

***In situ* differentiation of iridophore crystalotypes underlies zebrafish stripe patterning**

Dvir Gur^{#,1,2}, Emily Bain^{#,3}, Kory Johnson⁴, Andy J. Aman³, Amalia Pasoili¹, Jessica D. Flynn⁵, Michael C. Allen⁶, Dimitri D. Deheyn⁶, Jennifer C. Lee⁵, Jennifer Lippincott-Schwartz^{*,1}, David Parichy^{*,3}

¹ HHMI Janelia Research Campus, Ashburn, VA

² National Institute of Child Health and Human Development, NIH, Bethesda, MD

³ Department of Biology and Department of Cell Biology, University of Virginia, Charlottesville VA

⁴ Bioinformatics Section, National Institute of Neurological Disorder and Stroke, NIH, Bethesda, MD

⁵ National Heart, Lung and blood Institute of, NIH, Bethesda, MD

⁶ Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA

Co-first authors

* Co-Corresponding authors

Correspondence: lippincottschwartz@janelia.hhmi.org; dparichy@virginia.edu

Skin color patterns are ubiquitous in nature, evolve rapidly, and impact social behavior¹, predator avoidance², and protection from ultraviolet irradiation³. A leading model system for vertebrate skin patterning is the zebrafish⁴⁻⁷; its alternating blue stripes and yellow interstripes depend on guanine crystal-containing cells called iridophores that reflect light. It was suggested that the zebrafish's alternating color pattern arises from a single type of iridophore migrating differentially to stripes and interstripes⁷⁻⁹. When we tracked iridophores, however, we found they did not migrate between stripes and interstripes but instead differentiated and proliferated in place based on their micro-environment. RNA seq analysis further revealed stripe and interstripe iridophores had different transcriptomic states, while cryogenic scanning electron microscopy and micro-X-ray diffraction showed they had different guanine crystal organizations and responsiveness to norepinephrine, all indicating that stripe and interstripe iridophores are different cell types. Based on these results, we present a new model of skin patterning in zebrafish in which distinct iridophore crystalotypes containing specialized, physiologically responsive, subcellular organelles arise in stripe and interstripe zones by *in situ* differentiation. In this model, pattern phenotype depends not only on interactions among pigment cells that affect their arrangements, but also on factors that specify subcellular organization and physiological responsiveness of specialized organelles.

1 Introduction

2 Biological patterning is ubiquitous in nature, but mechanisms underlying its
3 establishment and maintenance have been well-documented in only a few instances that
4 are unlikely to represent the full spectrum of pattern-forming systems^{6,10}. Indeed,
5 patterning can arise in response to graded positional information or by self-organization
6 of interacting cells, and it can require alternative specification of cell-types from a
7 common progenitor or sorting-out of cells that are heterogeneous already. Elucidating
8 the mechanisms required to pattern cells in diverse tissues and organs is fundamental to
9 understanding development and how phenotypes evolve.

10 The alternating dark (blue) and light (yellow) pigmented stripe pattern of adult
11 zebrafish *Danio rerio* (**Fig. 1a**) is a useful model for dissecting patterning
12 mechanisms^{4,5,7,11,12}. Cells within the dark stripes include black pigment-containing
13 melanophores; cells in the light stripes (known as “interstripes”) include orange-pigment
14 containing xanthophores; and both dark stripes and light interstripes contain specialized
15 cells called iridophores^{13,14}. Iridophores are the major players for skin pattern
16 establishment and reiteration in zebrafish. They behave as reflective cells, exhibiting
17 angular-dependent changes in hue—iridescence—owing to membrane-bound reflecting
18 platelets of crystalline guanine¹⁴⁻¹⁶. In the light interstripes, iridophores have a cuboidal
19 shape and form an epithelial-like mat, presenting a “dense” morphological arrangement
20 (**Fig.1b**, fluorescence panel). In the dark stripes, by contrast, iridophores are sparse in
21 number and stellate in shape, and are sometimes referred to as having a “loose”
22 morphology⁸ (**Fig.1b**, fluorescence panel). The iridophore’s importance in skin patterning
23 has been demonstrated in experiments showing that genetically or experimentally
24 induced deficiencies in iridophores cause pattern defects, including alterations in primary
25 stripe positioning and boundary formation, and also lead to reductions or losses of
26 secondary interstripes and stripes¹⁷⁻²¹. In addition, an evolutionary truncation in
27 iridophore development leads to an attenuated stripe pattern in the zebrafish relative *D.*
28 *nigrofasciatus*²².

29 An elegant model explaining the iridophore’s role in stripe and interstripe formation
30 links pattern establishment and reiteration to changes in iridophore morphology,
31 proliferation and migration⁷⁻⁹ (**Fig. 1c**, left panel). Densely arranged iridophores are
32 proposed to first proliferate to fill the primary interstripe. Some of these cells then adopt
33 a loose shape and migrate out into the stripe zone where they continue to proliferate.
34 Subsequently, some loose iridophores reaggregate to adopt a dense morphology and
35 thereby initiate secondary interstripes. The iridophore shape transitions from dense-to-
36 loose and loose-to-dense are thought to resemble epithelial-to-mesenchymal transitions
37 (EMT) and mesenchymal-to-epithelial transitions (MET), respectively. Signals by
38 melanophores and xanthophores are proposed to determine the specific morphologies
39 adopted by iridophores. Consistent with this idea, quantitative models incorporating
40 proposed dynamic morphological changes of individual iridophores are able to produce
41 stripe patterning and robustness^{23,24}.

42 Key predictions of the above model, hereafter referred to as “morphogenetic
43 respecification”, are that some interstripe iridophores undergo an EMT-like
44 transformation and migrate out from the interstripe zone, while presumably maintaining

1 attributes (e.g., reflecting platelet composition and optical properties) other than their
2 overall shape. In testing these predictions through a variety of approaches, we found
3 that individual iridophores did not migrate out from the interstripe into the stripe. Instead,
4 iridophores assumed a particular morphology at the time of their differentiation according
5 to the presence or absence of melanophores and this morphology remained fixed
6 thereafter. We also observed that interstripe and stripe iridophores exhibited distinct
7 organizations of guanine-reflecting platelets (i.e., crystal types) conferring intrinsic
8 differences in color, and that only stripe-localized iridophores could modulate reflecting-
9 platelet spacings physiologically (blue → yellow). Furthermore, interstripe and stripe
10 iridophores had distinct transcriptomic states. Based on these results, we propose a new
11 model for stripe pattern formation in the adult zebrafish in which iridophore precursor
12 cells undergo “differentiation *in situ*” into distinct iridophore types (i.e., crystallotypes)
13 (**Fig. 1c**, right panel). This process would depend on factors in the iridophore
14 environment that impact the specification and subcellular organization of specialized
15 organelles within iridophore-precursors.

16

17 **Results**

18 ***Time-lapse imaging reveals iridophores do not migrate out from the interstripe***

19 Stripe pattern establishment and reiteration in the zebrafish has been proposed to occur
20 through morphogenetic respecification, in which iridophores differentiate to form a
21 primary interstripe and then these cells or their progeny migrate out to contribute to
22 stripes as well as secondary interstripes and stripes^{7-9,23} (**Fig. 1c**, left panel). Individual
23 cells would switch morphologies as appropriate to pattern context, undergoing
24 morphogenetic respecification via processes resembling EMT or MET.

25 To test this model, we examined iridophore behaviors by time-lapse imaging of
26 membrane targeted mCherry driven by regulatory elements of *pnp4a*^{22,25}. If
27 morphogenetic respecification accounts for variation in iridophore morphology and
28 patterning, then events resembling EMT or MET should be observable: densely packed
29 iridophores of the completed interstripe should delaminate to populate the stripe,
30 whereas loosely arranged iridophores of completed stripes should aggregate to initiate
31 new interstripes. In over 300 hours of recordings, we observed no instances in which
32 interstripe iridophores—having the dense morphology—delaminated from their
33 neighbors and assumed the loose morphology (**Fig. 1d**; 14,475 total cells, including
34 1,637 cells located at interstripe edges). Likewise, interstripe iridophores that divided
35 yielded daughter cells that remained in the interstripe (981 divisions including 160 at
36 interstripe edges; **Fig. 1d**, **Extended Data Fig. 1a**, **Supplementary Movies S1**, **S2**).
37 These observations do not support the morphogenetic respecification model.

38 A different way to produce cells in distinct locations having distinct morphologies
39 would be if iridophores populate interstripes and stripes by differentiating from a
40 progenitor not yet specified to type (**Fig. 1c**, right panel). Iridophore morphology in this
41 model would emerge by “differentiation *in situ*” in response to context-appropriate
42 signals, and that same morphology would be retained thereafter by the cells or their
43 progeny. A prediction of this model for iridophore patterning is that new cells should

1 begin to express markers of iridophore differentiation during pattern formation.
2 Consistent with this hypothesis, we frequently observed cells acquire or increase
3 *pnp4a:mem-mCherry* expression within developing stripes (**Extended Data Figs. 1b-e**,
4 **Supplementary Movies S2–S5**).

5 Quantitative image analyses of proliferation and migration further supported pattern
6 development by a mechanism of differentiation *in situ*. We found that proliferation of
7 loose iridophores within stripes was greater than dense iridophores within
8 interstripes²²(**Extended data Fig. 2a**). Moreover, iridophores within interstripes tended
9 to divide along an anterior–posterior plane, consistent with the known faster growth
10 along this axis than dorsoventrally²⁶ (**Extended Data Figs. 1b, 2b**). By contrast, planes
11 of division by stripe iridophores were more uniformly distributed, in keeping with a rapid
12 and relatively uniform occupancy of prospective stripe regions (**Extended data Figs. 1b-**
13 **e, 2b**). Similarly, movements of dense iridophores were negligible, whereas loose
14 morphology iridophores could migrate up to several cell diameters and these
15 movements tended to be biased away from the first interstripe (**Extended Data Figs. 2c**,
16 **2d, Supplementary Movie S5**).

17 ***Fate-mapping and repeated imaging support a model of differentiation in situ***

18 We next devised a further set of experiments to challenge the two models of iridophore
19 patterning by following the long-term fates of iridophores marked by photoconversion
20 (green → magenta) of nuclear-localizing *pnp4:nucEos* fluorescent protein. In this
21 experiment, photoconverted “old” iridophores will acquire white-colored nuclei over time
22 due to their having both newly synthesized nucEos^{un} (unconverted green) and
23 photoconverted nucEos^{conv} (magenta) in their nuclei; by contrast, “new” iridophores
24 formed from precursor cells will have only nucEos^{un} (green) in their nuclei. (**Fig. 2a**)^{27,28}.
25 We reasoned that if individual iridophores change their morphological states and migrate
26 out to contribute to both interstripes and stripes, as predicted by the morphogenetic
27 respecification model, then marking cells in one pattern element should later yield
28 marked cells in both pattern elements. On the other hand, if individual iridophores are
29 fixed for their morphological state and contribute only to interstripes or stripes, as
30 predicted by the differentiation *in situ* model, marked cells should retain their morphology
31 and be confined to their original pattern element.

32 Immediately after photoconverting a region in the interstripe zone, all iridophores in
33 this region had magenta nuclei, whereas iridophores in regions not targeted for
34 photoconversion, including a very few loose iridophores already present in the stripe
35 zone, had only green nuclei (**Figs. 2b**, post-photoconversion). After 7 days, only
36 iridophores in the interstripe zone had white nuclei, whereas newly-formed iridophores,
37 having green nuclei (indicative of their acquiring *pnp4* expression), could be seen mostly
38 in the stripe zone (**Figs. 2b**, After 7 day). The presence of white-colored nuclei in the
39 interstripe and their absence in the stripe clearly indicates that interstripe marked cells
40 did not migrate, favoring the model of differentiation *in situ*. Additionally, we found that
41 the formation of secondary interstripes was characterized by the development of cells
42 newly expressing *pnp4* within this region, suggesting differentiation with subsequent
43 proliferation rather than active aggregation (**Extended Data Fig. 3**).

1 The above analyses focused on a region in the middle of the flank. Because
2 iridophore behaviors may differ between anatomical regions, we extended our analyses
3 by examining distributions of *pnp4*:mem-mCherry+ cells in entire, individual fish imaged
4 daily over 33 d. These analyses also revealed extensive differentiation of iridophores
5 without indications of EMT (**Extended Data Figs. 4 and 5a,b Supplementary Movie**
6 **s6**). In some instances, patches of dense iridophores anteriorly appeared to split
7 between primary interstripe and ventral secondary interstripes, perhaps owing to rapid
8 expansion of the flank directly over the swim bladder (**Extended Data Fig. 5d**;
9 **Supplementary Movie S7**). In a minority of larvae (~20%), patches of 5–10 closely
10 associated iridophores developed anteriorly, dorsal to the primary interstripe. Cells in
11 these patches sometimes maintained their tight associations and became incorporated
12 into the secondary dorsal interstripe. In other instances, such cells were incorporated
13 instead into the stripe (**Extended Data Figs. 5c,e**). Contrary to the expectations of the
14 morphogenetic respecification model^{8,23}, the few of these cells that transitioned from a
15 nascent dense morphology to a loose morphology occurred already within prospective
16 stripe regions. These observations highlight subtle region-specific differences in
17 patterning events and suggest that, had state transitions occurred in a majority of cells or
18 over a broader anatomical area, as predicted in the morphological respecification model,
19 they should have been observed. That they were not observed lends further support to
20 the model of differentiation *in situ*.

21 ***Role of melanophores in iridophore pattern remodeling***

22 Because melanophores reside in stripe- but not interstripe-zones, we wondered whether
23 iridophore pattern remodeling (i.e., dense- versus loose- arrangement morphology) is
24 impacted by melanophore presence. Prior work has hinted at this possibility as mutants
25 for melanophore-inducing transcription factor (*mitfa*), which lack melanophores, have the
26 dense-morphology iridophores (characteristic of interstripe zones) over a broader area
27 than in wild-type fish^{18,29}. To explore this further, we used a temperature-sensitive allele,
28 *mitfa*^{vc7}, that allows conditional differentiation or ablation of melanophores^{28,30,31} (**Fig.**
29 **2c**), and then examined the phenotypes of iridophores in different skin areas.

30 Cells in a defined area were marked by nucEos photoconversion and followed over
31 time after shifting between temperature regimes in order to assess whether iridophores
32 in newly arising stripes, or regions newly devoid of stripes, were derived either from
33 previously differentiated or newly differentiated cells. When fish were shifted from
34 restrictive temperature, where they lacked melanophores, to permissive temperature,
35 where melanophore differentiation could occur, we found that pre-existing, dense
36 morphology iridophores receded and new iridophores differentiated into a loose
37 arrangement in regions where melanophore differentiation had occurred (**Fig. 2d**;
38 **Extended Data Fig. 6a**). Reciprocal temperature shifts to ablate melanophores led to a
39 similar loss of pre-existing loose iridophores (through population turnover) and the
40 differentiation of new dense iridophores (**Extended Data Fig. 6b**). Though we cannot
41 exclude the possibility that some pre-existing iridophores were incorporated into
42 remodeled pattern elements, these results suggest that the presence of melanophores

1 has a major effect on the pattern remodeling of iridophores, specifically, in promoting a
2 loose morphology.

3 ***Distinct crystal morphology and ultrastructural organization but shared chemistry*** 4 ***of loose and dense iridophores***

5 Differences in iridophore morphologies (dense/cuboidal vs loose/stellate) and our failure
6 to observe transitions between these two states, raised the possibility that iridophores of
7 dense/cuboidal morphology in interstripes and loose/stellate morphology in stripes
8 represent distinct cell subtypes, analogous to neuronal subtypes³². To test this
9 possibility, we evaluated the subcellular architecture, physiology and gene expression of
10 dense/cuboidal iridophores in stripes versus loose loose/stellate iridophores in
11 interstripes.

12 Because iridophores depend for their iridescence on stacks of membrane-bound
13 reflecting platelets consisting of crystalline guanine^{5,16}, we first asked whether numbers,
14 sizes or arrangements of these crystals differ between iridophores found in interstripe
15 versus stripe regions. To visualize guanine crystals *in situ* required a reagent that would
16 adhere to guanine crystals and so screened 12 cell-permeable dyes, chosen for their
17 ability to form both hydrogen bonds and pi-stacking interactions. We found that
18 Malachite Green efficiently bound guanine and therefore used it to examine guanine
19 crystal organization in iridophores from interstripe versus stripe regions. Incident
20 illumination images of the stripe zone showed blue, loosely distributed iridophores on top
21 of black melanophores, whereas images of the interstripe zone showed dense silvery
22 iridophores covered by yellow xanthophores (**Fig. 3a,b**, incident illumination). Notably,
23 Malachite Green labeling of guanine crystals within iridophores in these two zones
24 revealed tightly stacked arrays of crystals in loose iridophores from the stripe but
25 markedly disordered arrays of crystals in dense iridophores from the interstripe (**Fig. 3a-**
26 **b**, upper panels).

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

37 Beyond differences in crystal arrangements, the shapes and sizes of crystals
38 appeared to differ between loose iridophores in stripes and dense iridophores in
39 interstripes. To quantify these differences, we isolated skin separately from stripes (**Fig.**
40 **3c**, left panel) and interstripes (**Fig. 3c**, right panel) and extracted crystals for
41 transmission electron microscopy (TEM) and electron diffraction (ED) analyses. While
42 the crystals in cells from both tissue regions comprised plates of β -guanine (**Fig. 3d**),
43 crystals isolated from stripe iridophores were smaller (3.9 ± 0.4 μm vs. 5.3 ± 0.9 μm) and

1 had smaller aspect ratios than crystals from interstripe iridophores ($1.9 \pm 0.2 \mu\text{m}$ vs. 2.5
2 $\pm 0.3 \mu\text{m}$; $n=57, 60$) (**Fig. 3e**). *In situ* Raman spectroscopy of individual cells further
3 validated that crystals in loose iridophores in stripe zones and dense iridophores in
4 interstripe zones consist of β -guanine, and failed, within the accuracy afforded by these
5 measurements, to reveal other components, suggesting that differences in crystal
6 morphology are not related to their chemistry (**Extended Data Fig. 7**).

7 ***Iridophore subtypes differ in their optical properties***

8 Differences in colors reflected by stripe iridophores (blue) and interstripe iridophores
9 (silvery-yellow) have sometimes been ascribed to influences of pigments contained
10 within melanophores and xanthophores, respectively. Given the differences we observed
11 in reflecting platelet architectures of iridophores from stripes versus interstripes,
12 however, we reasoned that reflected spectra might be intrinsic properties of iridophore
13 subtypes. Consistent with this hypothesis, we found close matches between spectra
14 predicted from simulations based on a Monte Carlo transfer matrix³³ (with morphometric
15 data derived from cryo-SEM) and empirical reflectance spectra recorded for individual
16 cells by hyperspectral imaging microscopy³⁴ (**Fig. 3f, g**). Indeed, simulations for ordered-
17 crystal iridophores from stripes predicted a peak in the blue region at 450 nm
18 approaching unity reflection, whereas simulations for disordered-crystal iridophores from
19 interstripes, predicted a broad wavelength reflection. In addition, while the reflection from
20 the ordered-crystal iridophores was highly dependent on the angle of incident light, the
21 reflection from disordered-crystal iridophores was not (**Extended Data Fig. 8**).

22 We further found that intrinsic differences in iridophore optical properties could
23 generate a strong contrast in color between stripes and interstripes independent of
24 pigments in other cell types. This was manifested in fish that lack both melanin in
25 melanophores and carotenoids in xanthophores, owing to mutations in both *tyrosinase*
26 and *scarb1*, respectively²⁵. Here, differences in color between stripes and interstripes
27 (i.e., blue versus silvery-yellow) persisted even in the absence of other pigments (**Fig.**
28 **3h**). Together, these results demonstrate the intrinsic differences in optical properties
29 between ordered-crystal iridophores of stripes and disordered-crystal iridophores from
30 interstripes.

31 ***Disordered-crystal and ordered-crystal containing iridophores remain distinct*** 32 ***throughout development***

33 To map the structural organization of iridophores across the entire skin pattern, we used
34 synchrotron-based micro X-ray diffraction, which allows large areas to be scanned while
35 still providing information on orientations and anisotropy of crystal arrays at the level of a
36 single cell. In this system, high angular distribution diffractions having a full-ring signal
37 are indicative of crystal orientations that vary (i.e., disordered), whereas low angular
38 distribution diffractions having a punctuated-ring signal are indicative of crystals that are
39 consistently oriented (i.e., ordered)¹⁵. Dorso-ventral line scanning across the flank of the
40 fish demonstrated there were consistent differences in structural organization of stripe
41 vs. interstripe regions (**Fig. 4a**). Specifically, based on their (012) and (002) diffraction

1 planes^{15,35}, crystal plates in iridophores of the stripe zone were well oriented (i.e.,
2 ordered) (**Figs. 4a**, panels 1 and 3), whereas those in the interstripe zone were non-
3 aligned (i.e., disordered) (**Figs. 4a**, panels 2 and 4).

4 Our *mitfa*^{vc7} photoconversion results (see **Fig. 2c**) raised the possibility that
5 melanophores promote the differentiation of progenitors into iridophores with ordered
6 crystal arrays. We tested this idea using micro X-ray diffraction to evaluate the crystals
7 architecture in iridophores of two zebrafish mutants: null-allele *mitfa*^{w2} and *albino*. In
8 *mitfa*^{w2} mutants, melanophores are missing owing to a defect in their specification; in
9 *albino* mutants, melanophores are present but lack melanin^{29,36}. We reasoned that if
10 melanophores drive iridophore differentiation towards the ordered crystallotype, then
11 *mitfa*^{w2} mutants should be deficient in iridophores having ordered crystals, whereas
12 *albino* mutants should retain ordered iridophores, similar to the wild type. Line scans
13 across the flanks of *mitfa*^{w2} fish revealed mostly high angular distribution (012)
14 diffractions, typical of the disordered crystallotype (**Fig. 4b**). Scanning the entire fish,
15 showed some diffraction patterns corresponding to ordered iridophores, but these were
16 located towards the posterior and were a minor component of the diffractions (**Extended**
17 **Data Fig. 9**). The same analysis on *albino* fish revealed alternating diffraction patterns
18 similar to that seen in wild-type (**Fig. 4c**). These results suggest that melanophores
19 enhance the differentiation of ordered crystallotype iridophores.

20 We next examined the relative developmental timing of precursor differentiation into
21 disordered and ordered crystallotypes by assessing micro X-ray diffraction patterns over
22 ontogeny. In fish of 6.0 mm standardized standard length (SSL) and 6.5 SSL, which
23 have only a single interstripe and very few adult melanophores²⁶, we observed only
24 disordered crystallotype iridophores (having high angular distribution diffraction patterns
25 of the (012) plane) (**Fig. 4d**; **Extended Data Fig. 10**). When fish were ~6.9 SSL, with a
26 substantial complement of melanophores and loose iridophores, low angular distribution
27 diffraction patterns of the (002) plane, typical of ordered crystallotype iridophores,
28 became visible (**Fig. 4e**). These results supported the idea that precursor cells
29 differentiate into ordered crystallotype iridophores only after the differentiation of
30 disordered-crystallotype iridophores and in the presence of melanophores.

31 ***Ordered and disordered crystallotypes exhibit distinct transcriptomic signatures***

32
33 We next compared the transcriptomic signatures of ordered and disordered crystallotype
34 iridophores (i.e., from stripes and interstripes) by single cell RNA-sequencing (scRNA-
35 seq) (**Fig. 5a**). Dimensionality reduction followed by unsupervised clustering revealed six
36 clusters, three of which (clusters 4–6) contained cells expressing known markers of
37 iridophores³⁷ (i.e., *tfec*, *gpnmb*) (**Fig. 5b**). Expression of purine synthesis pathway
38 genes, markedly upregulated²⁵ in iridophores, further validated this initial assignment
39 (**Extended Data Fig. 11**). Next, we tested whether iridophore clusters recovered by
40 scRNA-seq corresponded to their anatomical sites of origin. Supporting this possibility,
41 98% of cells in cluster 5 originated from interstripes, and 85% of cells in cluster 4
42 originated from stripes (**Fig. 5c**). Cells of cluster 6 were split between interstripe (63%)
43 and stripe (37%) (**Extended Data Fig. 12**). Pseudotemporal ordering showed that

1 ordered-crystal iridophores and disordered-crystal iridophores were associated with
2 different branches in trajectories of inferred differentiation, whereas cluster 6 iridophores
3 spanned across branches (**Fig. 5d**). Several hundred loci were differentially expressed
4 between cells of clusters 4 and 5 (**Fig. 5e** and **Supporting information**), suggesting
5 candidate genes that may contribute to structural or other differences between stripe and
6 interstripe iridophores. We concluded from these analyses that iridophores of interstripe
7 and stripe zones are transcriptionally distinct.

8 9 ***Physiological color change differs between iridophore subtypes***

10 Color pattern can be influenced physiologically^{38,39}, as some types of pigment cells
11 disperse or contract pigment granules in response to endocrine and neuroendocrine
12 factors, including norepinephrine (NE)⁴⁰⁻⁴². We wondered whether differential
13 responses from the iridophore subtypes might contribute to this response. To test this,
14 we bathed isolated fish skin in 10 μ M NE solution. Upon NE treatment, interstripe-
15 derived iridophores exhibited a \sim 120 nm shift in peak reflection, whereas stripe-derived
16 iridophores exhibited only a minor change in their overall organization of crystals and
17 spectrum of reflected light measured by hyperspectral imaging (**Fig. 5f**). Viewing the
18 response to NE in the context of the whole tissue also revealed a preferential color
19 change in striped- versus interstripe-zones (**Fig. 5g**). These results support prior work
20 showing that iridophores of different shapes respond differently to NE⁴⁰, and further
21 demonstrate that only stripe iridophores change their color upon NE treatment. This
22 differential response, in conjunction with the known aggregation of granules within
23 melanophores and xanthophores^{41,42}, could contribute to the dramatic reduction in
24 contrast between stripe and interstripe zones under NE treatment, which causes the
25 prominent zebrafish stripe pattern to diminish (**Supplementary movie 8**).

26 **Discussion**

27 Pigmentation of teleost fish has become a valuable system for understanding pattern
28 formation in animals, including how changes in pattern-forming mechanisms lead to
29 phenotypic variation within and between species^{4,7,11}. In zebrafish, a widely accepted
30 model links dynamic changes in iridophore shape—between a dense morphology in
31 interstripes and a loose morphology in stripes—to establishment and reiteration of
32 pattern^{8,9,23}. In this model, iridophores of interstripes and stripes are similar cells that
33 have merely adopted different morphologies as they migrate into different regions. Our
34 findings of substantial differences in ultrastructure, physiology and transcriptomic state of
35 iridophore subtypes—and the absence of morphological transitions predicted for
36 individual iridophores—support an alternative model of differentiation *in situ* for how the
37 reiterated stripe pattern of zebrafish develops. In this model, iridophore precursors in
38 developing stripes and interstripes differentiate *in situ* into distinct iridophore
39 crystalotypes with different subcellular organization and physiological responsiveness
40 based on their micro- environment.

41 Strongly supporting a model of differentiation *in situ* was our finding of substantial
42 physiological disparities between iridophores of stripes and interstripes. In particular,

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

11 We found no evidence for another key prediction of the prior model, namely that
12 individual iridophores should undergo state transitions as cells originating in one pattern
13 element disperse to populate another. Photo-labeled iridophores observed over short or
14 long periods failed to migrate between interstripe and stripe zones, even when they were
15 challenged to undergo transitions in the context of pattern remodeling (stimulated by
16 changes in melanophore abundance). Instead, development and remodeling of
17 interstripes and stripes involved the *in situ* differentiation, subsequent proliferation, and
18 in some cases migration of iridophores with morphologies appropriate to their location
19 within the pattern. Only in a minority of fish, and in a small anatomical region, did we
20 observe patches of initially dense iridophores assume a loose arrangement. Such
21 behaviors occurred within prospective stripes, rather than at boundaries between
22 interstripes and stripes, as previously postulated, and involved cells that had not yet fully
23 differentiated.

24 These data all point to a model of stripe patterning that depends on differentiation *in*
25 *situ*. In this model, latent progenitors associated with the peripheral nervous system that
26 have transited to the skin during the larva-to-adult transformation expand clonally as
27 iridoblasts—not specified to subtype—and subsequently differentiate according to cues
28 in the microenvironment they encounter. Our data, together with those of others, suggest
29 that some of these signals depend on melanophores^{18,19,27,43}, promoting differentiation of
30 iridoblasts towards a state having ordered reflecting crystal platelets that are
31 physiologically responsive (blue ↔ yellow) in stripes, and away from an alternative state
32 of disordered crystal platelets lacking physiological responsiveness (silvery-yellow) in
33 interstripes. Additional signals from xanthophores, iridophores, and other cell types likely
34 contribute as well. Whether these events of specification unfold as iridoblasts expand
35 their territory within the plane of the skin hypodermis, or as they arrive at the hypodermis
36 after migrating from progenitor niches within the peripheral nervous system, remains to
37 be determined. Whichever mode of iridoblast morphogenesis holds true, our findings
38 highlight the importance of extrinsic factors that specify and promote the *in situ*
39 differentiation of iridophore subtypes during pattern establishment and reiteration. The
40 resulting iridophore subtypes likely allow the zebrafish to alter its skin patterning to make
41 it more or less distinctive, a trait crucial for the fish to be able to join shoals or obscure
42 itself^{1,44}.

43
44

1 **Acknowledgements**

2 We thank Gil Levkowitz and Dan Oron, for their help and advice, Ivo Zizak for his help
3 with the diffraction measurements at the μ -Spot beamline in BESSY II. We thank Kathy
4 Schaefer, Lihua Wang and Andrew Lemire, for their help with FACS, and scRNA-seq.
5 We thank Rick Huang and Zhiheng Yu, for their help with ED. We thank HZB for the
6 allocation of synchrotron radiation beamtime. We thank members of the Lippincott-
7 Schwartz lab for their support. This work was supported by Howard Hughes Medical
8 Institute to JLS and NIH R35 GM122471 to DMP. EJB was additionally supported by
9 NIH T32 HD007183, DG was additionally supported by the Human Frontiers Cross-
10 Disciplinary Postdoctoral Fellowship LT000767/2018, the European Molecular Biology
11 Organization Long Term Fellowship and the Rothchild Fellowship. Hyperspectral
12 analyses, MCA and DDD were supported by a Multidisciplinary University Research
13 Initiative (MURI) - Melanin Grant, AFOSR FA9550-18-1-0142 (to DDD).

14 **Author contributions**

15 Author contributions: D.G, E.J.B, D.D.D, J.C.L D.M.P., and J.L.-S. designed
16 research; D.G, E.J.B, A.J.A, A.P, J.D.F, M.C.A, performed research; D.G, E.J.B, K.J,
17 J.D.F, D.D.D, J.C.L D.M.P., and J.L.-S. analyzed data; and D.G, E.J.B, D.M.P., and J.L.-
18 S. wrote the paper.

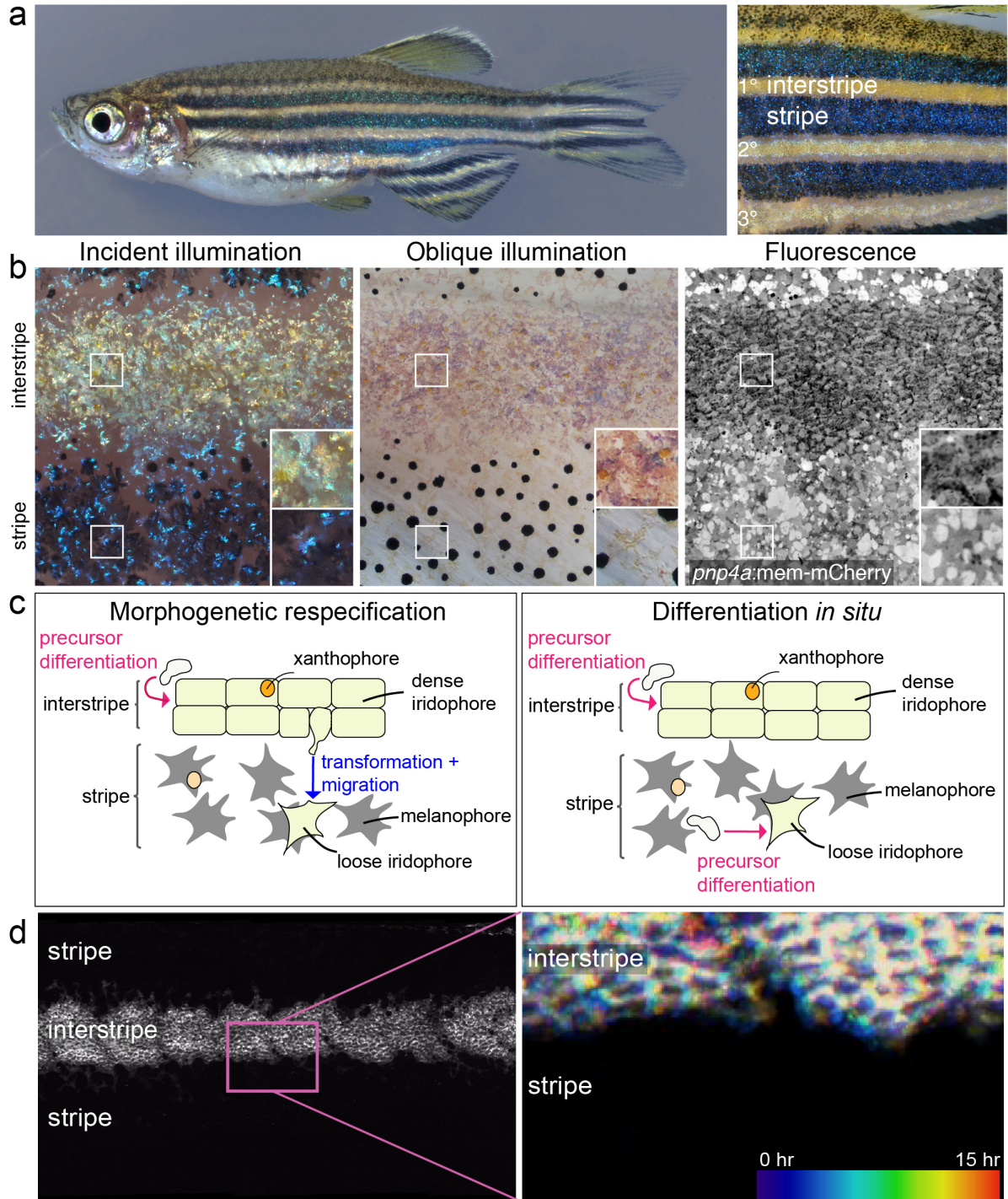
19 **References**

- 20
21
22 1 Rosenthal, G. G. & Ryan, M. J. Assortative preferences for stripes in danios. *Anim.*
23 *Behav.* **70**, 1063-1066, doi:<https://doi.org/10.1016/j.anbehav.2005.02.005> (2005).
- 24 2 Endler, J. A. in *Evolutionary Biology* (eds Max K. Hecht, William C. Steere, & Bruce
25 Wallace) 319-364 (Springer US, 1978).
- 26 3 Kapp, F. G. *et al.* Protection from UV light is an evolutionarily conserved feature of the
27 haematopoietic niche. *Nature* **558**, 445-448, doi:10.1038/s41586-018-0213-0 (2018).
- 28 4 Patterson, L. B. & Parichy, D. M. Zebrafish pigment pattern formation: insights into the
29 development and evolution of adult form. *Annu. Rev. Genet.* **53**, 505-530,
30 doi:10.1146/annurev-genet-112618-043741 (2019).
- 31 5 Schartl, M. *et al.* What is a vertebrate pigment cell? *Pigment Cell Melanoma Res.* **29**, 8-
32 14, doi:10.1111/pcmr.12409 (2016).
- 33 6 Schweisguth, F. & Corson, F. Self-Organization in Pattern Formation. *Dev. Cell* **49**, 659-
34 677, doi:10.1016/j.devcel.2019.05.019 (2019).
- 35 7 Irion, U., Singh, A. P. & Nusslein-Volhard, C. The Developmental Genetics of Vertebrate
36 Color Pattern Formation: Lessons from Zebrafish. *Curr. Top. Dev. Biol.* **117**, 141-169,
37 doi:10.1016/bs.ctdb.2015.12.012 (2016).
- 38 8 Singh, A. P., Schach, U. & Nusslein-Volhard, C. Proliferation, dispersal and patterned
39 aggregation of iridophores in the skin prefigure striped colouration of zebrafish. *Nat. Cell.*
40 *Biol.* **16**, 607-614, doi:10.1038/ncb2955 (2014).
- 41 9 Fadeev, A., Krauss, J., Frohnofer, H. G., Irion, U. & Nusslein-Volhard, C. Tight Junction
42 Protein 1a regulates pigment cell organisation during zebrafish colour patterning. *Elife* **4**,
43 doi:10.7554/eLife.06545 (2015).

- 1 10 Dahmann, C., Oates, A. C. & Brand, M. Boundary formation and maintenance in tissue
2 development. *Nat. Rev. Genet.* **12**, 43-55, doi:10.1038/nrg2902 (2011).
- 3 11 Kondo, S. & Watanabe, M. Black, yellow, or silver: which one leads skin pattern
4 formation? *Pigment Cell Melanoma Res.* **28**, 2-4, doi:10.1111/pcmr.12328 (2015).
- 5 12 Eskova, A., Frohnhöfer, H. G., Nüsslein-Volhard, C. & Irion, U. Galanin Signaling in the
6 Brain Regulates Color Pattern Formation in Zebrafish. *Curr. Biol.* **30**, 298-303.,
7 doi:<https://doi.org/10.1016/j.cub.2019.11.033> (2020).
- 8 13 Hirata, M., Nakamura, K. & Kondo, S. Pigment cell distributions in different tissues of the
9 zebrafish, with special reference to the striped pigment pattern. *Dev. Dyn.* **234**, 293-300,
10 doi:10.1002/dvdy.20513 (2005).
- 11 14 Hirata, M., Nakamura, K., Kanemaru, T., Shibata, Y. & Kondo, S. Pigment cell
12 organization in the hypodermis of zebrafish. *Dev. Dyn.* **227**, 497-503,
13 doi:10.1002/dvdy.10334 (2003).
- 14 15 Gur, D. *et al.* The Dual Functional Reflecting Iris of the Zebrafish. *Adv. Sci.* **5**,
15 doi:10.1002/advs.201800338 (2018).
- 16 16 Gur, D., Palmer, B. A., Weiner, S. & Addadi, L. Light manipulation by guanine crystals in
17 organisms: biogenic scatterers, mirrors, multilayer reflectors and photonic crystals. *Adv.*
18 *Funct. Mater.* **27**, doi:10.1002/adfm.201603514 (2017).
- 19 17 Krauss, J. *et al.* Endothelin signalling in iridophore development and stripe pattern
20 formation of zebrafish. *Biol. Open* **3**, 503-509, doi:10.1242/bio.20148441 (2014).
- 21 18 Frohnhofer, H. G., Krauss, J., Maischein, H. M. & Nusslein-Volhard, C. Iridophores and
22 their interactions with other chromatophores are required for stripe formation in zebrafish.
23 *Development* **140**, 2997-3007, doi:10.1242/dev.096719 (2013).
- 24 19 Patterson, L. B. & Parichy, D. M. Interactions with iridophores and the tissue environment
25 required for patterning melanophores and xanthophores during zebrafish adult pigment
26 stripe formation. *PLoS Genet.* **9**, e1003561, doi:10.1371/journal.pgen.1003561 (2013).
- 27 20 Parichy, D. M. *et al.* Mutational analysis of endothelin receptor b1 (rose) during neural
28 crest and pigment pattern development in the zebrafish *Danio rerio*. *Dev. Biol.* **227**, 294-
29 306, doi:10.1006/dbio.2000.9899 (2000).
- 30 21 Patterson, L. B., Bain, E. J. & Parichy, D. M. Pigment cell interactions and differential
31 xanthophore recruitment underlying zebrafish stripe reiteration and *Danio* pattern
32 evolution. *Nat. Commun.* **5**, 5299, doi:10.1038/ncomms6299 (2014).
- 33 22 Spiewak, J. E. *et al.* Evolution of Endothelin signaling and diversification of adult pigment
34 pattern in *Danio* fishes. *PLoS Genet.* **14**, e1007538, doi:10.1371/journal.pgen.1007538
35 (2018).
- 36 23 Volkening, A. & Sandstede, B. Iridophores as a source of robustness in zebrafish stripes
37 and variability in *Danio* patterns. *Nat. Commun.* **9**, 3231, doi:10.1038/s41467-018-05629-
38 z (2018).
- 39 24 McGuirl, M. R., Volkening, A. & Sandstede, B. Topological data analysis of zebrafish
40 patterns. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 5113-5124, doi:10.1073/pnas.1917763117
41 (2020).
- 42 25 Saunders, L. M. *et al.* Thyroid hormone regulates distinct paths to maturation in pigment
43 cell lineages. *eLife* **8**, e45181 (2019).

- 1 26 Parichy, D. M., Elizondo, M. R., Mills, M. G., Gordon, T. N. & Engeszer, R. E. Normal
2 table of postembryonic zebrafish development: staging by externally visible anatomy of
3 the living fish. *Dev. Dyn.* **238**, 2975-3015, doi:10.1002/dvdy.22113 (2009).
- 4 27 McMenamin, S. K. *et al.* Thyroid hormone-dependent adult pigment cell lineage and
5 pattern in zebrafish. *Science* **345**, 1358-1361, doi:10.1126/science.1256251 (2014).
- 6 28 Lewis, V. M. *et al.* Fate plasticity and reprogramming in genetically distinct populations of
7 Danio leucophores. *Proc. Natl. Acad. Sci. U.S.A* **116**, 11806-11811,
8 doi:10.1073/pnas.1901021116 (2019).
- 9 29 Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L. & Raible, D. W. nacre encodes
10 a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment
11 cell fate. *Development* **126**, 3757-3767 (1999).
- 12 30 Zeng, Z., Johnson, S. L., Lister, J. A. & Patton, E. E. Temperature-sensitive splicing of
13 mitfa by an intron mutation in zebrafish. *Pigment Cell Melanoma Res.* **28**, 229-232,
14 doi:10.1111/pcmr.12336 (2014).
- 15 31 Johnson, S. L., Nguyen, A. N. & Lister, J. A. mitfa is required at multiple stages of
16 melanocyte differentiation but not to establish the melanocyte stem cell. *Dev. Biol.* **350**,
17 405-413, doi:10.1016/j.ydbio.2010.12.004 (2011).
- 18 32 Zeng, H. & Sanes, J. R. Neuronal cell-type classification: challenges, opportunities and
19 the path forward. *Nat. Rev. Neurosci.* **18**, 530-546, doi:10.1038/nrn.2017.85 (2017).
- 20 33 Gur, D., Leshem, B., Oron, D., Weiner, S. & Addadi, L. The structural basis for enhanced
21 silver reflectance in Koi fish scale and skin. *J. Amer. Chem. Soc.* **136**, 17236-17242,
22 doi:10.1021/Ja509340c (2014).
- 23 34 Holzinger, A., Allen, M. C. & Deheyn, D. D. Hyperspectral imaging of snow algae and
24 green algae from aeroterrestrial habitats. *J. Photochem. Photobiol. B* **162**, 412-420,
25 doi:10.1016/j.jphotobiol.2016.07.001 (2016).
- 26 35 Gur, D. *et al.* Guanine-based photonic crystals in fish scales form from an amorphous
27 precursor. *Angew. Chem. Int. Ed.* **52**, 388-391, doi:10.1002/anie.201205336 (2013).
- 28 36 Dooley, C. M. *et al.* Slc45a2 and V-ATPase are regulators of melanosomal pH
29 homeostasis in zebrafish, providing a mechanism for human pigment evolution and
30 disease. *Pigment Cell Melanoma Res.* **26**, 205-217, doi:10.1111/pcmr.12053 (2013).
- 31 37 Lister, J. A., Lane, B. M., Nguyen, A. & Lunney, K. Embryonic expression of zebrafish
32 MiT family genes tfe3b, tfeb, and tfec. *Dev. Dyn.* **240**, 2529-2538,
33 doi:10.1002/dvdy.22743 (2011).
- 34 38 Gur, D. *et al.* Light-induced color change in the Sapphirinid copepods: tunable photonic
35 crystals. *Adv. Funct. Mat.* **26**, 1393-1399, doi:10.1002/adfm.201504339 (2016).
- 36 39 Gur, D. *et al.* The mechanism of color change in the Neon Tetra fish: a light-induced
37 tunable photonic crystal array. *Angew. Chem. Int. Edit.* **54**, 12426-12430,
38 doi:10.1002/anie.201502268 (2015).
- 39 40 Oshima, N. & Kasai, A. Iridophores involved in generation of skin color in the zebrafish,
40 Brachydanio rerio. *FORMA-TOKYO-* **17**, 91-101 (2002).
- 41 41 Fujii, R. The regulation of motile activity in fish chromatophores. *Pigment. Cell. Res.* **13**,
42 300-319 (2000).
- 43 42 Fujii, R. Cytophysiology of fish chromatophores. *Int. Rev. Cytol.* **143**, 191-255 (1993).

- 1 43 Mahalwar, P., Singh, A. P., Fadeev, A., Nusslein-Volhard, C. & Irion, U. Heterotypic
2 interactions regulate cell shape and density during color pattern formation in zebrafish.
3 *Biol. Open*. **5**, 1680-1690, doi:10.1242/bio.022251 (2016).
- 4 44 Engeszer, R. E., Wang, G., Ryan, M. J. & Parichy, D. M. Sex-specific perceptual spaces
5 for a vertebrate basal social aggregative behavior. *Proceedings of the National Academy
6 of Sciences of the United States of America* **105**, 929-933, doi:10.1073/pnas.0708778105
7 (2008).
- 8 45 Eom, D. S., Bain, E. J., Patterson, L. B., Grout, M. E. & Parichy, D. M. Long-distance
9 communication by specialized cellular projections during pigment pattern development
10 and evolution. *Elife* **4**, doi:10.7554/eLife.12401 (2015).
11
12
13



1

2

Figure 1. Anatomy, development and models of zebrafish adult pigment

3

patterning. (a) Left panel, an adult zebrafish showing light interstripes with intervening

4

dark stripes. Right panel, a closeup showing the primary interstripe (1°) which develops

5

first with stripes above and below, followed by secondary interstripes ventrally (2°)

6

and dorsally with additional stripes, and ultimately a tertiary (3°) interstripe and

7

stripe. **(b)** Closeups of first-forming (primary) interstripe and stripes, illustrating overall

8

features as well as morphologies and arrangements of iridophores. All panels are the

1 same location in a single animal. Left panel is incident illumination showing iridescence
2 of iridophore-reflecting platelets with yellowish tinge in the interstripe and bluish tinge in
3 the stripe. Center panel is oblique illumination revealing surface features and non-
4 iridescent colors of iridophores. Right panel is membrane-targeted mCherry (mem-
5 mCherry) driven at high levels in iridophores by regulatory elements of *purine nucleoside*
6 *phosphorylase 4a (pnp4a)* (Eom et al., 2015⁴⁵; Saunders et al., 2019²⁵; Spiewak et al.,
7 2018²²) to reveal cell boundaries and arrangements. Pixel values are inverted for easier
8 comparison to brightfield images. **(c)** Two models for iridophore patterning in interstripes
9 and stripes. In the morphogenetic respecification model (left panel), initially densely-
10 packed, cuboidal iridophores begin adopting a loose morphology as they and their
11 progeny migrate out to populate the prospective stripe. In the differentiation *in situ* model
12 (right panel), iridophores residing in interstripes and stripes are different cell types that
13 have differentiated ‘in place’ from a precursor population. Hence, loose iridophores in
14 stripes are not lineally related to dense iridophores in interstripes. **(d)** The flank of a 7.5
15 standardized standard length (SSL) *pnp4a: mem-mCherry* fish. Left panel; fluorescence
16 image showing the arrangement of labeled cells in the dense primary interstripe. Right
17 panel; pseudo-temporal coloring representation of a 15 hour time-lapse movie (zoomed
18 to the region outlined in ‘d’) revealing that interstripe iridophores migrate primarily in the
19 anteroposterior direction, with no apparent dorsoventral migration into the stripe region.
20
21
22
23
24

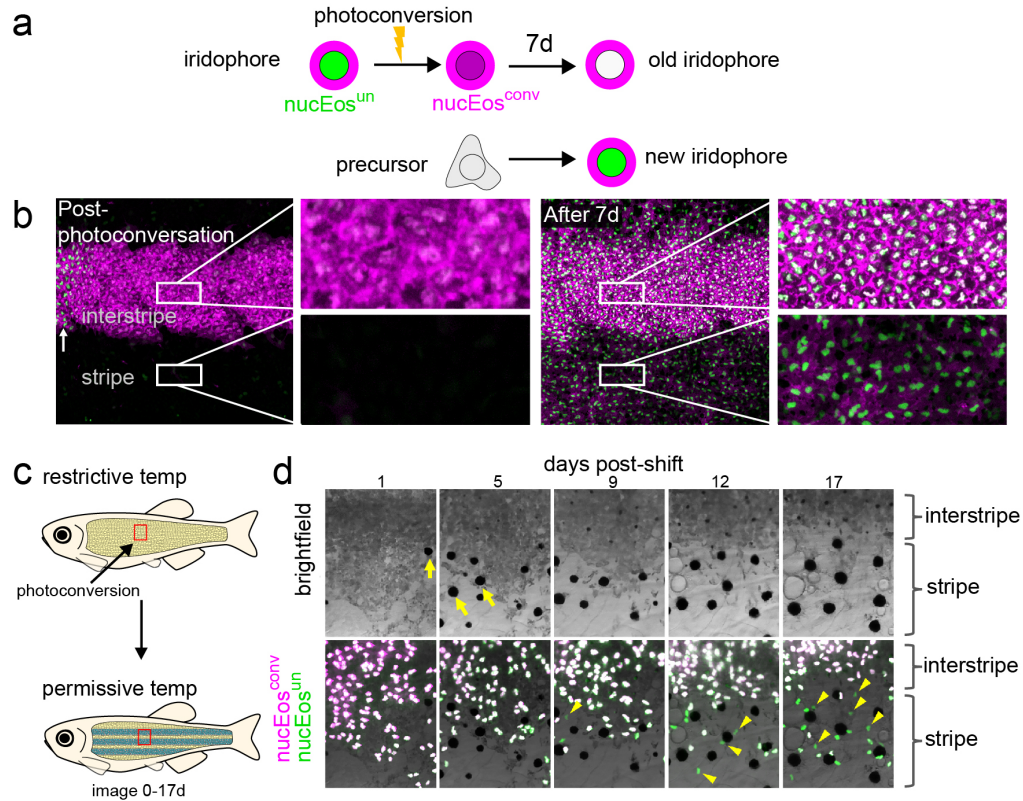
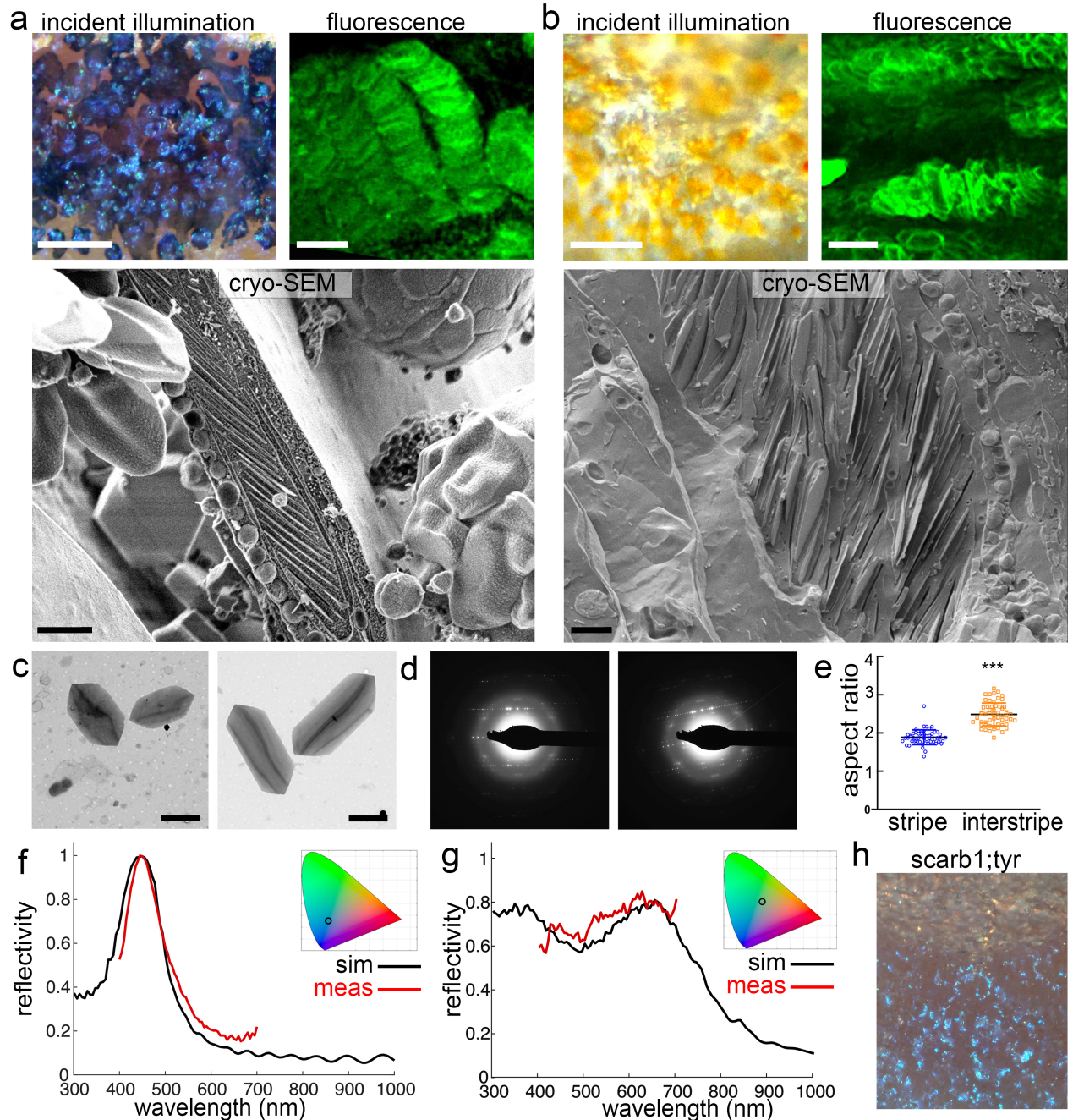


Figure 2. Photoconversion experiments to test models of pattern development and remodeling.

(a) Fish were created that have iridophores expressing nuclear localizing Eos ($nucEos^{un}$, green) and a membrane-targeted mCherry (mem-Cherry, magenta) driven by regulatory elements of *pnp4a*. Following photoconversion of an iridophore population, the converted nuclei will appear magenta ($nucEos^{conv}$). After 7 days, previously photoconverted nuclei will appear white (due to the combination of 'new' green proteins and 'old' magenta proteins), whereas nuclei of newly differentiated cells will appear green. (b) Tracking photoconverted iridophores in the interstripe revealed stripe iridophores do not derive from the interstripe population. Following photoconversion of a region in the primary interstripe of a fish at 7.5 SSL, all nuclei appeared magenta (Post-photoconversion), with surrounding mem-Cherry labeled plasma membrane magenta-colored as well (left panel). After seven days of additional development (8.6 SSL), at which time iridophores now populate the primary stripe, only nuclei with green signal are seen in the stripe zone, whereas interstripe nuclei are primarily white (right panel). In zoom-up images, interstripe iridophores are seen to have continued to proliferate and retained $nucEos^{conv}$, acquiring new $nucEos^{un}$ (making their nuclei white) (upper inset, right panel). Stripe iridophores, by contrast, lacked $nucEos^{conv}$ and expressed only $nucEos^{un}$ (making their nuclei green) (lower inset, right panel). (c) Use of a temperature-sensitive *mitfa*^{vc7} fish to examine the effect of conditional melanophore development on iridophore pattern remodeling. In this experimental setup, iridophores were labeled only with a nuclear-localizing Eos ($nucEos^{un}$, green), so that following photoconversion their converted nuclei would appear magenta ($nucEos^{conv}$). (d)

1 Brightfield (upper) and fluorescence superimposed on brightfield (lower) following
2 photoconversion and shift to permissive temperature to drive onset of melanophore
3 differentiation. Iridophores labeled by nucEos expression were photoconverted at the
4 beginning of the experiment and followed over 17 days to distinguish newly
5 differentiating iridophores (green nuclei) from previously differentiated iridophores
6 (white). As melanophores differentiated (see yellow arrows in top panel), the region of
7 dense morphology iridophores receded dorsally. This change was accompanied by
8 differentiation of new iridophores having green nuclei (see yellow arrowheads in bottom
9 panel) in the newly forming stripe.
10
11



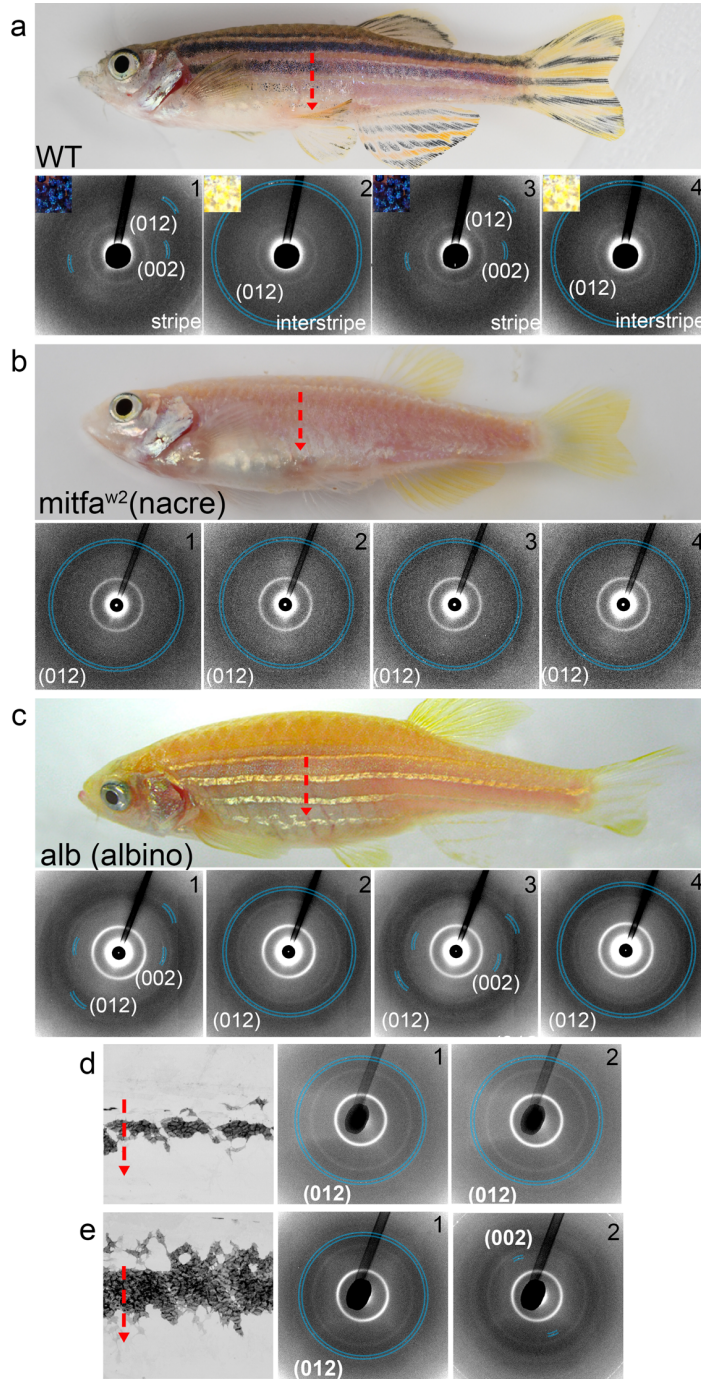
1
2
3
4
5
6
7
8
9
10
11

Figure 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 with highly ordered arrays of guanine crystals; and the Cryo-SEM image shows iridophore cytoplasm with highly disordered arrays of crystals.

(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

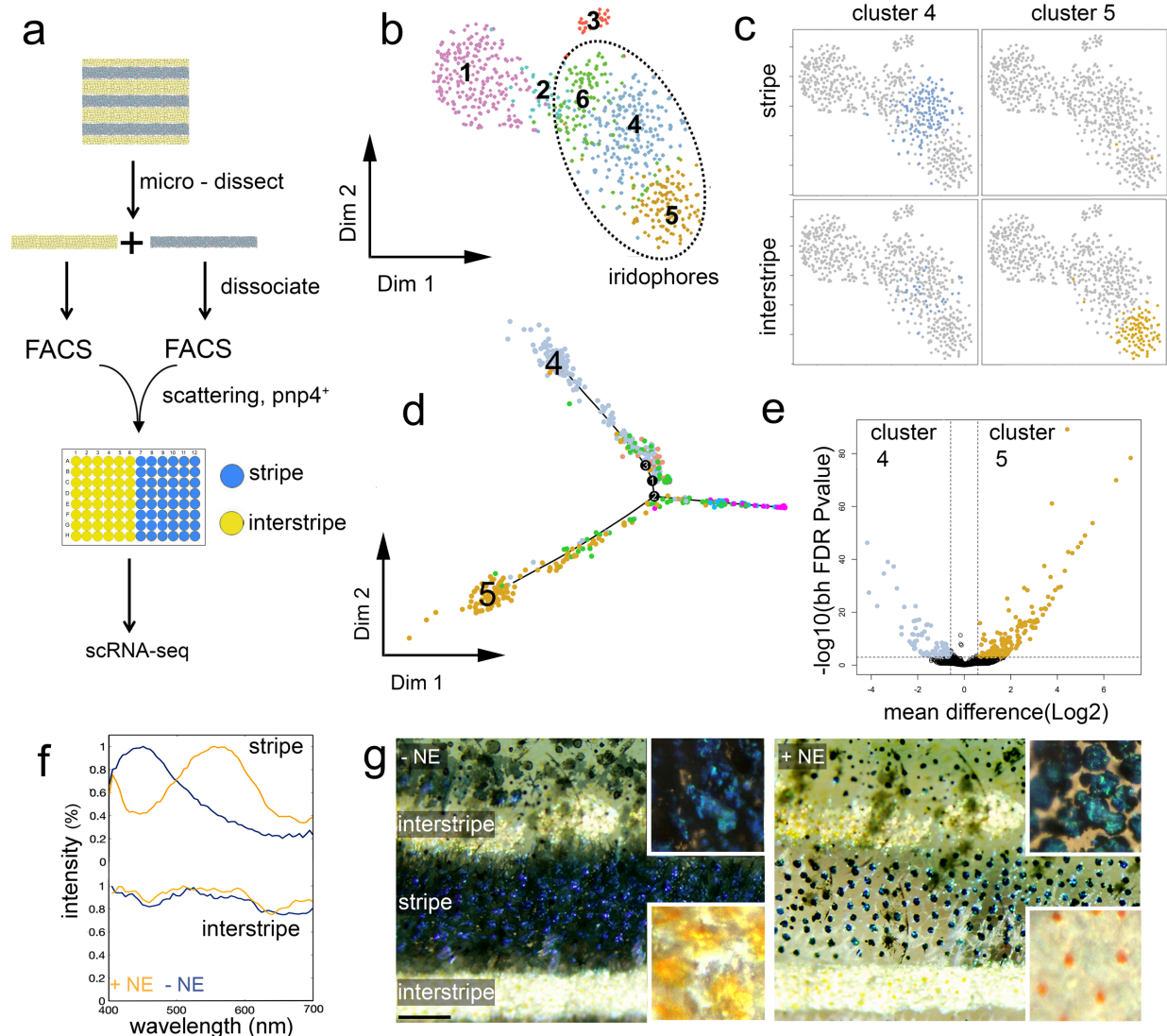
1 disordered arrays of guanine crystals; and the high pressure–frozen, freeze-fractured
2 cryo-SEM micrograph shows iridophore cytoplasm with disordered arrangements of
3 crystals. **(c-e)** TEM analysis of crystals isolated from iridophores from either the stripe or
4 the interstripe regions. (c), TEM micrographs of crystals isolated from stripe (left panel)
5 and interstripe regions (right panel); (d), TEM-based electron diffraction of crystals
6 isolated from stripe iridophores (left panel) and interstripe iridophores (right panel); (e)
7 graph of aspect ratio (length/width) of stripe iridophores (blue) and interstripe iridophores
8 (orange), $p < 0.0001$. **(f)** Simulated reflection (black) and measured reflection (red) from a
9 stripe iridophore. **(g)** Simulated reflection (black) and measured reflection (red) from an
10 interstripe iridophore. Insets in both (f) and (g) show the corresponding reflectance color
11 on a CIE (International Commission on Illumination) chromaticity space diagram. **(h)**
12 Incident illumination image of an adult fish lacking melanin in melanophores and
13 carotenoids in xanthophores due to mutations in *tyrosinase* and *scarb1*, respectively.
14 The image shows iridophore-type specific coloration is independent of melanin and
15 carotenoids, consistent with reflectance data obtained for stripe (f) and interstipe
16 iridophores (g).



1
2

3 **Figure 4. Disordered- and ordered- crystal-containing iridophores remain distinct**
4 **throughout development. (a)** Upper panel shows wildtype zebrafish with the red
5 vertical dotted arrow showing where X-ray diffraction measurements were made. Lower
6 panels 1 through 4 show X-ray diffraction pattern measurements in stripe and interstripe
7 regions, with upper left insets showing the incident illumination differences in these
8 regions. Diffraction patterns collected in the stripe regions (1 and 3) had low angular
9 distributions with a punctuated-like signal, indicating iridophore crystals in these regions

1 are parallel to one another. Diffraction patterns collected in the interstripe regions (2 and
2 4), by contrast, exhibited high angular distributions with a full-ring signal, indicating
3 iridophore crystals in these regions are not well aligned. **(b)** X-ray diffraction
4 measurements as in (a) made in *Mitfa-w2* mutant (nacre) fish using a vertical line scan
5 across the trunk of the fish. The typical diffraction pattern of the ordered stripe iridophore
6 is missing in this line scan, and the observed diffractions are of high angular distribution
7 (“full ring”). **(c)** X-ray diffraction measurements as in (a) made *albino* mutant (*alb*). The
8 overall diffraction pattern resembles that of wild type fish, with highly ordered diffraction
9 patterns of the (002) and (012) diffraction planes throughout the stripe region (1 and 3),
10 and high angular distribution of only the (012) diffraction plane throughout the interstripe
11 region (2 and 4). **(d)** X-ray diffraction patterns from a vertical line measured across the
12 trunk of a ~6 SSL wild-type zebrafish. Panels 1 and 2 show X-ray diffraction patterns
13 from areas in the 1° interstipe and adjacent to the 1° interstipe, respectively. Both
14 patterns show a high angular distribution of the (012) diffraction plane. **(e)** X-ray
15 diffraction patterns from a vertical line measured across the trunk of a ~6.9 SSL wild-
16 type zebrafish. Panels 1 and 2 show X-ray diffraction patterns from areas in the 1°
17 interstipe and adjacent to the 1° interstiperespectively. A low angular distribution
18 diffraction of the (002) plane (2) is visible just adjacent to the first interstripe region (1).
19
20
21



1
 2 **Figure 5. Disordered- and ordered- crystal-containing iridophores exhibit distinct**
 3 **transcriptomic signatures and response to stimuli. (a)** Experimental design of single
 4 cell RNA sequencing experiment. **(b)** Two-dimensional tSNE representation of the
 5 collected skin cell clusters (dashed ellipse marks iridophores). **(c)** Anatomical origin of
 6 iridophores clusters. **(d)** Pseudo-time trajectory of the collected skin cells. **(e)** A volcano
 7 representation of differentially expressed genes between clusters 4 (light blue) and 5
 8 (orange). **(f-g)** The response of an adult zebrafish skin pattern to norepinephrine (NE)
 9 stimuli. **(f)** is the optical response of individual iridophores from the stripe (upper panel)
 10 and interstripe (lower panel). In the stripe, the reflection peak of an ordered iridophore
 11 shifts from ~450 nm to ~570 nm upon NE treatment. In the interstripe, only minor
 12 changes in the reflection spectra occur in response to NE. **(g)** is the optical response of
 13 the relaxed, untreated fish (-NE) compared to the treated fish (+NE). Note the contrast
 14 between the blue stripe and the two-flanking yellow interstripes and how this changed
 15 with NE treatment. Before treatment, a deep blue color for the stripe region and a
 16 golden-yellow color for the interstripe region is observed. After NE treatment, the

1 contrast between the stripe and the interstripe is drastically reduced. This color change
2 arises because NE causes melanophores in the stripe to aggregate and blue iridophore
3 reflectance to shift from a dark-blue to green-yellow hue (see upper insets), while in the
4 interstripe, NE causes xanthophores to aggregate and silvery iridophores to have only a
5 minor color change (lower insets).

6

7

8