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

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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 Mitfa, 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 Northcutt, 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.

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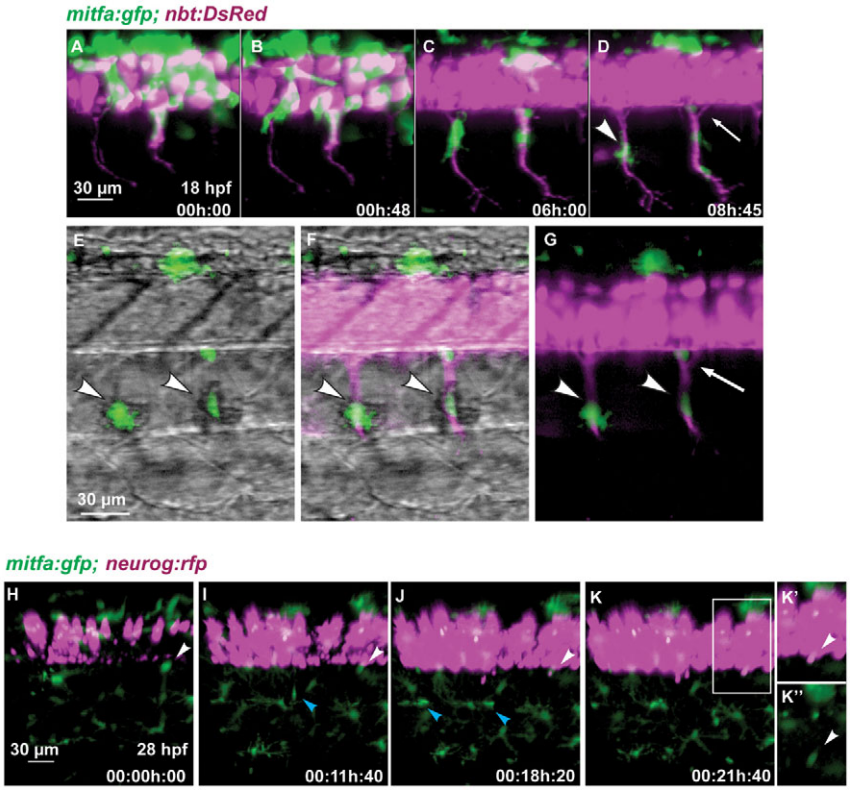

Fig. 1. Melanoblasts migrate along the motor neurons. (**A-G**) Time-lapse confocal imaging of a *Tg(mitfa:gfp;nbt:DsRed)* zebrafish embryo. Medially migrating GFP-positive cells (arrowhead in D) migrate along ventrally extending primary motor axons (DsRed) starting at 18 hpf. A portion of the area in D is shown in G, and in E,F with brightfield illumination. Arrowheads in E-G indicate melanoblasts melanising *in situ*. A GFP-positive cell remains at a ventral position of the neural tube (arrow in D,G). (**H-K″**) *Tg(mitfa:gfp;-8.4neurog1:nrfp)* embryo imaged starting at 28 hpf over a period of 22 hours. A GFP-positive cell (white arrowheads) located close to the ventral base of the neural tube remains at this position over the next 21 hours when the expression of nRFP marks the appearance of the DRG in the same region. A medially positioned cell (blue arrowhead in I) migrates out to the horizontal myoseptum and divides to form melanophores of the lateral stripe (blue arrowheads in J). (K′) Enlargement of the boxed region in K. (K″) Green channel only.

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)*^{w47} by James Lister, VCU Medical Center, Richmond, Virginia, USA; *Tg(−8.4neurog1:nRFP)*^{sb3}, *Tg(−4.9sox10:gfp)* by Robert Kelsh, University of Bath, Bath, UK; *Tg(nbt:DsRed)* by Madeleine van Drenth from the Max-Planck Institute for Developmental Biology, Tübingen, Germany. The following mutant alleles were used in this study: *alb*^{b4} (Streisinger et al., 1986), *erbb2*^{t20604} (*kiz*), *erbb3b*^{t21411} (*hps*) (Rojas-Muñoz et al., 2009), *mitfa*^{w2} (*nac*) (Lister et al., 1999), *kitlga*^{tc244b} (*slk*), *sox10*^{t3} (*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′-TCTCGTTCCATATGAAGAAGTCAA-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) was obtained from Life Technologies.

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 *alb* 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′-CATGTTCAACTATGTGTTAGCTTC-3′), *kitlga* 5.0 ng (*kitlga*-MO, 5′-CTGGATAACAACACTCACCACTTCT-3′) (Hultman et al., 2007) and *erbb3b* 2.0 ng (erbb3b, 5′-TGGGCTCGAACTGGGTGGAAACAA-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)^{sb3} 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).

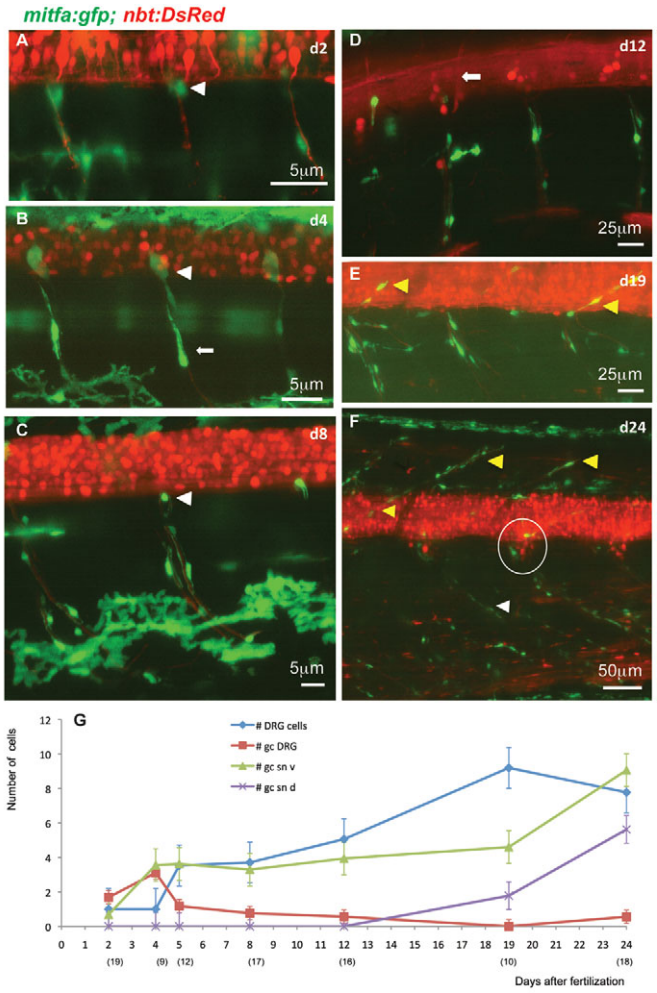

Fig. 2. Stationary MPs located at the DRGs give rise to MPs located along the spinal nerves. (**A-F**) Time-lapse confocal imaging of *Tg(mitfa:gfp;nbt:DsRed)* zebrafish individuals during larval development of consecutive ages. GFP-positive cells at the exit point of the spinal nerves (white arrowheads in A-C) appear to be stationary. Other GFP-positive cells of more elongated shapes are observed along the spinal nerves (e.g. arrow in B). Until 12 dpf (D), no GFP-positive cells can be seen along the dorsally extending spinal nerves (arrow in D), whereas at metamorphic stages (E,F) both the dorsally extending (yellow arrowheads) and the ventrally extending (white arrowhead) spinal nerves show an increased number of associated GFP-positive cells. The number of cells in each DRG (circled in F) has increased to about eight. (**G**) Average numbers of GFP-positive cells per hemisegment located at the DRGs, the ventral and dorsal spinal nerves as well as DRGs at increasing larval age (dpf). The numbers of hemisegments counted is indicated below the respective time points. gc DRG, green cells at DRG; gc sn v, green cells at spinal nerves ventral; gc sn d, green cells at spinal nerves dorsal.

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

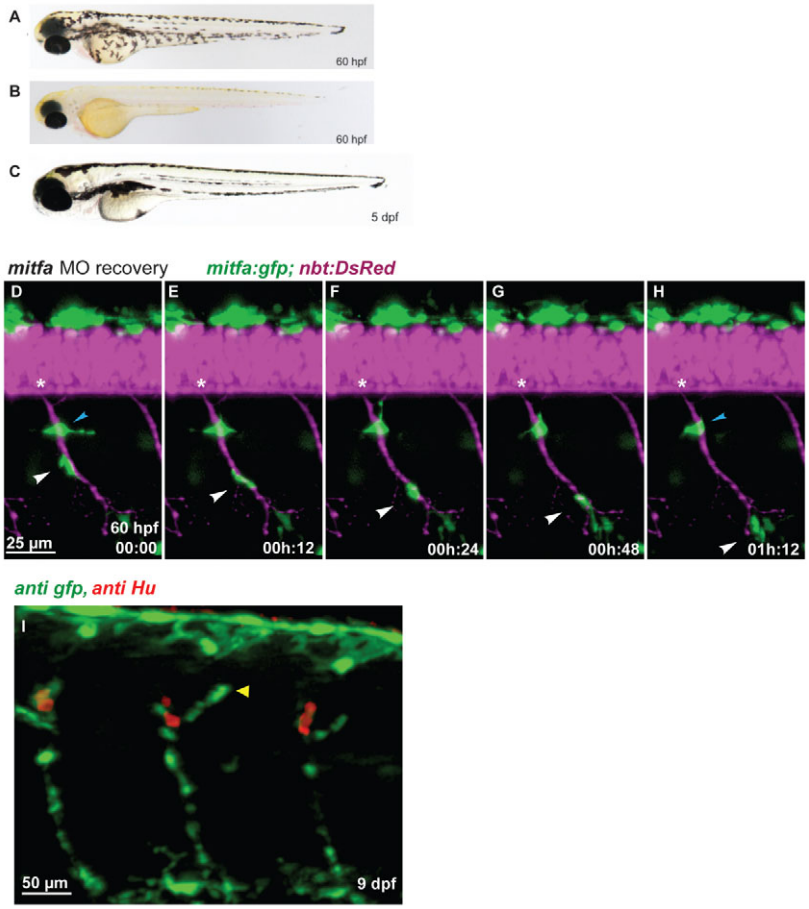

Fig. 3. Regenerating MPs emerge at the site of the DRGs and travel along the spinal nerves. (**A-C**) In contrast to wild type (A), *mitfa*-MO-treated zebrafish embryos (B) lack larval melanophore pigmentation until 60 hpf, but regenerate the larval melanophore stripes completely by day 5 (C). (**D-H**) *Tg(mitfa:gfp; nbt:DsRed)* embryo previously treated with *mitfa*-MO imaged for several hours starting at 60 hpf (digital sectioning). A GFP-positive cells (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 close association with primary motor neurons, labelled in Fig. 1A-G with a transgene expressing DsRed under the control of a neuron-specific promoter (*nβt: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 embryos do not show pigmented melanophores. Strikingly, in subsequent stages, larval melanophore pigmentation recovers and by 5 dpf the larval melanophore pattern is completely restored (Mellgren and Johnson, 2004) (Fig. 3A-C). This indicates that knockdown of Mitfa during early development affects melanoblasts committed to direct differentiation, whereas the melanoblast population that restores the pattern is derived from melanophore stem cells that did not require Mitfa early in development (Hultman and Johnson, 2010).

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.

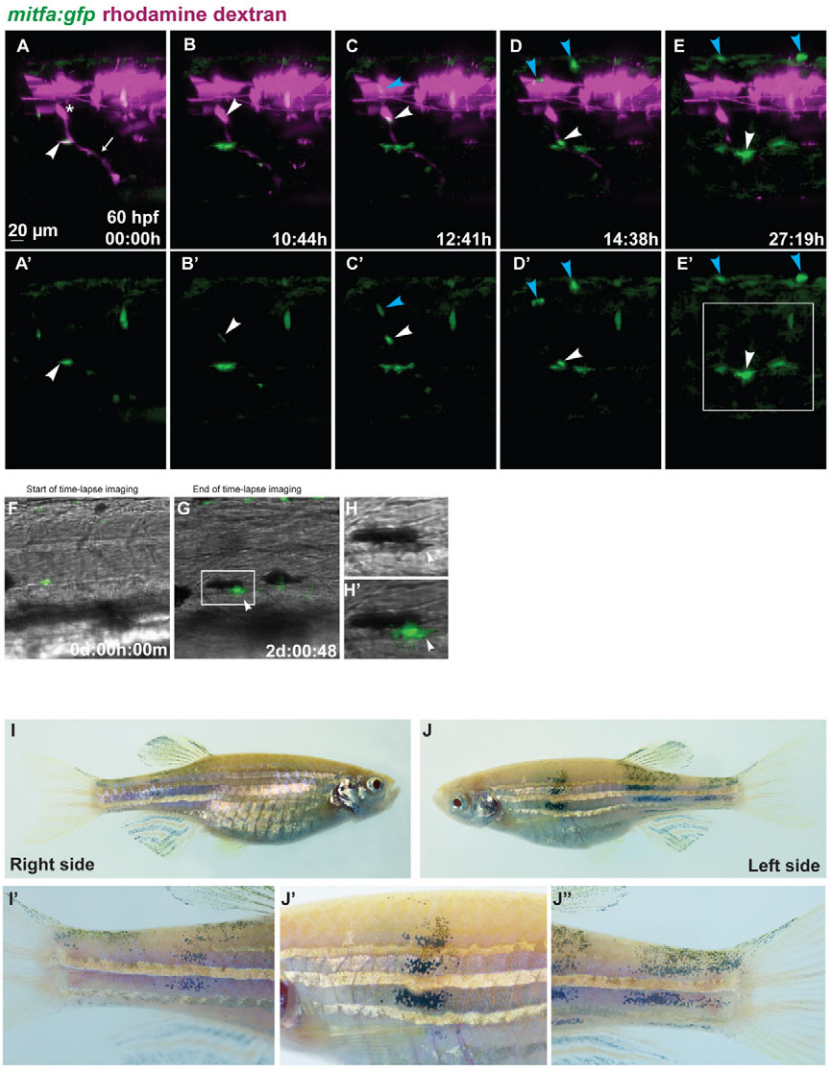

Fig. 4. Tracing individual MPs during regeneration in chimeric animals. Blastula transplantations were performed with *Tg(mitfa:gfp)* zebrafish embryos injected with rhodamine dextran as donors and *alb* embryos injected with *mitfa*-MO as recipients. (**A-E′**) Red and green channel are shown in A-E, 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 arrowheads 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′,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*, *kiz*) 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* fishes are devoid of melanophores (Budi et al., 2008) (Fig. 5A). Small molecule inhibitor studies indicated that ErbB3b/ErbB2 signalling is required during the first day of embryogenesis for the subsequent development of metamorphic melanoblasts (Budi et al., 2008). A close correlation between the position of defects along the anteroposterior pattern in the adult fishes and the time window of inhibition coinciding with 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 *nβt:DsRed*. In wild-type embryos, *sox10: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.

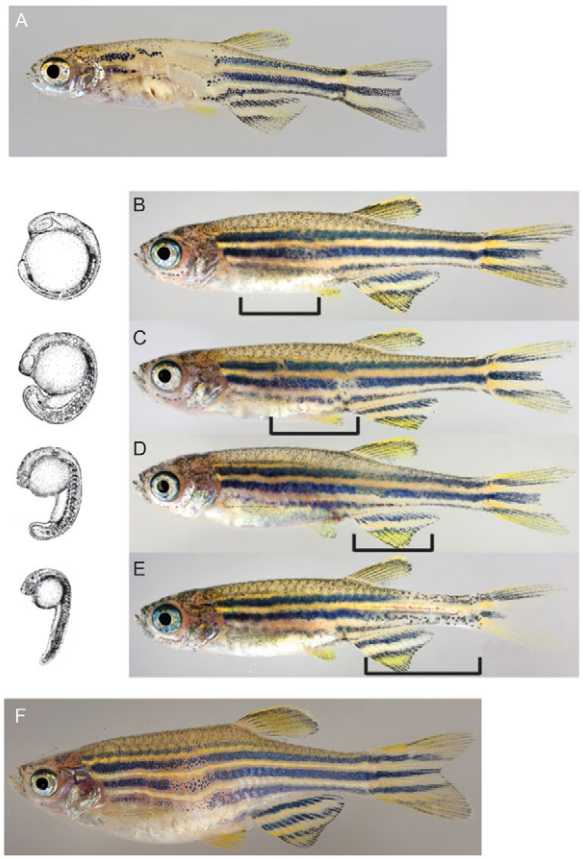

Fig. 5. The Role of ErbB signalling and the DRGs for adult melanophore pigmentation. (**A**) *hps/erbb3b*^{t21411} adult fish showing a dramatic regional reduction of melanophores. (**B-E**) Adult fish treated at successive stages of somitogenesis (left) with the ErbB inhibitor PD168393. The later the 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.

sparse like encodes Kit ligand a

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 melanophores 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 1. Quantification of the association of DRGs and GFP-positive cells as shown in Fig. 6F

	Count	
DRG staining	Control	*erb3b*MO+*mitfa*MO
Hu+; *mitfa*:GFP+	190	138
Hu+; *mitfa*:GFP–	40	30
Hu–; *mitfa*:GFP+	2	5
Hu–; *mitfa*:GFP–	4	77
Total	236	250

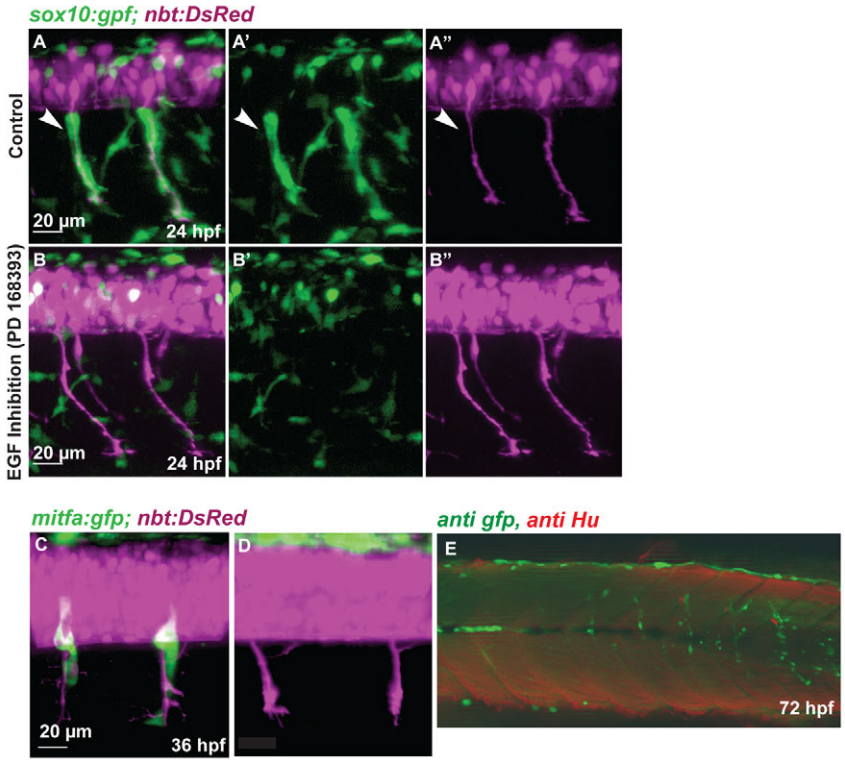

Fig. 6. NC migration along the ventromedial path is blocked by inhibition of ErbB receptors. (**A-B″**) *Tg(sox10:gfp*; *nbt:DsRed*) zebrafish embryos at 24 hpf. (A,B) Red and green channel (merge); (A′,B′) green channel; (A″,B″) red channel. Medial NC cells (green) covering the primary motor axons (white 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.

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

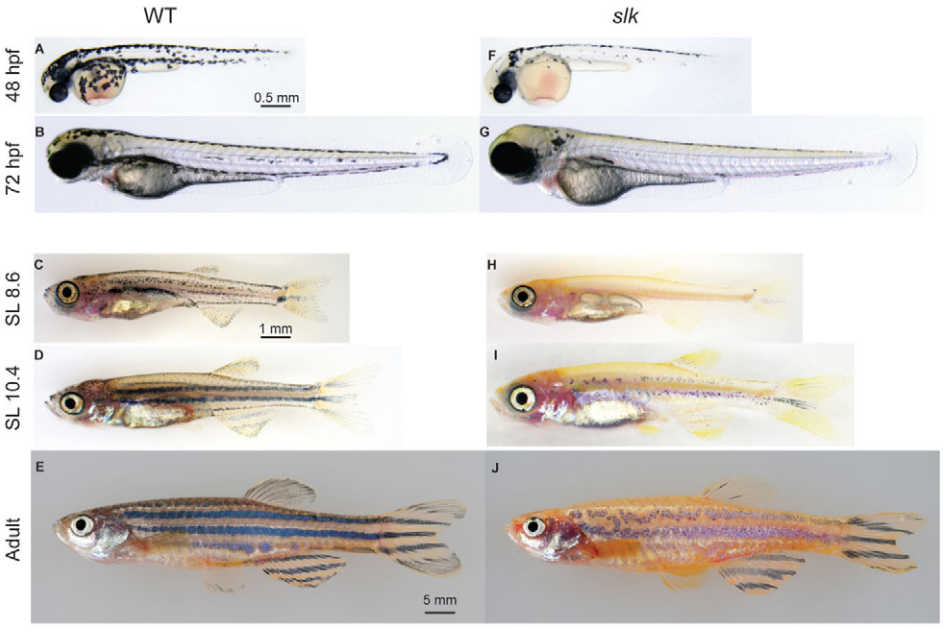

Fig. 7. The phenotype of *sparse like*^{tc244b} fish at different developmental stages. (**A-J**) The phenotype of wild-type (A-E) and *sparse like* (F-J) fish at different developmental stages. Unlike wild-type fish (C), *slk* fish are almost completely devoid of melanophores at the beginning of metamorphosis (H). Adult *slk* mutants (J) form stripes with strong reduction in melanophore number compared with wild-type siblings (E). SL, standard length.

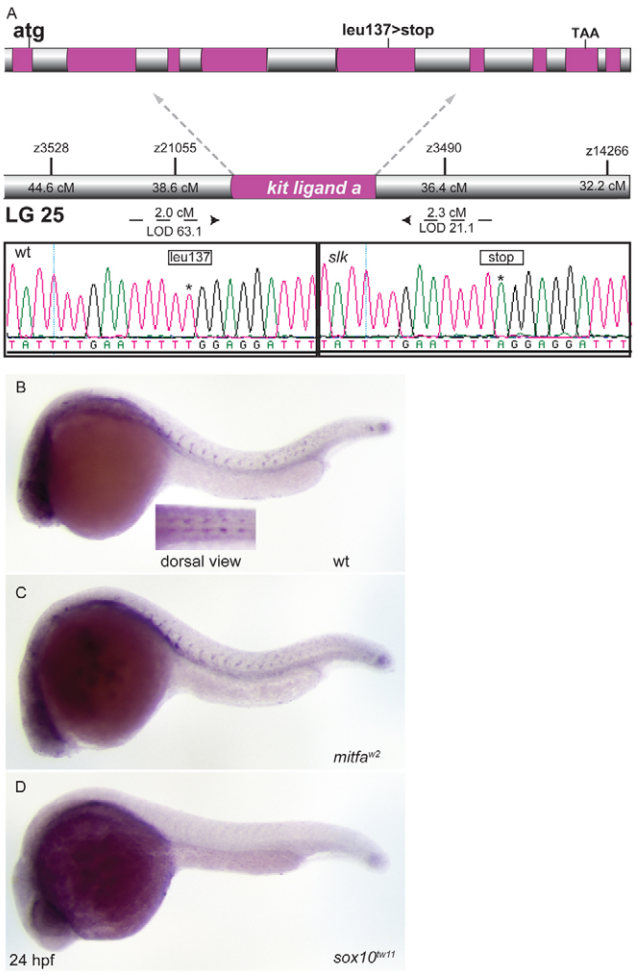

Fig. 8. *slk* encodes Kitlga and is expressed in NC cells. (**A**) *slk* mutants reveal a T to A base substitution in exon 5 of the *kitlga* transcript causing a premature stop codon. (**B-D**) *kitlga in situ* hybridisation. At 24 hpf, *kitlga* is expressed in NC cells migrating along the medial path. This expression domain is maintained in *mitfa* mutants (C) but not in *sox10* mutant embryos (D).

kitlga mutant embryos, embryonic MPs located at the DRG are missing or fail to function as stem cells. It also suggests that in normal development MPs are established during the first few days of embryogenesis when the MO knockdown is effective, under the control of Kitlga signalling.

In *slk* embryos, we do observe that *mitfa:gfp* cells undergo apoptosis before NC migration commences (Fig. 9C,D), reflecting the *slk* phenotype. This observation supports the notion that there is a population of melanoblasts in this dorsal location that is specified before migration along the dorsolateral route and that requires Kitlga signalling for its survival.

DISCUSSION

Identification of melanophore stem cells

Although the presence of embryonic melanophore stem cells (MSCs) had already been postulated (Budi et al., 2008; Johnson et al., 2011), their location remained elusive. We demonstrate that the embryonic MPs we observe during early development closely associated with the emerging DRGs are MSCs. These MSCs can be activated prematurely using MO knockdown of Mitfa to remove 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.

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

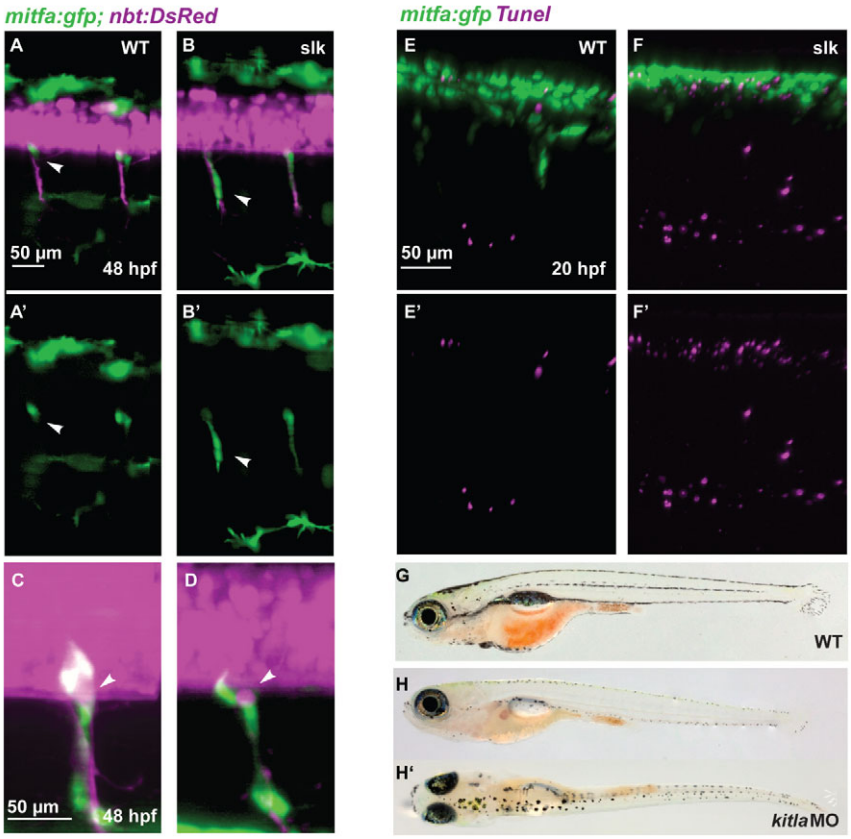

Fig. 9. Abnormal NC migration and apoptosis in *slk* embryos. (**A-B′**) GFP-positive cells in double transgenic *Tg(mitfa:gfp; nbt:DsRed)* wild-type (A,A′) and *slk* mutant (B,B′) embryos at 48 hpf. In the wild-type embryo, GFP-positive NC cells remain at the position of the DRG and have a rounded morphology (white arrowhead in A,A′) whereas in *slk* mutants they are stretched along the nerves and appear to be migratory (white arrowheads in B,B′). A′, B′ show green channel only. (**C**,**D**) Magnification showing *mitfa:gfp*-labelled cells at the exit point of the spinal nerves at 48 hpf of wild-type (C) and *slk* mutant (D) embryos. The arrowheads point to DRGs. (**E-F′**) TUNEL staining in *Tg*(*mitfa:gfp)* embryos in wild-type (E,E′) and *slk* (F,F′) embryos at 20 hpf. E′,F′ show red channel only. (**G-H′**) Wild type (G) and *slk* morphant (H,H′) at 20 dpf (5.7 mm SL).

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 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 *slk* 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

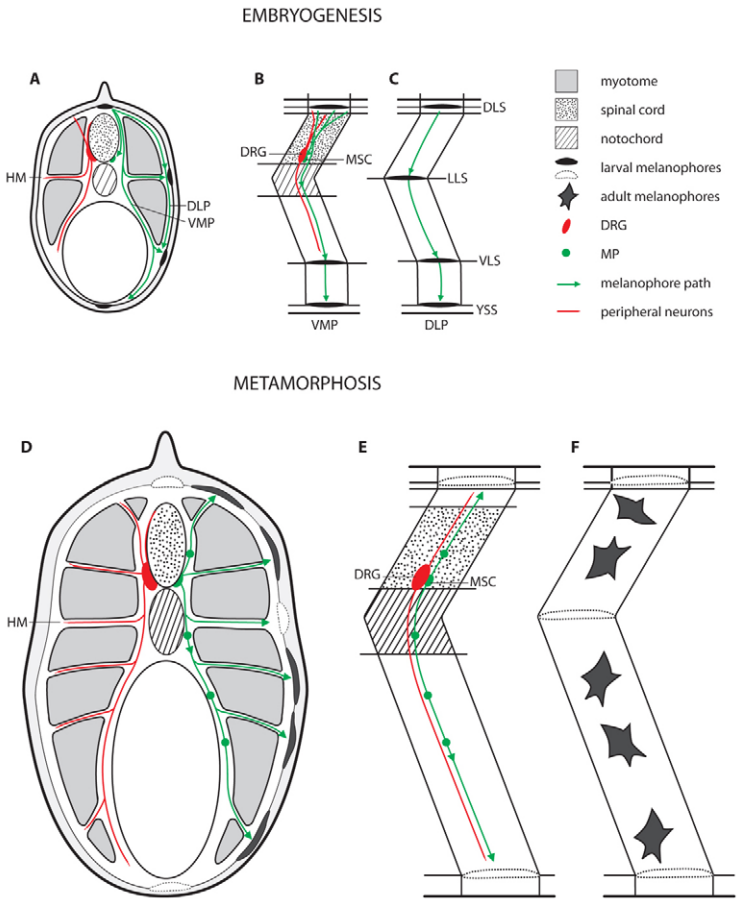

Fig. 10. Model of migration of melanophore progenitors during embryogenesis and metamorphosis. (**A**) Schematic cross-section through a trunk segment indicating the path of the peripheral neurons emerging from the DRG (left side). The migratory route taken by the melanoblasts along the ventromedial path (VMP) and the dorsolateral path (DLP) (right side). (**B**) Side view of the ventromedial path showing that melanoblasts contribute to the ventral larval stripes (VLS), they migrate through the horizontal myoseptum (HM) to form the lateral larval stripe (LLS). (**C**) Melanoblasts traveling along the dorsolateral path between somites and epidermis contribute to all three stripes. DLS, dorsal larval stripe; YSS, yolk sac stripe. (**D-F**) During metamorphosis adult melanophores arrive in the skin originating from melanophore progenitors (MPs) associated with the spinal nerves (red) that innervate the myotome via myosepta. These MPs are derived from embryonic melanophore stem cells (MSCs) located at the DRGs. The larval melanophores (dotted) later disappear.

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 the ventromedial path of NC migration has also been observed 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.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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