Leucophores are similar to xanthophores in their specification and differentiation processes in medaka

Tetsuaki Kimura^{a,b,1}, Yusuke Nagao^c, Hisashi Hashimoto^c, Yo-ichi Yamamoto-Shiraishi^d, Shiori Yamamoto^d, Taijiro Yabe^{b,e}, Shinji Takada^{b,e}, Masato Kinoshita^f, Atsushi Kuroiwa^d, and Kiyoshi Naruse^{a,b,g}

^aInteruniversity Bio-Backup Project Center, National Institute for Basic Biology, Okazaki 444-8787, Aichi, Japan; ^bDepartment of Basic Biology, School of Life Science, Graduate University for Advanced Studies (SOKENDAI), Okazaki, Aichi 444-8787, Japan; ^cBioscience and Biotechnology Center and Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan; ^dDivision of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan; ^eOkazaki Institute for Integrative Bioscience and National Institute for Basic Biology, National Institutes of Natural Sciences, Okazaki, Aichi 444-8787, Japan; ^fDivision of Applied Bioscience, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan; and ^gLaboratory of Bioresources, National Institute for Basic Biology, Okazaki 444-8585, Aichi, Japan

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Animal body color is generated primarily by neural crest-derived pigment cells in the skin. Mammals and birds have only melanocytes on the surface of their bodies; however, fish have a variety of pigment cell types or chromatophores, including melanophores, xanthophores, and iridophores. The medaka has a unique chromatophore type called the leucophore. The genetic basis of chromatophore diversity remains poorly understood. Here, we report that three loci in medaka, namely, *leucophore free* (*lf*), *lf-2*, and *white leucophore* (*wl*), which affect leucophore and xanthophore differentiation, encode solute carrier family 2, member 15b (*slc2a15b*), paired box gene 7a (*pax7a*), and solute carrier family 2 facilitated glucose transporter, member 11b (*slc2a11b*), respectively. Because *lf-2*, a loss-of-function mutant for *pax7a*, causes defects in the formation of xanthophore and leucophore precursor cells, *pax7a* is critical for the development of the chromatophores. This genetic evidence implies that leucophores are similar to xanthophores, although it was previously thought that leucophores were related to iridophores, as these chromatophores have purine-dependent light reflection. Our identification of *slc2a15b* and *slc2a11b* as genes critical for the differentiation of leucophores and xanthophores in medaka led to a further finding that the existence of these two genes in the genome coincides with the presence of xanthophores in nonmammalian vertebrates: birds have yellow-pigmented irises with xanthophore-like intracellular organelles. Our findings provide clues for revealing diverse evolutionary mechanisms of pigment cell formation in animals.

genome evolution | vertebrate body color | pigment cell variation | neural crest differentiation

In animals, body color is an important trait linked directly to fitness. Pigment cells in the skin, called chromatophores in poikilothermic vertebrates, produce pigments that give the body its color (1). Even though mammals and birds have only melanocytes, they can exhibit multiple body colorations because of the production of eumelanin (black or brown) and pheomelanin (yellow or red) in melanocytes and their subsequent secretion to the skin and hair or feathers. In teleosts, pigment cells are generally classified into six categories based on their hue: melanophores (black or brown), iridophores (iridescent), xanthophores (yellow), erythrophores (red), leucophores (white), and cyanophores (blue) (2). Both xanthophores and erythrophores frequently contain yellow and red pigments (pteridines and carotenoids) (3, 4). The distinction of the two chromatophores depends on the ratio of the pigments, and thus, their appearance. We refer to both xanthophores and erythrophores as xanthophores in this paper.

Whereas melanophores, iridophores, and xanthophores are widely distributed among poikilothermic vertebrates (fishes, amphibians, and reptiles), leucophores and cyanophores have been found in only a few fish species (5–7). Among the fish species, medaka has four types of pigment cells, including leucophores, melanophores, xanthophores, and iridophores. Leucophores have

been considered to be closely related to iridophores based on the primary pigment. Purines are the primary pigment of leucophores and iridophores (i.e., uric acid in leucophores and guanine in iridophores) (3, 8, 9). Melanin is the pigment of melanophores, and pteridines and carotenoids are the pigment of xanthophores. Additionally, in medaka embryos, leucophores are positioned along the dorsal midline of the trunk and are associated with melanophores in a very similar manner to that of iridophores in zebrafish embryos (10). On the other hand, leucophores are also reminiscent of xanthophores because medaka embryonic/larval leucophores as well as xanthophores contain pteridines in cytoplasmic organelles called pterinosomes (3). Leucophores appear to be orange, not white, during the embryonic and larval stages due to drosopterin, an orange pteridine, whereas xanthophores contain sepiapterin, a yellow pteridine (3, 11).

The pigment cells on the body surface of vertebrates are derived from neural crest cells (12). In fish, the neural crest cells generate more than three types of pigment cells (melanophores, xanthophores, and iridophores). In zebrafish, a considerable overlap was found between iridoblast and melanoblast markers, but not xanthoblast markers, and melanophores and iridophores arise from a common *mitfa*+ precursor (13). These facts suggest that melanophores/iridophores and xanthophores differ in the genetic basis of

Significance

Body color plays an important role in the diversity and speciation of vertebrates. In this paper, we revealed that three loci in medaka, *leucophore free* (*lf*), *lf-2*, and *white leucophore*, which affect leucophores and xanthophores, encoded solute carrier family 2, member 15b (*slc2a15b*), paired box gene 7a (*pax7a*), and solute carrier family 2 facilitated glucose transporter, member 11b (*slc2a11b*), respectively. The *pax7* is important transcriptional factor for xanthophore development in zebrafish. The function of the two solute carrier family (*SLC*) genes was unknown. We show that the presence of the *SLCs* was coupled with the presence of xanthophores in vertebrates. The results suggest that leucophores are similar to xanthophores in their specification and differentiation process, and *SLCs* contribute to the diversification of hues in the pigment cells in vertebrates.

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¹To whom correspondence should be addressed. E-mail: tekimura@nibb.ac.jp.

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their fate specification and differentiation. Studies on leucophores, which have characteristics similar to xanthophores as well as to iridophores in medaka, can be helpful in elucidating relationships between leucophores and other chromatophores. In this study, we investigated three medaka leucophore mutants through positional cloning and expression analyses and found that leucophores and xanthophores share the genetic basis of fate specification and differentiation, which is different from that of melanophores/iridophores.

Results and Discussion

Positional Cloning of Three Leucophore Mutants: *lf*, *lf-2*, and *wl*. In medaka, leucophores develop in two different regions. Firstly, leucophores appear beneath the midbrain/hindbrain (leucophore beneath the brain, LBB) by stage 26 (Fig. S1 A and B). Secondly, beginning at stage 33, an increasing number of leucophores appear on the dorsal surface along the midline (Fig. S1 C–E). At early stages, the LBBs are white, but they become orange by stage 33 (Fig. S1 B–E); however, leucophores on the body surface are orange from the beginning. Therefore, all embryonic/larval leucophores are orange after stage 33. Unlike the embryonic/larval leucophores, adult leucophores are white.

The *leucophore free* (*lf*) mutant has no visible leucophores throughout life (Fig. 1 A and B) (10, 14–17). The *lf-2* mutant has transient leucophores (LBBs) present beneath the brain, which disappear before hatching (Fig. 1 A and C) (10, 14, 15). These two mutants have no visible xanthophores in the embryo/larva, resulting in a pale appearance (Fig. 1 B and C). In the *white leucophore* (*wl*) mutant, the differentiation of both leucophores and xanthophores is affected; leucophores are prominent but appear white instead of orange, and no pigmented xanthophores are observed during the embryonic/larval stages (Fig. 1D). The

wl mutant has no obvious phenotype in adulthood (14, 15). As previously described, *lf* and *lf-2* have no phenotype during melanophore development, but *wl* results in the formation of some light black melanophores (Fig. S1 F–I). All three mutants have no phenotype in iridophores (Fig. 1).

In accordance with previous studies, our linkage analysis mapped the *lf* locus to chromosome 1, which was further narrowed to a candidate region of 85 kbp (Fig. S24) (16–18). Microinjection experiments showed that the BAC (ola1-136_M01) and fosmid (GOLWFno17_n04) clones, which cover this region, were able to rescue the *lf* phenotype (Fig. 1 E and F). Both the BAC and fosmid clones contained the gene solute carrier family 2 member 15 b (*slc2a15b*), suggesting that the loss of *slc2a15b* is responsible for the *lf* mutant phenotype. To test this possibility, we made a fosmid construct, GOLWFno17_n04-*slc2a15b*-GFP, by replacing exon 1 of *slc2a15b* with GFP cDNA, and subjected it to microinjection for a rescue experiment. GOLWFno17_n04-*slc2a15b*-GFP failed to rescue the *lf* phenotype. Further analysis revealed the deletion of a 703-bp sequence, including exons 8 and 9 of *slc2a15b* in the *lf* genome, presumably resulting in a truncated *slc2a15b* protein in *lf* mutants (Fig. S2 B and C). The data indicate that the gene at the *lf* locus is *slc2a15b*.

We assigned the *lf-2* locus to chromosome 5 by bulk segregation analysis (Fig. S34). Further linkage analysis showed that the *lf-2* locus was tightly linked with a marker, MM03D01K, located beside paired box gene 7a (*pax7a*) (Fig. S3B). Because *pax7a* orthologs in zebrafish are involved in xanthophore development (19), we assumed that *pax7a* was a candidate for *lf-2*. The microinjection of a BAC clone, ola-008A15, containing *pax7a* was able to rescue the *lf-2* phenotype (Fig. 1 G and H). In addition, the clone ola-008A15-*pax7a*-GFP, in which exon 1 of *pax7a* was replaced by GFP cDNA, failed to rescue the *lf-2* phenotype. Further analysis showed that exon 2 of *pax7a* in the *lf-2* genome was disrupted by a 1.8-kbp insertion having a *Toll* arm (20), and that the *lf-2* mutant expressed *pax7a* mRNA lacking the paired box, which was indispensable for DNA binding (Fig. S3 C–F) (21–24). The data indicate that the gene at the *lf-2* locus is *pax7a*.

We assigned the *wl* locus to chromosome 9 by bulk segregation analysis (Fig. S44). Further linkage analysis narrowed the *wl* genetic region to 533 kbp (Fig. S4B). Among the 18 genes annotated in this region, we identified solute carrier family 2, member 11b (*slc2a11b*), a gene closely related to *lf/slca15b*, as a strong candidate for *wl*. This locus has tandem-duplicated *slc2a11b* genes, which are indicated as *slc2a11b* (1 of 2) (Fig. S4B, light blue arrow) and *slc2a11b* (2 of 2) (Fig. S4B, orange arrow) in Ensembl genome annotation. The microinjection of a fosmid, GOLWFno599_n14, harboring *slc2a11b* (2 of 2) resulted in the development of some orange leucophores in *wl* mutants (Fig. 1I). Conversely, the *slc2a11b* (2 of 2)-disrupted fosmid clone GOLWFno599_n14-*slc2a11b*-GFP failed to rescue *wl*. Further analysis revealed that *slc2a11b* (2 of 2) in the *wl* genome has a 2-bp deletion in exon 3, resulting in a premature stop codon, generating a predicted truncated protein of 117 amino acids [compared with 511 amino acids in the wild-type SLC2a11b (2 of 2) protein] (Fig. S4C). The data indicate that the gene at the *wl* locus is *slc2a11b* (2 of 2) [*slc2a11b* (2 of 2) will henceforth be referred to as *slc2a11b*].

The Gene *pax7a* Is Upstream of *slc2a15b* and *slc2a11b* in the Trunk Surface. To understand the roles of the three causal genes in pigment cell development, we examined the spatiotemporal patterns of their expression, focusing on the neural crest cells and their derivatives by whole-mount in situ hybridization (WISH). From stage 21 (8 somites) to 26 (22 somites), *pax7a* expression was observed in the tectum, hindbrain, and dorsal side of the anterior neural tube (Fig. 2 A–D). At stage 26, *pax7a*-expressing cells were detected in dots on the lateral surface of the head and trunk

Fig. 1. Phenotypes of the mutants. (A) The wild-type larva had orange leucophores (triangles) and a yellow-colored body surface, although the outline of the xanthophores was not visible. (B) This *lf* mutant had no visible leucophores. (C) This *lf-2* mutant had no visible leucophores. (D) The *wl* mutant had white instead of orange leucophores (triangles). The *lf*, *lf-2*, and *wl* mutants all appeared pale due to the loss of pigmented xanthophores. (E–I) Rescued phenotypes. *lf* (E, stage 26) and *lf-2* (G, stage 40) mutants displayed no fluorescence under blue light (leucophores usually emit yellow fluorescence under blue light). Microinjection of BACs GOLWFno17_n04 and ola1-008A15 partially rescued leucophore formation in the *lf* (F) and *lf-2* (G) mutants, respectively, as evidenced by fluorescence (triangles). (I) Microinjection of BAC GOLWFno599_n14 partially rescued the orange pigmentation of leucophores (triangles) in the *wl* mutant (stage 40).

Fig. 2. Expression patterns. (A–D, M, and N) *pax7a*, (E–H, Q, and R) *slc2a15b*, and (I–L, O, P, S, and T) *slc2a11b*. In wild-type fish, *pax7a* was expressed in the tectum, hindbrain, and dorsal neural tube (A), which was not altered in the *lf-2* mutant (B). Cells expressing *pax7a* were detected in dots laterally on the surface of the head and trunk (C), whereas they were lost in the *lf-2* mutant (D). The dotted expression signal of *slc2a15b* was not affected in the head region of the *lf* mutant (E and F), whereas it was lost in the trunk region (G and H). Compared with wild-type fish (I and K), *slc2a11b* expression was not affected in either the head (J) or trunk (K) region in the *wl* mutant. (M–P) In the *lf* mutant, *pax7a* (M and N) and *slc2a11b* (O and P) mRNA were normally expressed. (Q–T) In the *lf-2* mutant, whereas expression of both *slc2a15b* and *slc2a11b* was unaffected in the head region (Q and S), their dotted signals were lost in the trunk (R and T). (A, B, E, F, I, J, M, O, Q, and S) Stage 21. (C, D, G, H, K, L, N, P, R, and T) Stage 26. (A, B, E, F, I, J, M, O, Q, and S) Dorsal view, and (C, D, G, H, K, L, N, P, R, and T) lateral view; anterior to the *Left*. Triangles indicate signals on body surface.

(Fig. 2C). The expression of *pax7a* in the neural crest cells seems to precede the appearance of leucophores and xanthophores. Compared with the wild-type fish, the *lf-2* mutant lacked the dotted expression of *pax7a* only in the trunk (Fig. 2C and D).

The *slc2a15b* was expressed in a salt-and-pepper fashion dorsal to the midbrain and hindbrain at stage 21 (Fig. 2E). By stage 26, the *slc2a15b* signal became more prominent in the LBB and on the dorsal surface of the trunk (Fig. 2G and Fig. S5A and B). In the *lf* mutant, the *slc2a15b* signal on the body surface became faint at stage 26 (Fig. 2G and H) and was undetectable by stage 35 (Fig. S5C and D). Thus, *slc2a15b* expression in *lf* is initiated normally (Fig. 2E and F) but not maintained, suggesting that *slc2a15b* is required for the maintenance of its own expression. solute carrier (SLC) family members are suggested to be involved in the uptake of pigmentary materials into pigment cell precursors, which implies that the loss of function of *slc2a15b* may cause the failure of differentiation but not the absence of precursor cells. To test this possibility, we examined the expression of *GTP cyclohydrolase 1* (*gch1*), a marker of leucophore and xanthophore precursors in medaka (25), in the *lf* mutant embryos. The expression pattern of *gch1* in *lf* was identical to that in wild-type fish (Fig. S5E and F), indicating that the *lf* mutant embryo has leucophore and xanthophore precursors, but they are not pigmented.

The expression pattern of *slc2a11b* was similar to that of *slc2a15b* (Fig. 2I–L). On the trunk surface, *slc2a11b* was expressed earlier than *slc2a15b*. There was no remarkable difference in the expression pattern of *slc2a11b* between the *wl* mutant and wild-type embryos. Consistent with our speculation that *slc2a11b* functions similarly to *slc2a15b*, the *wl* mutant embryos had leucophore and xanthophore precursors defective for orange or yellow pigmentation.

The defects of the three mutants, restricted to xanthophores and leucophores, suggest that expression of the causal genes *lf/slcl2a15b*, *lf-2/pax7a*, and *wl/slcl2a11b* may be exclusive to leucophores and xanthophores. To test this possibility, we established an EGFP transgenic medaka using the GOLWFno17_n04-*slc2a15b*-GFP fosmid (Fig. S6). The EGFP signal was detected only in leucophores and xanthophores in the transgenic medaka (Fig. S6D–G). We further examined the expression patterns of *lf-2/pax7a* and *wl/slcl2a11b* in the transgenic medaka and found that the expression of *lf-2/pax7a* and *wl/slcl2a11b* overlaps with the EGFP signals. The results indicate that all three causal genes are exclusively expressed in leucophores and xanthophores (Fig. S6H–O).

To elucidate the genetic relationship of *lf/slcl2a15b*, *lf-2/pax7a*, and *wl/slcl2a11b*, we examined the expression patterns of the three genes in the *lf* and *lf-2* mutants by WISH. In *lf* mutants, the expression of *pax7a* and *slc2a11b* was indistinguishable from that of wild-type fish at stages 21–26 (Fig. 2M–P). This suggests that the *lf/slcl2a15b* function is not epistatic to *pax7a*, and that, despite the presumed similarity of *slc2a11b* and *slc2a15b*, the expression of *wl/slcl2a11b* but not *lf/slcl2a15b* is independent of *lf/slcl2a15b*.

In *lf-2* mutants, both *lf/slcl2a15b* and *wl/slcl2a11b* expression were normal in a salt-and-pepper fashion on the surface of the head at stage 21 (Fig. 2Q and S). Interestingly, however, at stage 26 and later, their expression was severely reduced in the head and completely lost in the trunk of *lf-2* (Fig. 2R and T). The results indicate that *lf-2/pax7a* is dispensable for the initial expression of *lf/slcl2a15b* and *wl/slcl2a11b* in the head but not on the trunk surface. The expression of *lf-2/pax7a* itself was also lost at later stages in the trunk. The results imply that *pax7a* is required for leucophore and xanthophore development but not for the specification of LBB.

Fig. 3. Phylogenetic tree of amino acid sequence of SLC2A class II. The phylogenetic tree was constructed using the neighbor-joining method and displayed using MEGA5. Numbers indicate the percentage of replicate trees in which the associated clade clustered together in the bootstrap test (1,000 replicates). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Reptilia were excluded from this analysis due to short amino acid sequences with no information on expressed sequence tags (ESTs) of SLC2A genes.

The Presence of *slc2a15b* and *slc2a11b* Genes Is Coupled with the Presence of Xanthophores in Vertebrates. Both the causal genes of *lf/slca15b* and *wl/slca11b* are SLC2 genes that are members of the major facilitator superfamily of membrane transporters. SLC2 proteins appear to transport hexoses, polyols, or other small organic molecules, but the substrates for SLC2a15b and SLC2a11b remain unknown. To gain insight into their substrates, we performed a phylogenetic analysis. Because both *slc2a15b* and *slc2a11b* are categorized as class II sugar transport facilitators (26, 27), we constructed the phylogenetic tree using the ascidian and vertebrate orthologs of SLC2A5, SLC2A7, SLC2A9, SLC2A11, and SLC2A15 (Fig. 3). The resultant tree has six clades, SLC2A5/7, SLC2A9, SLC2A11A, SLC2A11B, SLC2A15, and ascidian SLC2As. Ascidiens have only SLC2A5/7-like genes. Lampreys have one SLC2A9-like and two SLC2A11-like genes.

It is noteworthy that SLC2A15 is similar to SLC2A9. SLC2A9 is a urate transporter (28, 29) and is associated with serum uric acid levels (30–35) and gout (32) in humans. Uric acid is a major component of white particles in leucophores, so there is a possibility that the *lf/slca15b* functions as a urate transporter. This hypothesis explains why leucophore differentiation does not occur in the *lf* phenotype, but does not explain why xanthophore differentiation is severely delayed. Thus, although the substrates of SLC2A15 are unclear, it is possible that SLC2A15 transports not

only urate but also some other substrate required for xanthophore differentiation.

Surprisingly, SLC2A11B was placed in a different clade than SLC2A11A. Paralogs originating through fish-specific genome duplication (FSGD) are usually placed in the same clade, but SLC2A11A and SLC2A11B were not. This means that *slc2a11a* and *slc2a11b* originated before FSGD and may merit revised subfamily designations in future revisions of the overall nomenclature of SLC2 family members.

The Zebrafish Mutation Project revealed that the zebrafish *slc2a11b* mutant has a differentiation defect in its xanthophores (www.sanger.ac.uk/cgi-bin/Projects/D_rerio/zmp/gene.pl?id=ENSDARG00000093395). Another study in zebrafish demonstrated that *slc2a15b* expression was faint (36), but *slc2a15a* was expressed in a salt-and-pepper pattern during neural crest-developing stages in zebrafish (37). Because zebrafish do not have leucophores, *slc2a15b*-expressing cells presumably correspond to xanthophore precursors in zebrafish. Our findings suggest that *slc2a11b* and *slc2a15* have important roles in xanthophore differentiation in teleosts.

In addition to phylogenetic analysis, our synteny analysis showed that mammals had lost SLC2A15 and SLC2A11B orthologs, although both were conserved in other jawed vertebrates (Fig. 3 and Fig. S7). To date, three other class SLC genes, SLC7A11, SLC24A5, and SLC45A2, have been reported to be involved in melanization (38–40). In contrast to SLC2A15 and SLC2A11B, the above-mentioned genes are conserved among jawed vertebrates. Namely, the loss of both orthologs in mammals is consistent with the loss of xanthophores. Our results suggest that both SLC2A15 and SLC2A11B are necessary for xanthophore differentiation. Supporting this finding, in the sea lamprey (*Petromyzon marinus*), which has two SLC2A11 orthologs but no SLC2A15, only melanophores and iridophores have been reported (41, 42). Assuming that birds have only melanocytes, the existence of both orthologs in birds seems contradictory to our hypothesis. This discrepancy may be resolved by the fact that birds use pteridines as a yellow pigment and have xanthophore-like cells in the iris (43, 44).

To confirm this possibility, we performed cloning and WISH of chicken SLC2A11B and SLC2A15, and found that SLC2A11B was expressed in the iris and feather bud (Fig. S8). A previous study showed that carotenoids and pteridines were used as pigments not only in the iris but also in feathers (45). The expression patterns of SLC2A11B support our idea that SLC2A11B plays a conserved role in xanthophore differentiation. Conversely, SLC2A15 expression was not detected in those tissues. The findings imply that SLC2A11B, but not SLC2A15, is necessary for yellow

Fig. 4. Model for pigment cell development. Chromatoblast cells were generated from neural crest cells through fate restriction. Leucophore and xanthophore development was driven by *pax7a*. Both leucophore and xanthophore precursor cells became positive for *slc2a15b* and *slc2a11b*. Yellow pigmentation was promoted by both *slc2a15b* and *slc2a11b*, and *slc2a15b* promoted leucophore differentiation.

pigmentation in birds, and that *SLC2A11B* has a conserved role for xanthophore differentiation in vertebrates.

Previous studies showed that ascidians have orange pigment cells deriving from rudimentary neural crest cells (46–48). Although not confirmed by expression analysis with xanthophore markers, the orange pigment cells probably correspond to vertebrate xanthophores because *Ciona* has been shown to be equipped with orthologous genes for the pteridine synthetic enzymes of vertebrate xanthophores (49). Our phylogenetic analysis showed that ascidians had neither *SLC2A11B* nor *SLC2A15* orthologs. This suggests that in ascidians, both *SLC2A11B* and *SLC2A15* are dispensable for xanthophore differentiation or that the two ascidian *SLC2A* genes transport the same substrates as *SLC2A11B* and *SLC2A15*. To further reveal the conserved role of *SLC* genes in vertebrates and invertebrates, it will be necessary to investigate the substrate specificity of *SLC2A* families and to compare the loss-of-function phenotypes of coloration in their pigment cells.

Our results shed light on previously unidentified functions of *SLC* genes and suggest that the duplication of *SLC* genes in the vertebrate genome is critical to the production of six kinds of pigment cells. FSGD is likely related to the presence of the two extra chromatophores (leucophores and cyanophores) in fish. A study of *SLC* gene expression in cyanophores would also be of merit.

Leucophores Are Similar to Xanthophores in Their Specification and Differentiation Processes. The positional cloning of *lf-2* demonstrates that *pax7a* is required for the formation of leucophores as well as xanthophores (Fig. 4). We showed that *lf-2/pax7a* encodes an upstream transcription factor necessary for *lf/slsc2a15b* and *wl/slsc2a11b* expression in the leucophores and xanthophores of the body surface and that *wl/slsc2a11b* has a role in the xanthophore differentiation of teleosts. This demonstrates that leucophores are similar to xanthophores rather than to iridophores in their specification and differentiation processes. It is consistent with a previous morphological observation that leucophores have pigment granules similar to the pterinosomes of xanthophores rather than to the reflecting platelets of iridophores (7–9, 50, 51).

Materials and Methods

Fish Strains and Rearing Conditions. The medaka were reared at 26.0 °C on a 14-h light/10-h dark cycle. The *lf*, *lf-2*, and *wl* mutants have been described previously (10, 14, 15). T5 medaka, which are quintuple mutants for *lf*, *wl*, *colorless melanophore*, *guanine-less*, and *l^p*, were used to map the *lf* and *wl* loci (52). Because all mutants were derived from a southern Japanese population, the inbred strain HNI-II, derived from a northern Japanese population, was used as a reference to generate the mapping panels (53–56). All medaka strains were obtained from the National BioResource Project (NBRP) Medaka (www.shigen.nig.ac.jp/medaka/).

Mapping. F₁ fish from the mapping cross were intercrossed to generate F₂ fish. Bulk segregation analyses were processed as previously described (22). The mutant loci were mapped by scoring for recombination with PCR-length polymorphism markers as described previously (57). The marker sets used for mapping are shown in Table S1.

Phenotypic Rescue. One-cell stage embryos from mutant crosses were injected with 50 ng/μL of BAC or fosmids. All eggs were incubated at 28 °C until hatching.

BAC and Fosmid Modification. BAC and fosmid clones were modified as described previously (58). The primers used in the construction of gene disruptions are shown in Table S1.

cDNA Analysis. RNeasy (Qiagen) and ReverTra Ace (Toyobo) were used to synthesize cDNA from embryos. The primers used for RT-PCR are shown in Table S1. Both wild-type and mutant cDNA were electrophoresed using 1.5% agarose gels in tris-acetate buffer.

Mutation Identification. The cDNA were treated with ExoSAP-IT (Affymetrix) and sequenced directly. The sequencing of mutants was performed on PCR fragments amplified from genomic DNA prepared from single embryos. The sequences were analyzed with BLAT using the University of California, Santa Cruz Genome Bioinformatics website (<http://genome.ucsc.edu>).

Whole-Mount in Situ Hybridization of Medaka. The following EST clones containing full-length cDNA were obtained from NBRP Medaka: *oleb56b02* (*slc2a15b*), *oleb63k15* (*slc2a11b*), and *oleb22l23* (*pax7a*). The partial sequences of the clones were cloned into TOPO-II (Invitrogen) via PCR. The primers used for PCR are shown in Table S1. Digoxigenin (DIG)-labeled RNA probes were generated using the DIG RNA labeling kit (Roche). WISH was performed as described previously (59).

Syntenic Analysis. Syntenic analyses were performed using Genomicus (www.genomicus.biologie.ens.fr/genomicus-72.01/cgi-bin/search.pl) (60).

Phylogenetic Analysis. Phylogenetic analyses were conducted by the neighbor-joining method with 1,000 bootstrap replicates using MEGA version 5.2.2 (61–64). Amino acid sequence data were obtained from GenBank and Ensembl.

Whole-Mount in Situ Chicken Hybridization. Fertilized chicken eggs were purchased from the Yamagishi farm and incubated at 38.5 °C. Embryos were staged according to Hamburger and Hamilton (65). For the isolation of chicken *SLC2a* cDNA fragments, PCR was performed using stage (St.) 36 eye cDNA and the primers are shown in Table S1. For chicken *SLC2A15* probes, the 5' region of the coding domain, upstream of the XcmI site, was used. RNA probes were labeled with digoxigenin. WISH was performed following Yamamoto-Shiraishi and Kuroiwa (66). Fixed eyes were treated with 6% (wt/vol) H₂O₂ in methanol overnight under a fluorescent lamp, and BM purple (Roche) was used for the coloring reaction. After WISH, samples were sectioned with a surgical knife and the cut surfaces were photographed.

Relative Gene Expression Analysis by Real-Time Quantitative PCR. RNA was isolated from the whole body of St. 18 chicken embryos (excluding the head), St. 30 eyes, and St. 36 eyes and back skin. Reverse transcription was performed with oligo dT, and the primers shown in Table S1 were used in the real-time quantitative PCR (qPCR) analyses for absolute quantification of the *SLC11b* and a housekeeping gene in reverse transcription, *GAPDH*. Statistical analyses were performed as described (67), and a one-tailed t test was used.

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