Title Zebrafish adult pigment stem cells are multipotent and form pigment cells by a progressive fate restriction process
Subtitle Clonal analysis identifies shared origin of all pigment cell types

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Abbreviations: CNS, central nervous system; dpf, days post fertilisation; DRGs, dorsal root ganglia; hpf, hours post fertilisation; PNS, peripheral nervous system.

Summary
Skin pigment pattern formation is a paradigmatic example of pattern formation. In zebrafish the adult body stripes are generated by coordinated rearrangement of three distinct pigment cell-types, black melanocytes, shiny iridophores and yellow xanthophores. A stem cell origin of melanocytes and iridophores has been proposed although the potency of those stem cells has remained unclear. Xanthophores, however, seemed to originate predominantly from proliferation of embryonic xanthophores. Now, data from Singh et al. shows that all three cell-types derive from shared stem cells, and that these cells generate peripheral neural cell-types too. Furthermore, clonal compositions are best explained by a progressive fate restriction model generating the individual cell-types. The numbers of adult pigment stem cells associated with the dorsal root ganglia remain low, but progenitor numbers increase significantly during larval development up to metamorphosis, likely via production of partially-restricted progenitors on the spinal nerves.

Introduction
The problem of pattern formation – how specific cell-types are organised into cohesive functional units – is a fundamental problem of developmental biology. At the forefront of systems in which to identify the mechanistic basis for pattern formation is the field of pigment pattern formation, which aims to identify the mechanisms ensuring that pigmentation in the skin is correctly distributed. This process is all-the-more fascinating because 1) these patterns are tremendously variable even between closely-related species and hence can be fast-evolving, and 2) they are often stunningly beautiful.
Zebrafish as a model for studying pigment pattern formation

The zebrafish has rapidly become the model system par excellence for study of this phenomenon, principally because its naturally striking pattern of bold horizontal stripes is increasingly well-understood at both cellular and genetic levels [1-6]. Unlike in mammals, fish pigmentation revolves around the problem of distributing six or more differently-coloured cell-types within the skin – the pattern formed is essentially a pointillist painting of just a few colours [2,7]. In zebrafish, the striped pattern is generated by three distinct cell-types, black melanophores (=melanocytes; M), yellow xanthophores (X) and shiny iridophores (I). Iridophores contain membrane-bound guanine crystals which form reflective platelets, and depending upon the spacing and arrangement can generate a variety of hues [7]. Adult zebrafish show two discrete, but still poorly understood, variants of iridophores, one blue, one silver. Consequently, the adult pattern is formed from a pallet of four colours. These cells are arranged in a distinctive pattern, with melanocytes restricted to longitudinal stripes, and associated with low density xanthophores and blue iridophores, whereas the silvery-yellow interstripes consist of dense silver iridophores combined with dense xanthophores.

Beautiful studies of both wild-type and mutant pigment patterning using a diversity of tools including transgenic labelling, chemical, genetic and laser ablation, and timelapse imaging, have shown that cell-cell interactions are crucial for stripe formation. Pigment cells appear throughout the skin, but melanophore-xanthophore interactions help segregate and maintain the distribution of melanophores in discrete stripes [8-14]. Until recently, the role for iridophores was largely ignored, but important studies from the Nuesslein-Volhard and Parichy groups have shown that iridophore differentiation at the horizontal myoseptum on the mid-flank of the fish, coupled with their extensive capacity for proliferation and tendency to spread in both dorso-ventral and antero-posterior directions, drives the establishment of the first interstripe and acts as a prepattern to orient the entire patterning process [15-18]. Furthermore, reorganisation of these iridophores into dense and loose arrangements, working together with melanophore-iridophore interactions to generate the interstripes, is likely to help define the positions of the melanophore stripes [4,15].

Origins of adult zebrafish pigment cells

Whilst the cellular basis of pigment pattern formation in the metamorphic larva is relatively well-understood, a significant gap in our understanding concerns the origin of the different pigment cell-types in the adult. Previous work has suggested that adult xanthophores originate from reversion of embryonic xanthophores to a proliferative state, but have indicated too that a second source must exist to explain the fish’s capacity for xanthophore regeneration [19-21]. In contrast, the origin of most adult melanophores and iridophores is considered to be de novo in the adult, with most embryonic cells dying and being replaced with cells from one or more adult progenitors, presumably adult pigment stem cells. However, the location of these stem cells and other progenitors and, especially their potency, has only begun to be addressed. Melanophore origins have been linked to the peripheral nervous system, especially the dorsal root ganglia (DRGs) and spinal nerves [22,23]. During both metamorphosis and regeneration of ablated embryonic melanocytes, melanocyte progenitors are identified as cells expressing an early melanoblast reporter, microphthalmia-related transcription factor a (mitfa), a master regulator of melanocyte development [24,25]. An mitfa:GFP reporter transgene has been shown to label a small subset of cells in the DRGs which appear to become activated during ontogenetic or regenerative production of melanocytes, and the same reporter remains active in specified melanocyte progenitors migrating along peripheral nerves [22,23]. Although mitfa function is not required for formation of the melanocyte stem cells, it is required for melanocyte differentiation from them [26]. These melanocyte stem cells are set-aside in the embryonic stages, in a process that requires ErbB signaling between 9 and 48 hpf; the exact role
of ErbB signaling is somewhat unclear, since the mutant and inhibitor studies reveal widespread defects in migration of neural crest cells and formation of the peripheral nervous system [22,27,28]. Likewise, adult iridophores have been reported to arise from adult iridophore stem cells located in the DRGs [15]. The relationship between these two stem cell-types has been controversial, and their potential to form other cell-types has not been explored.

In a recent paper, the Nuesslein-Volhard lab uses modern molecular tools (Cre-lox recombination) and a classical approach (clonal analysis) to address this problem of the nature of the adult pigment stem cells [29]. Since key deductions are based upon interpretations of clones induced at one stage but not assessed until much later, it is worth considering exactly how these clones are formed. Singh and colleagues use Cre-lox recombination to label their clones genetically, at four different stages during pigment cell progenitor development. They studied fish combining two transgenes, a tissue-specific Tg(sox10: ER12-Cre) with a colour switch reporter Tg(β-actin2:loxP-STOP-loxP-DsRed-express); Cre-mediated recombination activates expression of the DsRed-express marker under the ubiquitous promoter of the β-actin2 gene. Temporal specificity resulted from the focused application of an inducer, which binds the estrogen response element (ER12), activating the Cre recombinase, during only a 1-3 hr time window. The Cre driver construct is based upon a sox10 promoter fragment that labels all neural crest cells at mid-embryonic stages (around 24 hours post fertilisation (hpf)), but becomes prominently focused on glial expression in both the PNS (neural progenitors/satellite glia and Schwann cells) and CNS (oligodendrocytes) in early larval stages (from 3 days post fertilisation (dpf)) [30-37]. This would be expected to be the case at later stages too, and indeed Parichy and colleagues reported expression in putative peripheral glia of early metamorphic larvae, a little older than those used here [23].

**Adult pigment cell origins revealed**

**Clonal analysis of adult pigment cells**

Singh and colleagues induced clones in neural crest cells at 16 hpf, in early larva before metamorphosis (5 dpf), at the beginning of metamorphosis (15 dpf) and during metamorphosis (21 dpf) when iridophores start to appear in the skin. They studied DsRed-labelled pigment cell clones in the skin of near-adult fish, distinguishing different cell-types unambiguously by their distinctive morphology, colour and location. Given that the prominent expression of the Tg(sox10:Cre) driver is in glial or glial-like cells at the older stages (from 5 dpf onwards) assessed, it is reasonable to assume that the labelled clones represented the progeny of adult pigment cell progenitors. For clones generated at 16 hpf, the interpretation is more subtle since labelled cells would be either premigratory or early migrating NCCs, so in theory an individual labelled cell at these stages may generate both embryonic xanthophores and one or more adult pigment stem cells.

Analysis of the types of pigment cell which each of these clones gives rise to leads to conclusions relating to the multipotency of adult pigment stem cells. Accurately determining the components of a single clone is thus vital to the interpretation of these results; at the time of labelling, was there a single cell, or might the progeny of two cells be erroneously amalgamated, potentially invalidating the conclusion of multipotency? The authors carefully report the total numbers of clones, the number of animals in which these were induced and (for treatments at 15 dpf) the total number of fish treated. Using the total number of fish treated and the number in which clones were induced, under the assumption of independent clone induction events, we can use maximum likelihood estimation to infer the parameter of a Poisson distribution for the probability of the number of clones induced at 15dpf. From this we can calculate that we would expect to see only 87 individual clones. In fact, the authors report 112 individual clones, just outside the 95% confidence interval for this estimate. If anything, our analysis suggests it is more likely that a number of single clones are
being erroneously reported as two separate clones, potentially due to the spatial separation of a single cell’s progeny in the adult, rather than two clones being erroneously reported as one. Thus, the degree to which clones are multipotent is, perhaps, more likely underestimated, rather than the opposite, strengthening the authors’ conclusion of multipotency. Similarly, the spatial spread of a single clone is potentially underestimated.

The multipotency of adult pigment stem cells

This conclusion enhances the impact of the most exciting discovery from this work, the multipotency of the adult pigment progenitor cells. At all stages examined, the proportion of clones that give two or more pigment cell-types is above 50%, and those that include all three pigment cell-types (‘MIX’ clones) is also high (>40%). Furthermore, most clones also included neurons and/or glia. This was to be expected given that the Cre-expressing cells labelled at each stage either are (those labelled from 5 dpf onwards) or would generate glial cells (those labelled at the earlier stage, 16 hpf), including satellite glia of the peripheral ganglia, consistent with previous findings [15,22,23]. This firmly establishes the peripheral DRG as a melanocyte and iridophore stem cell niche [15], and importantly suggests that these hypothesised melanophores and iridophore stem cells are one and the same. Given the likely close relationship between these two pigment cells in the embryo [38,39], this conclusion will not be controversial, but informs the search for definitive markers of the adult pigment stem cell, which has so far been elusive.

Another novel observation concerned the dual origin of xanthophores, and confirmed a prediction made in an earlier paper [21]. Previous studies had shown that adult xanthophores were generated through dedifferentiation and proliferation of embryonic xanthophores, and had contrasted this with the adult pigment stem cell origin of melanocytes and iridophores [19,20]. However, it had also been observed that xanthophores can regenerate after ablation of embryonic/early larval cells, suggesting a second source, likely from a multipotent stem cell [20,21]. Singh et al. observe xanthophores in many of their clones, including many multipotent (MIX) clones, and so conclude that there is a significant contribution from adult pigment stem cells during generation of the normal pigment pattern.

Although not discussed by the authors, it is worth considering whether progenitor cells might remain highly multipotent until final differentiation, as suggested by the classic direct fate restriction model proposed for embryonic neural crest development [40,41] and recently supported in mouse [42]. Each clone analysed shows only what a progenitor’s offspring became, and so only gives an imperfect readout of its true potential. For a large dispersed clone it might be reasonable to assume that the fate restriction observed and the cell’s potency are very closely related. However, for small clones, especially those clustered in a small region, this assumption is weaker. In this context, the relative frequency of apparently bipotent clones becomes highly significant, with an IX (i.e. iridophore and xanthophore) clone seen only once (0.89% of clones), compared with MI (10% of clones) and MX (3%). The authors interpret their data in the context of a progressive fate restriction model, arguing convincingly that multipotent MIX progenitors segregate MI and MX progenitors en route to specification of individual pigment cell fates. This argument is strongly supported by the relative scarcity of IX bipotent clones. Although the different ratios of MI, MX and IX clones could be reproduced in a direct fate restriction model, it would require the frequency of differentiation of multipotent MIX progenitors into iridoblasts and xanthoblasts to be low, which would lead in turn to low proportions of fate-restricted iridophore- and xanthophore-only clones (Fig. 1). However, the authors observe fate-restricted iridophore and xanthophore clones at frequencies (c. 20%) similar to or greater than the frequency of melanophore-only clones (around 10%), suggesting that a progressive fate restriction model is most appropriate.
When and where does progressive fate restriction occur (Fig. 2)? Assuming that the ganglia-associated stem cells are multipotent (MIX, but also neural), then we might ask whether segregation of partially-restricted Mi and MX clones occurs in the ganglia, or only on migration along the peripheral nerves. This latter idea is favoured by the authors; a consequent prediction is that such bipotent clones might well include glial cells (Schwann cells), but would be unlikely to include neurons. Conversely, under the first suggestion such clones might include both neurons and glial cells. The authors note that most (50-80%) bipotent clones include neural cells, but do not distinguish clones containing neurons from those containing glia. Hence, whether the segregation of bipotent clones occurs before or after cells leave the ganglial niche is an important question for future studies.

In an embryonic pigment cell context, where there is a long-standing proposal that all pigment cells share a common progenitor, the chromatoblast, that is fate-restricted to this subset of fates alone [43], it is surprising that apparently bipotent pigment cell progenitors also share neural derivatives. Although the chromatoblast model has not yet been rigorously tested, study of gene expression in sox10 mutants provides support for a multipotent pigment cell progenitor [44]. More recently, work from our lab has indicated that these embryonic progenitor cells might also have neural potential (Nikaido et al., in prep.). The data from Singh et al. clearly converge on a similar idea for these adult pigment cells, and build on an earlier paper by the same authors which showed a shared clonal origin for adult iridophores and DRG neurons and glia [15].

Do adult pigment stem cells become more abundant as the larva grows?

Another key question is how many adult pigment stem cells there are? The only proposed direct marker is expression of GFP in an mitfa:GFP transgenic line, noted as labelling small rounded cells closely-associated with each DRG [22]. However, it is not clear whether this stably labels all pigment stem cells. The number of these cells remains roughly constant at one or less per ganglion during larval development (5-24 dpf) [22]. As Singh et al. note, the adult pigment stem cells of one hemisegment may derive from as few as one 16 hpf neural crest cell, but this could still mean >1 stem cell per hemisegment, since the labelled progenitor may generate a clone incorporating two or more adult pigment stem cells. Furthermore, the interpretation of the number of stem cells and their potency is also complicated by the authors’ observations of peripheral ganglial growth between 5 and 21 dpf. In zebrafish, just like other vertebrates, the numbers of both neurons and glial cells/progenitors in the peripheral ganglia increases significantly [45], a result confirmed by Singh et al. using a recently generated transgenic line [46]. Many of these DRG cells are likely glial/progenitor cells, and thus expected to include the adult pigment stem cells. Does the number of adult pigment stem cells also increase proportionately? Data from the mitfa:GFP labelling studies indicates that the pigment cell stem cells in the DRG are not proliferating [22], consistent with Singh et al. showing that the frequency of labelling an adult MIX clone is approximately constant. Yet, Singh et al. show clearly that the clone-span and clone size both decrease between 5 dpf and 21 dpf, as has also been shown in studies of wild-type clones in albino mutant chimaeras [21]. Indeed, although at this stage many clones contribute extensively throughout the dorsoventral axis, others are much more focused; the impression is of an apparently stochastic contribution from multiple stem cell clones to generate the full complement of a hemisegment (Fig. 3). Consistent with this, at later stages when the expectation is that a clone will be restricted to a single progenitor cell, the extent of the MIX clones suggests that they cover less of the skin (and therefore are only part of the complement of that hemisegment), so that a normal hemisegment does derive from multiple, but probably a small number of, stem cells. However, this is very variable and stem cells often contribute to pigment cells in more than one segment, intermingling with clones from other stem cells. These observations can be reconciled by suggesting that adult pigment stem cells in the peripheral ganglia remain approximately constant,
but their proliferation generates a series of partially-restricted and fate-restricted progenitors (on the spinal nerves) that are accumulating throughout the 5-21 dpf time period. Thus, the clonal contribution of progenitors to the adult pigment pattern at later stages is inevitably, on average, smaller due to the accumulating number of pigment cell progenitors. Consistent with the idea that clone size is limited by the presence of pigment cells derived from other (unlabelled) clones, this wild-type situation contrasts with those where one or more pigment cell-types cannot be formed; under these circumstances, clones of the individual pigment cell-types expand significantly compared to those in a wild-type host [18,21,47].

Conclusions (300)
The authors’ key conclusion, that multipotent adult pigment stem cells generate fate-restricted clones by a progressive fate-restriction model, is here deduced from an elegant study of adult clonal contributions and distributions and frequent, but not continuous, observation of specific individual clones. Since these patterns form over periods of weeks, direct observation of lineage relationships within labelled clones is technically highly challenging - in some cases, the authors felt able to identify the same cells from day to day, but usually they did not and their interpretation is suitably cautious. Technological breakthroughs in long-term maintenance of anaesthesia and in high-resolution imaging of growing individuals might allow a true lineage study, allowing direct assessment of the lineage segregation problem. Extension of these studies to consider the origins of fin pigment cells would make a fascinating comparison, especially in view of the suggestion that bipotent melanophores-xanthophore clones segregate early from iridophore clones [48,49]. The demonstration of the multipotency of the adult pigment stem cells informs the search for molecular markers of these cells. The identification of xanthophores as a regular component of these clones opens the way to consideration of the relative frequency and timing of xanthophore origins from these stem cells and from embryonic xanthophores. The data support a progressive fate restriction model of adult pigment cell development and integrating this model with the anatomical location of the stem cells (peripheral ganglia) and partially-restricted progenitors (peripheral nerves, perhaps?) will be crucial for dissecting the mechanistic basis for fate restriction. Subsequent integration of this knowledge with the cell biology and genetics of pigment cell development promises to reinforce the position of the zebrafish as the model of choice for study of pigment pattern formation.

Figure legends

**Figure 1.** Modelling the ratios of ‘bipotent’ pigment cell clones. Upper panels) Proposed lineage diagrams under **A**: progressive and **B**: direct fate restriction models. The solid coloured circles labelled M,I,X in the fate restriction schematics represent the expected proportions of homogeneous clones of M, I and X respectively required to allow the proportion of IX clones to be small in comparison to MI and MX clones. Lower panels) Venn diagrams indicate the proportions of all clone types (NB neural fate contributions are ignored). Both models are constrained to produce small proportions of IX clones compared to MI and MX clones (see Venn diagrams). To produce small numbers of IX clones under the direct fate restriction model the rate of production of I and X restricted clones must be much smaller than the rate of production of M restricted clones, leading to proportionally larger numbers of M-only clones than I-only or X-only clones. Under the progressive fate restriction model the proportions of X-, I- and M-only clones are similar, whereas in the direct fate restriction model there are considerably more M-only clones than any other types. This latter condition provides a much better match to the observations of Singh et al.
Figure 2. Schematic representation of likely location of adult pigment stem cells and partially restricted progenitors. Adult pigment stem cells (green) are proposed to be localized to the dorsal root ganglia (purple), but give rise to two classes of progenitors, each restricted to two pigment cell types: MX (brown) and MI (dark blue). These cells disperse along the dorsal, medial and ventral projections of the spinal nerves, and give rise to unipotent progenitors. These cells - melanoblasts (M; black), iridoblasts (I; light blue) and xanthoblasts (X; yellow) - are shown on the distal regions of the nerves, and reach the skin where they may proliferate before generating the adult pigment pattern. Neural Tube (NT), Notochord (NC), Dorsal (DA) and Posterior Cardinal Vein (PCV).

Figure 3. Schematic representation of the contributions of stem cell descendants to the adult pigment pattern. Colour shading represents different clones, contributing randomly to the complement of one or more hemisegments (delineated by the underlying muscle blocks indicated by rotated V shapes). Thus, some clones contribute to more than one hemisegment, while others have only either a dorsal or ventral contribution. The pigment cell complement of one hemisegment is often derived from more than one stem cell. First light interstripe (X0), first dorsal dark stripe (D) and first ventral dark stripe (V).

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