On the embryonic origin of adult melanophores: the role of ErbB and Kit signalling in establishing melanophore stem cells in zebrafish

Christopher M. Dooley¹,², Alessandro Mongera¹, Brigitte Walderich¹ and Christiane Nüsslein-Volhard¹,*

SUMMARY

Pigment cells in vertebrates are derived from the neural crest (NC), a pluripotent and migratory embryonic cell population. In fishes, larval melanophores develop during embryogenesis directly from NC cells migrating along dorsolateral and ventromedial paths. The embryonic origin of the melanophores that emerge during juvenile development in the skin to contribute to the striking colour patterns of adult fishes remains elusive. We have identified a small set of melanophore progenitor cells (MPs) in the zebrafish (Danio rerio, Cyprinidae) that is established within the first 2 days of embryonic development in close association with the segmentally reiterated dorsal root ganglia (DRGs). Lineage analysis and 4D in vivo imaging indicate that progeny of these embryonic MPs spread segmentally, giving rise to the melanophores that create the adult melanophore stripes. Upon depletion of larval melanophores by morpholino knockdown of Mita, the embryonic MPs are prematurely activated; their progeny migrate along the spinal nerves restoring the larval pattern and giving rise to postembryonic MPs associated with the spinal nerves. Mutational or chemical inhibition of ErbB receptors blocks all early NC migration along the ventromedial path, causing a loss of DRGs and embryonic MPs. We show that the sparse like (slk) mutant lacks larval and metamorphic melanophores and identify kit ligand a (kitlga) as the underlying gene. Our data suggest that kitlga is required for the establishment or survival of embryonic MPs. We propose a model in which DRGs provide a niche for the stem cells of adult melanophores.

KEY WORDS: Kit ligand a, ErbB, Dorsal root ganglia, Melanophore stem cells

INTRODUCTION

Colour patterns are prominent features of many animals; they evolve rapidly and vary between closely related species, providing an important target for both natural and sexual selection (Darwin, 1871; Roulin, 2004; Hoekstra et al., 2006; Protas and Patel, 2008).

In vertebrates, all pigment cells of the body are derived from the neural crest (NC) except the pigmented retinal epithelium. The NC is an embryonic population of pluripotent migratory cells that contribute to a variety of organs and tissues. The NC is largely responsible for the emergence of many of the vertebrate-specific characteristics involved in head and body shape, protection and pattern diversity that evolved in this phylum over 550 million years (Gans and Northcott, 1983).

The pigment patterns observed in birds and mammals are due to variations in melanin synthesis within a single type of chromatophore, the melanocyte. However, anamniotes and reptiles display several chromatophore types. In zebrafish, black melanophores, yellow xanthophores and silvery iridophores are arranged in distinct patterns in the hypodermis during larval and adult stages.

Although the early melanophores contributing to the larval pattern can be traced back directly to the migrating NC, the ontogeny of the melanophores that compose the continuously growing and expanding adult pattern during metamorphosis weeks later remains unclear. As the NC is no longer present, these cells must arise from hypothetical NC-derived stem cells that are set aside in the embryo (Rawls et al., 2001; Parichy et al., 2003; Yang and Johnson, 2006; Budi et al., 2011). These cells are quiescent during embryonic development but start to proliferate during juvenile development (Quigley et al., 2004; Mellgren and Johnson, 2004; Hultman et al., 2009).

Several zebrafish mutants in which melanophores are reduced or completely absent have been collected (Streisinger et al., 1986; Haffter et al., 1996; Kelsh et al., 1996; Lister et al., 1999). Mutations in the colourless (cls) gene, which encodes the HMG-box transcription factor Sox10, eliminate all three types of chromatophores as well as glia and the peripheral nervous system (Kelsh et al., 1996; Dutton et al., 2001). Mutations in genes such as nacre (nac; mitfa – Zebrafish Information Network), sparse (spa; kita – Zebrafish Information Network) and sparse-like (slk) specifically affect melanophores of the body whereas the other chromatophore types are present (Kelsh et al., 1996; Haffter et al., 1996; Lister et al., 1999). mitfa mutant fish lack all body melanophores throughout their lives. mitfa encodes a helix-loop-helix transcription factor (Elworthy et al., 2003). mitfa is expressed in melanoblasts at all stages of migration and differentiation (Lister et al., 1999). Interestingly, mitfa morphants, although completely deficient in early larval stripe formation, do recover and adopt normal larval and adult pigmentation (Mellgren and Johnson, 2004). This finding and evidence from temperature-sensitive mitfa alleles indicate that mitfa is not required for establishing or maintaining melanophore stem cells (Johnson et al., 2011). In juvenile fish, a population of postembryonic MPs expressing mitfa was described in the myotomes and associated with the Schwann cells of the spinal nerves and the DRGs (Budi et al., 2011). Although the proliferation and migration of these MPs ultimately gives rise to pigmented melanophores in the skin, the embryonic origin of postembryonic MPs remains unknown.
In adult *erbb3b* (also known as *hypersensitive*, *hps* and *picasso*) mutants, melanophores are drastically reduced but the larval melanophore pattern appears normal (Whitfield et al., 1996; Budi et al., 2008; Hultman et al., 2009; Hultman and Johnson, 2010). Mutations in *erbb3b* as well as in *erbb2* (*kitzelig*, *kiz*) not only cause defects in pigmentation, but also in the peripheral nervous system and lateral line glia (Lyons et al., 2005; Rojas-Muñoz et al., 2009). Morpholino and small molecule inhibitor studies show that both regenerating and adult melanophores require ErbB3b signalling during the first day of embryogenesis when NC cells delaminate and migrate (Budi et al., 2008; Hultman et al., 2009; Johnson et al., 2011; Budi et al., 2011). These findings support a model in which ErbB signalling is required in the early embryo to establish both the peripheral nervous system and the melanophore stem cells that later generate the adult melanophore pattern (Hultman et al., 2009; Hultman and Johnson, 2010).

*kita* mutant fish display a severe melanophore reduction. In larvae and juveniles, melanophores are almost completely absent. The adult fish display median longitudinal stripes that contain only ~15% of the normal number of melanophores (Johnson et al., 1995; Parichy et al., 1999; Hultman et al., 2007). In mice, mutations in *Kit* (also known as dominant-white spotting), in addition to a pigment phenotype, lead to an early failure in haematopoiesis, and homozygotes are embryonic lethal (Geissler et al., 1988). Similar phenotypes are observed in *Steel* (*Kitl*) mutant mice, which carry lesions at the *Kitlg* locus encoding the ligand of Kit. In mice, zebrafish and humans, *Kitlg* expression is required both for the migration and the survival of melanocyte precursors as well as later in the epidermis, where melanocyte precursors disperse throughout the entire embryo (Huang et al., 1992; Wehrle-Haller et al., 2001; Rawls and Johnson, 2003; Gu et al., 2009; Gu et al., 2011).

The *sparse like* (*slk*) mutant (Kelsh et al., 1996) displays a phenotype similar to that of *kita* mutants. We have positionally cloned *slk* and show that it encodes the zebrafish Kit ligand a. Both *kita* and *slk* (*kitlga*) mutants are viable and fertile; they display no defects in haematopoiesis, germ cell development or osteoclast development. The mutant phenotype of both genes suggests that larval and the majority of adult melanophores are Kitlga signalling dependent.

We present evidence for a distinct population of MPs associated with the DRGs. They require *slk* (*kitlga*) to function as stem cells. These melanophore stem cells are quiescent during larval life and activated in juveniles to give rise to the postembryonic MPs that distribute in a segmental fashion along the spinal nerves. Precocious activation is observed in embryos depleted of melanophores by *mitfa*-morpholino (MO) knockdown. Furthermore, their establishment, although not requiring Mitfa, depends on ErbB signalling in early embryonic development, which is required for migration of all NC cells including the MPs and DRG precursors along the ventromedial path. Our data suggest that DRGs serve as a niche for stem cells that generate melanophores contributing to the pigment pattern of adult fishes.

**MATERIALS AND METHODS**

**Zebrafish husbandry and stocks**

Zebrafish were maintained as described (Brand et al., 2002). Transgenic lines were provided by the following people: *Tg*(mitfa:gfp)*w* by James Lister, VCU Medical Center, Richmond, Virginia, USA; *Tg*(−8.4neurog1:nRFP)*b* by Robert Kelsh, University of Bath, Bath, UK; *Tg*(nbt:DsRed)*b* by Madeleine van Drenth from the Max-Planck Institute for Developmental Biology, Tübingen, Germany. The following mutant alleles were used in this study: *alb*<sup>b</sup> (Streisinger et al., 1986), *erbb2<sup>20660b</sup>* (*kiz*), *erbb3<sup>17447</sup>* (*hps*) (Rojas-Muñoz et al., 2009), *mitfa<sup>2</sup>* (*nac*) (Lister et al., 1999), *kitlga<sup>2344b</sup>* (*slk*), *sox10<sup>b</sup>* (*cls*).

**Mapping and cloning of sparse like**

*slk* was mapped following Geisler et al. (Geisler et al., 2007) between markers z21055 (2.0 cM; LOD 63.1) and z3490 (2.3 cM; LOD 21.1) on
chromosome 25. *kitlga* maps within the same interval. RNA was isolated from 48 hours post fertilisation (hpf) *slk* embryos using TRIzol (Life Technologies, Frankfurt, Germany) and processed into cDNA with Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics Deutschland, Mannheim, Germany). The *kitlga* transcript was amplified, subcloned into pGEM-T (Promega, Mannheim, Germany) and sequenced.

**RNA in situ hybridisation**

A RT-PCR-based approach was used to generate probes for RNA *in situ* hybridisation. The PCR was performed using an antisense primer containing a T7-promoter sequence on its 5'-end. The DNA oligonucleotides used were: *kitlga*: 5'-TCTGTTCTGAAATGGAAGACTCAA-3' and 5'-TAATACGACTCACTATAGGTCAGATATCCCCACATCTAATGG-3' (Hultman et al., 2007). The PCR product included the target sequence flanked by the T7-promoter sequence, enabling us to synthesise an RNA riboprobe by *in vitro* transcription of the PCR product using T7-RNA polymerase (Fermentas, St Leon-Rot, Germany). Whole-mount *in situ* hybridisation (Thisse and Thisse, 2008) and antibody staining (Schulte-Merker, 2002) were performed as described. Anti-HuC/HuD (Honjo et al., 2008) and phospho-ERK antibody (Cell Signaling Technology, Frankfurt, Germany) were used to stain developed melanophores. The data were analysed using IMARIS 6.1 software (Bitplane A6, Zurich, Switzerland).

**Generation of chimeric fish**

Rhodamine dextran (Life Technologies) was injected into Tg(*mitfa:gfp*) donor eggs at the one-cell stage and about ten cells were transplanted at the blastula stage into alb recipients of the same age. The *alb* recipients had been injected at the one-cell stage with *mitfa* MO. Chimeric embryos were scored at 3 days post-fertilisation (dpf) and imaged using the LSM5 Live, Carl Zeiss Microimaging, Jena, Germany. Chimeras displaying donor-derived melanophores at the horizontal myoseptum were individually raised to adulthood and scored for darkly pigmented melanophores in the pale background.

**Morpholino injection**

MO injections were carried out as described (Nasevicius and Ekker, 2000) with the following amounts: *mitfa* 5.0 ng (*mitfa*-MO, 5’-CATGTCTAACAATGTGTTAGCTTC-3’), *kitlga* 5.0 ng (*kitlga*-MO, 5’-CTGGATAACAACACTCACCTTCT-3’) (Hultman et al., 2007) and erbbb3b 2.0 ng (erbbb3b, 5’-TGGGTCGAACTTGTTGGAAAAAC-3’) (Budi et al., 2008). All MOs were obtained from Gene Tools LLC, Philomath, USA.

**Inhibitor treatment**

Embryos at somitic stages were dechorionated and placed in 10 μM of ErbB inhibitor (PD168393, Calbiochem, Merck KGaA, Darmstadt, Germany) at 28°C for 4 hours. Embryos were mounted in agarose (also containing 10 μM PD168393) and imaged for 24 hours.

**DRG ablation**

Tg(~8.4neurog1:nrfp)embryos at 3 dpf were anaesthetised and mounted in 0.6% low temperature melting agarose on glass-bottom dishes. Embryos were mounted laterally and four to eight DRGs of consecutive metamers were ablated using a 351-nm 100-Hz pulsed laser line on a FV1000 Olympus confocal microscope (Olympus Europa Holding, Hamburg, Germany).

**TUNEL assay**

Embryos were dechorionated and then fixed in 4% paraformaldehyde overnight at 4°C. Apoptosis staining was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics Deutschland) according to the manufacturer’s protocol.

**Imaging**

Embryos were dechorionated, anaesthetised in 0.004% tricaine and mounted in 0.6% low temperature melting agarose on glass-bottom dishes at 28°C. Live imaging was performed using a LSM5 Live confocal microscope (Carl Zeiss Microimaging). The data were analysed using IMARIS 6.1 software (Bitplane A6, Zurich, Switzerland). In some imaging experiments, the medial crest population was identified after removing the superficial optical sections with the function ‘Crop 3D’. Adult fish were imaged using a Canon EOS 5d mark II (Canon Deutschland, Krefeld, Germany).

**RESULTS**

**Stationary MPs are associated with DRGs**

A transgenic line expressing GFP under the control of the *mitfa* promoter (Lister, 2002) allows the 4D imaging and tracing of melanoblasts in early development. *mitfa:gfp* is initially broadly expressed among NC cells. Subsequently, its expression becomes more and more restricted to melanoblasts although it is also observed in other chromatophore types and glia (Curran et al., 2009). In the trunk, NC cells migrate along the ventromedial path...
in close association with primary motor neurons, labelled in Fig. 1A-G with a transgene expressing DsRed under the control of a neuron-specific promoter (nßt:DsRed) (supplementary material Movie 1). We identified some of the migrating GFP-positive cells as melanoblasts because they undergo melanisation while still associated with primary motor axons (Fig. 1E,F). We observe round stationary cells remaining at the motor axon exit point (Fig. 1G, arrow).

We followed mitfa:gfp-labelled cells together with RFP-labelled cells of neuronal origin under the control of a neurogenin promoter (neurog:rfp). Melanophores forming the lateral stripe also originate from mitfa:gfp cells following the ventromedial path at ~40 hpf, turning into the horizontal myoseptum and migrating towards the skin (Fig. 1H-K, blue arrowheads). Fig. 1K shows the appearance of the DRG precursor cell at 48 hpf located next to a mitfa:gfp-labelled cell that resided at this position for at least 21 hours.

The larval set of melanophores has differentiated by 3 dpf and GFP expression has largely ceased. Interestingly, at later time points we still observe GFP-labelled cells located close to the DRGs (Fig. 2A-C, arrowheads) and along the ventral motor neurons (Fig. 2B, arrow). Their elongated shape suggests that they are, at least in part, Schwann cells. Schwann cells along the lateral line also retain the mitfa:gfp label until late larval stages (data not shown).

Imaging larvae until metamorphosis (Fig. 2A-F) shows that the number of stationary cells at the site of the DRG expressing mitfa:gfp remains at the level of zero to one labelled cell per hemisegment. The number of labelled cells along the ventral spinal nerve (Fig. 2B, arrow) remains about four throughout larval development (Fig. 2G). No labelled cells along the dorsal spinal nerves (Fig. 2D, arrow) can be observed until metamorphosis, when the number of mitfa:gfp-labelled cells increases along the spinal nerves both dorsally and ventrally (Fig. 2E,F, arrowheads). This suggests that these are MPs emerging from the stationary cells at the DRG.

We propose that the DRG-associated stationary cells include melanophore stem cells producing MPs that later give rise to adult melanophores.

Recovering melanoblasts emerge at the DRG and migrate along the spinal nerves
Larval melanophores can regenerate if chemically or genetically ablated (Yang and Johnson, 2006; Hultman et al., 2009; Tryon et al., 2011). To investigate whether these newly formed melanophores originated from the putative DRG-associated stem cells, we depleted the directly differentiating melanophores with a mitfa-morpholino. (Mellgren and Johnson, 2004) and traced melanophore renewal by 4D in vivo imaging in the Tg(mitfa:gfp) line.

In the first 60 hpf, knockdown of Mitfa faithfully phenocopies the nac mutant phenotype as the mitfa-MO-treated zebrafish embryos lack larval melanophore pigmentation until 60 hpf, but regenerate the larval melanophore stripes completely by day 5 (C). (D-H) Tg(mitfa:gfp; nbt:DsRed) embryo previously treated with mitfa-MO imaged for several hours starting at 60 hpf (digital sectioning). A GFP-positive cell (white arrowhead) migrates along the spinal nerve. Another cell remained stationary (blue arrowhead) but was later observed to migrate away. Asterisks indicate the position of the DRG. (I) In mitfa morphant larvae (9 dpf), the regenerating MPs form a string of mitfa-positive cells along the spinal nerves associated with the DRGs both dorsally (arrowhead) and ventrally.
In mitfa-MO embryos between 60 and 80 hpf, new mitfa:gfp-labelled melanoblasts appear. In time-lapse movies, we captured melanoblasts originating at the site of the DRG and migrating along the spinal nerves (Fig. 3D-H). We were able to follow mitfa:gfp-labelled cells in mitfa-MO individuals until late larval stages. Fig. 3I shows a stereotyped pattern of GFP-positive cells associated with DRG neurons (red) notably following both the dorsal (arrowhead) and ventral projections. This pattern resembles that observed later in juvenile wild-type fish, when mitfa:gfp-positive cells appear in close contact with the spinal nerves (Fig. 2E,F) (Budi et al., 2011).

To trace the migration and cell lineage of individual regenerating melanoblasts, we transplanted cells from mitfa:gfp embryos into albino (slc45a2 – Zebrafish Information Network) mutant embryos treated earlier with mitfa-MO. This enabled us to select embryos in which donor cells (rhodamine-tracer positive) contributed to the DRGs. We imaged GFP-positive cells appearing at the location of donor-derived DRGs for 28 hours starting at 60 hpf. Fig. 4A-E (see also supplementary material Movies 2, 3) shows the migration of GFP-positive cells dividing several times while travelling along the sensory projections labelled in red. These cells originated from a single unlabelled cell that resides close to a DRG, begins to express GFP and undergoes a division giving rise to one daughter cell that remains at this location. The other daughter cell moves away, divides again and melanises once it reaches the ventral side of the myotome. In this clone, the migration of individual progeny of silent MPs could be traced along DRG-derived neurons to a location in the ventral stripe as well as into the dorsal stripe to differentiate into darkly pigmented melanophores (Fig. 4F-H).

We traced several GFP-labelled melanoblasts (n=8) that originate from the site of the DRGs, migrate along the spinal nerves and give rise to melanophores of larval stripes. We thus identified the MPs residing close to the DRG as progenitors of regenerating melanoblasts. From these transplantation experiments we selected chimeric animals displaying donor-derived melanophores located at the horizontal myoseptum and individually raised these to adulthood. In three out of five such cases, adult melanophores developed at the same location as the regenerated larval pigmentation (Fig. 4I,J). The chimeras displayed donor-derived melanophores in vertical streaks often spanning the entire pattern from dorsal to ventral. This indicates that MPs that give rise to the regeneration of the larval pigmentation also generate the adult melanophore pattern.

We conclude that in mitfa-MO-treated embryos the absence of differentiated melanophores induces premature activation of embryonic MPs located in close proximity to DRGs. This results in proliferation and migration of MPs that first restore a larval pattern, and eventually produce adult melanophores.

---

Fig. 4. Tracing individual MPs during regeneration in chimeric animals. Blasto transplantation were performed with Tg(mitfa:gfp) zebrafish embryos injected with rhodamine dextran as donors and alb embryos injected with mitfa-MO as recipients. (A-E') Red and green channel are shown in A-E, green channel only in A'-E'. Starting at 60 hpf (A,A'), we imaged a clone appearing at the site of a rhodamine-labelled DRG (asterisk) from which a peripheral axon extends ventrally (arrow). The arrowhead in A,A' points to a GFP-positive cell located at the ventral side of the myotome. Another labelled cell appears at 70 hpf at the site of the DRG (arrowhead in B,B'). This cell divides, and the two daughter cells migrate dorsally (blue arrowhead) and ventrally (white arrowhead) along a spinal nerve (C,C'). The dorsally migrating cell divides once (blue arrowhead in D-E'), its progeny arrive at the dorsal side of the larva. The ventrally migrating cell divides once (white arrowheads in D-E'). (F-H') The daughter cells, after reaching a position along the ventral stripe close to a cell that arrived there earlier, melanise (arrowheads in G-H'). Complete time-lapse imaging is shown in supplementary material Movies 2, 3. The region marked in E' is shown in more detail in G, and the boxed area in G is enlarged in H,H'. (I-J') Chimeric adult fish that had developed several donor-derived melanophores at the lateral stripe in late larval stages. These display vertical streaks of donor-derived melanophores spanning the entire flank from dorsal to ventral, including the fins, enlarged in I,J'. These streaks appeared in the same rostrocaudal position as the larval melanophores.
ErbB receptors are required for the migration of all NC cells along the ventromedial path

To learn more about the role of DRGs in melanophore development, we examined mutants of the neuregulin receptors ErbB3b (also known as hypersensitive, hps) and ErbB2 (kitzelig, kita) that lack DRGs and glia (Lyons et al., 2005; Honjo et al., 2008; Budi et al., 2008; Rojas-Muñoz et al., 2009). Whereas erbb2 mutants are larval lethal, the erbb3b alleles are hypomorphs with variable expressivity and viability and mutant fish may survive to adulthood. Strikingly, large portions of the flank of erbb3b fish are devoid of melanophores (Budi et al., 2008) (Fig. 5A). Small molecule inhibitor studies indicated that ErbB3b/ErbB2 signalling is required during the developmental stage at which NC delaminates and migrates in the respective segments was shown by Budi et al. (Budi et al., 2008) in sparse (kita) mutant embryos for Kit-independent melanophores. We confirmed this correlation for wild type (Fig. 5B-E).

Honjo et al. (Honjo et al., 2008) reported that the lack of DRGs in erbb3b mutant embryos resulted from a failure of the NC cells to pause during migration towards the ventral side of the embryo. We performed 4D time-lapse imaging of NC labelled with sox10:gfp and neural tissue labelled with nßt:DsRed. In wild-type embryos, soxx10:gfp-positive NC cells, after reaching the ventral side of the neural tube, continue to migrate in the conspicuous segmental streams along the primary motor neurons towards the ventral side of the embryo (Fig. 6A-A’). By contrast, no migration occurs in embryos treated with small molecule inhibitors of ErbB receptors, and NC cells are arrested dorsally to the neural tube (Fig. 6B-B’). The motor neurons remain uncovered by glial cells. A close-up at 36 hpf shows that embryonic mitfa:gfp-positive MPs are absent in inhibitor-treated embryos (Fig. 6C,D). Imaging of mitfa:gfp cells in the erbb2 mutants showed that no NC migration occurs along the ventromedial path (supplementary material Movie 4). In erbb3b mutant embryos, some segments are completely devoid of migrating NC, whereas in others migration is normal (data not shown). Notably, migration of NC cells along the dorsolateral path is normal in erbb mutants or inhibitor-treated embryos (Fig. 6B’; supplementary material Movie 4). These observations indicate that ErbB signalling is required for NC migration specifically along the ventromedial path.

We investigated the recovery of melanoblasts in partial MO knockdowns of ErbB3b in mitfa morphants. We observed that some segments completely recovered the stream of GFP-positive cells associated with the DRG neurons, whereas others had a complete lack of such cells (Fig. 6E). There is a strong correlation between the presence of DRGs and recovered mitfa:gfp-positive cells whereas segments lacking a DRG do not usually display them (Table 1).

To examine further the role of DRGs, eight DRGs were laser ablated in wild-type larvae at 3 dpf. We observed a lower number of melanophores in the corresponding segments of the flank of the respective adult fishes and, thus, mild phenocopies of the hps/picasso mutant phenotype in eight out of ten cases (Fig. 5F).

These experiments support the notion that DRGs provide a niche for melanophore stem cells.

**sparse like encodes Kitlga**

We next investigated the role of Kitlga signalling in the establishment of melanophore progenitors. spa (kita) is required for the survival, migration and differentiation of melanoblasts at all stages of development. We identified the mutant sparse like (slk), (Kelsh et al., 1996) (Fig. 7) as affected in kit ligand a (kitlga) (Hultman et al., 2007). In both spa and slk mutants, late larval and metamorphic melanoblasts are absent (Fig. 7F-H). A population of Kitlga signalling-independent melanophores appears in adult fish (Fig. 7J). The mutant slk carries a stop codon in the kitlga gene (Fig. 8A). kitlga is expressed at very low levels in early embryos at the site of premigratory NC (Hultman et al., 2007) (data not shown). Its expression persists in segmental patches in cells under the epidermis on the dorsal side as well as in segmentally repeated expression domains close to the notochord (Hultman et al., 2007). This kitlga expression domain is absent in sox10 mutant embryos and includes a subset of neural crest at the site of the developing PNS (Fig. 8B-D).

<table>
<thead>
<tr>
<th>DRG staining</th>
<th>Control</th>
<th>erb3bMO+;mitfaMO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hu+; mitfa;GFP+</td>
<td>190</td>
<td>138</td>
</tr>
<tr>
<td>Hu+; mitfa;GFP−</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Hu−; mitfa;GFP+</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Hu−; mitfa;GFP−</td>
<td>4</td>
<td>77</td>
</tr>
<tr>
<td>Total</td>
<td>236</td>
<td>250</td>
</tr>
</tbody>
</table>

Fig. 5. The Role of ErbB signalling and the DRGs for adult melanophore pigmentation. (A) hps/erbb3b(Kitlga) adult fish showing a dramatic regional reduction of melanophores. (B-E) Adult fish treated at successive stages of somitogenesis (left) with the ErbB inhibitor PD168393. The later the inhibitor was applied, the more posterior is the position of the defect (brackets). (F) Adult fish in which eight consecutive DRGs were laser-ablated at 3 dpf.
Kitlga signalling is required for embryonic MP formation

We imaged the site of DRG formation in slk embryos at 48 hpf. Fig. 9A-D shows the presence of mitfa:gfp-labelled cells located close to the progenitors of the DRGs. However, these cells often appear elongated and detached in slk mutants (Fig. 9B,D, arrowhead), suggesting that the stationary MPs are reduced or missing but mitfA:gfp-positive glia cells are retained. We observed the mitfa:gfp-labelled cells located along the spinal nerves in wild-type (Fig. 2) and slk mutant (data not shown) fish until metamorphosis. Although reduced in number, we do not observe a significant qualitative difference, presumably because the labelled cells are mostly glia. Interestingly, like in wild type, in slk mutants labelled cells along the dorsal spinal nerve are not observed before metamorphosis (data not shown). This indicates that in slk mutants, despite the absence of melanophores there is no precocious activation of MPs to produce regenerating melanoblasts, as seen in mitfa-MO-treated embryos (Fig. 3I). Embryos treated with kitlga MO do not show melanophore pigmentation and faithfully copy the mutant phenotype until late stages of larval development (Fig. 9G,H). Thus, in contrast to mitfa, kitlga morphants do not regenerate the larval melanophore population. This indicates that in

Fig. 6. NC migration along the ventromedial path is blocked by inhibition of ErbB receptors. (A-B*) Tg(sox10:gfp; nbt:DsRed) zebrafish embryos at 24 hpf. (A,B) Red and green channel (merge); (A*,B*) green channel; (A**,B**) red channel. Medial NC cells (green) covering the primary motor axons (white arrowheads, A-A**) are absent after treatment with the ErbB inhibitor PD168393 (B-B*). (C,D) Confocal images of 36 hpf Tg(mitfa:gfp; nbt:DsRed) embryos. (C) Wild-type embryo. (D) Embryo treated with ErbB inhibitor at 16 hpf. (E) Tg(mitfa:gfp) embryos were injected with a double MO combination against mitfa and erbb3b. Larvae were stained at 8 dpf using anti-GFP (green) and anti-HU (red) antibody (white arrowheads). The association of DRGs (red) and GFP-positive cells was quantified (Table 1). In double mitfa and erbb3b knockdowns, of 82 metamers lacking HU positive cells only five (6%) develop a string of GFP-positive cells.

Fig. 7. The phenotype of sparse like<sup>c244b</sup> fish at different developmental stages. (A-J) The phenotype of wild-type (A-E) and sparse like (F-J) fish at different developmental stages. Unlike wild-type fish (C), slk fish are almost completely devoid of melanophores at the beginning of metamorphosis (H). Adult slk mutants (J) form stripes with strong reduction in melanophore number compared with wild-type siblings (E). SL, standard length.
the apoptosis before NC migration commences (Fig. 9C,D), reflecting that MSCs are located at the site of the DRGs, we do not exclude additional sites. Some of the mitfa:gfp-positive cells along ventral spinal nerves in larval stages (Fig. 2) might also function as MPs.

**Kitlga signalling specifically affects melanophore stem cells**

Our data indicate that the Kitlga pathway is specifically required for a progenitor population of melanophores whereas other NC-derived cell types are not affected. In kitlga embryos, the DRGs are formed and mitfa:gfp-positive cells are seen close to them and along the spinal nerves. Most, if not all, of these cells are presumably glial cells, and from the morphology we cannot unequivocally distinguish embryonic MPs and glia cells. These cells may include progenitors of Kitlga-independent adult melanophores that also migrate along the ventromedial path (Budi et al., 2008). The failure of the melanophore population to regenerate after kitlga-MO treatment means that they do not function as MSCs, and that MSCs require Kitlga signalling very early for their establishment. We observe cells that undergo apoptosis at a dorsal location, indicating that they are specified as larval melanophores. The progeny of activated MSCs can be traced as they express mitfa:gfp and are mitotically active. They can regenerate all components of the larval melanophore pattern. Moreover, during migration along spinal nerves they form a chain of GFP-expressing cells, closely resembling postembryonic MPs reported by Budi et al. (Budi et al., 2011). Melanoblasts originating from these MPs migrate into the skin, melanise and arrange themselves in the striped pattern of the adult (Fig. 4). During normal development, activation of the quiescent melanophore stem cells occurs in late larval and juvenile stages (Fig. 2), giving rise to the adult melanophore pattern.

**MSCs require ErbB signalling and are associated with the DRGs**

Our live imaging confirms that NC migration along the ventromedial path occurs in close association with the extending primary motor axons (Banerjee et al., 2011). Melanoblasts of the larval pattern also migrate along this path, as shown by the direct tracing of mitfa:gfp cells that undergo melanisation (Fig. 1E,F). Mutations in erbb2b or treatment with drugs inhibiting ErbB receptors inhibit migration of NC cells along the ventromedial path. The close correlation between the position of adult pigmentation defects along the rostrocaudal axis and the time window of inhibiting migration of NC cells (Budi et al., 2008) (Fig. 5B-E) strongly supports the notion that the MSCs are also affected by the inhibitor. Both glia and melanoblasts express ErbB receptors (Lyons et al., 2005; Budi et al., 2008). Unlike glia, which require ErbB signalling during migration, proliferation and differentiation, in the case of the melanophores, once this migration has occurred, ErbB signalling is no longer required for the development of adult melanophores.

The stem cells we have identified are associated with the DRGs of the peripheral nervous system. ErbB-dependent NC migration also affects DRGs when perturbed. The progenitor cells of the DRGs settle at the exit point of the primary motor neurons adjacent to the ventral spinal cord (Carney et al., 2006). We found a close association of MSCs with the DRGs. Through ablation in the embryo we have provided evidence of the role of DRGs in the establishment of melanophore stem cells either as a niche, a source for the peripheral nerves on which MPs travel to the surface, or both.

So far, although we have ample evidence from lineage tracing that MSCs are located at the site of the DRGs, we do not exclude additional sites. Some of the mitfa:gfp-positive cells along ventral spinal nerves in larval stages (Fig. 2) might also function as MPs.
melanoblasts prior to migration. Presumably most of these cells are melanoblasts destined to migrate along the dorsolateral route. kitlga is expressed early in NC cells located at the site of the future DRG and associated glia. It is tempting to speculate that, in addition to survival and migration, Kitlga has the function of a niche factor of MSCs. In this context, it is interesting to note that in mice, kit ligand (also known as steel factor) provides a niche factor for MSCs. In this context, it is interesting to note that in mice, kit ligand (also known as steel factor) provides a niche factor for survival and migration of primordial germ cells (Gu et al., 2009).

We note that at the early time point, kitlga function distinguishes the various NC fates as in silk mutants only the melanophores are strongly reduced but there is no evidence that glia, peripheral nervous system or other chromatophore types are affected.

**Melanophore progenitors migrate along a vertical path**

In zebrafish, 4D imaging allows tracing of migrating cells and tissue with high resolution in time and space during embryonic and larval life. The premature activation of quiescent stem cells after mitfa-MO knockdown has enabled us to study the origin and migration of postembryonic regenerating MPs already in late larval stages. Regenerating melanophores are responsive to ErbB inhibition in the same time window in early development as adult melanophores (Hultman and Johnson, 2010; Johnson et al., 2011). Tracing regenerating melanophores in larval and adult chimeras confirmed the notion that both populations arise from the same stem cells (Fig. 4).

During metamorphosis, MPs originate at the DRGs and move along the spinal nerves both dorsally and ventrally. These cells populate individual segments with MPs that form a stream of labelled cells (Fig. 2E,F). In chimeric animals, during metamorphosis donor MPs populate the skin of albino recipients with melanophores in vertical streaks running from dorsal to ventral (Fig. 4; data not shown). This suggests that a segmental melanophore streak results from a single or a very small number of stem cells being seeded and that these can give rise to the entire population of MPs of a segment by spreading along the segmental spinal nerves, whereas little lateral spreading occurs. Fig. 10 illustrates schematically the paths taken by melanophore progenitors during embryogenesis (Fig. 10A-C) and metamorphosis (Fig. 10D-F).

**Distinct classes of melanophore progenitors**

On the basis of these observations, several classes of MPs can be distinguished. On the first day of development, a small number of MSCs that reside close to the DRGs in each segment is established. These are normally quiescent throughout larval life and do not require mitfa. They are dependent on Kitlga signalling for their function as MSCs. The development of MSCs depends on migration of NC cells along the ventromedial path and the establishment of the DRGs. This process requires ErbB2/3b receptor signalling during the first 18-24 hours of development.

In later larval and juvenile stages, another class of MPs appears as progeny of activated MSCs. These postembryonic MPs express mitfa:gfp, originate from the DRGs, proliferate and migrate along the spinal nerves. We do see melanising cells along the peripheral nerves in adult fishes, and propose that the MPs observed in extra hypodermal space (Budi et al., 2011) are in fact associated with nerve bundles running along myosepta. Both classes of MPs are present in mitfa mutants.

The DRG-associated MSCs give rise to the melanophores of the adult pattern. We presume but have not yet directly shown that these...
have the capacity of self-renewal, as anticipated from stem cells. Failure in the establishment of MSCs cannot easily be repaired, as seen in the long-lasting effects of the early transient inhibition of the receptors of the ErbB signalling system, the DRG ablation, and the failure to recover the melanophore pattern in kitlga morphants. We propose that stem cell maintenance depends on the microenvironment provided by the DRG and associated glia as a stem cell niche, and speculate that one function of Kitlga signalling is to attract and to maintain them in this niche. The postembryonic MPs may be regarded as transient amplifying cells as they proliferate and provide the adults with the necessary supply of melanophores during stripe formation, growth and regeneration.

It is important to note that most, but not all, adult melanophores originate from these Kitlga signalling-dependent stem cells. During adulthood, spa and slk mutants develop a striped pattern displaying melanophores that do not require Kitlga signalling. Budi et al. (Budi et al., 2011) presented evidence for the existence of melanophore progenitor cells that develop from cells co-expressing foxd3 and mitfa, possibly shared Schwann cell progenitors. A Schwann cell precursor origin of melanocytes derived from nerve innervation is also described in amniotes such as mice and chicken (Adameyko et al., 2009). Clonal analysis in adult zebrafish indicates that some melanophores share a lineage with iridophores (A. Singh, A.M. and C.N.-V., unpublished). Kitlga signalling-independent adult melanophores, however, require ErbB signalling at the same early time interval as the Kit-dependent MSCs (Budi et al., 2008), suggesting that these melanophore progenitors also travel along the ventromedial path. We do not yet know the embryonic origin of adult xanthophores or iridophores.

Although dorsolateral migration holds for some of the larval melanophores in zebrafish, adult melanophores arise from migration along nerves in a similar manner to the melanocyte progenitors described by Adameyko et al. (Adameyko et al., 2009) in mouse and chicken. We present evidence for a stem cell population that resides at the DRGs and that appears to be specific to melanophores. Although we expect the embryonic origin of adult melanophores to be common to fishes, it will be interesting to ascertain whether stem cells with a similar origin are also present in the amniotic vertebrates.

Acknowledgements
Sören Alsheimer, Ajeet Singh, Virgilio Failla and Christian Liebig provided excellent support in confocal microscopy and image processing. We thank Andrey Fadeev for assistance in adult fish photography. Robert Geisler and Ines Gehring provided technical support in the mapping of slk. We thank Jana Krauss and Ajeet Singh for discussion, and Robert Kelsh, Darren Gilmour, Simon Perathoner for valuable comments on earlier versions of the manuscript.

Funding
This research was funded by the Max-Planck Society, Germany.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.087007/-/DC1

References


