In situ differentiation of iridophore crystallotypes underlies zebrafish stripe patterning

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Skin color patterns are ubiquitous in nature, impact social behavior, predator avoidance, and protection from ultraviolet irradiation. A leading model system for vertebrate skin patterning is the zebrafish; its alternating blue stripes and yellow interstripes depend on light-reflecting cells called iridophores. It was suggested that the zebrafish's color pattern arises from a single type of iridophore migrating differentially to stripes and interstripes. However, here we find that iridophores do not migrate between stripes and interstripes but instead differentiate and proliferate in-place, based on their micro-environment. RNA-sequencing analysis further reveals that stripe and interstripe iridophores have different transcriptomic states, while cryogenic-scanning-electron-microscopy and micro-X-ray diffraction identify different crystal-array architectures, indicating that stripe and interstripe iridophores are different cell types. Based on these results, we present an alternative model of skin patterning in zebrafish in which distinct iridophore crystallotypes containing specialized, physiologically responsive, organelles arise in stripe and interstripe by in-situ differentiation.
Biological patterning is ubiquitous in nature, but mechanisms underlying its establishment and maintenance have been well-documented in only a few instances that are unlikely to represent the full spectrum of pattern-forming systems. Indeed, patterning can arise in response to graded positional information or by self-organization of interacting cells, and it can require alternative specification of cell types from a common progenitor or sorting out of cells that are heterogeneous already. Elucidating the mechanisms required to pattern cells in diverse tissues and organs is fundamental to understanding development and how phenotypes evolve.

The alternating dark (blue) and light (yellow) pigmented stripe pattern of adult zebrafish Danio rerio (Fig. 1a) is a useful model for dissecting patterning mechanisms. Cells within the dark stripes include black pigment-containing melanophores; cells in the light stripes (known as “interstripes”) include orange pigment-containing xanthophores; and both dark stripes and light interstripes contain specialized cells called iridophores. Iridophores are the major players for skin pattern establishment and reiteration in the zebrafish. In the dark stripes, by morphogenetic-to-epithelial transitions (MET), respectively.Signals from dense-to-loose and loose-to-dense are thought to affect platelet spacings

Iridophores undergo an EMT-like transformation and migrate out from the interstripe into the stripe. Instead, iridophores within stripes were more uniformly distributed, in keeping with a rapid and relatively uniform occupancy of prospective stripe regions (Supplementary Figs. 1b–e and Supplementary Movies S2–S5).

A key prediction of the above model, hereafter referred to as “morphogenetic respecification,” is that some interstripe iridophores undergo an EMT-like transformation and migrate out from the interstripe zone to adopt a new, loose morphology and arrangement. In testing this prediction through a variety of approaches, we found that individual iridophores did not migrate out from the interstripe into the stripe. Instead, iridophores assumed a particular morphology at the time of their differentiation according to the presence or absence of melanophores, and this morphology remained fixed thereafter. We also observed that interstripe and stripe iridophores exhibited distinct organizations of guanine-reflecting platelets (i.e., crystal types) conferring intrinsic differences in color, and that only stripe-localized iridophores could modulate reflecting platelet spacings physiologically (blue → yellow). Furthermore, interstripe and stripe iridophores had distinct transcriptomic states. Based on these results, we propose an alternative new model for stripe pattern formation in the adult zebrafish, in which iridophore precursor cells undergo “differentiation in situ” into distinct iridophore types (i.e., crystallotytypes; Fig. 1c, right panel). This process would depend on factors in the iridophore environment that impact the specification and subcellular organization of specialized organelles within iridophore precursors.

Results

Time-lapse imaging reveals iridophores do not migrate out from the interstripe. Stripe pattern establishment and reiteration in the zebrafish has been proposed to occur through morphogenetic respecification, in which iridophores differentiate to form a primary interstripe and then these cells or their progeny migrate out to contribute to stripes, as well as secondary interstripes and stripes (Fig. 1c, left panel). Individual cells would switch morphologies as appropriate to pattern context, undergoing morphogenetic respecification via processes resembling EMT or MET.

To test this model, we examined iridophore behaviors by time-lapse imaging of membrane-targeted mCherry (mem-Cherry) driven by regulatory elements of pnp4a. If morphogenetic respecification accounts for variation in iridophore morphology and patterning, then events resembling EMT or MET should be observable. That is, densely packed iridophores of the completed interstripe should delaminate to populate the stripe, whereas loosely arranged iridophores of completed stripes should aggregate to initiate new interstripes. In over 300 h of recordings, we observed no instances in which interstripe iridophores—having the dense morphology—delaminated from their neighbors and assumed the loose morphology (Fig. 1d; 14,475 total cells, including 1637 cells located at interstripe edges). Likewise, interstripe iridophores that divided yielded daughter cells that remained in the interstripe (981 divisions, including 160 at interstripe edges; Supplementary Fig. 1a, and Supplementary Movies S1 and S2). These observations are inconsistent with the morphogenetic respecification model.
**Fig. 1 Anatomy, development, and models of zebrafish adult pigment patterning.**

**a** Left panel, an adult zebrafish showing light interstripes with intervening dark stripes. Right panel, a closeup showing the primary interstripe (1°) which develops first with stripes above and below, followed by secondary interstripes ventrally (2°) and dorsally with additional stripes, and ultimately a tertiary (3°) interstripe and stripe. **b** Closeups of first-forming 1° interstripe and stripes, illustrating overall pattern features, as well as morphologies and arrangements of iridophores. All panels are the same location in a single animal. Left panel is incident illumination showing iridescence of iridophore-reflecting platelets with yellowish tinge in the interstripe and bluish tinge in the stripe. Center panel is oblique illumination revealing surface features and non-iridescent colors of iridophores. Here, the fish has been treated with epinephrine to contract melanin granules of melanophores and pigment within xanthophores toward cell centers, thereby better revealing iridophore morphologies. Right panel is membrane-targeted mCherry (mem-Cherry) driven at high levels in iridophores by regulatory elements of purine nucleoside phosphorylase 4a (pnp4a), revealing iridophore cell boundaries and arrangements. Pixel values are inverted for easier comparison to bright field images. Example shown is representative of >20 individual fish examined. **c** Two models for iridophore patterning in interstripes and stripes. In the morphogenetic respecification model (left panel), initially densely packed, cuboidal iridophores begin adopting a loose morphology as they and their progeny migrate out to populate the prospective stripe. In the differentiation in situ model (right panel), iridophores residing in interstripes and stripes are different cell types that have differentiated “in place” from a precursor population. Hence, loose iridophores in stripes are not lineally related to dense iridophores in interstripes. **d** The flank of a 7.5 standardized standard length (5SL) pnp4a:mem-Cherry fish. Left panel, fluorescence image showing the arrangement of labeled cells in the dense primary interstripe. Right panel, pseudo-temporal coloring representation of a 15 h time-lapse movie (zoomed to the region outlined in **d**) revealing that interstripe iridophores migrate primarily in the anteroposterior direction, with no apparent dorsoventral migration into the stripe region. Example image is representative of time-lapse videos from a total of 10 individuals during primary stripe formation at 7.0–7.5 5SL, as well as 15 individuals during secondary stripe formation at 10.0–12.0 5SL. Scale bars, **a** 2 mm, **b** 500 µm, **d** 500 µm.
Effect of conditional melanophore development on iridophore pattern remodeling. For this experiment, iridophores were labeled only with a nuclear-localizing Eos (nucEos\textsuperscript{un}, green; nucEos\textsuperscript{conv}, magenta); after photoconversion nuclei appear magenta, or white as new nucEos\textsuperscript{un} was produced. Inset, right panel). Example shown is representative of a total of eight individual fish examined.

Inset, right panel). Example shown is representative of a total of 12 individuals across two independent experiments. Scale bars, b, 100 \(\mu\)m, d, 50 \(\mu\)m.

Several cell diameters and these movements tended to be biased away from the first interstripe (Supplementary Fig. 2c, d and Supplementary Movie S5).

Fate-mapping and repeated imaging support a model of differentiation in situ. We devised a further set of experiments to challenge the two models of iridophore patterning by following the long-term fates of iridophores marked by photoconversion (green \(\rightarrow\) red) of nuclear-localizing \(pnp4a\):nucEos fluorescent protein. In this experiment, photoconverted “old” iridophores will acquire white-colored nuclei over time due to their having both newly synthesized nucEos\textsuperscript{un} (unconverted green) and photoconverted nucEos\textsuperscript{conv} (red, here displayed as magenta) in their nuclei; by contrast, “new” iridophores developed from precursor cells will have only nucEos\textsuperscript{un} (green) in their nuclei (Fig. 2a)\textsuperscript{25,26}.

We reasoned that if individual iridophores change their morphological states and migrate out to contribute to both interstripes and stripes, as predicted by the morphogenetic respecification model, then marking cells in one pattern element should later yield marked cells in both pattern elements. On the other hand, if individual iridophores are fixed for their morphological state and contribute only to interstripes or stripes, as predicted by the differentiation in situ model, marked cells should retain their morphology and be confined to their original pattern element.

Immediately after photoconverting a region in the interstripe zone, all iridophores in this region had magenta nuclei, whereas iridophores in regions not targeted for photoconversion, including a very few loose iridophores already present in the stripe zone, had only green nuclei (Fig. 2b, post-photoconversion). After 7 days, only iridophores in the interstripe zone had white nuclei, having green nuclei (see yellow arrowheads in bottom panel) in the newly forming stripe.

Example shown is representative of a total of 12 individuals across two independent experiments. Scale bars, b, 100 \(\mu\)m, d, 50 \(\mu\)m.
whereas newly formed iridophores, having green nuclei (indicative of their acquiring pnp4a expression), could be seen mostly in the stripe zone (Fig. 2b, after 7 day). The presence of white-colored nuclei in the interstripe and their absence in the stripe indicates that interstripe marked cells did not migrate, favoring the model of differentiation in situ. In addition, we found that the formation of secondary interstripes was characterized by the development of cells newly expressing pnp4a within this region, suggesting differentiation with subsequent proliferation rather than active aggregation of widely dispersed cells12 (Supplementary Fig. 3).

The above analyses focused on a region in the middle of the flank. Because iridophore behaviors may differ between anatomical locations, we extended our analyses by examining distributions of pnp4a:mCherry+ cells in entire, individual fish imaged daily over 33 days. These analyses also revealed extensive differentiation of iridophores without indications of EMT (Supplementary Figs. 4 and 5a, b, and Supplementary Movie S6). In some instances, patches of dense iridophores anteriorly appeared to split between primary interstripe and ventral secondary interstripes, perhaps owing to rapid expansion of the flank directly over the swim bladder (Supplementary Fig. 5d and Supplementary Movie S7). In a minority of larvae (~20%), patches of five to ten closely associated iridophores developed anteriorly, dorsal to the primary interstripe. Cells in these patches sometimes maintained their tight associations and became incorporated into the secondary dorsal interstripe. In other instances, such cells were incorporated instead into the stripe (Supplementary Fig. 5c, e). Contrary to the expectations of the morphogenetic re specification model12,20, the few of these cells that transitioned from a nascent dense morphology to a loose morphology occurred already within prospective stripe regions. These observations highlight subtle region-specific differences in patterning events and suggest that, had state transitions occurred in a majority of cells or over a broader anatomical area, as predicted in the morphological re specification model, they should have been observed. That they were not observed lends further support to the model of differentiation in situ.

Role of melanophores in iridophore pattern remodeling. Because melanophores reside in stripe zones but not interstripe zones, we wondered whether iridophore pattern remodeling (i.e., dense- versus loose-arrangement morphology) is impacted by melanophore presence. Prior work has hinted at this possibility as mutants for melanophore-inducing transcription factor (mitfa), which lack melanophores, have the dense morphology iridophores (characteristic of interstripe zones) over a broader area than wild-type fish14,27. To explore this further, we used a temperature-sensitive allele, mitfa°C7, that allows conditional differentiation or ablation of melanophores26,28,29 (Fig. 2c), and then examined the phenotypes of iridophores in different skin areas.

To assess whether iridophores in newly arising stripes, or regions newly devoid of stripes, were derived either from previously differentiated or newly differentiated cells, we marked cells by nucEos photoconversion, shifted fish between temperature regimes, and followed marked cells over time. When fish were shifted from restrictive temperature, in which they lacked melanophores, to permissive temperature, in which melanophore differentiation could occur, we found that preexisting, dense morphology iridophores receded, presumably owing to death, migration from the region, or both, and that new iridophores differentiated into a loose arrangement in regions where melanophore differentiation had occurred (Fig. 2d and Supplementary Fig. 6a). Reciprocal temperature shifts to ablate melanophores led to a similar loss of preexisting loose iridophores and the differentiation of new dense iridophores (Supplementary Fig. 6b). Though we cannot exclude the possibility that some preexisting iridophores were incorporated into remodeled pattern elements, these results suggest that the presence of melanophores has a major effect on the pattern remodeling of iridophores, specifically, in promoting a loose morphology.

Distinct crystal morphology and ultrastructural organization, but shared chemistry of loose and dense iridophores. Differences in iridophore morphologies (dense/cuboidal versus loose/stellate) and our failure to observe transitions between these two states, raised the possibility that iridophores of dense/cuboidal morphology in interstripes and loose/stellate morphology in stripes represent distinct cell subtypes, analogous to neuronal subtypes20. To test this possibility, we evaluated the subcellular architecture, physiology, and gene expression of dense/cuboidal iridophores in stripes versus loose/stellate iridophores in interstripes.

Because iridophores depend for their iridescence on stacks of membrane-bound reflecting platelets consisting of crystalline guanine4,11, we first asked whether numbers, sizes, or arrangements of these crystals differ between iridophores found in interstripe versus stripe regions. To visualize guanine crystals in situ required a reagent that would adhere to guanine crystals, and so we screened 12 cell-permeable dyes, chosen for their ability to form both hydrogen bonds and pi-stacking interactions31. We found that malachite green efficiently bound guanine and therefore used it to examine guanine crystal organization in iridophores from interstripe versus stripe regions. Incident illumination images of the stripe zone showed blue, loosely distributed iridophores on top of black melanophores, whereas images of the interstripe zone showed dense silvery iridophores covered by yellow xanthophores (Fig. 3a, b, incident illumination). Notably, malachite green labeling of guanine crystals within iridophores in these two zones revealed remarkably stacked arrays of crystals in loose iridophores from the stripe, but markedly disordered arrays of crystals in dense iridophores from the interstripe (Fig. 3a, b, upper panels).

To assess the ultrastructural organization of iridophores under near-physiological conditions, in which crystal organization and cytoplasmic spacing are likely to be retained, we used cryogenic scanning electron microscopy (cryo-SEM). Crystal arrays of loose iridophores from stripes were remarkably ordered, with 20–30 layers of parallel crystals having an average thickness of 27 ± 7 nm (n = 82), neatly separated by thin layers of cytoplasm of average thickness 131 ± 24 nm (n = 91; Fig. 3a, lower panel). By contrast, crystal arrays of dense iridophores from interstripes were disordered, varying in both orientations and spacings between crystals (Fig. 3b, lower panel), with 30–40 crystals per cell, and a similar average crystal thickness of 25 ± 8 nm (n = 130) and an average cytoplasm spacing of 186 ± 81 (n = 145).

Beyond differences in crystal arrangements, the shapes and sizes of crystals appeared to differ between loose iridophores in stripes and dense iridophores in interstripes. To quantify these differences, we isolated skin separately from stripes (Fig. 3c, left panel) and interstripes (Fig. 3c, right panel), and extracted crystals for transmission electron microscopy (TEM) and electron diffraction analyses. While the crystals in cells from both tissue regions comprised plates of β-guanine (Fig. 3d and Supplementary Fig. 7), crystals isolated fromstripe iridophores were smaller (3.9 ± 0.4 versus. 5.3 ± 0.9 µm; n = 60) and had smaller aspect ratios than crystals from interstripe iridophores (1.9 ± 0.2 versus 2.5 ± 0.3 µm; n = 57; Fig. 3e). In situ Raman spectroscopy of individual cells further validated that crystals in loose iridophores...
Fig. 3 Loose versus dense iridophores have distinct crystal morphologies and ultrastructural organizations, but shared chemistry. (a) Loose iridophores in stripe region viewed by incident illumination, fluorescence, and high pressure–frozen, freeze-fractured cryo-SEM (Cryo-SEM). The incident illumination image shows blue iridophores on top of black melanophores; the fluorescent image reveals malachite green (MG) labeled iridophores (pseudo colored green) with highly ordered arrays of guanine crystals; and the Cryo-SEM image shows iridophore cytoplasm with highly disordered arrays of crystals. \( n = 5 \) adult fish for incident illumination and fluorescence, and \( n = 4 \) adult fish for cryo-SEM. (b) Dense iridophores in interstripe region viewed by incident illumination, fluorescence, and Cryo-SEM. The incident illumination image shows silvery iridophores covered by yellow xanthophores; the fluorescent image reveals MG-labeled iridophores with disordered arrays of guanine crystals (pseudo colored green); and the high pressure–frozen, freeze-fractured cryo-SEM micrograph shows iridophore cytoplasm with disordered arrangements of crystals. \( n = 5 \) adult fish for incident illumination and fluorescence, and \( n = 4 \) adult fish for cryo-SEM. (c–e) TEM analysis of crystals isolated from iridophores from either the stripe or the interstripe regions of adult fish (\( n = 4 \) adult fish). (c) TEM micrographs of crystals isolated from stripe (left panel) and interstripe regions (right panel); (d) TEM-based electron diffraction of the crystals shown in Supplementary Fig. 7, isolated from stripe iridophores (left panel) and interstripe iridophores (right panel); (e) graph of aspect ratio (length/width) of stripe iridophores (blue) and interstripe iridophores (orange), \( n = 60 \) for stripe isolated crystals and \( n = 57 \) for interstripe isolated crystals. Data are represented as mean ± SEM. The \( p \) value, \( p < 0.0001 \), was determined using two-tailed Mann–Whitney test. (f) Simulated reflection (black) and measured reflection (red) from a stripe iridophore. (g) Simulated reflection (black) and measured reflection (red) from an interstripe iridophore. Insets in both (f) and (g) show the corresponding reflectance color on a CIE (International Commission on Illumination) chromaticity space diagram. (h) Incident illumination image of an adult fish lacking melanin in melanophores and carotenoids in xanthophores due to mutations in tyrosinase and scarb1, respectively. The image shows iridophore-type-specific coloration is independent of melanin and carotenoids, consistent with reflectance data obtained for stripe (f) and interstripe iridophores (g). Scale bars, (a, b) (left panels) 50 \( \mu m \), (a, b) (right panels) 4 \( \mu m \), (a, b) (bottom panels) 1.5 \( \mu m \), (c) 2 \( \mu m \).
in stripe zones and dense iridophores in interstripe zones consist of β-guanine, and failed, within the accuracy afforded by these measurements, to reveal other components, suggesting that differences in crystal morphology are not related to their chemistry (Supplementary Fig. 8).

**Iridophore subtypes differ in their optical properties.** Differences in colors reflected by stripe iridophores (blue) and interstripe iridophores (silvery-yellow) have sometimes been ascribed to influences of pigments contained within melanophores and xanthophores, respectively. Given the differences we observed in reflecting platelet architectures of iridophores from stripes versus interstripes, however, we reasoned that reflected spectra might be intrinsic properties of iridophore subtypes. Consistent with this hypothesis, we found close matches between spectra predicted from simulations based on a Monte Carlo transfer matrix (with morphometric data derived from cryo-SEM) and empirical reflectance spectra recorded for individual cells by hyperspectral imaging microscopy. Indeed, simulations for ordered-crystal iridophores from stripes predicted a peak in the blue region at 450 nm approaching unity reflection, whereas simulations for disordered-crystal iridophores from interstripes predicted a broad wavelength reflection. In addition, while the reflection from the ordered-crystal iridophores was highly dependent on the angle of incident light, the reflection from disordered-crystal iridophores was not.

We further found that intrinsic differences in iridophore optical properties could generate a strong contrast in color between stripes and interstripes independent of pigments in other optical properties could generate a strong contrast in color between stripes and interstripes independent of pigments in other cell types. This was manifested in fish that lacked both melanin in melanophores and carotenoids in xanthophores, owing to mutations in *tyrosinase* and *scarb1*, respectively. Here, differences in color between stripes and interstripes (i.e., blue versus silvery-yellow) persisted even in the absence of other pigments (Fig. 3h). Together, these results demonstrate the intrinsic differences in optical properties between ordered-crystal iridophores of stripes and disordered-crystal iridophores from interstripes.

**Disordered-crystal and ordered-crystal-containing iridophores remain distinct throughout development.** To map the structural organization of iridophores across the entire skin pattern, we used synchrotron-based micro X-ray diffraction, which allows large areas to be scanned, while still providing information on orientations and anisotropy of crystal arrays at the level of a single cell. In this system, high-angular distribution diffractions having a full-ring signal are indicative of crystal orientations that vary (i.e., disordered), whereas low-angular distribution diffractions having a punctuated-ring signal are indicative of crystals that are consistently oriented (i.e., ordered). Dorsoventral line scanning across the flank of the fish demonstrated there were consistent differences in structural organization of stripe versus interstripe regions (Fig. 4a). Specifically, based on their (012) and (002) diffraction planes, crystal plates in iridophores of the stripe zone were well oriented (i.e., ordered; Fig. 4a, panels 1 and 3, and Supplementary Fig. 10), whereas those in the interstripe zone were nonaligned (i.e., disordered; Fig. 4a, panels 2 and 4, and Supplementary Fig. 10).

Our *mitfa* photoconversion results (see Fig. 2c) raised the possibility that melanophores promote the differentiation of progenitors into iridophores with ordered-crystal arrays. We tested this idea using micro X-ray diffraction to evaluate the crystals architecture in iridophores of two zebrafish mutants: null-allele *mitfa* and *albino*. In *mitfa* mutants, melanophores are missing owing to a defect in their specification; in *albino* mutants, melanophores are present but lack melanin. We reasoned that if melanophores drive iridophore differentiation toward the ordered crystallotype, then *mitfa* mutants should be deficient in iridophores having ordered crystals, whereas *albino* mutants should retain ordered iridophores, similar to the wild type. Line scans across the flanks of *mitfa* fish revealed mostly high-angular distribution (012) diffractions, typical of the disordered crystallotype (Fig. 4b and Supplementary Fig. 11). Scanning the entire fish showed some diffraction patterns corresponding to ordered iridophores, but these were located toward the posterior and were a minor component of the diffractions (Supplementary Fig. 12). The same analysis on *albino* fish revealed alternating diffraction patterns similar to that seen in wild type (Fig. 4c and Supplementary Fig. 13). These results suggest that melanophores enhance the differentiation of ordered-crystallotype iridophores.

Next, we examined the relative developmental timing of precursor differentiation into disordered and ordered crystallotypes by assessing micro X-ray diffraction patterns over ontogeny. In fish of 6.0 mm standardized standard length (SSL) and 6.5 SSL, which have only a single interstripe and very few adult melanophores, we observed only disordered-crystallotype iridophores (having high-angular distribution diffraction patterns of the (012) plane; Fig. 4d, and Supplementary Figs. 14 and 15). When fish were ~6.9 SSL, with a substantial complement of melanophores and loose iridophores, low-angular distribution diffraction patterns of the (002) plane, typical of ordered-crystallotype iridophores, became visible (Fig. 4e, see white arrows, and Supplementary Fig. 16). These results supported the idea that precursor cells differentiate into ordered-crystallotype iridophores only after the differentiation of disordered-crystallotype iridophores and in the presence of melanophores.

**Ordered and disordered crystallotypes exhibit distinct transcriptomic signatures.** We next tested whether stripe and interstripe iridophores also have distinct transcriptomic signatures by single-cell RNA-sequencing (scRNA-seq; Fig. 5a). Dimensionality reduction followed by unsupervised clustering revealed five clusters (Fig. 5b, Supporting file—Table 1), three of which (clusters c1, c3, and c5) contained cells expressing high levels of known markers of iridophores (i.e., *gpnmb*, *pnp4a*) (Supplementary Figs. 17 and 18a, b), as well as genes that were recently identified as iridophore markers (i.e., *alx4a*, *alx4b*) (Supplementary Figs. 17 and 18c, d). The expression levels of these iridophore markers, as well as other markers for pigment cells, were lower in clusters c2 and c4 (Supplementary Fig. 17), suggesting these clusters potentially represent cells that are not fully differentiated.

Next, we tested whether any of the iridophore clusters recovered by scRNA-seq were originated specifically from either the stripe or the interstripe locations. We found that cluster c5 was mostly stripe specific, as 85% of cells in cluster c5 originated from stripes, whereas cluster c3 was mostly interstripe specific, as 84% of cells in cluster c3 originated from interstripes (Fig. 5c). Cells of the third iridophores cluster, c1, were shared between the interstripe (44%) and stripe (56%; Supplementary Fig. 19). Several hundred loci were differentially expressed between cells of clusters c3 and c5 (Fig. 5d and Supporting file—Table 2), highlighting candidate genes that may contribute to structural or other differences between stripe and interstripe iridophores. For example, it was recently shown using immuno-labeling that Tight Junction Protein 1a (tjp1a) is highly expressed only in dense iridophores (found in the interstripe), and that knocking out *tjp1a* results in the disruption of the stripe pattern due to dense
In line with these findings, we found that tjp1a is highly expressed in cluster c3 (mostly interstripe iridophores) and expressed at lower levels in cluster c5 (mostly stripe iridophores). Other interesting genes that were differentially expressed at high levels were retinol dehydrogenase 5 (rdh5) and retinol dehydrogenase 10a (rdh10a), upregulated in cluster c3 and cluster c5, respectively. These genes code for proteins that catalyze key oxidation–reduction reactions in the visual cycle, and could be related to the presence of visual pigments in these cells and their reported ability to sense light. We also performed a gene-set enrichment analysis of the differentially expressed genes using FishEnrichr (Supplementary...
Table 1 and Supplementary Table 2). The gene enrichment analysis highlighted many differentially regulated pathways, including responses to purine-containing compounds, and cAMP pathways (Supplementary Table 1), which were both upregulated in cluster c5 (mostly stripe iridophores) and might be involved in iridophore environmental sensing and reaction to specific stimuli (see below). Another set of differentially enriched pathways were related to the cytoskeleton, including pathways for all three main cytoskeleton components, actin filaments, intermediate filaments, and microtubules (Supplementary Table 2), consistent with roles in mediating differences in structural organization and crystal array architectures between stripe and interstripe iridophores. We concluded from these analyses that iridophores of interstripe and stripe zones are transcriptionally distinct.

Physiological color change differs between iridophore subtypes. Color pattern can be influenced physiologically41,42, as some types of pigment cells disperse or contract pigment granules in response to endocrine and neuroendocrine factors, including norepinephrine (NE)43–45. We wondered whether morphological differences between iridophore subtypes might be accompanied by physiological differences as well. To test this, we bathed isolated fish skin in 10 µM NE solution. Upon NE treatment, stripe-derived iridophores exhibited a ~120 nm shift in peak reflectance, whereas interstripe-derived iridophores did not exhibit gross changes in reflected light spectra, measured by hyperspectral imaging (Fig. 5e). Viewing the response to NE in the context of the whole tissue also revealed a reduction in the color differences between the stripe and the interstripe locations (Fig. 5f). These results support prior work showing that iridophores of different shapes respond differently to NE45, and further demonstrate that only stripe iridophores change their color upon NE treatment. This differential response, in conjunction with the known aggregation of granules within melanophores and xanthophores44,45, could contribute to the dramatic reduction in contrast between stripe and interstripe zones under NE treatment, which causes the prominent zebrafish stripe pattern to diminish (Supplementary Movie 8).

Discussion

Pigmentation of teleost fish has become a valuable system for understanding pattern formation in animals, including how changes in pattern-forming mechanisms lead to phenotypic variation within and between species5,6. In zebrafish, a widely accepted model implicates dynamic changes in iridophore shape—between a dense morphology in interstripes and a loose morphology in stripes—to establishment and reiteration of pattern12,19,20,22. In this model, iridophores of interstripes and stripes are similar cells that have adopted different morphologies, as they migrate into different regions. Using diverse tools, we found no evidence for morphological transitions by individual iridophores. Rather, our data support an alternative model of differentiation in situ for how the reiterated stripe pattern of zebrafish develops. In this model, iridophore precursors in developing stripes and interstripes differentiate based on their microenvironment into distinct iridophore crystallotypes, with different subcellular organization and physiological responsiveness.

We found several structural disparities between iridophores of interstripes and stripes; iridophores of interstripes had larger reflecting crystal platelets that were disordered, whereas those of stripes had smaller crystal platelets that were uniformly stacked and oriented. The colors of these cells differed as well: iridophores in interstripes were silvery-yellowish, and iridophores in stripes were blue, and these differences were autonomous properties of the cells, not a consequence of pigments contained in other pigment cells with which iridophores associate. Physiological responses also differed: disordered-crystal platelets of interstripe iridophores were refractory to NE, whereas, ordered-crystal platelets of stripe iridophores changed their cellular organization upon NE treatment. Finally, iridophores from interstripes and stripes had distinct gene expression profiles.

We found no evidence that individual iridophores undergo state transitions, as would be expected if cells originating in one pattern element disperse to populate another. Photolabeled iridophores observed over short or long periods failed to migrate between interstripe and stripe zones, even when they were challenged to undergo transitions in the context of pattern remodeling (stimulated by changes in melanophore abundance). Instead, development and remodeling of interstripes and stripes involved de novo differentiation, subsequent proliferation, and in some cases migration of iridophores with morphologies appropriate to their location within the pattern. Only in a minority of fish, and in a small anatomical region, did we observe patches of initially dense iridophores assume a loose arrangement. Such behaviors occurred within prospective stripes, rather than at boundaries between interstripes and stripes, as previously postulated5,12, and involved cells that had not yet fully differentiated.

The apparent conflict between our observations and those of prior studies5,12,19 may reflect differences in how iridophores and their progenitors have been visualized. Previous analyses used a sox10:CreER22 transgene that can mark most or all postembryonic neural crest derivatives, including multipotent progenitors in the peripheral nervous system, and cells transiting from the peripheral nervous system to the hypodermis of the skin12,46. In this
case, clonal spread over the flank from interstripe-to-stripe, and stripe-to-interstripe, is indeed apparent as progeny of individual progenitors migrate to the skin or expand their numbers at the hypodermis. If individual cells appear to switch between dense and loose morphologies, these may represent iridophore precursors transiently associated with one pattern element or another, but not yet committed to crystallotype. By contrast our analyses employed pnp4a transgenes, expressed as iridophores begin to differentiate and our photoconversion approach, tracking individual pnp4a expressing cells, allowed us to map the fate of differentiated iridophores during development. We postulate that pnp4a-labeling allowed us to distinguish later stage, committed iridophores or iridoblasts from earlier stage iridoblasts or earlier progenitors that had not yet become fate restricted. These data all point to a model of stripe patterning that depends on differentiation in situ. In this model, latent progenitors associated with the peripheral nervous system that have transited to the skin during the larva-to-adult transformation3 expand clonally as early iridoblasts—not specified to subtype—and subsequently differentiate according to cues in the microenvironment they encounter. Our data, together with those of others, suggest that some of these signals depend on melanophores14,15,25,47, promoting differentiation of iridoblasts toward a state having ordered reflecting crystal platelets that are
Physiologically responsive (blue → yellow) in stripes, and away from an alternative state of disordered-crystal platelets lacking physiologically responsiveness (silver-yellow) in interstripes. Additional signals from xanthophores, iridophores, and other cell types likely contribute as well. Whether these events of specification unfold as iridoblasts expand their territory within the plane of the skin hypodermis, or as they arrive at the hypodermis after migrating from progenitor niches within the peripheral nervous system, remains to be determined. Whichever mode of iridoblast morphogenesis holds true, our findings highlight the importance of extrinsic factors that specify and promote the in situ differentiation of iridophore subtypes, during pattern establishment and reiteration. The resulting iridophore subtypes likely allow the zebrafish to alter its skin patterning to make it more or less distinctive, a trait crucial for the fish to be able to join shoals or obscure itself48,49.

**Methods**

**Fish stocks, rearing conditions, transgene expression, and CRISPR/Cas9 mutagenesis.** We reared zebrafish under standard long period conditions (14 L:10 D at −28°C as stated and in accordance to how HMMI-IFRC ethical committee via animal Care and Use protocol 16-137 and UVA IACUC protocol 4170. Staging followed50. Stocks were wild type WT(AAB), a derivative of ABp51; Tg(pnpx4a:nucEos)m851.50, with a membrane-tethered temperature-sensitive mTurquoise2ycling to the apical membrane to allow control over the activity of the mCherry expression on all sides), dense (surrounded by other iridophores, bright mCherry expression), or edge that lacked both carotenoid pigmentation and mCherry expression, or crystals between quartz coverslips (Electron Microscopy Sciences, Cat. No. 16004-422). All data were presented using a MIDL® hyperspectral imaging system (Lightform Inc. with PARISS software v1.14), which acquires instantaneously 380–980 nm spectra from each pixel of a line of pixels when pixel size is 1.25 × 1.25 μm². The hyperspectral imager was mounted on a Nikon Eclipse 80i microscope. Spectral calibration was performed using a MIDL® Hg®/Ag® wavelength calibration lamp (Lightform Inc.) with a diameter better than 2 mm. The confocal cells spectra were acquired using spectroscopic reflectance and transmittance, using a tungsten halogen light source with a Nikon NCB11 filter, and a 20 × 0.50 NA objective. Reflectance spectra were normalized using a standard silver mirror (Thorlabs Inc.). All spectra were smoothed with a running average of three data points and plotted using Matlab (2019a).

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**Time-lapse imaging and analyses.** Pigment cells were imaged ex vivo in their tissue environment on a laser spinning disc microscope (above)50. We imaged the entire flank between gut and caudal fin peduncle of Tg(pnpx4a:nucEos) fish every 5 min for 15 h, unless otherwise noted. To capture rare behaviors over large areas, images were typically collected as multiple tiles for each specimen, then stitched computationally using Zen Soft Imaging software. Supplemental Movies SI–SS illustrate regions of interest from larger views. Analyses focused on the area including the primary interstripe, ventral primary stripe, and ventral secondary interstripe (where applicable). All iridophores in these regions were included in proliferation and migration analyses. Proliferating iridophores were evident as single cells that rounded up and then divided to generate adjacent daughter cells. We measured the plane of proliferation using the angle tool in ImageJ from the center of each daughter cell in the frame immediately following cytokinesis. To analyze migration, we used ImageJ to determine start and end points of all stripe-associated iridophores that moved 21 cell diameter over the course of each time-lapse movie. Tracings were examined from fish at stages 7.0 SSL (N = 5), 7.5 SSL (N = 5), 10.0 SSL (N = 11), and 12.0 SSL (N = 4) were included in cell behavioral analyses. Iridophores were classified as dense (surrounded by other iridophores, bright mCherry expression on all sides), loose (stellate, dim mCherry expression), or edge (contacting other dense iridophores over ~75% of the cell perimeter with the remainder open to the prospective stripe region). Total cells analyzed was 7.0 SSL — 630 dense, 57 loose, 359 edge; 7.5 SSL—1230 dense, 338 loose, 423 edge; 10.0 SSL—411 dense, 2047 loose, 523480 edge; 12.0 SSL—1657 dense, 1508 loose, 186 edge.

**Temperature shift experiments.** We injected mitfA-EGFP; Tg(pnpx4a:nucEos) with pnpx4a:nucEos and Toz2 mRNA using standard methods. We raised the fish at the primary reiterative temperature (30 °C) to induce progenitor differentiation in the primary interstripe, primary stripes, and ventral secondary interstripe developed at permissive temperature. We then photoconverted mosaically expressed patches of pnpx4a:nucEos as described above, and shifted fish to the opposite temperature.

**Synchrotron-based micro wide X-ray diffraction.** Fish (larvae or adults) were euthanized in MS222 (E10521, Sigma-Aldrich) and were immersed in a physiological buffer (PBS, P3813, Sigma-Aldrich, 136 mM osm/kg). For adult fish, fresh skin sections were obtained and mounted on a lead tape between two kapton windows. For larvae fish, entirely freshly euthanized fish were mounted on a lead tape between two kapton windows. In situ wide X-ray diffraction (WAXD) was collected at the 7-ID-S beamline, in the synchrotron radiation facility BESSY II, Helmholtz-Zentrum Berlin für Materialien und Energie, Berlin, Germany. Samples were mounted on a y−z scanning table and scans of the sample in areas of interest were performed. The microbeam was defined by a toroidal mirror and a pinhole of either 10 or 30 μm diameter close to the sample, providing a beam size of ~10 × 10 μm² or 30 × 30 μm² at the sample position. Line scans were performed across the flank of the fish, with two to four data points collected for each interstripe, and three to six data points collected for each stripe. An energy of 15 keV (λ = 0.82656 Å) was selected by a Mo/BC multilayer monochromator. The 2D SAXS/WAXD patterns were measured by using a MarMosaic 2 D detector (Bruker) placed at a sample−detector distance of 286 mm. The position center in the detector and the sample−detector distance were calibrated, using a powder X-ray diffraction pattern of synthetic guanine powder standard (Sigma-Aldrich). Radial integration of the 2D scattering patterns was performed using and DSD54. The data were normalized with respect to the primary beam monitor (ionization chamber) and corrected for background caused by pinhole and air scattering.

**Hyperspectral imaging.** Fish were euthanized in MS222 (E10521, Sigma-Aldrich), then relevant skin portions were dissected and placed in PBS solution (P3813, Sigma-Aldrich) at room temperature. Spectral measurements were performed using a PARISS® hyperspectral imaging system (Lightform Inc. with PARISS software v1.14), which acquires instantaneously 380–980 nm spectra from each pixel of a line of pixels when pixel size is 1.25 × 1.25 μm². The hyperspectral imager was mounted on a Nikon Eclipse 80i microscope. Spectral calibration was performed using a MIDL® Hg®/Ag® wavelength calibration lamp (Lightform Inc.) with a 20 μm diameter better than 2 μm. The confocal cells spectra were acquired using spectroscopic reflectance and transmittance, using a tungsten halogen light source with a Nikon NCB11 filter, and a 20 × 0.50 NA objective. Reflectance spectra were normalized using a standard silver mirror (Thorlabs Inc.). All spectra were smoothed with a running average of three data points and plotted using Matlab (2019a).

**TEM.** Fish were euthanized in MS222 (E10521, Sigma-Aldrich) then relevant skin portions were dissected and placed in PBS (P3813, Sigma-Aldrich) solution at room temperature. Tissue was mechanically dissected using a scalpel and then sonicated for 10 min. The obtained suspension was centrifuged at 4000 RPM, after which the pellet was discarded, and the supernatant was kept and vortexed for 1 min. This process was repeated twice. A total of 10 μl drop of obtained suspension was placed onto a copper slot TEM grid coated with Formvar/ carbon and was allowed to settle for 5 min. After which the grid was carefully blotted dry with help of a piece of filter paper. The TEM grids were imaged in a Tecnai Spirit electron microscope (FEI, Hillsboro, OR) operating at 80 kV equipped with an Ultrascan 4000 digital camera (Gatan Inc, CA).

**Raman microspectroscopy.** Samples were prepared by sandwiching prepared tissues or crystals between quartz coverslips (Electron Microscope Sciences, Cat. No. 72355-02) and glass microscope slides (VWR, Cat. No. 16004-422). All data were collected at RT using a home-built Raman microscope50. We used a 514-nm line of an argon-ion laser (CVI Melles Griot, 35-307-M41-200), which was passed through a clean-up filter (Semrock, LL01-514-25) and then directed into a modified inverted microscope (Olympus IX71). Excitation light (~30 mW at the sample) was detected by a 10× objective sample using a Raman microspectrometer (Horiba Scientific). Spectral data was acquired using a 50-μm entrance/exit slit, coupled to a 320-mm focal length (Semrock, LP02-514RE-25) to remove any residual excitation light or Rayleigh scattering, and then directed into a 320-mm focal length (β/α aperture) imaging spectrometer (Horiba Scientific, IH2R 320) through a 0.4 μm pinhole (Thorlabs) and dispersed using a 200 mm doublet lens and detected by a 500 × 500 pixel detector. We collected for 10–15 s acquisitions (~500 × 3700 cm⁻¹) with high gain enabled on a liquid nitrogen cooled, back illuminated deep-depletion CCD array (Horiba Scientific, Symphony II, 1024 × 256 px, 26 mm × 26 mm, 6.6 mm, 1 MHz repetition rate). Bright field images were collected using a USB 2.0 camera (IDS, UI-1220-C). Daily calibration of imaging spectrometer was done using neat cyclohexane (20 μl in a sealed capillary tube), Bandpass and accuracy were found to be <12 and ± 1 cm⁻¹, respectively. All Raman spectra were corrected by applying a baseline polynomial fit (Lab Spec 6 software).
Isolation of cells for scRNA-seq. Fish were euthanized in MS222 (E10521, Sigma-Aldrich) and the stripe and interstriped regions were microdissected from fish expressing both pro-panmuc-mCherry and pro-panmuc-Eos. Stripes and interregion samples were enzymatically dissociated separately with Liberase (0.25 mg/ml in PBS, LIBIDL-RO, Roche) at 25 °C for 15 min followed by manual trituration with increasingly narrower flame polished glass pipette for 3 min at a time for three times. Cells suspensions were then filtered through a 70 μm nylon cell strainer to obtain a single-cell suspension. Liberated cells were resuspended in 1% BSA (A2153, Sigma-Aldrich)/5% FBS (F2442, Sigma-Aldrich) in dPBS before FACS purification. This was done for samples collected from four different fish that were processed in two different cycles, combining skin samples from two different fish for each cycle. The flow cytometry experiments were performed on a BD FACSAria II SOPR sorter (BD Biosciences, San Jose, CA, USA) coupled with BD FACS Diva software (BD Biosciences) for instrument operation, data acquisition, and analysis. A 637 nm and a 488 nm laser were utilized for fluorophore excitation and a 100-μm nozzle was used to generate single droplets under 20 PSI sheath pressure. The applied settings were as follows: forward light scatter (FSC) detector photo-multiplier (PMT) gain setting = 60 V, 1.5 μm neutral density filter, side light scatter (SSC) detector PMT gain setting = 90 V; FSC threshold = 10,000; PE-Texas Red (PE TRX Red) channel PMT gain setting = 280 V; fluorescein isothiocyanate channel PMT gain setting = 280 V (an exemplification the gating strategy is provided in Supplemental Fig. 2b). Sample dilutions and flow rate were adjusted to optimal event recordings for 96-well plate single cell sorts (below 500 processed events per sec). The population of zebrafish skin iridophores was designated based on their FSC and SSC characteristics and back-gating on fluorescence. Control zebrafish skin samples were used to gate out the highly auto fluorescent cells among the members of this population. Fluorescently labeled from either the stripe or interstriped regions were sorted separately with cells by high throughput and Eos expression were collected into 3 μl of smart-scrub lysis buffer (0.2% Triton X-100 (Sigma), 0.1 U/μl RNase inhibitor (NEB)) using “single cell” indexing sorted mode. Five indexed 96-well plates were collected for each skin band. After sorting, the samples were spun down at 3166 x g at 4 °C for 2 min in an Eppendorf table top 5810 R centrifuge (Eppendorf AG, Hamburg, Germany) and stored at −80 °C until further processing.

scRNA-seq library preparation. Four plates of each skin band were selected for sequencing. cDNA was prepared from sorted cells as described previously by Cembrowski et al.37 with minor modifications. Reverse transcription, PCR, purification, tagmentation, and library quantification were performed as described. Libraries from eight plates were pooled equimolar and were sequenced on a NextSeq 550 high-output flowcell with 25 bases in read 1 to read the 1 bp spacer, 8 bp barcode, 10 bp UMI as described in Cembrowski et al., 8 bases in the i7 index read, and 50 bases in read 2 (cDNA). phiX control library (Illumina) was spiked in at a final concentration of 15% to improve color balance in read 1. Libraries were sequenced to an average depth of 39,875,610 to 8,449,306 reads (mean ± standard deviation (SD)).

scRNA-seq analysis. Sequencing adapters were trimmed from the reads with Cutadapt v2.10 (Martin, 2011) prior to alignment with STAR v2.7.5a (Dobin et al., 2013) to the GRCz11.94 genome assembly from Ensembl (ensemble.org). Gene counts were generated with the STARsololo algorithm using the following additional parameters: --soloType CB_UMI Simple--soloCBwhiteList smartscrbc_whiteList.txt--soloStart 2--soloCBlen 8--soloUMLstart 10--soloUMLElen 10--soloR0length 20--soloCellFilter None--soloCellFilter None. The full set of 384 barcodes designed for this assay was used as the whitelist (Supplemental file "q.index.tex.txt"). The full description of Cutadapt and STAR parameters is provided in Supplemental file "pipeline_parameters.txt". Gene counts for the subset of barcodes used in each library were extracted using custom R scripts. Raw counts representing the enumerated expression for 32,618 features for 768 cells was read into R (v3.6.1) in table form with features in rows and cells in columns. This matrix was then converted into a single-cell experiment object using the "SingleCellExperiment" function supported in the "SingleCellExperiment" library (v1.8.0). Features observed to not have at least one cell removed. Calculation of quality control metrics and screening for low quality cells was accomplished using a combination of functions supported in the "scatter" library (v1.14.6). Specifically, the "perCellQCMetrics" function was used to calculate metrics per cell, the "perFeatureQCMetrics" function used to calculate metrics per feature, and the "quickPerCellQC" function used to identify cells to low quality. In addition, the "plotCotData" function was used to visually inspect the number of detected features per cell by the total number of counts to define a range per number of features (>200, <1750) and counts (>0.45e5, <1.45e5) a cell must satisfy to not be considered low quality. The union set of cells identified as low quality from each experiment was recalculated and counts for surviving features were performed in two different cycles, combining skin samples from two different fish for each cycle. The flow cytometry experiments were performed on a BD FACSAria II SOPR sorter (BD Biosciences, San Jose, CA, USA) coupled with BD FACS Diva software (BD Biosciences) for instrument operation, data acquisition, and analysis. A 637 nm and a 488 nm laser were utilized for fluorophore excitation and a 100-μm nozzle was used to generate single droplets under 20 PSI sheath pressure. The applied settings were as follows: forward light scatter (FSC) detector photo-multiplier (PMT) gain setting = 60 V, 1.5 μm neutral density filter, side light scatter (SSC) detector PMT gain setting = 90 V; FSC threshold = 10,000; PE-Texas Red (PE TRX Red) channel PMT gain setting = 280 V; fluorescein isothiocyanate channel PMT gain setting = 280 V (an exemplification the gating strategy is provided in Supplemental Fig. 2b). Sample dilutions and flow rate were adjusted to optimal event recordings for 96-well plate single cell sorts (below 500 processed events per sec). The population of zebrafish skin iridophores was designated based on their FSC and SSC characteristics and back-gating on fluorescence. Control zebrafish skin samples were used to gate out the highly auto fluorescent cells among the members of this population. Fluorescently labeled from either the stripe or interstriped regions were sorted separately with cells by high throughput and Eos expression were collected into 3 μl of smart-scrub lysis buffer (0.2% Triton X-100 (Sigma), 0.1 U/μl RNase inhibitor (NEB)) using “single cell” indexing sorted mode. Five indexed 96-well plates were collected for each skin band. After sorting, the samples were spun down at 3166 x g at 4 °C for 2 min in an Eppendorf tabletop 5810 R centrifuge (Eppendorf AG, Hamburg, Germany) and stored at −80 °C until further processing.

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**Author contributions**

D.G., E.B., D.D.D., J.C.L., D.P., and J.L.-S. designed research; D.G., E.B., A.J.A., A.P., J.D.F., and M.C.A., performed research; D.G., E.B., K.J., J.D.F., D.D.D., J.C.L., D.P., and J.L.-S. analyzed data; and D.G., E.B., and J.L.-S. wrote the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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