Fate plasticity and reprogramming in genetically distinct populations of *Danio* leucophores

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Understanding genetic and cellular bases of adult form remains a fundamental goal at the intersection of developmental and evolutionary biology. The skin pigment cells of vertebrates, derived from embryonic neural crest, are a useful system for elucidating mechanisms of fate specification, pattern formation, and how particular phenotypes impact organismal behavior and ecology. In a survey of *Danio* fishes, including the zebrafish *Danio rerio*, we identified two populations of white pigment cells—leucophores—one of which arises by transdifferentiation of adult melanophores and another of which develops from a yellow–orange xanthophore or xanthophore-like progenitor. Single-cell transcriptomic, mutational, chemical, and ultrastructural analyses of zebrafish leucophores revealed cell-type–specific chemical compositions, organelle configurations, and genetic requirements. At the organismal level, we identified distinct physiological responses of leucophores during environmental background matching, and we showed that leucophore complement influences behavior. Together, our studies reveal independently arisen pigment cell types and mechanisms of fate acquisition in zebrafish and illustrate how concerted analyses across hierarchical levels can provide insights into phenotypes and their evolution.

pigmentation | neural crest | transdifferentiation | evolution | zebrafish

Vertebrate pigmentation contributes to ecological interactions and is often a target of selection during adaptation and speciation (1–3). Teleost fishes are among the most phenotypically diverse of vertebrate taxa, and their spectacular array of pigment phenotypes support a variety of behaviors, from attracting mates to predator avoidance, social aggregation, and aggressive interactions. In contrast to birds and mammals that have only a single pigment cell type, the melanocyte, teleosts develop multiple pigment cell classes, including black melanophores, yellow–orange xanthophores, and iridescent iridophores. Pigment patterns reflect the relative abundance and spatial locations of these chromatophores. Decades of work have contributed to understanding developmental and genetic bases of black, yellow, and iridescent pigmentation and the cellular interactions underlying pattern formation (4).

Teleosts also develop several additional classes of chromatophores, including white or yellow–white "leucophores" (5). Our knowledge of fate specification, genetic requirements, physical and chemical properties, and behavioral roles of these cells remains fragmentary. Nevertheless, the presumptive origin of all these chromatophores in a common precursor cell population—the embryonic neural crest—and the distinctiveness of these chromatophores in fish that display them suggest an opportunity to dissect phenotypic diversification ranging from genetic mechanisms and cell-fate plasticity to organismal interactions.

Zebrafish *Danio rerio* has emerged as a preeminent laboratory model for studying neural crest development and pigment pattern formation, and comparisons of zebrafish and other *Danio* species have provided insights into the evolution of pattern-forming mechanisms (4, 6–8). Here we investigate physical properties, genetic mechanisms, and cell lineage of leucophores in zebrafish and its relatives. We show that *Danio* fishes have two distinct classes of leucophores with independent developmental origins and cellular architectures. We further identify lineage-specific requirements and pathways modulated in these cells by genetic, chemical, and single-cell transcriptomic analyses, and we show that leucophores potentially impact behaviors at the whole-organism level. Our findings suggest that white pigmentation in *Danio* has resulted from phenotypic convergence in neural crest sublineages, revealing an unexpected mode of pigment cell evolution.

Results

Dual Classes and Origins of Leucophores in *Danio*. We evaluated leucophore distribution across nine species representing multiple subclades within the genus. Leucophores containing orange and white pigment were evident in anal fins of seven species, including zebrafish, and less prominently in dorsal fins of two species (Fig. 1*A*

Significance

A foundational question in biology is how phenotypically similar traits arise. Here we identify two distinct white pigment cell populations, leucophores, that arise from independent progenitors in zebrafish and its relatives. Remarkably, one of these leucophore populations develops from previously differentiated, black melanophores. These different leucophores exhibited distinct pigment chemistries, cytological features, gene expression profiles, and genetic requirements, and whole-animal experiments implicated these cells in behavioral interactions. Our several approaches provide insights into pigment cell complements and origins in zebrafish and contribute to our understanding of form and function in the spectacular pigment patterns of teleost fishes.

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The authors declare no conflict of interest.

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Data deposition: The scRNA-Seq data reported in this paper have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database, https://www.ncbi.nlm.nih.gov/geo/ (accession no. GSE130526).

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and *B*, *Left*, and *SI Appendix*, Fig. S1). Similar to xanthophores, these cells contained pteridines and carotenoids (*SI Appendix*, Fig. S2 *A*–*C*). During development, pronounced yellow–orange pigmentation was evident first, with a white halo of pigmentation becoming gradually more distinct over several days (*SI Appendix*, Fig. S2 *D* and *E*). These findings are consistent with some shared characteristics and potentially lineage origins of leucophores and xanthophores, as inferred from visual observations and genetic analyses of medaka fish (9–11). Given the temporal order of pigment deposition in *Danio*, we refer to these cells as "xantholeucophores."

In addition to xantholeucophores, a population of leucophores has been noted in zebrafish as "white cells" at the distal edges of dorsal and caudal fins (12). Across species, eight of nine *Danio* exhibited leucophores lacking orange coloration at the margin of the dorsal fin, in the dorsal and ventral lobes of the caudal fin, and, in two species, within the anal fin (Fig. 1 *A* and *B* and *SI Appendix*, Fig. S1). These cells had reflective but opaque white material that was variably contracted toward the cell body or dispersed in cellular processes (Fig. 1*C*) and lacked pteridines and carotenoids (*SI Appendix*, Fig. S2 *A* and *B*). Because the cells sometimes contained melanin (Fig. 1 *B*, *Right*, and *SI Appendix*, Fig. S1 *B* and *C*) we refer to them as "melanoleucophores."

In zebrafish, melanoleucophores and xantholeucophores expressed distinct pigment cell reporters (Fig. 1*D* and *SI Appendix*, Fig. S3). Melanoleucophores expressed a *tyrosinase related protein 1b* (*tyrp1b*) transgene that marks melanophores, whereas xantholeucophores expressed an *aldehyde oxidase 5* (*aox5*) transgene that marks xanthophores (13). Both melanoleucophores and xantholeucophores expressed a reporter for *purine nucleoside phosphorylase 4a* (*pnp4a*), which is expressed strongly in iridophores and at lower levels in other pigment cells (14, 15)

Whereas xantholeucophores somewhat resembled leucophores of medaka (9), melanoleucophores appeared to be a cell type of unknown origin. We hypothesized that melanoleucophores and melanophores share a lineage, given shared expression of the *tyrp1b* transgene and the sporadic melanin found in melanoleucophores. To test this hypothesis, we labeled cells mosaically (16) using a *mitfa:tdTomato* transgene expressed by pigment cell precursors and melanophores (17). Each of 64 labeled clones containing melanoleucophores also included melanophores, indicating that the cells share a common progenitor or that one cell type transdifferentiates from the other (Fig. 1*E*). To distinguish between these possibilities, we labeled cells with photoconvertible Eos (*pnp4a:nlsEos*) and followed individual cells as they differentiated (13). These analyses demonstrated that melanoleucophores arise directly from melanophores, accumulating white material and losing melanin over several days (Fig. 1*F*). Consistent with this finding, >95% of newly differentiating melanoleucophores, just beginning to acquire white pigment, contained melanin (143 of 150 cells in 10 larvae). Together, these observations indicate that *Danio* develop leucophores of two varieties, with distinct morphologies and origins.

Distinct Requirements for Specification and Morphogenesis. The origins of xantholeucophores from xanthophore-like cells, and melanoleucophores from melanophores, suggested that their development would involve genes required for these respective lineages. Colony-stimulating

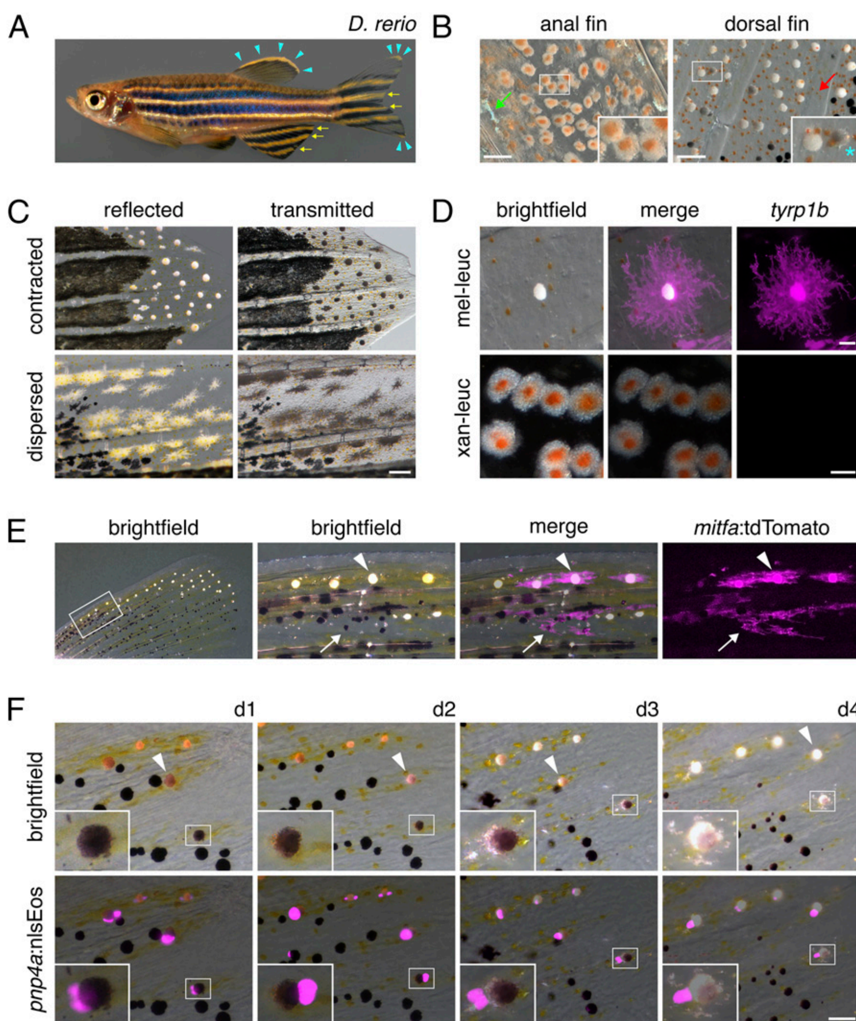

Fig. 1. Leucophore appearance and origins in zebrafish. (*A*) Pigment cells containing white pigment. Arrows, leucophores containing yellow–orange pigment. Arrowheads, white cells lacking orange pigment. (*B*, *Left*) Leucophores with white and orange pigment. (*B*, *Right*) Cells containing white pigment. (*B*, *Insets*) Higher magnification, including gray melanin-containing cell (asterisk). Green arrow, iridescent iridophore. Red arrow, orange pigment of xanthophore. Fish were treated with epinephrine to contract pigment granules. (*C*) Leucophores at tips of caudal fins under reflected and transmitted illumination. (*D*) Melanoleucophores but not xantholeucophores expressed *tyrp1b*:palm-mCherry. (*E*) Labeled clones contained melanoleucophores (arrowhead) and melanophores (arrow). (*F*) Acquisition of white pigment by a melanophore over 4 d (boxed cell; *Inset*). Arrowhead, a second cell that transits distally. (Scale bars: *B*, 50 μm; *C*, 100 μm; *D*, *Upper*, 20 μm and *Lower*, 10 μm; *E* and *F*, 40 μm.)

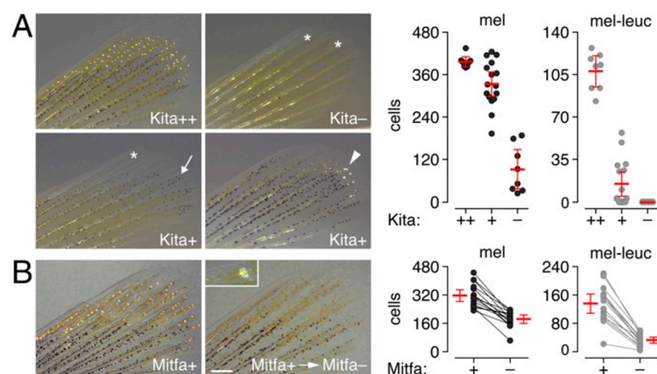

Fig. 2. Distinct requirements for Kita and Mitfa. (*A*) $kita^{j1e99}$ at permissive temperature (Kita++) had wild-type complements of melanophores and melanoleucophores, but at restrictive temperature (Kita–) were deficient for melanophores and lacked all melanoleucophores (asterisks). At intermediate temperature (Kita+), melanophores were abundant but locations normally harboring melanoleucophores were devoid of these cells (asterisk, *Left*), populated by melanophores (arrow, *Left*), or had fewer melanoleucophores compared with permissive temperature (arrowhead, *Right*). Plots, cell numbers for individual fish ($F_{2,29}$ = 65.2 and 119.2, respectively, $P < 0.0001$; bars, means ± 95% CI). (*B*) $mitfa^{vc7}$ fish at permissive temperature (Mitfa+) developed melanophores and melanoleucophores, but many of each died on transition to restrictive temperature (Mitfa–). Panels show same fish before and 2 d after transition. (*B*, *Inset*) White pigment containing cellular debris indicative of cell death [e.g., ref. 50]. Plots, cell numbers for individual fish before and after transition (paired t_{15}= −8.6 and −10.9, respectively, $P < 0.0001$; bars, means ± 95% CI). Controls maintained at permissive temperature exhibited 1% increases in numbers of each cell type over this period (paired t_{15} = 3.2, $P < 0.05$ and paired t_{15} = 2.2, P = 0.06, respectively). (Scale bar: 250 μm).

factor-1 receptor-a (Csf1ra) and Kit-a (Kita) promote the development of xanthophores and melanophores, respectively (18, 19). Consistent with our expectation, *csf1ra* mutants lacked xantholeucophores but retained melanoleucophores, and *kita* mutants lacked melanoleucophores but retained xantholeucophores (*SI Appendix*, Figs. S4 *A*, *B*, and *D*). Thus, different types of leucophores have different genetic requirements. Leukocyte tyrosine kinase (Ltk) is required by iridophores but not xanthophores or melanophores (20), and *ltk* mutants retained both leucophore classes (*SI Appendix*, Fig. S4*A*).

We further asked if melanoleucophores have distinct requirements for pathways essential to melanophore development. Because different levels of Kit signaling can promote different cellular outcomes (21, 22), we speculated that melanoleucophores and melanophores might have different requirements for Kita activity. We therefore compared phenotypes of the temperature-sensitive allele $kita^{j1e99}$ at permissive, restrictive, and intermediate temperatures (23). At permissive temperature, normal complements of both cell types were present; at restrictive temperature, both cell types were missing (Fig. 2*A*). At intermediate temperature, 91% of melanophores but only 14% of melanoleucophores developed (Fig. 2*A*). These findings, and the presence of melanophores in regions normally occupied by melanoleucophores, support a model in which melanoleucophore differentiation requires more Kita signaling than melanophore development or maintenance.

The Melanocyte-inducing transcription factor (Mitf) is necessary for fate specification, melanogenesis, and survival of melanocytes in mammals, and its homolog, Mitfa, has conserved roles in melanophores (24, 25). Mutants for *mitfa* lacked melanophores and melanoleucophores (*SI Appendix*, Fig. S4*C*). To test if differentiated melanoleucophores themselves require Mitfa or if their deficit results only from a lack of their melanophore progenitors, we employed the temperature-sensitive allele $mitfa^{vc7}$ (26, 27). We reared fish at permissive temperature to allow melanophore and melanoleucophore differentiation and then shifted the fish to restrictive temperature to curtail Mitfa availability. This condition resulted in the loss of 42% of melanophores and 76% of melanoleucophores within 2 d (Fig. 2*B*). Thus, Mitfa is necessary for survival of differentiated melanoleucophores in addition to development of their melanophore progenitors.

Distinct Organelle Ultrastructures and Chemistry of Melanoleucophore and Xantholeucophore White Coloration. To address physical and chemical bases for white coloration, we examined spectral properties of leucophores. Both melanoleucophores and xantholeucophores reflected across a wide spectrum (*SI Appendix*, Fig. S5*A*). Moreover, both cell types lacked the angular-dependent change of hues—iridescence—of iridophores, which results from stacked arrangements of crystalline guanine reflecting platelets within membrane-bound organelles (5, 28, 29) (Fig. 3*A*). Accordingly, we predicted and then observed by transmission electron microscopy (TEM) that both cell types lack ordered reflecting platelets. Melanoleucophores exhibited variably shaped organelles distinct from reflecting platelets and melanosomes (Fig. 3 *B* and *C*). Xantholeucophores were similar to leucophores of other species and xanthophores of zebrafish (*SI Appendix*, *Materials and Methods*, *TEM*) in harboring round organelles—carotenoid vesicles containing yellow–orange pigment—as well as irregular organelles indistinguishable from pterinosomes, which contain yellow or colorless pteridines (Fig. 3*D* and *SI Appendix*, Fig. S5*B*).

We used mass spectrometry to assay purine contents of fin tissue containing different pigment cell classes. Guanine but not other purines was detected in excess in tissues specifically containing melanoleucophores (Fig. 3*E* and *SI Appendix*, Fig. S5 *C* and *D*). Raman spectroscopy of individual melanoleucophores revealed crystalline β-guanine, the metastable phase of guanine, typical of iridophores and other biological systems in which crystallization is

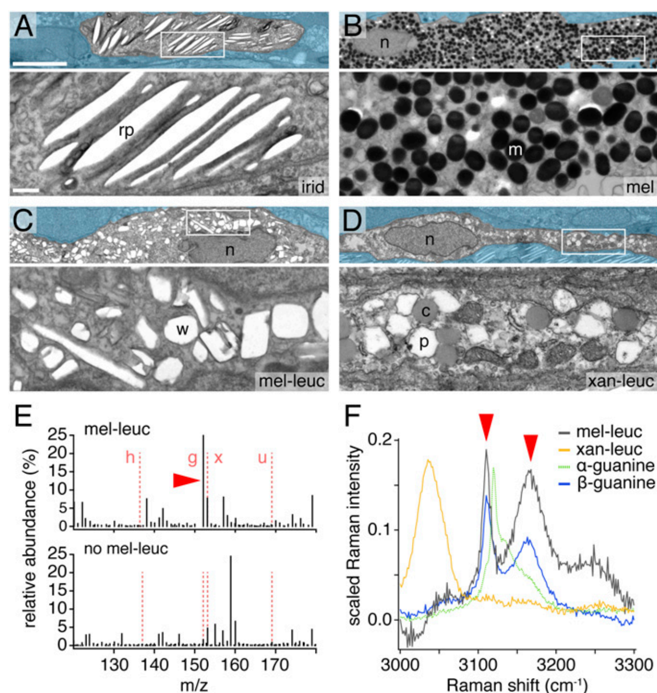

Fig. 3. Ultrastructural and chemical characteristics of melanoleucophores and xantholeucophores. (*A*–*D*) Fin iridophores (*A*) exhibited reflecting platelets (rp) and melanophores had typical melanosomes (m). Melanoleucophores (*C*) contained irregularly shaped and arranged organelles (w) presumptively containing white material, whereas xantholeucophores (*D*) contained presumptive pterinosomes (p) and carotenoid vesicles (c), without other organelles likely to harbor white pigment. (*Upper*) Low magnification with adjacent cells masked. (*Lower*) Boxed regions. n, nucleus. (*E*) Mass spectrometry of fin tissue containing melanoleucophores (*Upper*) revealed more abundant guanine (arrowhead) compared with fin tissue without melanoleucophores (*Lower*; h, hypoxanthine; g, guanine; x, xanthine; u, uric acid). (*F*) Representative Raman spectra of melanoleucophores (gray; n = 19 cells) and xantholeucophores (orange; n = 14) compared with α-guanine and β-guanine. High energy peak pattern (arrowheads) indicates β-guanine is present in melanoleucophores but is not detectable in xantholeucophores. (Scale bars: *Upper* 5 μm, *Lower* 500 nm).

regulated (30, 31) (Fig. 3*F*). Tissue harboring xantholeucophores had somewhat increased guanine content (*SI Appendix*, Fig. S5*E*) although crystalline forms of guanine were not detectable (Fig. 3*F*). This suggests that other factors contribute to white pigmentation in these cells. Together, these analyses indicate distinct ultrastructural and chemical bases for white pigmentation of melanoleucophores and xantholeucophores.

Melanoleucophores and Xantholeucophores Differentially Modulate Pigment Synthesis Pathways. We hypothesized that differentiation of melanoleucophores from melanophores would involve a switch from melanin to purine synthesis and therefore analyzed transcriptomes of individual pigment cells during melanoleucophore development. Dimensionality reduction and clustering followed by empirical validation of cluster assignments identified melanophores and melanoleucophores, as well as xanthophores and xantholeucophores (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S6). Supporting a developmental switch in pigmentation pathways, melanoleucophores exhibited higher expression of genes for de novo purine synthesis, but lower expression of genes for melanin synthesis, compared with melanophores (Fig. 4 *C* and *D*). Pseudotemporal ordering of cells (32) along a differentiation trajectory (Fig. 4 *B*, *Right*) likewise revealed an inverse relationship in gene expression between purine and melanin synthesis pathways (Fig. 4 *E* and *F*). This sequence raised the possibility that acquisition of white pigment might depend on prior melanization; that mutants with unmelanized melanophores develop melanoleucophores allowed us to reject this model (*SI Appendix*, Fig. S7*A*).

Compared with melanoleucophores and melanophores, xantholeucophores and xanthophores occupied more distinct locations in transcriptomic space (Fig. 5 *B*, *Left*) (33). A differentiation trajectory likewise revealed few cells of intermediate states (*SI Appendix*, Fig. S7*B*), suggesting that differentiating xantholeucophores were not well represented and that xanthophores (from dorsal fin) were an inadequate proxy for early states of xantholeucophore differentiation. Genes involved in purine synthesis, carotenoid processing, and pteridine synthesis were all expressed at higher levels in xantholeucophores than in nonpigment cells (*SI Appendix*, Fig. S7 *C* and *D*).

Physiological Responses and Behavioral Implications of *Danio* Leucophores. We sought to understand how leucophores might impact phenotype at the organismal level. During ambient background color adaptation, zebrafish and other species physiologically contract or disperse pigment-containing organelles within pigment cells. Typically, melanosomes of melanophores are contracted on a light background, yielding a lighter fish, but are dispersed on a dark background, yielding a darker fish. Leucophores of medaka and *Fundulus* have the opposite response of melanophores (34–36). Given the distinct properties of melanoleucophores and xantholeucophores in *Danio*, we predicted that they would have different physiological responses as well. Under standard conditions, guanine deposits of *Danio* melanoleucophores were often contracted whereas pigment deposits of xantholeucophores were often expanded (*SI Appendix*, Fig. S1 *B*, *Top*). Melanoleucophores responded, as did melanophores, by contracting pigment organelles centripetally in the light and dispersing them peripherally in the dark (Fig. 5 *A* and *B*). Xantholeucophores displayed the opposite response. Thus, melanoleucophores and xantholeucophores have opposing physiological responses during background adaptation.

Finally, we asked whether social behavior might be influenced by melanoleucophores, given their prominent position on the fins, which are held erect during aggressive and courtship interactions (37). To test if zebrafish can perceive and respond to tissue containing melanoleucophores, we presented fish with alternative shoals having intact melanoleucophores (sham-manipulated) or excised melanoleucophores (*SI Appendix*, Fig. S8 *A* and *B*). Overall, test fish spent significantly more time in proximity to shoals having intact melanoleucophores (Fig. 5*C*). The first approach of test fish to stimulus shoals also varied with melanoleucophore status (*SI Appendix*, Fig. S8*C*). These differences are consistent with a model in which fish attend to the presence of melanoleucophores and raise

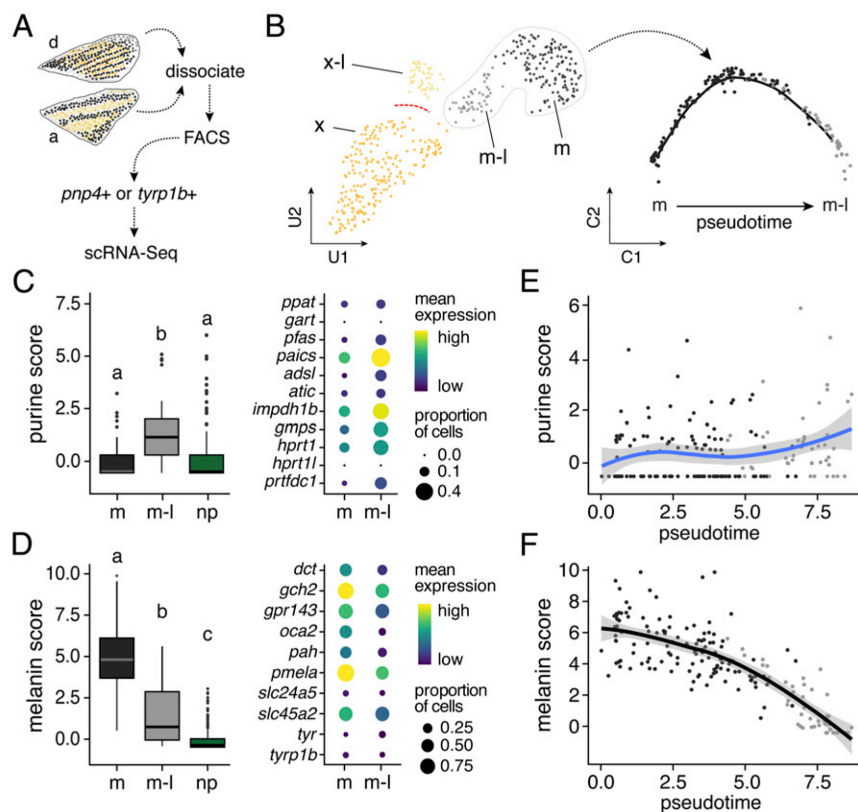

Fig. 4. Pigmentation pathway switch during melanoleucophore maturation. (*A*) Experimental design. d, dorsal fin. a, anal fin. (*B*) 2D UMAP representation (*SI Appendix*, *Materials and Methods*) of pigment cell clusters (*Left*; x, xanthophores; x-l, xantholeucophores; m, melanophores; m-l, melanoleucophores) and differentiation trajectory (*Right*) of melanophores to melanoleucophores. Red dotted line, cell-free space. (*C*) Aggregate score for de novo purine synthesis genes was greater in melanoleucophores than melanophores or nonpigment cells (np). Box plots, medians with interquartile ranges; different letters indicate differences significant in post hoc comparison ($P < 0.0001$; shared letters, $P = 0.2$). Bubble plots show individual gene expression. (*D*) Melanin synthesis gene expression was reduced in melanoleucophores compared with melanophores. (*E* and *F*) Purine and melanin synthesis gene expression scores for individual cells arranged in pseudotime (arbitrary scale) from melanophore (*Left*) to melanoleucophore (*Right*). Line and shaded region, smoothed average with 95% CI.

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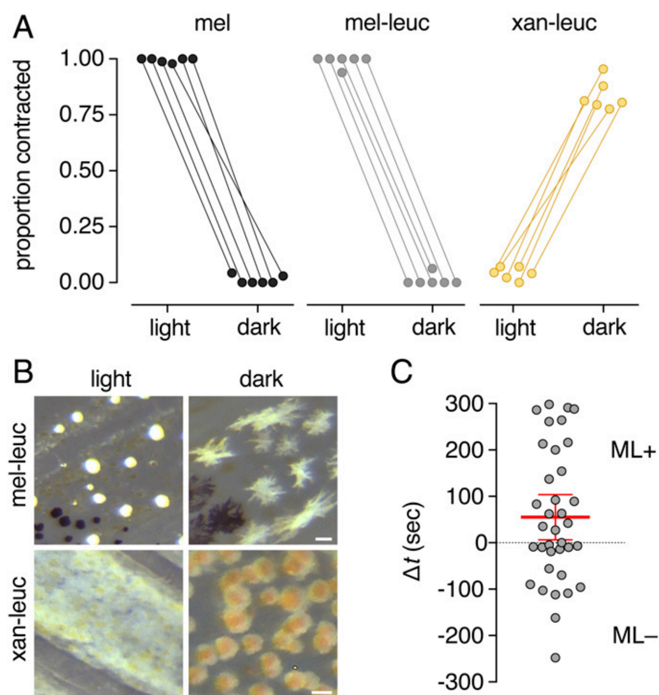

Fig. 5. Background adaptation and behavioral responses. (*A* and *B*) Melanophores and melanoleucophores exhibited the same background adaptation responses whereas xantholeucophores had opposite responses. Points connected by line indicate cells of a single fish ($n = 26$–133 cells, median = 62 cells). (*C*) Whole-fish behavioral response to stimulus fish with intact melanoleucophores (sham-manipulated, ML+) or excised melanoleucophores (ML–). If test fish cannot perceive a phenotypic difference between shoals, or do not respond to it, they should spend equal times with each stimulus type (difference in time spent, $\Delta t = t_{intact} - t_{ablated} = 0$). If test fish perceive and respond to a phenotypic difference, they should spend more time in association with one shoal than the other ($\Delta t \neq 0$). Longer times spent on average with ML+ over ML– shoals indicated a preference for fish retaining melanoleucophore-containing fin tissue (red bars, means ± 95% CI; $\Delta t = 55.0 \pm 48.7$ s; null hypothesis $\Delta t = 0$, two-tailed $t_{35} = 2.29$, $P = 0.03$). Times did not differ with sex of test fish, sex of stimulus fish, or their interaction (ANOVA, all $P \geq 0.18$). (Scale bars: 20 μm).

the possibility of these cells contributing to social interactions in the wild.

Discussion

Our observations that melanoleucophores transdifferentiate from melanophores while xantholeucophores arise from cells resembling xanthophores suggest that a *Danio* ancestor evolved two types of leucophores independently (*SI Appendix*, Fig. S9*A*). These findings, in conjunction with an iridophore requirement for white bars of the clownfish *Amphiprion* (38), suggest that each of the three major chromatophore lineages can contribute to a white pigmentary phenotype.

An unresolved question in the evolution of morphology is the extent to which similar but independently evolved phenotypes depend on the same (parallel) or different (convergent) mechanisms. In melanoleucophores, the deposition of guanine crystals suggests a co-option of purine synthesis and biomineralization mechanisms similar to those of iridophores. However, melanoleucophore crystals are displayed in organelles having morphologies and arrangements differing from those of iridescent and noniridescent (white) iridophores, revealing parallelism in pigmentary composition (guanine crystals) but convergence in cellular architectures to generate a white phenotype (*SI Appendix*, Fig. S9*A*). Melanoleucophores of *Danio* and leucophores of medaka also seem to have arrived independently at a white phenotype through convergence in cell lineage (melanophore vs. shared progenitor with xanthophores or erythrophore), pigmentary composition (guanine vs. uric acid), and ultrastructure (10, 11, 39).

By contrast, our analyses of *Danio* xantholeucophores, which might be expected to resemble medaka leucophores, failed to reveal substantial deposition of either guanine or uric acid, suggesting that these cells have converged on a means of generating a white phenotype that differs from both melanoleucophores and noniridescent iridophores. White material in xantholeucophores might be pteridines (39, 40), given their abundance in these cells. A definitive characterization, and determination of potential roles for small amounts of purines, will require additional analyses.

Beyond evolutionary considerations, this study provides insights into plasticity in cell fate. Over 60 y ago it was observed that amphibian xanthophores can transform into melanophores in the context of interspecies transplantation (41). Likewise, dedifferentiation followed by redifferentiation occurs during regeneration and can be induced experimentally (42–44). Whether cells convert from one differentiated state to another—i.e., transdifferentiate—during normal development has been less clear. Our demonstration that melanoleucophores develop directly from melanophores identifies a natural example of transdifferentiation, corroborating hints that cell states are not as definitive as once thought (45) and highlighting the potential of teleost chromatophores for understanding cell-state dynamics.

The mechanisms responsible for melanoleucophore and xantholeucophore fate specification are not yet known. Nevertheless, our findings do reveal some functions of known pigmentary genes during the development of these cells. The dependence of xantholeucophores and melanoleucophores on xanthogenic *csf1ra* and melanogenic *kita*, respectively, supports a model in which leucophore deployment is constrained by prior lineage requirements (*SI Appendix*, Fig. S9*A*). Our analysis also uncovered a role for *mitfa* in xantholeucophore development, and conditional genetic analyses indicate roles for *kita* and *mitfa* in melanoleucophores, independent of roles in melanophores. It remains for future studies to interrogate how these and other "classic" pigmentation genes integrate with pathways required for the white pigmentary phenotype—including up-regulation of de novo purine synthesis, down-regulation of melanin synthesis, and factors underlying melanin depletion. An intriguing possibility is that *mitfa* itself plays a role, analogous to functions in fate specification or phenotype switching in melanocytes, melanophores, and melanoma (46).

Finally, white pigmentation is thought to be ecologically relevant: white bars of clownfish contribute to species recognition (47), a yellow–white bar in the caudal fin of male Goodeinae fishes is linked to mating success (48), and white pigmentation in fins of the guppy *Poecilia reticulata* likely enhances honest signaling in sexual selection (49). In our study, melanoleucophores and xantholeucophores responded physiologically during background adaptation, suggesting consequences for the avoidance of predation. Moreover, individual fish preferred to associate with stimulus shoals having intact melanoleucophores and differentially approached these shoals depending on sex and melanoleucophore complement. These observations, the prominent position of melanoleucophores, and their likely visibility in habitats experienced by zebrafish (6) all raise the possibility that *Danio* leucophores contribute to social interactions in the wild. In this regard, differences in fin-specific complements of these cells among *Danio* species may be especially interesting.

Materials and Methods

Fish Rearing, Lineage Analysis, and Temperature-Shift Experiments. Zebrafish and other *Danio* species were housed at ∼28 °C, 14 h light:10 h dark and fed rotifers, *Artemia*, and flake food. Clonal labeling and Eos-fate mapping followed (13, 16). Fish were anesthetized in MS222 before imaging or fin clipping. Protocols were approved by Institutional Animal Care and Use Committees of the University of Virginia and University of Washington.

Physical and Chemical Analyses. Measurement of reflectance and transmittance used a PARISS hyperspectral imaging system mounted on a Nikon Eclipse 80i microscope. TEM used standard methods. Guanine content was analyzed on an Agilent HPLC with photodiode array detector and single quadrupole mass spectrometer after extracting purines in 1 M NaOH and also using a custom-built Raman microscope.

Single-Cell RNA-Sequencing and Analysis. Cells expressing *tyrp1b*:palm-mCherry or *pnp4a*:palm-mCherry were isolated by fluorescence-activated cell sorting, captured in a Chromium controller (10X Genomics), and sequenced on an Illumina NextSeq 500. Visualization used UMAP (33) with clusters validated using cells of specific phenotypes collected manually and tested by RT-PCR. Trajectory analysis used Monocle (v2.99.1). Gene sets for signature scores were manually curated from the literature and ZFIN: The Zebrafish Information Network.

Physiological and Behavioral Response Testing. Fish were tested for background adaptation in black or white beakers under constant light for 5 min. Behavioral assays were tested for the effects of sham manipulation or melanoleucophore excision as well as for the sex of both test and stimulus fish.

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