 5'-CTSYAGGAC AACAGCATGYTTG-3' was used successfully in combination with guppy-specific <i>kita</i> reverse primers. Subsequently, we designed primers in the UTRs for full-ORF amplification.</p>				
Csf1ra	Transcriptome database	5'-RACE PCR primers (reverse):		5'-GATCTGAGGTGTGAAGG CAAC -3' (forward)
		5'-CTATCCGTACATATTCATCTG TCTCC-3' (reaction 1)	5' UTR forward: 5'-CCTCCTTCA AGCTGAAGACATC-3'	5'-CCTTCAACATAGTTGG AGTCATTC- 3' (reverse)
		5'-CAGTGTAGAAGCACTTGTTAC GTTCC-3' (reaction 2)	3' UTR reverse: 5'-ACCGACTCA GCCTTGAACAG-3'	5'-GTCTAGACATCCAGAGCA TCCTGAC- 3' (forward)

We detected an incomplete *csf1ra* transcript in our guppy transcriptome database (SHARMA, E., A. KÜNSTNER, B. A. FRASER, M. HOFFMANN, V. A. KOTTLER, G. ZIPPRICH, D. WEIGEL, and C. DREYER, unpublished data) and carried out a nested 5'-RACE PCR to obtain the missing 5' end of the wild-type *csf1ra* transcript. Based on the full-length cDNA sequence of *csf1ra*, we designed primers in the UTRs for full-ORF amplification.

Rapid amplification of cDNA ends (RACE)

First-strand cDNA for 5'/3'-RACE PCR was prepared with BD SMART PCR cDNA Synthesis Kit (Clontech) according to the manufacturer's instructions. The RACE PCR product of reaction 1 was diluted 50-fold to serve as template in reaction 2 (nested RACE PCR). Reverse (5'-RACE PCR) and forward (3'-RACE PCR) gene-specific primers were combined with the forward (5'-RACE PCR) and reverse (3'-RACE PCR) primer of the BD SMART PCR cDNA Synthesis Kit, respectively. Both PCR reactions were carried out with Advantage 2 Polymerase Mix (Clontech) as described before.

Accession numbers

^aGenBank EU218897 (stickleback, *Gasterosteus aculeatus*); Ensembl ultracontig72 (medaka, *Oryzias latipes*)

^bGenBank EU218897 (stickleback), AB285216 (fugu, *Takifugu rubripes*), AY929068 (zebrafish, *Danio rerio*), FJ907418 (goldfish, *Carassius auratus*); Ensembl GSTENT10006132001 (tetraodon, *Tetraodon nigroviridis*)

^cEnsembl MEDAKA1:4:1429800:1459632:1 (medaka), FUGU4:scaffold_13:1529100:1545623:1 (fugu)

Literature cited

TRIPATHI, N., M. HOFFMANN and C. DREYER, 2008 Natural variation of male ornamental traits of the guppy, *Poecilia reticulata*. *Zebrafish* **5**: 265-278.

Table S2 List of primers used for real-time quantitative PCRs

Gene	Primers	Efficiency
<i>Gapdh</i>	Forward: 5'-ACATCAAGAAGGTTGTGAAAGCTG-3' Reverse: 5'-ATCAAAGATGGAGGAGTGAGAATC-3'	85.3%
<i>Csf1ra</i>	Forward: 5'-AACTGGAGGAGGAGCAGGTAATC-3' Reverse: 5'-GTGACACTTAGGCTTGTCATACG-3'	96.4%
<i>Csf1rb</i>	Forward: 5'-TTGACGAGTGACATGTTGCCTC-3' Reverse: 5'-ATCATCATCCTCTTCTCTGCTCTG-3'	97.6%
<i>Kita</i>	Forward: 5'-AAGAGAGCTGCAGATGGTGAC-3' Reverse: 5'-CGTCAGCTTCGAGGTACAGG-3'	98.2%
<i>Kitb</i>	Forward: 5'-ACCAAGAACGTGTACTGACTC-3' Reverse: 5'-CTACACACAGAGCTAAGCCTCC-3'	95.0%

Table S3 List of primers used for genomic PCRs

Gene	Primers	Purpose	Remarks
<i>Kita</i>	Forward exon 6: 5'-TGTCTCTGAACG TTAGCATGGAG-3' Reverse in 36 bp 'exon' of insert: 5'- AAGCTTGTCAAGGAATCTGGTATGG-3'	Amplification of <i>kita</i> ^{insert} (quick PCR test)	
<i>Kita</i>	Forward exon 6: 5'-TGTCTCTGAACGTTAGCATGGAG-3' Reverse exon 7: 5'-ACACGGAGAAGTTCTGCTTTACC-3'	Amplification of <i>kita</i> ^{wt} (quick PCR test)	Elongation time of 2 min not sufficient to amplify <i>kita</i> ^{insert}
<i>Csf1ra</i>	Forward exon 16: 5'-ACATTGACGACCTGCTGAGATTC-3' Reverse exon 17: 5'-CCTTCACAACATAGTTGGAGTCATTC-3'	Amplification of <i>csf1ra</i> ^{wt} and <i>csf1ra</i> ^{indel}	

Table S4 Sequences and description of six *kita* splice variants found in *golden* mutants

Sequences of variants (V) ^a	Description	Predicted protein
<p>>V1 gatgtcgggagttacaatcgctagccggaaacgacaaaggaacagtagatgtccgtgtggctggacgtt tatgaacgggggtttatcagctcagcagaaatccaacaacagaacctccacgtccgctcggcgacagttgt ctctgaacgttagctaggcgatcctcaaaagccgcgccgctcctggagcttctgttactaccaataat ctcacatacccataccagattccttgacaagcttcatgggagcagaacctcagaacaccagcgaccacgtcat caccacgcacagcagggtacagctacatcagcgagttgaggctggctcggctcaaagcgacagaagggtg gcatttataccttgaagcctcaacggcgacggcggtaaagcagaacttctcctgtttgtatcagtaag cctgcgatcatagatcatggggcccagtgagtgagcaggtgcaactgtgtggctgaaggctaccctgcccc agatcaaattggtactactcggagaagcatgctgtcaggtgctcctgcaaaagaacgccaccaggaggagc gcagcgtcatgacctatgtcgggaagcaccagctcgggaagagggtggagagctgggtcaacgtcaga aaacagttcagtactctggagtgctgccaccggggacggagagcaagcctacatactgttt</p>	17+36 bp extra	358 aa, with the last 15 new
<p>>V2 gatgtcgggagttacaatcgctagccggaaacgacaaaggaacagtagatgtccgtgtggctggacgtt tatgatctcacataccataccagattccttgacaagcttcatgggagcagaacctcagaacaccagcgacca cgtcatcaccagcagcagggactacagctacatcagcgagttgaggctggctcggctcaaagcgacag aagggtggcatttataccttgaagcctcaacggcgacggcggtaaagcagaacttctcctgtttgttatc agtaagcctgcgatcatagatcatggggcccagtgagtgagcaggtgcaactgtgtggctgaaggctaccct gccccagatcaaatgggtactactcggagaagcatgctgtcaggtgctcctgcaaaagaacgccaccag gaggagcgcagctcatgacctatgtcgggaagcaccagctcgggaagagggtggagagctgggtcaa cgtcagaaaaacagttcagtactctggagtgctgccaccggggacggagagcaagcctacatactgttt</p>	lacks 124 bp of exon 6, 36 bp extra	311 aa, with the last 9 new
<p>>V3 gatgtcgggagttacaatcgctagccggaaacgacaaaggaacagtagatgtccgtgtggctggacgtt tatgaacgggggtttatcagctcagcagaaatccaacaacagaacctccacgtccgctcggcgacagttgt ctctgaacgttagctaggcgatcctcaaaagccgcgccgctcctggagcttctgttactaccaataact acatcagcgagttgaggtggctcggctcaaaagcgacagaagggtggcatttataccttgaagcctcaacg gcgacgcggcggtaaagcagaacttctcctgtttgttatcagtaagcctgcgatcatagatcatggggccc agtggatggacaggtgcaactgtgtggctgaaggctaccctgccccagatcaaatgggtactactcggaga acatgctgtcaggtgctcctgcaaaagaacgccaccaggagagcgcagcgtcatgacctatgtcggg aagcaccagctcgggaagagggtggagagctgggtcaacgtcagaaaaacagttcagtactctggagtgct cgccaccggggacggagagcaagcctacatactgttt</p>	17 bp extra, lacks 63 bp of exon 6	354 aa, with the last 11 new
<p>>V4 gatgtcgggagttacaatcgctagccggaaacgacaaaggaacagtagatgtccgtgtggctggacgtt tatgaacgggggtttatcagctcagcagaaatccaacaacagaacctccacgtccgctcggcgacagttgt ctctgaacgttagctaggcgatcctcaaaagccgcgccgctcctggagcttctgttactaccaatagt gctcctgcaaaagaacgccaccaggagagcgcagcgtcatgacctatgtcgggaagcaccagcttcg ggaagagggtggagagctgggtcaacgtcagaaaaacagttcagtactctggagtgctgccaccggggac ggagagcaagcctacatactgttt</p>	17 bp extra, lacks 63 bp of exon 6 and complete exons 7 and 8	364 aa, with the last 21 new
<p>>V5 gatgtcgggagttacaatcgctagccggaaacgacaaaggaacagtagatgtccgtgtggctggacgtt tatgtcatcagcaggttgggctggctcggctcaaagcgacagaagggtggcatttataccttgaagcctc caacggcgacggcggtaaagcagaacttctcctgtttgttatcagtaagcctgcgatcatagatcatggg ggccagtgagtgagcaggtgcaactgtgtggctgaaggctaccctgccccagatcaaatgggtactactcg agaagcatgctgtcaggtgctcctgcaaaagaacgccaccaggagagcgcagcgtcatgacctatgt cgggaagcaccagctcgggaagagggtggagagctgggtcaacgtcagaaaaacagttcagtactctggag tgctgcgccaccggggacggagagcaagcctacatactgttt</p>	lacks complete exon 6	307 aa, with the last 5 new
<p>>V6 gatgtcgggagttacaatcgctagccggaaacgacaaaggaacagtagatgtccgtgtggctggacgtt tatgtgctcctgcaaaagaacgccaccaggagagcgcagcgtcatgacctatgtcgggaagcaccag gcttcgggaagagggtggagagctgggtcaacgtcagaaaaacagttcagtactctggagtgctgccaccg ggagcggagagcaagcctacatactgttt</p>	lacks complete exons 6, 7, and 8	317 aa, with the last 15 new

^aForward primer in exon 5 and reverse primer in exon 9 of *kita* were used for amplification; primer sequences are given in MATERIALS AND METHODS.