Contribution of *sox9b* to pigment cell formation in medaka fish

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Abstract

SoxE-type transcription factors, Sox10 and Sox9, are key regulators of the development of neural crest cells. Sox10 specifies pigment cell, glial, and neuronal lineages, whereas Sox9 is reportedly closely associated with skeletogenic lineages in the head, but its involvement in pigment cell formation has not been investigated genetically. Thus, it is not fully understood whether or how distinctly these genes as well as their paralogs in teleosts are subfunctionalized. We have previously shown using the medaka fish *Oryzias latipes* that pigment cell formation is severely affected by the loss of *sox10a*, yet unaffected by the loss of *sox10b*. Here we aimed to determine whether Sox9 is involved in the specification of pigment cell lineage. The *sox9b* homozygous mutation did not affect pigment cell formation, despite lethality at the early larval stages. By employing *sox10a*, *sox10b*, and *sox9b* mutations, compound mutants were established for the *sox9b* and *sox10* genes and pigment cell phenotypes were analyzed. Simultaneous loss of *sox9b* and *sox10a* resulted in the complete absence of melanophores and xanthophores from hatchlings and severely defective iridophore formation, as has been previously shown for *sox10a*$^{-/-}$; *sox10b*$^{-/-}$ double mutants, indicating that Sox9b as well as Sox10b functions redundantly with Sox10a in pigment cell development. Notably, leucophores were present in *sox9b*$^{-/-}$; *sox10a*$^{-/-}$ and *sox10a*$^{-/-}$; *sox10b*$^{-/-}$ double mutants, but their numbers were significantly reduced in the *sox9b*$^{-/-}$; *sox10a*$^{-/-}$ mutants. These findings highlight that Sox9b is involved in pigment cell formation, and plays a more critical role in leucophore development than Sox10b.

Keywords: medaka fish, *Oryzias latipes*, SoxE transcription factor, melanophore, melanocyte, iridophore, xanthophore, leucophores, cell fate, functional redundancy

Introduction

Sry (sex determining region Y)-box (Sox) proteins are high-mobility group (HMG) transcription factors that regulate various developmental processes in animals (Wegner, 1999). The Sox family, characterized by the presence of an HMG-type DNA binding domain in the N-terminus and a transactivation domain in the C-terminal, includes the SoxE subfamily, comprising Sox8, Sox9, and Sox10 (Wegner, 1999). Sox9 and Sox10 have emerged as key regulators of neural crest cells (NCCs); expression of these are overlapping in NCCs, but expression of Sox9 precedes that of Sox10 in frogs, chicks, mice, and others (Simoes-Costa & Bronner, 2015).

Sox10 specifies pigment cell, as well as neural cell in neural crest derivatives: glial cells In the peripheral nervous system (Kuhlbrodt et al., 1998; Weider & Wegner, 2017), sensory neurons (Carney et al., 2006; Delfino-Machin et al., 2017), and enteric neurons (Elworthy, Pinto, Pettifer, Cancela, & Kelsh, 2005). In mice, Sox10 activates *Mitf* expression, and Mitf in turn promotes melanocyte differentiation by driving *Dopa chrome tautomerase* expression, and thereby melanin synthesis (Hou, Arnheiter, & Pavan, 2006).

In general, teleosts, including zebrafish, have three types of pigment cells, melanophores, iridophores, and xanthophores, but a few species, including medaka (Japanese rice fish), contain leucophores as an additional fourth type (Hashimoto, Goda, & Kelsh, 2021). To date, all pigment cell types are believed to originate from the common stem cell, chromatoblasts, which are derived from NCCs, although there is ongoing controversy about the exact identity of these progenitors (see Nikaido et al., doi.org/10.1101/2021.06.17.448805). Since Sox10 loss-of-function in zebrafish *sox10*$^{-/-}$ *(colorless)* mutants results in severe depletion of all pigment cell types (Dutton et al., 2001; Kelsh, 2006), Sox10 activity is vital for fate specification of each pigment cell type.

Sox9 may be best known as a regulator of chondrocyte differentiation (Lefebvre, 2019). For instance, Sox9 inactivation in the neural crest lineage results in severe craniofacial defects in mice (Mori-Akiyama et al., 2003). Sox9 functions as an early neural crest specifier, and its expression is maintained in migratory NCCs within the branchial arches, reflecting its crucial role in cartilage formation (Mori-Akiyama et al., 2003). In zebrafish,

both *sox9b* and *sox10* are expressed in early NCCs, and thus in the progenitors of all pigment cells (Hashimoto et al., 2021). Thus, it is conceivable that both Sox9 and Sox10 might have partially redundant functions in the development of one or more pigment cell types. In frogs, overexpression of Sox9 or Sox10 has equivalent effects on neural crest formation (Taylor & Labonne, 2005). Overexpression of Sox9 induces the formation of melanophores and rescues neural crest formation in zebrafish *sox10*$^{-/-}$ *(colorless)* mutant embryos (Lee et al., 2016). Similarly, in zebrafish, injection of a fruit fly or lamprey *soxE* expression plasmid partially rescues melanogenesis in *sox10*$^{-/-}$ *(colorless)* mutant embryos (Lee et al., 2016). More directly, Greenhill and colleagues have reported that zebrafish *sox10*$^{-/-}$ *(colorless)* mutants occasionally exhibit residual melanin expression (Greenhill, Rocco, Vibert, Nikaido, & Kelsh, 2011). They showed that morpholino-mediated knockdown of Sox9b suppressed the expression of residual melanin. These findings suggest that Sox9b is a component of the gene regulatory network, not only for early neural crest specification, but also for pigment cell development, perhaps through supportive action of Sox10.

While zebrafish has a single *sox10* gene, medaka has two teleost paralogs of *sox10*, *sox10a*, and *sox10b*, and their simultaneous loss results in defects of melanophore, iridophore, and xanthophore specification, as seen in zebrafish *sox10* mutants (Nagao et al., 2018). Surprisingly, the number of leucophores, which are absent in zebrafish, was unaffected in medaka, although their distribution was shifted to the head (Nagao et al., 2018). Thus, although leucophores are proposed to share a progenitor with xanthophores (Kimura et al., 2014; Nagao et al., 2014), these findings suggest that leucophore fate may be less dependent on Sox10a and Sox10b than xanthophore fate. This finding led to the hypothesis that residual SoxE activity may promote leucophore fate in medaka.

To address this possibility, we first investigated the expression of candidate *soxE* genes, *sox8*, *sox9a*, and *sox9b*, in the neural crest. Based on the results, phenotypic analyses of medaka *soxE* mutants were conducted, comparing *sox10a*$^{-/-}$*, sox9b*$^{-/-}$ double, and *sox10a*$^{-/-}$*; sox10b*$^{-/-}$*; sox9b*$^{-/-}$ triple mutants. We found that *sox9b* acted on pigment cell fate specification in a manner comparable to that of *sox10b*. Additionally, the effect of *sox9b* in leucophore specification was stronger than that of *sox10b*. Thus, this study uncovered

a previously unappreciated function of Sox9 in vertebrate pigment cell development.

Materials and Methods

Ethics

The animal work in this study was approved by the Nagoya University Animal Experiment Committee and was conducted in accordance with the Regulations on Animal Experiments at Nagoya University.

Medaka strains

The Nagoya strain of the medaka fish *Oryzias latipes* was used as the wild type (WT). The *sox9b*^{K136X} TILLING mutant was a gift from Prof. M. Tanaka, Nagoya University (Nakamura et al., 2012). The *sox10a*^{E2del16} and *sox10b*^{E1del7} mutant strains have been described previously (Nagao et al., 2018). The *sox10a*$^{E2del16/E2del16}$, *sox10b*$^{E1del7/E1del7}$, and *sox9b*$^{K136X/K136X}$ mutants are considered null mutants (Nakamura et al., 2012, Nagao et al., 2012) (see Suppl. Fig. 1), and are therefore designated as *sox10a*$^{-/-}$, *sox10b*$^{-/-}$, and *sox9b*$^{-/-}$, respectively. The *sox10a*$^{-/-}$ mutants hatched and survived at approximately 9–10 days post fertilization (dpf) but died thereafter. The *sox10b*$^{-/-}$ mutants appeared normal throughout their lives. Some of the *sox9b*$^{-/-}$ mutants, but not all, hatched and survived at approximately 9–10 dpf, but died thereafter.

Mating

Homozygotes for single, double, or triple *soxE* mutations were obtained by crossing heterozygotes for the corresponding mutation(s).

Genotyping

Mutations in *sox10a*, *sox10b*, and *sox9b* were detected using polymerase chain reaction fragment length polymorphism by polyacrylamide gel electrophoresis (PAGE), as previously described (Nagao et al., 2018; Nakamura et al., 2012). To detect the 16 bp *sox10a*^{E2del16} deletion mutation, the following primer set was used: 5′-CTCCCTCTAGGCTGCTGAACGAGA-3′ and 5′-GAGACCCTGCGCCCACATTGTGAT-3′. For the *sox10b*^{E1del7} 7 bp deletion mutation, the following primer set was used to amplify genomic DNA fragments: 5′-GAATTCAATGTCCAGGGAGGAGCAGAGCCT-3′ and 5′-GTCGTCGGATTTGGCGGAAGAACA-3′. These fragments were subsequently digested with

*Msp*I (New England Bio Labs, Beverly MA, USA), which only cuts the WT allele, and then separated using PAGE. To detect the *sox9b*^{K136X} point mutation, the following primer set was used: 5'-GTGCATTAGAGACGCGGTGTCCCAAGTGCT-3' and 5'-TAAGGAGCCTCCAAAGTTTTCCAAGAGTTC-3'. The resulting DNA fragments were digested with *Pvu*II (New England Bio Labs, Beverly MA, USA), which only cuts the mutant allele.

Whole mount *in situ* hybridization and plastic sectioning

Whole mount *in situ* hybridization and plastic sectioning were performed as previously described (Nagao et al., 2014). The digoxigenin (Roche Diagnostics GmbH, Mannheim, Germany)-labeled antisense riboprobe was synthesized from the plasmid harboring full- or partial-length open reading frame of *sox10a*, *sox10b*, *sox9a, sox9b* or *sox8* cDNA using SP6 or T7 polymerase (Promega, Madison, WI, USA) after restriction enzyme digestion (New England Bio Labs, Beverly MA, USA). For plastic sectioning, the stained samples were embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany) and sectioned at 10 μm-thickness.

Microscopy

Melanophores were subjected to a combination of bright and dark-field illumination under a stereomicroscope (MZ APO, Leica Microsystems, Wetzlar, Germany). Leucophores and iridophores were identified in a dark field. Xanthophores were identified by detecting their autofluorescence using UV light exposure with a DAPI filter (Imager.D1, Carl Zeiss, Oberkochen, Germany).

Results and Discussion

To identify candidate *soxE* gene(s) involved in pigment cell development, the *sox8*, *sox9a*, and *sox9b* expression patterns were examined using *in situ* analysis. During the early to mid-somite stages, when pigment cell progenitors are established in the trunk, *sox9b* shows *in situ* signals highly similar to those of *sox10a* and *sox10b* (Fig. 1). *sox9b* mRNA was observed in the premigratory NCCs and migrating cells, most prominently between somites and the neural tube, while *sox9a* mRNA showed little evidence of localized expression and *sox8* mRNA was observed mostly in the placode, somite, and neural tube. Thus, *sox9b* was identified as a strong candidate for residual SoxE activity underlying medaka pigment cells, especially leucophores, and fate specification in the absence of

Sox10a and Sox10b. Therefore, we further investigated the role of *sox9b*.

To determine whether *sox9b* functions in pigment cell development in medaka, a *sox9b* loss-of-function mutant, *sox9b*^{K136X}, was used. This mutant had a nonsense mutation in exon 2 and was predicted to generate a truncated Sox9b protein lacking the C-terminus of the HMG DNA-binding domain and the transactivation domain (Suppl. Fig. 1) (Nakamura et al., 2012). The homozygous mutant *sox9b*$^{-/-}$ exhibited normal pigmentation at the hatching stage (Fig. 2, A1, A3, and A6 vs. B1, B3, and B6 for leucophores; A2 and A4 vs. B2 and B4 for melanophores; A5 and A7 vs. B5 and B7 for iridophores; and A8 vs. B8 for xanthophores; see also Suppl. Table 1), apparently with a severe defect in jaw formation (Suppl. Fig. 2).

As previously shown by Nagao et al. (2018) (Nagao et al., 2018), although the medaka *sox10b*$^{-/-}$ single mutant shows no overt pigment cell phenotype, *sox10a*$^{-/-}$ single mutant hatchlings show a reduction in all pigment cell types except for leucophores (Fig. 2, A1, A3 and A6 vs. C1, C3, and C6) compared to that of WT siblings (Fig. 2, A2 and A4 vs. C2 and C4 for melanophores; A7 vs. C7 for iridophores [those in the iris of A5 and C5 look unaltered]; and A8 vs. C8 for xanthophores). The *sox10a*$^{-/-}$*; sox10b*$^{-/-}$ double mutants showed a more severe reduction in the numbers of melanophores (Fig. 2, E2 and E3), iridophores (Fig. 2, E4 and E6), and xanthophores (Fig. 2, E7), but retained leucophores in the dorsal head region (Fig. 2, E5). Thus, while *sox10b* was dispensable for pigment cell development, it acted redundantly with *sox10a* in pigment cell specification. To determine whether *sox9b* functions redundantly in a similar manner to *sox10b*, *sox10a*$^{-/-}$*; sox9b*$^{-/-}$ double mutants were studied. Simultaneous loss of Sox10a and Sox9b resulted in almost complete absence of melanophores, iridophores, and xanthophores, in addition to a severe reduction in leucophores in the trunk, although some double mutants retained a considerable number of leucophores in the head (Fig. 2): Melanophores (D2 and D3) and xanthophores (D7) were absent from the body surface, iridophores were lost from the dorsal surface of the yolk (D6), but occasionally remained partially in the iris (D4), while leucophores remained in the head to anterior trunk region with numbers varying between individuals (D1 and D5). These phenotypes are reminiscent of those in *sox10a*$^{-/-}$*; sox10b*$^{-/-}$ double mutants (Fig. 2 E1 and E5, Suppl. Fig. 3). The *sox10b*$^{-/-}$*; sox9b*$^{-/-}$ double mutants showed no apparent pigment cell phenotypes,

but showed defective jaw formation, presumably attributed to Sox9b loss.

Next, the number of leucophores over the dorsal surface of the head and the dorsal midline of the body were quantified in the *sox9b*$^{-/-}$ single mutant and the *sox10a*$^{-/-}$*; sox9b*$^{-/-}$ and *sox10a*$^{-/-}$*; sox10b*$^{-/-}$ double mutants (Fig. 3). Compared to that of the wild-type Nagoya strain, *sox10a*$^{-/-}$*; sox9b*$^{-/-}$ double mutant larvae exhibited a significant reduction in leucophore numbers, while *sox10a*$^{-/-}$*; sox10b*$^{-/-}$ double mutant or *sox9b*$^{-/-}$ single mutant larvae were indistinguishable from WT. These data suggested that leucophore specification required SoxE activity, with greater dependence on Sox9b than Sox10b.

Finally, whether leucophores differentiated in the absence of *sox10a*, *sox10b*, and *sox9b* was determined. Triple heterozygous fish were crossed, and out of their offspring, more than 200 embryos without melanophores were collected, which represent the population at least doubly homozygous for *sox10a* and either *sox10b* (Fig. 4, A) or *sox9b* (Fig. 4, B), and presumably contained triple homozygotes of these *soxE* genes. Assuming that a third mutation of either *sox9b* or *sox10b* may be homozygous in a Mendelian fashion, the *soxE* triple homozygosity was expected to appear in approximately one quarter of the 200 embryos obtained. At 6 dpf, when leucophores had become visible in siblings, the presence of leucophores in the embryos was determined and the embryos were genotyped. *sox10a*$^{-/-}$*; sox10b*$^{-/-}$*; sox9b*$^{+/-}$ compound mutants retained leucophores on the dorsal head region (Fig. 4, C), while *sox10a*$^{-/-}$*; sox10b*$^{+/-}$*; sox9b*$^{-/-}$ compound mutants also retained leucophores (Fig. 4, D), but in a few cases, there were few or no leucophores (Fig. 4, E). Importantly, no triple homozygotes were observed at 6 dpf, although the double homozygous embryos (> 200) examined should have included approximately one-quarter triple homozygotes, assuming that they were viable. We further collected and genotyped more than one hundred 2 dpf-embryos without melanophores, obtained from a cross of triple heterozygous fish. Again, no triple homozygotes were found in these embryos, so it was concluded that the triple homozygous mutation must be lethal during early developmental stages.

In conclusion, this study revealed that, in medaka, Sox9b plays a role in pigment cell specification to an extent similar to that of Sox10b. By considering the severity of

pigment cell phenotypes in the single and compound mutants, we concluded that while Sox10a is a pivotal player in pigment cell specification, both Sox10b and Sox9b functioned in a partially redundant manner to Sox10a. For melanophore, iridophore, and xanthophore specification, Sox10b and Sox9b appeared to be equally important. In contrast, for leucophore specification, Sox9b appeared to be more influential than Sox10b. Finally, analysis of the triple *soxE* compound mutants revealed that only those with *sox9b* and *sox10a* homozygosity occasionally show an absence of leucophores. Furthermore, this analysis suggests an essential role of *soxE* in viability of the early embryo, which may shed light on a new function of *soxE* in early embryogenesis.

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Figure legends

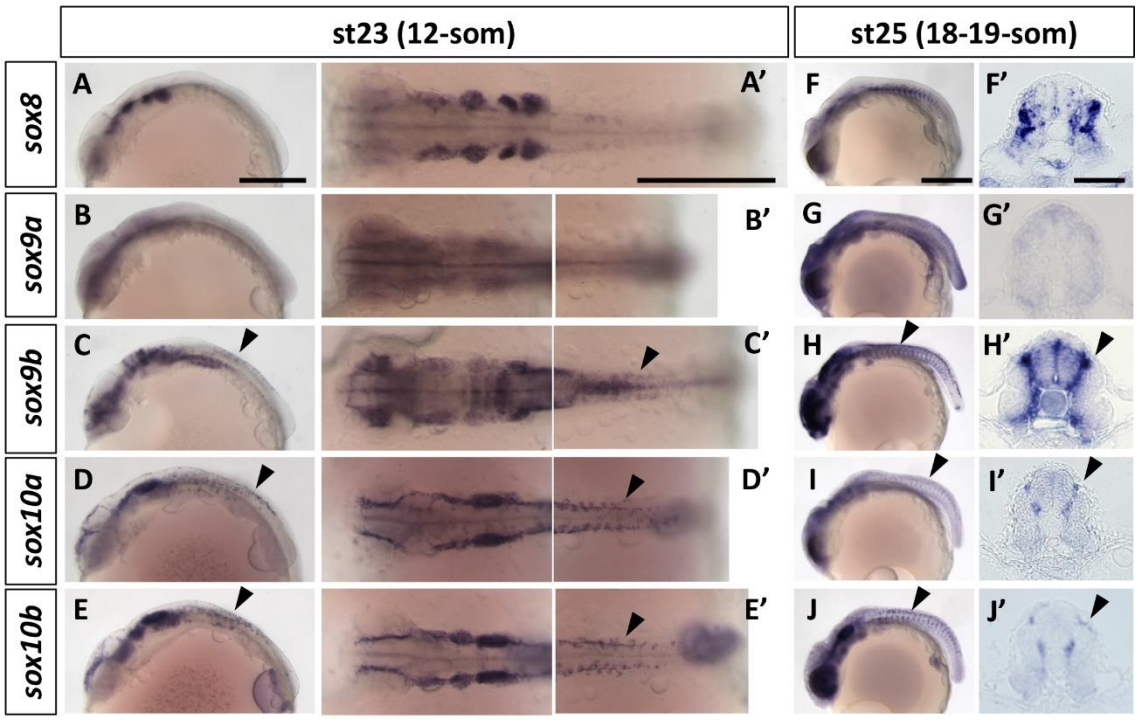


Fig. 1 Expression pattern comparison of the *soxE*-group genes

In situ hybridization of *sox8* (A, F), *sox9a* (B, G), *sox9b* (C, H), *sox10a* (D, I), and *sox10b* (E, J) mRNAs at stage 23 (12-somites, A to E) and stage 25 (18–19-somites, F to J) in wild-type (WT) embryos. Arrowheads indicate signals in the premigratory neural crest cells (NCCs). *sox9b, sox10a*, and *sox10b* mRNA expression is detected in the NCCs, in contrast to *sox8* and *sox9a*.

Scale bars: (A, A', F) 0.2 mm, also applied to B, C, D, E, B', C', D', E', G, H, I, and J; (F') 20 μm, also applied to G', H', I, and J'.

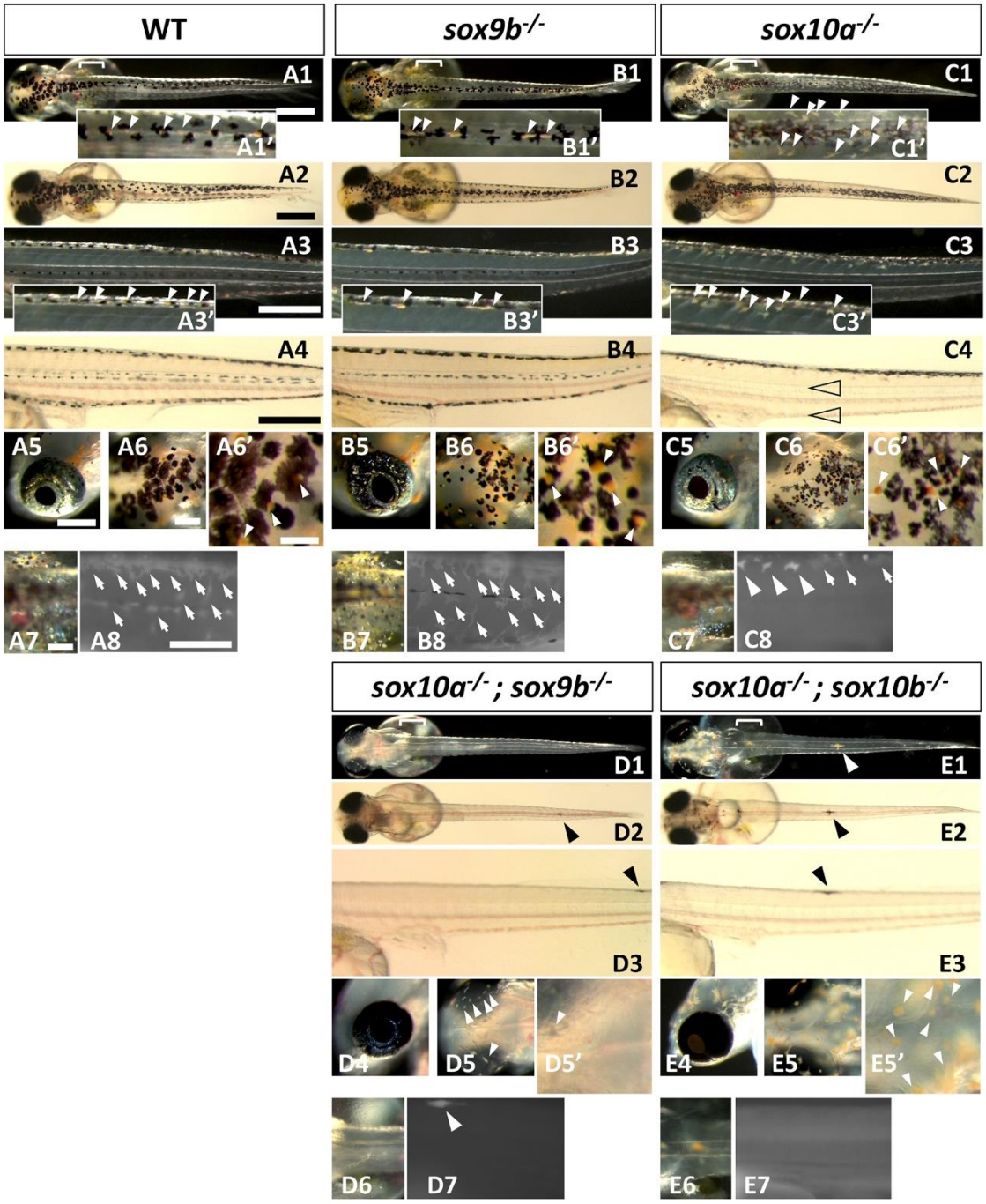


Fig. 2 Pigment cell phenotypes of *soxE* mutants

Pigments of wild-type (WT) and mutant hatchlings were observed at 7 days post fertilization (dpf). (A1–A8) WT, (B1–B8) *sox9b*$^{-/-}$ mutants, (C1–C8) *sox10a*$^{-/-}$ mutants, (D1–D7) *sox10a*$^{-/-}$*; sox9b*$^{-/-}$ mutants, and (E1–E7) *sox10a*$^{-/-}$*; sox10b*$^{-/-}$ mutants. Magnified images are numbered with a prime (’). Arrows indicate xanthophores, and arrowheads indicate leucophores. The square brackets in A1, B1, C1, D1, and E1 indicate where A7, B7, C7, D6, and E6 correspond to.

In the WT hatchlings, melanophores and black cells are observed dorsally in the head and form three horizontal stripes (dorsal, lateral, and ventral) at the trunk (A1, 2, 4, and 6). Leucophores, which appear yellowish to orange (A1, 3, and 6) in emitted light and blackish or brownish in transmitted light (A2 and 4), are also in the head and along the dorsal and ventral stripes and are associated with melanophores. In contrast, iridophores, shiny cells, are in the iris and dorsally on the yolk sac (A5 and 7) and xanthophores are evident on the lateral surface of the body through emission of autofluorescence under UV light (A8).

sox9b$^{-/-}$ mutant hatchlings appear normal in pigmentation. Melanophores are scattered on the head and form three stripes on the body (B1, 2, 4, and 6); leucophores are associated with melanophores all along the dorsal and ventral stripes (B1, 3, and 6); iridophores are observed both in the iris and on the yolk sac (B5 and 7); xanthophores are located laterally on the body (B8). In *sox10a* mutants, melanophores are severely reduced in the lateral and ventral stripes, more severely in the posterior body (C1, 2, 4; open arrowheads in C4 indicate the absence of two stripes (lateral and ventral) of melanophores), and xanthophores are nearly absent (C8), while leucophores and iridophores are unaffected (C1, 3, and 6, and C5 and 7, respectively).

In the *sox10a; sox9b* and *sox10a; sox10b* double homozygous null mutants, similar pigment cell phenotypes are observed. Melanophores and xanthophores are completely lost (D1, 2, 3, 5, E1, 2, 3, 5, and D7, E7, respectively), whereas leucophores are nearly absent from the body (D1 and E1) but remain in the head in considerable numbers (D5 and E5), while iridophores are lost from the yolk sac (D6 and E6), but remain in the iris in residual numbers (D4 and E4).

Scale bars: A1–5, 0.5 mm, also applied to B1–5, C1–5, D1–4, and E1–4; A6–8, 0.25 mm, also applied to B6–8, C6–8, D5–7, and E5–7.

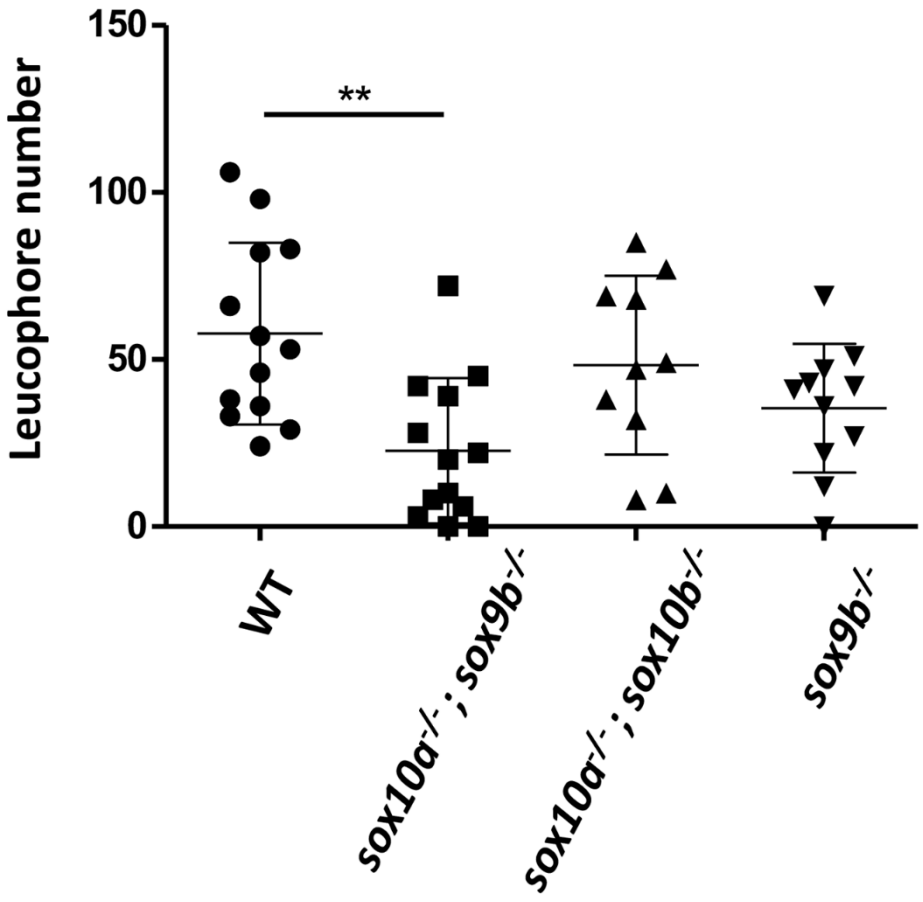


Fig. 3 Quantitation of leucophore numbers in *sox10a*; *sox9b* and *sox10a*; *sox10b* double homozygous mutants

Leucophores on the head and along the dorsal midline were counted for wild-type (WT), *sox10a*; *sox9b* double mutants, and *sox10a*; *sox10b* double homozygous mutants. Compared to WT, the counts are significantly reduced in the *sox10a*; *sox9b* double mutants (*p = 0.0029319*), but not in the *sox10a*; *sox10b* double mutants (*p = 0.7828484*). Please note that the counts in *sox9b* single homozygous mutants are not significantly different from those in WT (*p = 0.1215848*). **Significance was tested using one-way ANOVA and Tukey HSD. Plot graphs were drawn using PRISM software (version 5).

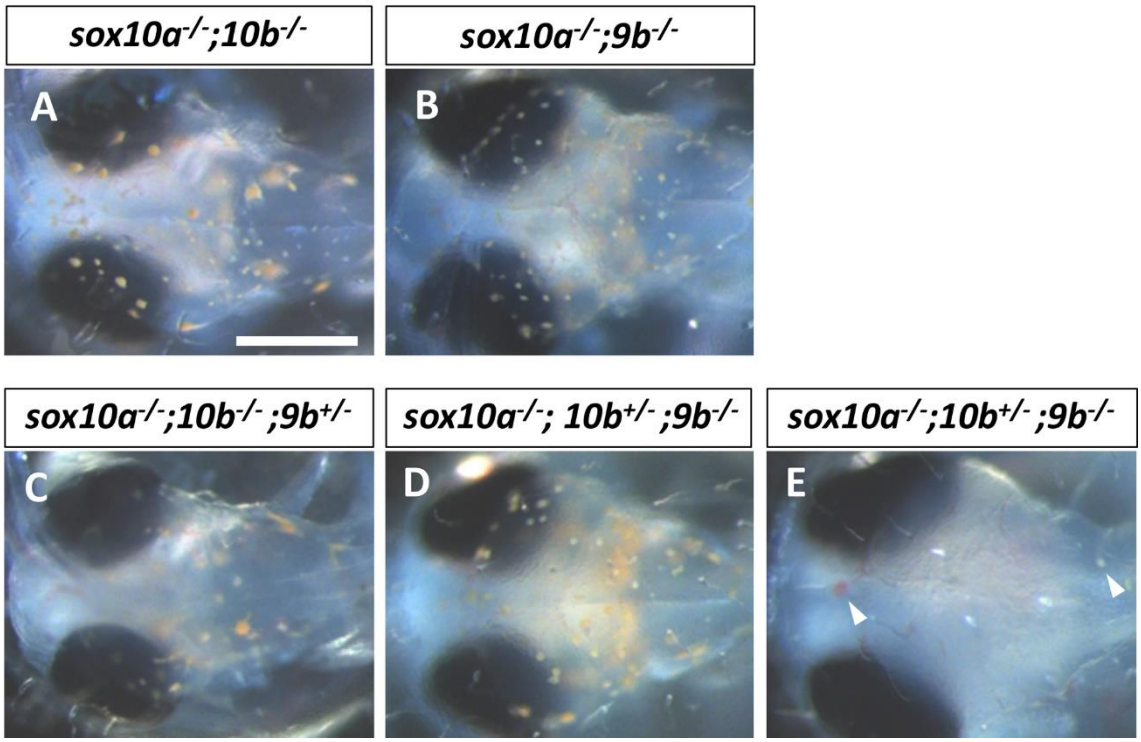
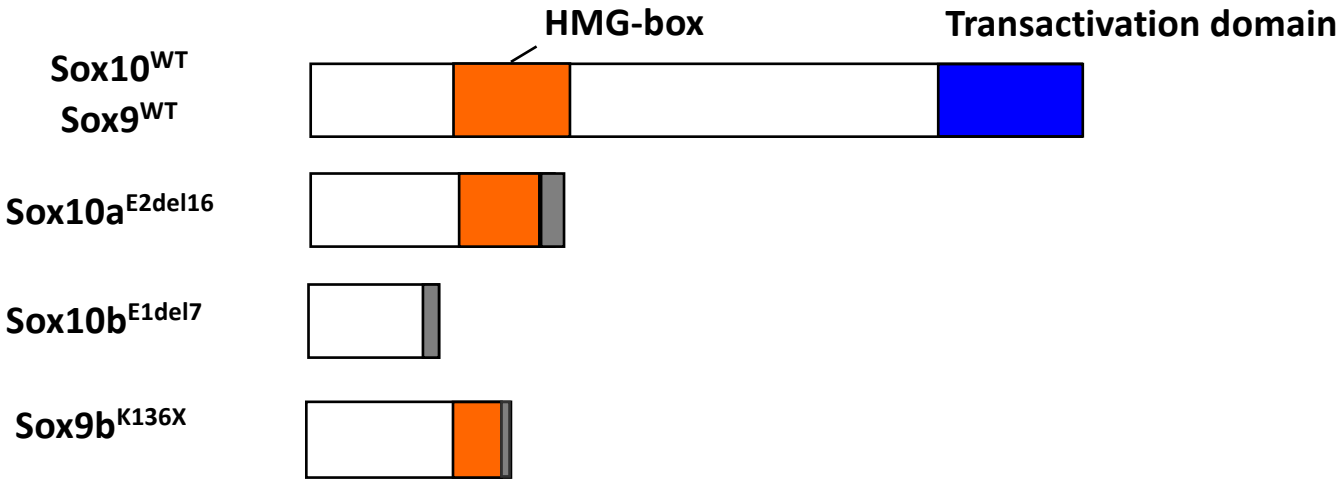


Fig. 4 Leucophore phenotype in the compound *sox10a*; *sox10b*; *sox9b* triple mutants

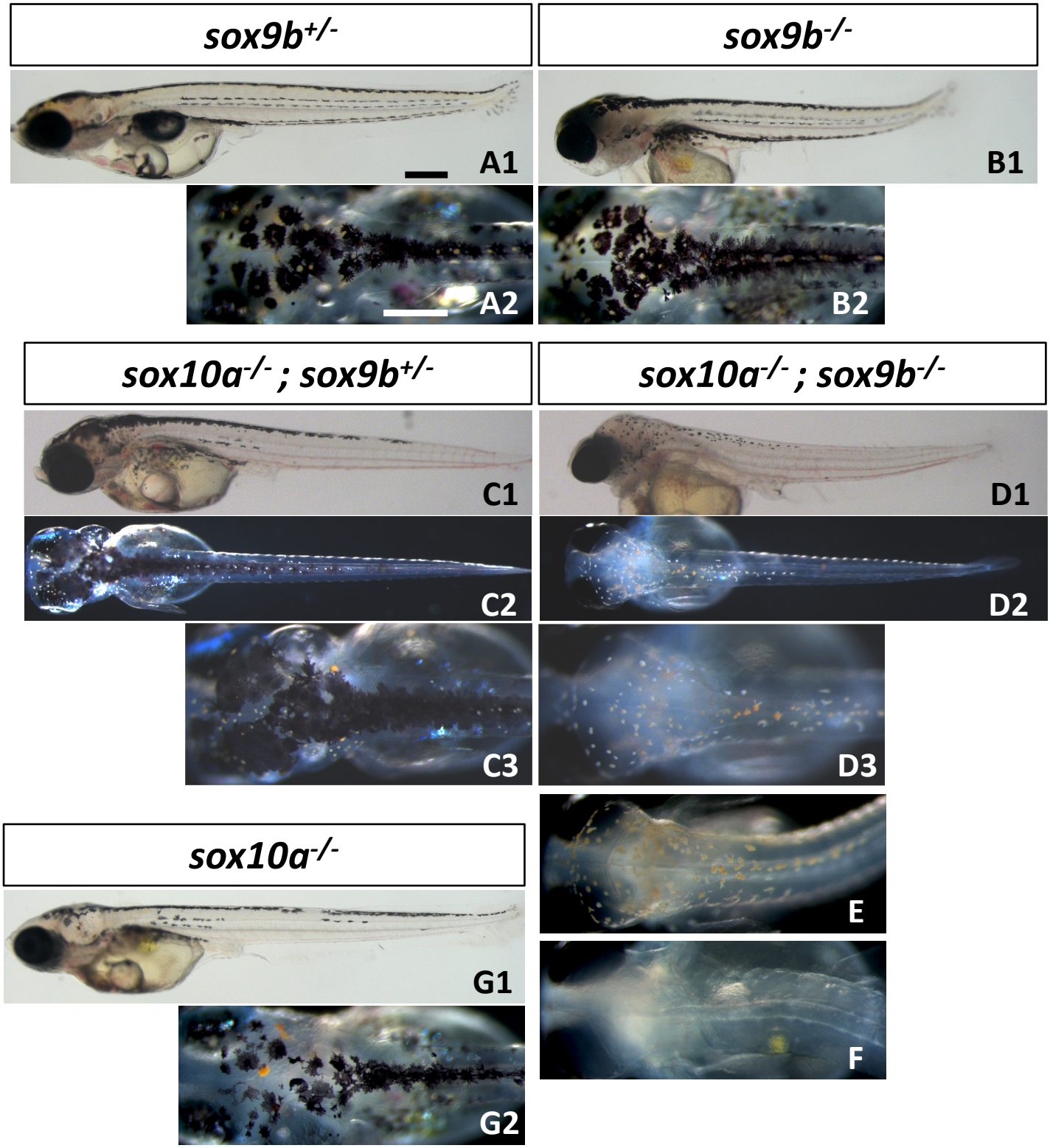
The 5-day post fertilization (dpf) embryos were obtained from *sox10a*; *sox10b*; *sox9b* triple heterozygous parents. Those without melanophores, which are presumably at least doubly homozygous for *soxE* mutations (*sox10a* and either *sox10b* or *sox9b*), were photographed and genotyped. As previously described, most double mutants, *sox10a*$^{-/-}$; *sox10b*$^{-/-}$ (A) and *sox10a*$^{-/-}$; *sox9b*$^{-/-}$ (B) have a considerable number of leucophores on their heads. The *sox10a*$^{-/-}$; *sox10b*$^{-/-}$; *sox9b*$^{+/-}$ (C) and *sox10a*$^{-/-}$; *sox10b*$^{+/-}$; *sox9b*$^{-/-}$ (D) compound mutants retained a few leucophores. In a few cases, *sox10a*$^{-/-}$; *sox10b*$^{+/-}$; *sox9b*$^{-/-}$ embryos have little or no leucophores. The mutant in (E) has two leucophores on the head (indicated by arrowheads). Importantly, there are no triple homozygous survivors of *sox10a, sox10b,* and *sox9b* in the 2 dpf or 5 dpf offspring of the *sox10a*; *sox10b*; *sox9b* triple heterozygous parents.

Notably, leucophores appear yellowish to orange at this stage.

Scale bars: (A) 0.5 mm, also applied to B, C, D, and E.



Suppl. Fig. 1 Schematic of predicted primary structures of WT and mutant Sox9b, Sox10a and Sox10b proteins.
None of the mutant Sox proteins has a complete HMG-type DNA binding domain (red) nor a transactivation domain (blue). Gray box indicates a de novo amino acid sequence following the frame-shift due to the deletion or point mutation.



Suppl. Fig. 2 Phenotype in the compound *sox10a*; *sox9b* mutants

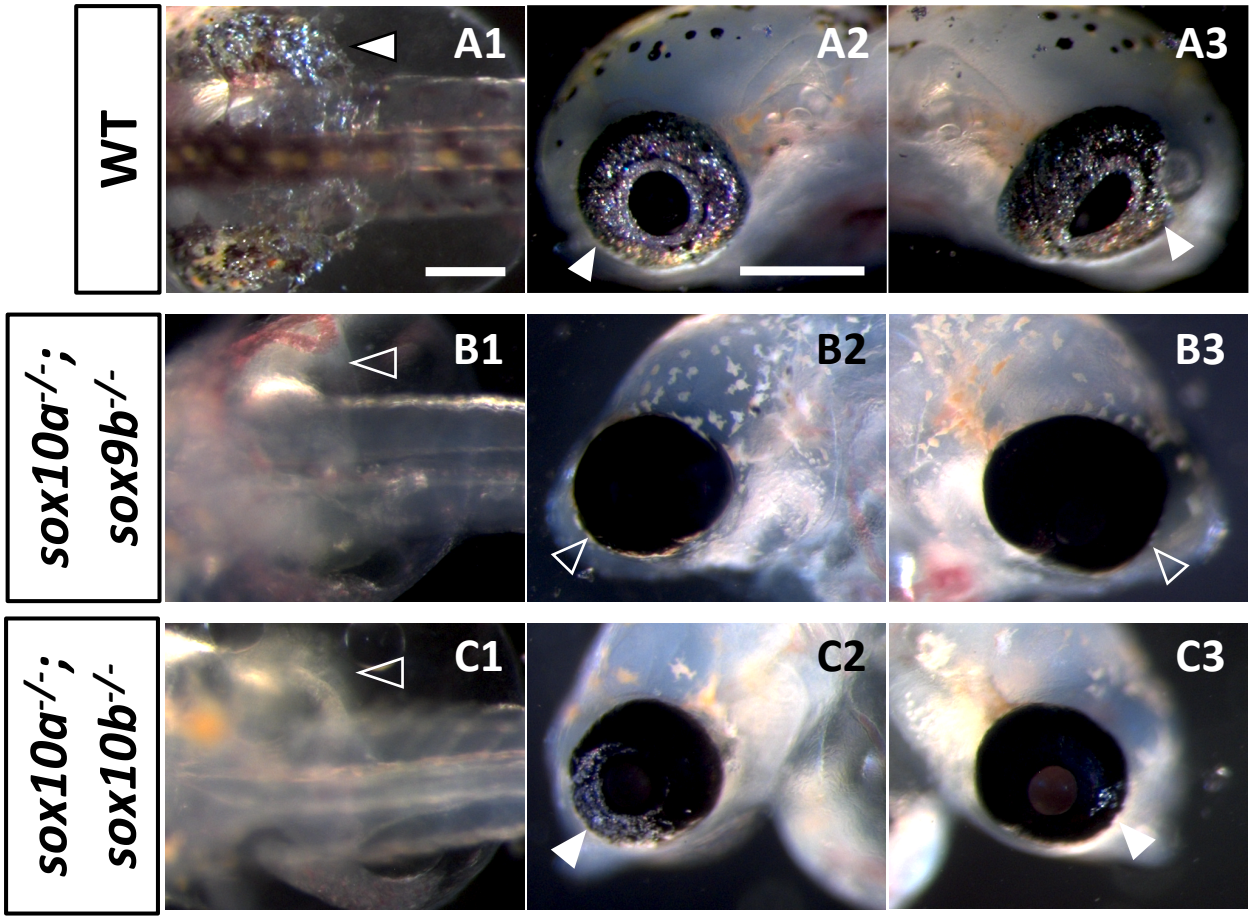
(A1-A2) *sox9b*$^{+/-}$ mutant. (B1-B2) *sox9b*$^{-/-}$ mutant. (C1-C3) *sox10a*$^{-/-}$*; sox9b*$^{+/-}$ mutant. (D1-D3, E, F) *sox10a*$^{-/-}$*; sox9b*$^{-/-}$ mutants. (G1-G2) *sox10a*$^{-/-}$ mutant. 9dpf larva.

Pigmentation looks normal in *sox9b* heterozygote (A1, 2) and homozygote (B1, 2) while *sox9b* homozygote shows defective jaw formation and body elongation (B1).

In the *sox10a*$^{-/-}$*; sox9b*$^{+/-}$ mutant, melanophores are mostly lost from the two ventral stripes (C1), and leucophores are reduced posteriorly in the tail region (C2, 3). In the *sox10a*$^{-/-}$*; sox9b*$^{-/-}$ mutant, melanophores are completely absent (D1), and location of leucophores become severely shifted to the anterior (D2, 3). Two additional *sox10a*$^{-/-}$*; sox9b*$^{-/-}$ mutants are shown: one retains abundant leucophores on the head (E) as also seen in the individual in D3, while the other has none of these (F), suggesting considerable variation in leucophore number (formation) in this genotype. Loss of *sox10a* alone results in partial but not complete absence of melanophores (G1, 2).

Please note that leucophores look yellowish to orange at this stage.

Scale bars: (A1-2) 0.5 mm, also applied to B1-2, C1-3, D1-3, E, F and G1-2.



Suppl. Fig. 3 Iridophore phenotype in *sox10a*; *sox9b* and *sox10a*; *sox10b* mutants
(A1-A3) WT. (B1-B3) *sox10a*$^{-/-}$*; sox9b*$^{-/-}$ mutant. (C1-C3) *sox10a*$^{-/-}$*; sox10b*$^{-/-}$ mutant.
Iridophores on the dorsal yolk (A1) are lost in the *sox10a*; *sox9b* (B1) and the *sox10a*; *sox10b* (C1) double homozygous mutants. Iridophores in the iris (A2, 3) are sometimes partially retained in the double homozygous mutants: almost complete absence in the *sox10a*; *sox9b* homozygote (B2, 3); partial retainment in the *sox10a*; *sox10b* homozygote (C2, 3). Compare these phenotypes with those in Fig.1 D4, E4.
White arrowheads indicate the presence of iridophores. White-outlined arrowheads indicate the absence of iridophores.
Please note that leucophores look yellowish to orange at this stage.
Scale bars: (A1) 0.25 mm, also applied to B1 and C1; (A2) 0.5 mm, also applied to A3, B2-3 and C2-3.

Supplementary Table 1. Summary of pigment cell phenotypes in *soxE* compound mutants

sox10a	*sox10b*	*sox9b*	Melanophore	Xanthophore	Iridophore	Leucophore
+/+ +/+ +/+ +/+	+/+ +/+ -/- -/-	+/+ -/- +/+ -/-	Forms three stripes (dorsal, lateral, and ventral) in the trunk; dorsally in the head	Dorso-laterally scattered over the trunk; dorsally in the head	On the dorsal surface over the yolk sac; in the iris	Along the trunk dorsal midline; dorsally in the head
-/-	+/+	+/+	Severely reduced in the lateral and ventral stripes; more severely reduced in the posterior body; relatively unaffected in the head	Reduced in the posterior body; relatively unaffected in the head	Apparently unaffected	Reduced in the posterior body; relatively increased in the head
-/-	-/-	+/+	Absent	Absent	Absent from the yolk sac; absent or a few in the iris	Nearly absent from the trunk; increased in the head
-/-	+/+	-/-	Absent	Absent	Absent from the yolk sac; absent or a few in the iris	Nearly absent from the trunk; increased in the head Note that the total leucophore number is significantly smaller than that in the above genotype (*sox10a* $^{-/-}$; *sox10b* $^{-/-}$; *sox9b* $^{+/+}$).

The wild-type phenotypes and locations of pigment cells focused on in this study (at 7 days post fertilization, hatching stage) are shown in the second row.