Pax7 is required for establishment of the xanthophore lineage in zebrafish embryos

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ABSTRACT The pigment pattern of many animal species is a result of the arrangement of different types of pigment-producing chromatophores. The zebrafish has three different types of chromatophores: black melanophores, yellow xanthophores, and shimmering iridophores arranged in a characteristic pattern of golden and blue horizontal stripes. In the zebrafish embryo, chromatophores derive from the neural crest cells. Using pax7a and pax7b zebrafish mutants, we identified a previously unknown requirement for Pax7 in xanthophore lineage formation. The absence of Pax7 results in a severe reduction of xanthophore precursor cells and a complete depletion of differentiated xanthophores in embryos as well as in adult zebrafish. In contrast, the melanophore lineage is increased in pax7a/pax7b double-mutant embryos and larvae, whereas juvenile and adult pax7a/pax7b double-mutant zebrafish display a severe decrease in melanophores and a pigment pattern disorganization indicative of a xanthophore-deficient phenotype. In summary, we propose a novel role for Pax7 in the early specification of chromatophore precursor cells.

INTRODUCTION

Most animals have evolved characteristic color patterns serving as protection from harmful radiation, species recognition, and intraspecies communication and sexual attraction. The pigment pattern in zebrafish (Danio rerio) is a result of the arrangement of different types of pigment cells, or chromatophores, originating from the neural crest cells (Dushane, 1934; Kelsh et al., 1996). Zebrafish have three different types of chromatophores: black melanophores, yellow xanthophores, and shimmering iridophores. The chromatophores are arranged in two distinct age-dependent patterns—the embryo/early larvae pattern and the adult pattern—separated by a metamorphic interphase (Kimmel et al., 1995; Parichy and Spiewak, 2015). The chromatophores in the embryo/early larvae are directly derived from neural crest cells (Kelsh et al., 1996; Parichy, 2006), whereas adult melanophores and iridophores originate from specific progenitor cell populations that both reside at the dorsal root ganglia (DRG; Budi et al., 2011; Dooley et al., 2013; Singh et al., 2014). Adult xanthophores are derived from larval xanthophores that proliferate during the metamorphic period (Mahalwar et al., 2014); however, adult xanthophores formed independently of larval xanthophores have also been identified (McMenamin et al., 2014). The neural crest is a group of multipotent precursor cells that give rise to several different cell types in the vertebrate embryo, such as neurons and glia cells, craniofacial cartilage, and pigment cells (Le Douarin et al., 2004; Mayor and Theveneau, 2013; Simoes-Costa and Bronner, 2015). The paired box homeodomain transcription factors Pax3 and Pax7 are closely related and have important roles during neural crest development (Monsoro-Burq, 2015). Pax3 mutant mice have severely reduced neural crest and thus show defects in all neural crest derivatives (Auerbach, 1954; Epstein et al., 1993; Tremblay and Gruss, 1994). In contrast, although Pax7 is expressed in neural crest cells in mouse (Jostes et al., 1990; Mansouri et al., 1996; Lang et al., 2003; Murdoch et al., 2012), no obvious neural crest phenotype is observed in Pax7 mutant mice except for facial malformations, indicating a cephalic neural crest defect (Mansouri et al., 1996). In chick, Pax7 is required for neural crest specification during gastrulation (Basch et al., 2006), and in medaka embryos, pax7a has been implied to be essential for the development of a shared xanthophore/leucophore progenitor cell derived.
from the neural crest (Kimura et al., 2014; Nagao et al., 2014). Furthermore, Pax7 is expressed by myoblasts in amniotes (Lacosta et al., 2005). Morpholino-based antisense RNA knockdown studies in zebrafish suggest that Pax3 is required for the specification of enteric neurons and xanthoblasts and that in the absence of Pax3, the number of xanthophores is decreased in correlation with an increase in melanophore cell number (Minchin and Hughes, 2008). Expression of Pax7 has been reported in cells of the xanthophore lineage, and knockdown experiments suggest that Pax7 is involved in the xanthophore pigmentation process but dispensable for the formation of the xanthophore lineage (Minchin and Hughes, 2008). The precise role of Pax7 in the specification of neural crest–derived lineages in zebrafish remains unclear.

The zebrafish genome contains two pax7 genes—pax7a and pax7b. In this study, we introduce two new zebrafish lines mutant for pax7a and pax7b. We present evidence for a previously unknown role for Pax7 in the establishment of the xanthophore lineage in which xanthophores are lost and the embryonic/larval melanophore lineage is expanded in the absence of Pax7. Moreover, in addition to a continuous absence of xanthophores, juvenile and adult pax7a/pax7b double-mutant zebrafish display a melanophore phenotype with a significant decrease in the number of melanophores and a defective pigment-patterning process. In summary, we present a new role for Pax7 in the zebrafish pigmentation process.

RESULTS
Generation of pax7a and pax7b mutant zebrafish
Using transcription activator-like effector nucleases (TALENs) targeting exon 1 of the pax7a and pax7b loci, respectively, we generated mutant zebrafish lines exhibiting premature stop codons and dys-functional proteins (Figure 1, A and B). The TALENs introduce double-strand DNA breaks that, when repaired, result in random nucleotide deletions and insertions. Initially, five different pax7a and six different pax7b mutation variants were identified in the F1 generation, and strains carrying frameshift-causing deletions, one for each gene, were used to establish stable mutant lines (Figure 1, A and B). Immunofluorescence staining using an antibody detecting Pax7 (Kawakami et al., 1997) on F2 zebrafish embryos 24 h postfertilization (hpf) confirmed the lack of Pax7 epitopes in pax7a/pax7b double-mutant embryos (Figure 1C).

The melanophore lineage is expanded in pax7 mutant embryos and larvae
The embryonic/early larval zebrafish has distinct stripes of melanophores spanning in an anterior-to-posterior manner along the most dorsal, medial, and ventral parts of the body and covering the ventral part of the yolk sac. The iridophores can be found sparsely intermingled with the melanophores, whereas xanthophores are widely scattered along the head and the most dorsal part of the fish, resulting in a yellow tone (Kimmel et al., 1995; Kelsch et al., 1996; Quigley and Parichy, 2002; Parichy and Turner, 2003b). The first visually detectable pigment cells in zebrafish embryos are black melanophores (Kimmel et al., 1995). At 3 d postfertilization (dpf), pax7a and pax7b single-mutant embryos have normal numbers, whereas pax7a/pax7b double-mutant embryos have significantly more melanophores on the crown of the head than do wild-type (wt) siblings (Figure 2A). A similar melanophore phenotype is found in zebrafish pax7a/pax7b double-mutant larvae at 6 dpf, in which the number of melanophores is significantly higher than in wt siblings on both the crown of the head and in the dorsal larval melanophore stripe (Figure 2, B–E). Moreover, the melanophores of pax7a/pax7b double-mutant larvae appear smaller (Figure 2, D and E). No consistent iridophore phenotype is observed in any of the pax7 mutant larvae (Figure 2, D and E).

To elucidate the role of Pax7 in melanophore formation, we analyzed the expression patterns of several chromatophore markers in wt and pax7a/pax7b double-mutant embryos at 24 hpf. No obvious effect in expression of the neural crest marker sox10 was detected except for a somewhat disorganized expression pattern mainly in the midbrain–hindbrain region of the pax7a/pax7b double-mutant embryos (Figure 3A). mitfa (microphthalmia-associated transcription factor a) is predominantly expressed in melanoblasts and possibly in xanthoblasts (Lister et al., 1999; Minchin and Hughes, 2008), and dct (dopachrome tautomerase) is a melanophore-specific gene (Kelsch et al., 2000). A significant increase in mitfa+ and dct+ cells is observed in pax7a/pax7b double-mutant embryos compared with wt siblings at 24 hpf (Figure 3, B–D), which agrees with the increase in melanized melanophores detected in pax7a/pax7b double mutants at older stages (Figure 2).

Pax7 is required for proper xanthophore formation
To analyze xanthophore development in the absence of functional Pax7, we analyzed the expression patterns of several xanthophore lineage markers in pax7a/pax7b double-mutant embryos at 24 hpf (Figure 4). The expression of gch2 is dramatically reduced in pax7a/pax7b double-mutant embryos compared with wt siblings (Figure 4A). gch2 encodes GTP cyclohydrolase 2 and is specifically expressed in xanthoblasts and to some extent in melanoblasts (Parichy et al., 2000; Pelletier et al., 2001; Minchin and Hughes, 2008). The zebrafish colony-stimulating factor 1 receptor (csf1r) is expressed by premigratory and migratory xanthophore precursors and is required for embryonic xanthophore precursor morphogenesis (Parichy et al., 2000). Compared to wt siblings, pax7a/pax7b double-mutant embryos have a severe reduction of csf1r+ neural crest cells; only a few, weakly stained csf1r+ cells can be detected in the head region and in the premigratory neural crest region along the most dorsal part of the trunk (Figure 4B). A similar expression pattern is detected for the xanthophore differentiation marker xanthine dehydrogenase (xdh) in pax7a/pax7b double mutants (Figure 4C). In agreement with previous studies (Parichy et al., 2000), double in situ hybridization shows that several chromatoblasts in wt embryos coexpress xdh and mitfa (Supplemental Figure S1), implying a certain amount of chromatoblast plasticity at this stage, which is also true for pax7a/pax7b double-mutant embryos (Supplemental Figure S1).

To visualize differentiated xanthophores at early stages, before any yellow color is obvious, UV light can be used to induce pteridine autofluorescence (Kelsh et al., 1996; Le Guyader and Jesuthasan, 2002). Subjecting wt embryos at 48 hpf to UV light (340–380 nm) results in strongly fluorescing xanthophores in the trunk (Figure 4D); however, no similar autofluorescing cells are detected when pax7a/pax7b double-mutant embryos are treated similarly (Figure 4D). Taken together, in the absence of functional Pax7, the pool of chromatophore precursor cells expressing xanthophore lineage markers is severely reduced, and there is an absence of differentiated xanthophores.

pax7a overexpression can rescue the xanthophore phenotype
To detect more mature xanthophores, we reared embryos in methylene blue, which has been reported to specifically label the pteridine pigment organelles found in terminally differentiated xanthophores (Le Guyader and Jesuthasan, 2002). At 5 dpf, the wt embryos display a yellow tone along the most dorsal part of the body, as well as methylene blue–colored, stellate-shaped xanthophores along the
midline and on the head (Figure 5A). However, in agreement with previous results, no yellow tone was detected, and no methylene blue–stained xanthophores were ever observed in the \(pax7a/pax7b\) double-mutant embryos (Figure 5B).

To rescue \(pax7\) expression in the chromatophore precursor population at the time point of chromatophore specification, we injected a construct in which 4118 base pairs upstream of the translational start of \(sox10\) drives the expression of \(pax7a\) specifically in the neural crest cells into \(pax7a/pax7b\) double-mutant embryos. Previous studies showed that a region 1252 base pairs upstream of the \(sox10\) transcriptional start (3731 base pairs upstream of the translational start) contains all regulatory elements sufficient to drive a mosaic GFP expression resembling the expression pattern and timing of the \(sox10\) gene (Dutton et al., 2008). At 5 dpf, several methylene blue–stained xanthophores can be identified in the \(pax7a/pax7b\) double mutants injected with \(sox10:pax7a\) (Figure 5C), and sporadic xanthophores can be detected in the same fish at adult stages (Supplemental Figure S2), suggesting that Pax7 is capable of rescuing the xanthophore population. To test whether Pax7 alone is sufficient to switch all chromatophore precursor cells into cells of the xanthophore lineage, we injected \(pax7a\) mRNA into wt embryos. General \(pax7a\) overexpression often results in defective eye formation and other somatic malformations; however, no decrease in melanophore cell number (Supplemental Figure S3A) or overrepresentation of xanthophores was detected at 6 dpf (Supplemental Figure S3B).

Other neural crest–derived cell lineages form normally in \(pax7a/pax7b\) double mutants

Neural crest cells are multipotent stem cells that differentiate into several different cell types. In addition to pigment cells, the neural crest gives rise to craniofacial cartilage and bone structures, most of the cephalic peripheral nervous system, DRG, and surrounding satellite glia, as well as the enteric neurons of the digestive tract (Mayor and Theveneau, 2013). At 3 dpf, no chondrocyte phenotype is observed in the developing cranial structures of \(pax7a/pax7b\) double-mutant embryos, as indicated by Alcian blue staining (Supplemental Figure S4A). Furthermore, normal formation of the craniofacial skeleton in \(pax7a/pax7b\) double-mutant zebrafish was confirmed using calcine to stain calcified bone (Supplemental Figure S4, B and C). To analyze whether the enteric nervous system forms normally in

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**FIGURE 1:** Generation of \(pax7a\) and \(pax7b\) mutants using TALENs. Schematic overview of TALEN-induced deletions resulting in frameshift mutations in genes (A) \(pax7a\) and (B) \(pax7b\) on chromosome 11. Black arrows represent the left and right TALEN sequences, and the gray box represents the spacer sequence indicating the FokI cut site. Purple indicates the insertion of mismatched nucleotides, and red indicates mismatched amino acids. (C) Lateral view of 24-hpf wt embryo and \(pax7a/pax7b\) double-mutant trunk immunofluorescently analyzed using a Pax7 antibody (red). DAPI is used to visualize nuclei.
the absence of Pax7, we analyzed HuC/D expression in embryos 4 dpf; the formation of enteric neurons in the gut of pax7a/pax7b double-mutant embryos seemed unaffected (Supplemental Figure S5). In addition, HuC/D-positive DRG appear to form normally in double-mutant embryos (Supplemental Figure S5). Thus involvement of Pax7 was only observed in the neural crest cell population, which differentiates into chromatophores.

The expanded melanophore lineage is not maintained in juvenile and adult pax7a/pax7b double mutants

At juvenile stages, the zebrafish enters a metamorphic phase in which the embryonic/larval pigment pattern is transformed into an adult pigment pattern with characteristic thick, dark stripes separated by lighter interstripes spanning the anterior–posterior axis of the fish (Parichy and Turner, 2003b; Parichy, 2006; Hirata et al., 2005). To assess whether Pax7 plays a role in the metamorphic pigment-pattern-forming process, we analyzed the pigment of juvenile zebrafish at 30 dpf. We counted the number of melanophores on the flank just above the pectoral fins. Compared to wt siblings, a significant decrease of melanophores can be detected in the pax7a/pax7b double-mutant fish, whereas pax7a and pax7b single mutants do not show any significant difference in melanophore number (Figure 6). To investigate how the melanophores behave during the metamorphic period in the absence of Pax7, we repeatedly observed the pigment pattern of juvenile zebrafish from 25 to 46 dpf (Supplemental Figure S6B). Monitoring single melanophores over time showed that almost all melanophores identified at 25 dpf are still present at 46 dpf in wt zebrafish. During this period, new melanophores appear, which become organized into dark, melanophore-rich stripes separated by xanthophore-rich interstripes (Supplemental Figure S6A). In pax7a/ pax7b double mutants, the majority of melanophores observed at 25 dpf subsequently disappear, and although new melanophores appear over time, their presence does not compensate for the severe loss observed at 46 dpf (Supplemental Figure S6B). The majority of the melanophores in the pax7a/pax7b double mutants are at first organized much like the wt into dorsal, medial, and ventral stripes, as well as covering the yolk sac; however, with time, this organization is disrupted, the melanophores in the pax7a/pax7b double mutants fail to organize into a striped pattern, and new melanophores appear in a scattered pattern (Supplemental Figure S6B).

Melanophores and a low number of iridophores and stellate-shaped xanthophores predominantly make up the dark stripes in adult zebrafish, whereas interstripes consist of large numbers of iridophores and round xanthophores (Figure 7A; Mahalwar et al., 2015; Singh and Nusslein-Volhard, 2015). In comparison to wt zebrafish (Figure 7A), the pigment pattern of pax7a and pax7b single-mutant zebrafish appears to be unaffected (Figure 7B and C). However, similar to juvenile stages, adult pax7a/pax7b mutant zebrafish display a severe depletion of melanophores (Figure 7D). In addition, a defect in the arrangement of the residual melanophores is observed in pax7a/pax7b mutant zebrafish, in which melanophores are found scattered all over the flank, although a general tendency of stripe formation can be distinguished (Figure 7D). Furthermore, in agreement with embryonic/larval stages, no xanthophores are detected in juvenile and adult pax7a/pax7b double-mutant zebrafish (Figures 6A and 7D). The iridophores in juvenile and adult zebrafish appear unaffected by the absence of Pax7 (Figures 6A and 7).
DISCUSSION

In this study, we assess the involvement of Pax7 in zebrafish pigment formation from embryonic to adult stages. By generating pax7a and pax7b mutant zebrafish lines, we demonstrate an early role for Pax7 in the development of neural crest–derived chromatophore precursor cells. In the absence of Pax7, there is a severe depletion of xanthophore precursor cells, a complete lack of differentiated xanthophores, and an expansion of the embryonic/larval melanophore lineage. In contrast, adult Pax7 mutants have a dramatic reduction in melanophore and disrupted pigment pattern.

Expression of Pax7 was shown in the zebrafish neural crest cells specifically in the xanthophore lineage (Minchin and Hughes, 2008). Knocking down pax7a using morpholinos suggests that Pax7a is dispensable for xanthoblast fate specification and required for the xanthophore pigmentation process, in which pax7a morphants have a reduction of yellow pigment but a normal number of methylene blue–colored xanthophores (Minchin and Hughes, 2008). However, in pax7a/pax7b double-mutant zebrafish embryos, we observe a severe reduction in expression of the xanthophore lineage markers gch2, xdh, and csf1r and a complete lack of methylene blue–colored and autofluorescing xanthophores (Figures 4 and 5), suggesting a deficiency of differentiated xanthophores. Thus, in the absence of Pax7, chromatophore precursor cells expressing xanthophore lineage markers are still present but severely reduced in number. There is, however, no formation of a xanthophore population. This suggests that Pax7 is involved in establishing a proper population of xanthoblasts and xanthophores; whether this is via xanthophore differentiation, xanthoblast survival, or chromatophore fate decision remains to be determined.

In contrast to the decrease in xanthophore precursors, a significant increase in cells expressing the melanophore lineage markers mitfa and dct, as well as in melanized melanophores, is observed in zebrafish embryos and larvae (Figures 2 and 3). This could imply that Pax7 is involved in instructing chromatophore precursor cells into adopting a xanthophore cell fate and that, in the absence of functional Pax7, these precursors take on a melanophore fate. However, the possibility cannot be excluded that the melanophore phenotype observed in pax7a/pax7b double-mutant embryos and larvae is a nonautonomous effect caused or enhanced by the absence of Pax7 and the xanthophore deficiency. There are studies indicating that Pax7 is expressed in some melanophores of the dorsal stripe at 48 hpf (Lacosta et al., 2007), which could imply that Pax7 might act directly on the melanophore lineage.

We find that a proportion of the chromatophores coexpress xdh and mitfa (Supplemental Figure S1), suggesting a certain plasticity of this population, agreeing with previous studies indicating the presence of pluripotent chromatophore precursor cells (Bagnara et al., 1979; Parichy et al., 2000; Parichy and Turner, 2003a; Pelletier et al., 2001; Minchin and Hughes, 2008; Curran et al., 2010). A model has been proposed in which melanophores and iridophores share a common mitfa+ precursor cell in zebrafish (Curran et al., 2010). Furthermore, morpholino-based knockdown data suggest that Pax3 drives a xanthophore/melanophore fate switch in chromatophore precursor cells by which there is a loss of xanthophores and an increase in melanophores in the absence of Pax3 (Minchin and Hughes, 2008). In addition, several studies show coexpression of melanoblast and xanthoblasts markers (Parichy et al., 2000; Parichy and Turner, 2003a; Pelletier et al., 2001; Minchin and Hughes, 2008), and a common melanophore/xanthophore lineage has been observed in the fin of the zebrafish (Tu and Johnson, 2010, 2011). In medaka embryos, leucophores and xanthophores have similar specification and differentiation processes (Kimura et al., 2014). In contrast, with the exception of the earliest-migrating neural crest cells, most zebrafish neural crest cells are proposed to be lineage restricted, producing

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lineage. However, because the pax7a rescue is mosaic and a low number of xanthophores were formed, we were not able to assay how the melanophore cell number was affected in this experiment.

Pax7 mutant mice display facial malformations in the nose and the maxilla (Mansouri et al., 1996). We analyzed the craniofacial cartilage and skeleton in several pax7a/pax7b double-mutant embryos and larvae, and although we observed individual variations in both mutant and wt zebrafish, we could not confirm any consistent defects caused by the absence of functional Pax7 (Supplemental Figure S4).

In addition, we found no obvious effect on the development of neural crest–derived enteric neurons and DRG in pax7a/pax7b double-mutant embryos (Supplemental Figure S5). Our study show a specific role for Pax7 in the neural crest–derived pigment lineage; however, we do not exclude a possible function for Pax7 in the development and specification process of other neural crest derivatives.

In contrast to the increase in melanophore cell number detected in pax7a/pax7b double-mutant zebrafish embryos and early larvae, pax7a/pax7b double-mutant juvenile and adult zebrafish display a severe reduction in melanophores and a disrupted melanophore organization compared with wt (Figures 6 and 7 and Supplemental Figure S6). We analyzed the pigment pattern of pax7a/pax7b mutant zebrafish during the larval-to-adult metamorphic period and found that when the analysis was initiated (25 dpf), the melanophore arrangement in pax7/pax7 mutants did not differ drastically from wt (Supplemental Figure S6). However, with time, the majority of the original melanophores in the pax7a/pax7b double mutant disappeared and possibly died, and although new melanophores appeared, there was still a dramatic reduction compared with wt zebrafish, and no stripe organization was observed (Supplemental Figure S6). This indicates that Pax7 either has a direct effect on the adult melanophore lineage or the melanophore shortage observed is a nonautonomous effect, possibly caused by the xanthophore deficit. In csf1r mutant zebrafish, xanthophores fail to develop, and the number of melanophores is reduced. Furthermore, the melanophore pattern is disrupted during the metamorphic period, similar to the pax7a/pax7b double mutant (Odenthal et al., 1996; Parichy et al., 2000; Parichy and Turner, 2003a; Maderspacher and Nusslein-Volhard, 2003; Frohnhofe et al., 2013). In addition, xanthophores are important for melanophore survival (Parichy et al., 2000; Parichy and Turner, 2003a), and proper communication between all three chromatophore types is necessary for normal pigment pattern formation (Maderspacher and Nusslein-Volhard, 2003; Frohnhofe et al., 2013; Irion et al., 2014; Eom et al., 2015). Hence the melanophore phenotype that we detect in juvenile and adult pax7a/pax7b double-mutant zebrafish is likely caused by the absence of xanthophores and further indicates that there is a
lack of xanthophores and not only a xanthophore pigmentation deficiency. To conclude, we present a model (Figure 8) in which either functional Pax7a or Pax7b is required for the establishment of xanthophores in zebrafish. Furthermore, our data show that in the absence of Pax7, there is an expansion of the embryonic and larval melanophore lineage, suggesting that Pax7 is involved in regulating the chromatophore specification process, possibly via a common xanthophore/melanophore precursor cell. In addition, the absence of Pax7 and subsequent lack of xanthophores result in a dramatic melanophore phenotype in juvenile and adult pax7a/pax7b double-mutant zebrafish.

MATERIALS AND METHODS

Zebrafish strains and maintenance
Embryos, larvae, and adult fish were maintained from wt zebrafish (D. rerio). Mutant lines used were pax7aumu3 and pax7bumu4. Zebrafish were maintained by standard procedures at the Umeå University Zebrafish Facility. All animal experiments were approved by the Umeå djurförsöksetiska nämnd, Dnr: A13-15.

Generation of pax7a and pax7b mutants using TALENs
TALENs were created using the TAL Effector Kit 2.0 (Cermak et al., 2011) and the RCScript-GoldyTALEN destination vector (Bedell et al., 2012), both available from Addgene (Cambridge, MA; 1000000024 and 38142). TAL Effector-Nucleotide Targeter (TALE-NT) 2.0 (Doyle et al., 2012) was used for designing the TALENs, with NN repeat-variable diresidues to target guanines. The TALEN pair targeting pax7a binds to the sequences 5′-GGAATGGCTACTTTACCAGGAACAG-3′ (left) and

FIGURE 5: Xanthophores can be rescued by specifically expressing pax7a in the neural crest cells of pax7a/pax7b double mutants. (A) Wt sibling, (B) pax7a/pax7b double mutant, and (C) pax7a/pax7b double mutant injected with a sox10:pax7a construct at the one-cell stage and reared in embryo medium with methylene blue until 5 dpf (n = 8). Boxes indicate area of magnification on the right. Arrowheads indicate a subset of methylene blue–colored xanthophores. Scale bar, 200 μm.

FIGURE 6: The number of melanophores is decreased in pax7a/pax7b double-mutant fish during metamorphosis. (A) Pigment phenotype of wt, pax7a−/−, pax7b−/−, and pax7a/pax7b double-mutant juvenile zebrafish at 30 dpf. Box indicates area of melanophore quantification presented in C. (B) Enlargement of area counted in C. (C) Number of melanophores in wt (n = 6), pax7a−/− (n = 8), pax7b−/− (n = 6), and pax7a/pax7b double-mutant (n = 10) juvenile zebrafish at 30 dpf. The pax7a/pax7b double-mutant zebrafish differs from all other groups with p < 0.001. Comparisons between groups by one-way ANOVA or Kruskal–Wallis ANOVA on ranks with Student’s–Newman–Keuls or Dunn’s post hoc contrast test for parametric and nonparametric data. Scale bar, 2 mm (A), 200 μm (B).
5′-GGGTAGTTCCGAGGCTGTGCTG-3′ (right), and the TALEN pair targeting pax7b binds to the sequences 5′-AGAATGTCATCCTTACCGGGAAC-3′ (left) and 5′-AGTTCTGTCCTGGAGCCGGTCGCA-3′ (right). From 100 to 200 pg of in vitro–generated mRNA (using the T3 mMessage mMachine Kit; Ambion, Austin, TX) of the respective TALEN pair was injected into one-cell-stage embryos. F₀ fish with mutated loci were identified using enzyme restriction digestion of a specific enzyme cut site incorporated in the TALEN spacer sequence of each TALEN pair. We used TaqI for identification of pax7a mutant embryos and BstUI for pax7b mutant embryos before verification by sequencing.

Immunofluorescence
Zebrafish embryos were fixed in 4% paraformaldehyde (PFA) overnight and then stored in 100% methanol at −20°C. Embryos were stepwise rehydrated in PBT (phosphate-buffered saline [PBS] + 0.1% Tween 20) and then acetone cracked for 7 min at −20°C, followed by extensive PBT washing before incubation in blocking solution (PBT + 1% blocking reagent) at room temperature for 1 h. After blocking, embryos were incubated in primary antibody diluted in blocking solution with overnight rocking at 4°C. Embryos were washed 4 × 30 min in PBDTT (PBS, 1% dimethyl sulfoxide, 0.1% Tween 20, 0.5% Triton X-100) and incubated in secondary antibody diluted in blocking solution with overnight rocking at 4°C. Embryos were washed 4 × 30 min in PBDTT and then analyzed. Primary antibodies used were anti-Pax7 (1:10; DSHB, Iowa City, IA; Kawakami et al., 1997), HuC/D (1:200; 16A11; Thermo Fisher Scientific, Waltham, MA), and the secondary antibody used was goat anti-mouse Alexa Fluor 594 (1:500; Molecular Probes, Eugene, OR). 4′,6-Diamidino-2-phenylindole (DAPI) was used to visualize nuclei.

Whole-mount in situ hybridization
Zebrafish embryos were fixed in 4% PFA overnight. Whole-mount in situ hybridization was performed as described previously (Thisse et al., 1993) with minor changes; 1% blocking reagent (Roche, Basel, Switzerland) was used instead of 2% sheep serum and 2 mg/ml bovine serum albumin. Digoxigenin-labeled and fluorescein-labeled RNA probes were detected using 5-bromo-4-chloro-3′-indoly-phosphate/nitro blue tetrazolium (Roche) and fast red (Roche). RNA probes were dct (Gene Bank accession number BC129260), gch2 (Gene Bank accession number BC071298), mitfa (Gene Bank accession number BC056318), sox10 (Dutton et al., 2001), and xdh and csf1r (Parichy et al., 2000). Embryos were analyzed blindly and genotyped postanalysis.

Craniofacial stainings
To detect cartilage, Alcian blue staining was performed. Zebrafish embryos were fixed in 4% PFA overnight and then stored in 100%
methanol at ~20°C. Embryos were dehydrated and washed in PBT before being bleached for 2 h in 30% H₂O₂. After further PBT washes, embryos were transferred to Alcian blue solution (1% concentrated hydrochloric acid, 70% ethanol, 0.1% Alcian blue) and incubated overnight. Embryos were washed in acidic alcohol (5% concentrated hydrochloric acid, 70% ethanol), stepwise rehydrated to H₂O, and successively cleared in 20% glycerol with 0.25% KOH and 50% glycerol with 0.25% KOH before imaging. To visualize calcified bone structures, zebrafish were stained with calcein as described previously (Du et al., 2001).

Constructs and microinjections
pax7a RNA (Gene Bank accession number BC163523.1) was generated using the mMESSAGE mMACHINE Kit (Ambion). RNA was microinjected into one-cell-stage embryos at a concentration of 400 ng/μl. The sox10,pax7a promoter construct was generated using 4118 base pairs of the region upstream of the translational start of sox10 (National Center for Biotechnology Information reference sequence NC_007114.6) and cloning it in-frame with the translational start of pax7a (Gene Bank accession number BC163523.1) or gfp as a control. The construct was injected into one-cell-stage embryos at a concentration of 50 ng/μl.

Cell counts and statistics
To calculate the number of mita" and dct" cells, we photographed embryos after in situ hybridization and counted positive cells in the region anterior to the first somite on one side of the embryo; positive cells in the eye were excluded. The average number of positive cells is presented, and Student’s t test was used to calculate significance. Error bars indicate SEM. For melanophore counts on the crown of the head of 3-dpf embryos and 6-dpf larvae, melanophores on a specific region from the midbrain–hindbrain region to the most anterior part of the dorsal larval melanophore stripe were counted. For more posterior melanophore counts on the dorsal larval melanophore stripe, the dorsal stripe melanophores on somites 8–12 were counted. For melanophore counts during juvenile stages, melanophores in a defined area on the flank of the fish, just above the pectoral fin, were counted. Statistical analyses were performed on SigmaStat, version 3.5 (Systat Software, San Jose, CA). Comparisons between four groups of zebrafish genotypes were analyzed by one-way analysis of variance (ANOVA) or Kruskal–Wallis ANOVA on ranks. Whenever significant, post hoc contrast analyses were performed by Student’s–Newman–Keuls test or Dunn’s test for parametric and nonparametric data. p < 0.05 was considered significant.

Image acquisition and processing
For live imaging, zebrafish were sedated using tricaine mesylate and then placed on a 2% agarose plate and imaged using a Nikon (Tokyo, Japan) SMZ1500 stereomicroscope and a Nikon D5200 digital camera. The same imaging setup was used when imaging embryos stained using in situ hybridization; however, embryos were imaged in 80% glycerol. Immunofluorescently stained embryos were mounted in 80% glycerol and scanned using a Nikon A1 confocal microscope. To visualize early xanthoblasts, live embryos were sedated in embryo medium with tricaine mesylate (ethyl 3-amino benzoate methanesulfonic acid) and subjected to ultraviolet light as previously described (Le Guyader and Jesuthasan, 2002). Fiji was used for z-stack processing and image stitching, and Adobe Photoshop CS4 was used in some instances to enhance contrast and adjust color balance. Comparable sets of images were processed identically.

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