Development 135, 2603-2614 (2008) doi:10.1242/dev.019299

Embryonic requirements for ErbB signaling in neural crest development and adult pigment pattern formation

Erine H. Budi, Larissa B. Patterson and David M. Parichy*

Vertebrate pigment cells are derived from neural crest cells and are a useful system for studying neural crest-derived traits during post-embryonic development. In zebrafish, neural crest-derived melanophores differentiate during embryogenesis to produce stripes in the early larva. Dramatic changes to the pigment pattern occur subsequently during the larva-to-adult transformation, or metamorphosis. At this time, embryonic melanophores are replaced by newly differentiating metamorphic melanophores that form the adult stripes. Mutants with normal embryonic/early larval pigment patterns but defective adult patterns identify factors required uniquely to establish, maintain or recruit the latent precursors to metamorphic melanophores. We show that one such mutant, *picasso,* lacks most metamorphic melanophores and results from mutations in the ErbB gene *erbb3b*, which encodes an EGFR-like receptor tyrosine kinase. To identify critical periods for ErbB activities, we treated fish with pharmacological ErbB inhibitors and also knocked down *erbb3b* by morpholino injection. These analyses reveal an embryonic critical period for ErbB signaling in promoting later pigment pattern metamorphosis, despite the normal patterning of embryonic/early larval melanophores. We further demonstrate a peak requirement during neural crest migration that correlates with early defects in neural crest pathfinding and peripheral ganglion formation. Finally, we show that *erbb3b* activities are both autonomous and non-autonomous to the metamorphic melanophore lineage. These data identify a very early, embryonic, requirement for *erbb3b* in the development of much later metamorphic melanophores, and suggest complex modes by which ErbB signals promote adult pigment pattern development.

KEY WORDS: Erbb, *erbb3*, HER3, Melanophore, Metamorphosis, Stem cell, Zebrafish

INTRODUCTION

The generation of adult form remains an enduring problem. An interesting system for studying the salient genetic and cellular mechanisms is the larva-to-adult transformation, or metamorphosis, of amphibians and fishes (Moran, 1994; Parichy, 1998; Webb, 1999; Brown and Cai, 2007). In zebrafish, *Danio rerio*, metamorphosis includes dramatic changes in the digestive, excretory and sensory systems; the peripheral nervous system; the skeleton, fins and integument; as well as physiology and behavior (Cubbage and Mabee, 1996; Brown, 1997; Elizondo et al., 2005; Tingaud-Sequeira et al., 2006; Engeszer et al., 2007).

The pigment pattern is another, particularly accessible, trait altered at metamorphosis (Kelsh, 2004; Parichy, 2006). The early larval pigment pattern develops in the embryo from neural crest-derived pigment cells, or chromatophores; this pattern is completed by 5 days post-fertilization (dpf) and comprises stripes of black melanophores with intervening yellow xanthophores. This pattern persists until metamorphosis (~14 dpf) when melanophores begin to differentiate outside of the early larval stripes. During the next 2 weeks, new adult stripes begin to form as some metamorphic melanophores migrate to sites of adult stripe formation and other melanophores differentiate already at these sites. The result is a juvenile pigment pattern with two 'primary' stripes of melanophores, bordering an 'interstripe' of xanthophores.

Embryonic/early larval chromatophores and metamorphic chromatophores might have commonalities as well as differences. For example, several mutants lack chromatophore types or pigments both before and after metamorphosis (Lister et al., 1999; Parichy et al., 2000b; Lamason et al., 2005). Others exhibit defects in the adult but not in the embryo (Parichy et al., 2000a; Iwashita et al., 2006; Watanabe et al., 2006). Mutants in this latter class are interesting because they identify genes uniquely required to establish, maintain or recruit latent precursors that contribute to adult form. Included among these are two mutants, *puma* and *picasso*, each having a normal early larval pigment pattern but fewer metamorphic melanophores (Fig. 1A,B) (Parichy and Turner, 2003b; Parichy et al., 2003; Quigley et al., 2004). Whereas *puma* is required autonomously to metamorphic melanophore precursors during pigment pattern metamorphosis, the cellular and temporal requirements for *picasso* are not known.

Here, we show that *picasso* is allelic to *erbb3b*, which encodes an epidermal growth factor receptor (EGFR)-like tyrosine kinase. *erbb3b* is one of two zebrafish orthologues of human epidermal growth factor receptor 3 (*HER3*, *ERBB3*) and part of a larger family that includes *EGFR* (*ERBB1*), *ERBB2* and *ERBB4* (Citri and Yarden, 2006; Stein and Staros, 2006). Ligands for ErbB receptors include Egf and neuregulins 1, 2 and 3. The receptors form dimers with individual monomers exhibiting different activities and ligand specificities: for example, ErbB3 lacks endogenous kinase activity, while ErbB2 lacks its own high affinity ligand. Whereas several heterodimers are possible, only a subset seems to have biological significance, with ErbB3 acting with ErbB2 (Graus-Porta et al., 1997; Jones et al., 1999; Oda et al., 2005) and potentially with Egfr (Soltoff et al., 1994; Frolov et al., 2007; Poumay, 2007). ErbB receptors function in glial morphogenesis (Lyons et al., 2005; Britsch, 2007), and their misregulation is associated with a variety of cancers (Breuleux, 2007; Sergina and Moasser, 2007).

In this study, we find that metamorphic melanophores express *erbb3b*, suggesting an autonomous activity that occurs late, during the larva-to-adult transformation. Nonetheless, we show that *erbb3b*

Department of Biology, Institute for Stem Cell and Regenerative Medicine, University of Washington, Box 351800, Seattle, WA 98195-1800, USA.

*Author for correspondence (e-mail: dparichy@u.washington.edu)

Accepted 19 March 2008

functions both autonomously and non-autonomously to the metamorphic melanophore lineage. We also identify a major critical period for ErbB signals during embryonic neural crest cell migration, 2 weeks before metamorphosis, indicating a novel role for ErbB signals in establishing precursors to adult chromatophores. Finally, we demonstrate cryptic requirements for ErbB signals during metamorphosis itself, suggesting redundant functions with other pathways at this later stage. Our study provides new insights into the development of adult form and the genetic requirements of a trait expressed before and after metamorphosis.

MATERIALS AND METHODS

Fish stocks

Fish were maintained at 26-28°C, 14L:10D (Westerfield, 2000). *picasso* mutants were recovered in screens for *N*-ethyl-*N*-nitrosourea-induced mutations and mapped using the partially inbred strains AB^{wp} and wik^{wp}.

Cell transplantation

Chimeric embryos were generated by transplanting cells at blastula stages (3.3-3.8 hours post-fertilization) and then were reared through metamorphosis (Parichy and Turner, 2003a).

Pharmacological Erbb inhibitor treatments

Stock solutions of AG1478 [4-(3-chloroanilino)-6,7-dimethoxyquinazoline; Calbiochem] or PD158780 (4-[(3-bromophenyl)amino]-6-(methylamino)-pyrido[3,4-d]pyridimine; Calbiochem) were diluted in DMSO. Fish were treated with 3 μM of either drug in 10% Hanks solution. To facilitate penetration, 0.5% DMSO was added to all media and embryos were dechorionated prior to treatment. Fish were reared in agar-lined Petri dishes or glass beakers and solutions were changed daily. Fish reared in either drug throughout development invariably died prior to formation of the adult pigment pattern, so could not be analyzed.

Morpholino injection

A splice-blocking morpholino against *erbb3b* [TGGGCTCGCA-ACTGGGTGGAAACAA (Lyons et al., 2005)] was obtained from GeneTools (Eugene, OR). One- or two-cell embryos were injected with 300-500 pg and reared through formation of the adult pigment pattern.

PCR, genotyping, and sequencing

For RT-PCR, metamorphosing larvae were euthanized and rinsed in 10% Hanks solution, after which tissues were dissected and placed in dissociation medium (1 mg/ml collagenase type IV; 0.1 mM epinephrine; 2 mg/ml bovine serum albumin; 0.1 mg/ml trypsin inhibitor) (Clark et al., 1987). Cells were picked and transferred to 5% fetal bovine serum in PBS for 10 minutes, then picked and extracted for RNA. cDNAs were synthesized with the Superscript III CellsDirect cDNA Synthesis System (Invitrogen) and RT-PCRs were performed using the following primers (forward, reverse): *erbb3b*, ACTCCCTAAAAATCCCTGTGG, GGCGAAGGTGTTGA-AGTAAT; *erbb2*, CACCGGAAGTTTACTCACCAA, GATCTCCAAC-ATTTGACCAT; *erbb3a*, TGACTCCATCCACTACTGCTG, TTCTT-CACCAGCACCTCTGTT; *egfr*, CCGTTGGTGTGTGTTTTGAG, GCTTTTCAGGAGGGAGACTTTC; *dct*, ACCTGTGACCAATGAG-GAGATT, TACAACACCAACACGATCAACA; β-actin, GTTTTCC-CCTCCATTGTT, GGTGTTGAAGGTCTCGAACA; *erbb4*, CTGCTG-CTCAACTGGTGTGT, CCAGTGCCATCACAGCTTCT.

For genotyping $picasso^{wp.r2e2}$, we amplified genomic DNA (pcs-wpr2e2*: TTGGTTACCATTGTGGTTGTTT, TCTTCATGGTAGCTCAGAAAC-ATC) from individual embryos and digested PCR products with *Rsa*I restriction enzyme. The wild-type amplicon cuts with *Rsa*I at position 219, whereas the mutant allele does not cut.

In situ hybridization

Analyses of mRNA distributions in embryos followed standard protocols (Parichy et al., 2000b). In situ hybridization on larvae followed (Elizondo et al., 2005), but overnight incubation was used for probes and antibodies (a detailed protocol is available at http://protist.biology.washington.edu/dparichy/). For analyses of gene expression in families segregating *picasso* mutant alleles, individual embryos or larvae were imaged after staining then transferred to DNA extraction buffer and processed to determine genotypes retrospectively.

Immunohistochemistry

Trunks of 12 dpf larvae were fixed in 4% PFA in PBS for 6 hours at room temperature then permeabilized by washing overnight in deionized water. Specimens were equilibrated in PDTX (PBS containing 1% DMSO and 0.3% Triton X-100) three times for 30 minutes each, then blocked with 5% goat serum in PDTX for 4 hours. Larvae were incubated overnight at 4°C with primary antibody mAB 16A11 (Marusich et al., 1994; Henion et al., 1996) against HuC/D (1:200 in blocking solution), washed in PDTX, incubated with secondary antibody (Alexa Fluor 568; Molecular Probes), then washed and visualized.

Image analyses and statistical methods

Embryos or larvae were viewed with Olympus SZX-12 or Zeiss Lumar stereomicroscopes, or with Zeiss Axioplan 2 or Zeiss Observer compound microscopes. Digital images were collected with Zeiss Axiocam cameras using Zeiss Axiovision and corrected for contrast and color balance when necessary.

Statistical analyses were performed with JMP 7.0 (SAS Institute, Cary, NC). For counts of melanophores, individual cells were distinguished from one another by treating fish with epinephrine to contract melanosomes towards cell bodies. Densities of melanophores were determined by counting melanophores within a rectangular region delimited by: anteriorly, the anterior margin of the dorsal fin insertion; posteriorly, the posterior margin of the anal fin insertion; dorsally, the posterior margin of the dorsal fin insertion; ventrally, the posterior margin of the anal fin insertion. To control for variation in larval development stage, we tested for effects of larval size (measured as flank height at the posterior margin of the anal fin, hpa) as a covariate in analyses (Parichy and Turner, 2003b), and retained this factor if $P<0.05$, though analyses without the co-factor yielded qualitatively equivalent results. Analyses of melanophore densities were treated as multifactorial analyses of variance or covariance with replicates as blocks. Residuals in all analyses were confirmed to be normally distributed and homoscedastic. Least squares means (correcting for size, replicate variation, or both) are presented in figures below, with significant differences assessed by Tukey-Kramer comparisons to preserve an experiment-wide $\alpha=0.05$.

For analyses of embryonic critical periods for Erbb signals in *kit* mutant larvae (see below), adult pigment patterns were scored qualitatively for stripe disruption. Breaks in stripes were considered present when three or fewer melanophores were present over a defined anterior-posterior length, as scaled by hpa (above): stripes exhibiting breaks ≤0.5 hpa were scored '0'; breaks between 0.5 and 1 hpa were scored '1'; breaks >1 hpa were scored '2'. Dorsal and ventral stripes were scored individually, then summed to generate a 'stripe break score' of 0-4. To test for differences among treatment groups, we compared ordinal scores using non-parametric Wilcoxon tests and contingency table analyses. Both methods yielded equivalent results; for simplicity, we present only the former (complete analyses available on request).

RESULTS

Metamorphic melanophore development requires *erbb3b*

To learn when *picasso* mutants first exhibit pigment pattern defects, we examined embryos and early larvae, and we imaged individual fish daily from early larval stages through formation of the adult pigment pattern. Pigment cell complements of embryos and early larvae were normal (Fig. 1C,D). Subsequently, *picasso* mutants largely failed to develop metamorphic melanophores, particularly in the mid-trunk, and instead retained early larval melanophores even as adults (Fig. 1E-L). In the posterior trunk of *picasso* mutants, seemingly more complete melanophore stripes form (Fig. 1B). Posterior stripes in *picasso* mutants appear more complete because more embryonic/early larval melanophores become situated in the

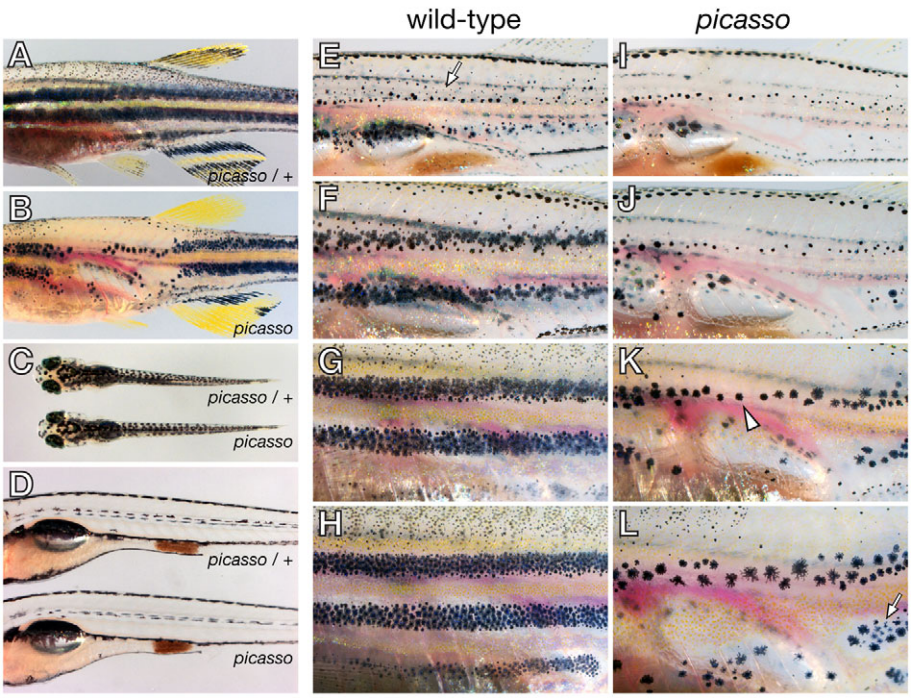

Fig. 1. Defective adult pigment pattern but normal embryonic/early larval pigment pattern of *picasso* mutants. (**A**) Wild-type adult pigment pattern of *picasso* heterozygote. (**B**) Defective pigment pattern of *picasso* homozygote. (**C**,**D**) Pigment patterns of wild-type and mutant siblings were indistinguishable at 5 dpf. (**E-H**) Repeated images of wild-type (*picasso*/+) larvae revealed normal development of initially dispersed metamorphic melanophores that organized into stripes (arrow, E), as well as metamorphic melanophores that developed already at sites of stripe formation. (**I-L**) *picasso* mutant larvae develop very few metamorphic melanophores (arrow, L), and instead many embryonic/early larval melanophores (arrowhead, K) persisted into the adult. (E,I) 17 dpf. (F,J) 23 dpf. (G,K) 31 dpf. (H,L) 40 dpf.

position of adult stripes in this region, and because more metamorphic melanophores differentiate near these cells when compared with the mid-trunk (Fig. 2).

We mapped *picasso* to chromosome 23 in the vicinity of *erbb3b* and found that *picasso* failed to complement an excess neuromast phenotype of an *erbb3b*-null allele (all of these alleles are recessive and homozygous viable, though weaker than wild type) (Lyons et al., 2005). *erbb3b* cDNAs had premature stop codons in each of two *picasso* alleles (Fig. 3), demonstrating that the *picasso* phenotype arises from mutations in *erbb3b*.

In embryos, *erbb3b* is expressed in neural crest cells and glia (Lyons et al., 2005). In metamorphosing larvae, we similarly found *erbb3b* expression in glia (Fig. 4A). To determine whether *erbb3b* might be expressed in other tissues below the threshold of detection by in situ hybridization, we used RT-PCR. We detected *erbb3b* transcripts in both isolated melanophores and in juvenile fin (comprising melanophores, melanophore precursors, bone, skin, vasculature and other cell types; Fig. 4C). We also detected the *erbb3b* paralogue, *erbb3a*, in glia and fin, though not in metamorphic melanophores (Fig. 4B,C). As ErbB receptors act as heterodimers, we tested where other ErbB genes are expressed (Fig. 4C): *erbb2* was expressed in metamorphic melanophores and in fin; *egfr* was not expressed in metamorphic melanophores, although it was expressed in fin; and we could not detect *erbb4* in melanophores or in fin (data not shown). *erbb2* and *egfr* are also widely expressed in zebrafish embryos (Goishi et al., 2003; Lyons et al., 2005).

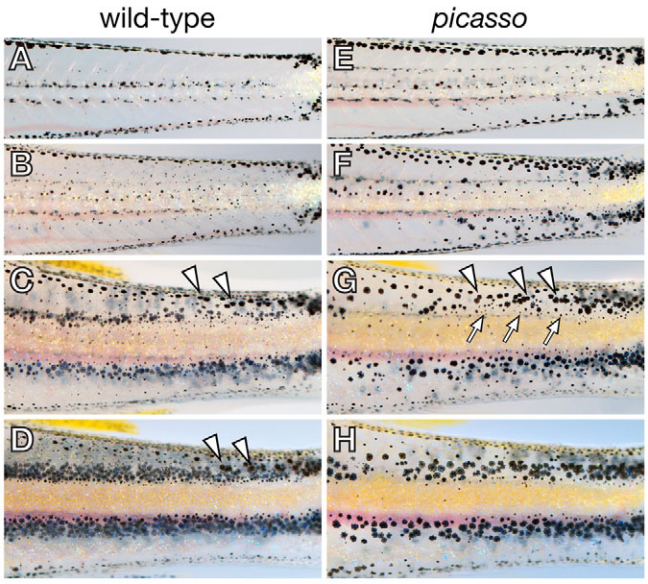

Fig. 2. Regulative posterior adult stripe formation in the *picasso* mutant. Adult pigment pattern development of wild-type (A-D) and *picasso* mutant (E-H) larvae. (**A-D**) In wild type, most embryonic/early larval melanophores remained in a dorsal position, though a few were incorporated into the adult stripe posteriorly (arrowheads in C,D). (**E-H**) In *picasso* mutants, more embryonic/early larval melanophores (e.g. arrowheads in G) were incorporated into an incomplete stripe. Additional metamorphic melanophores differentiated (e.g. arrows in G) where persisting embryonic/early larval melanophores contributed to the adult stripe. (A,E) 14 dpf. (B,F) 16 dpf. (C,G) 20 dpf. (D,H) 24 dpf.

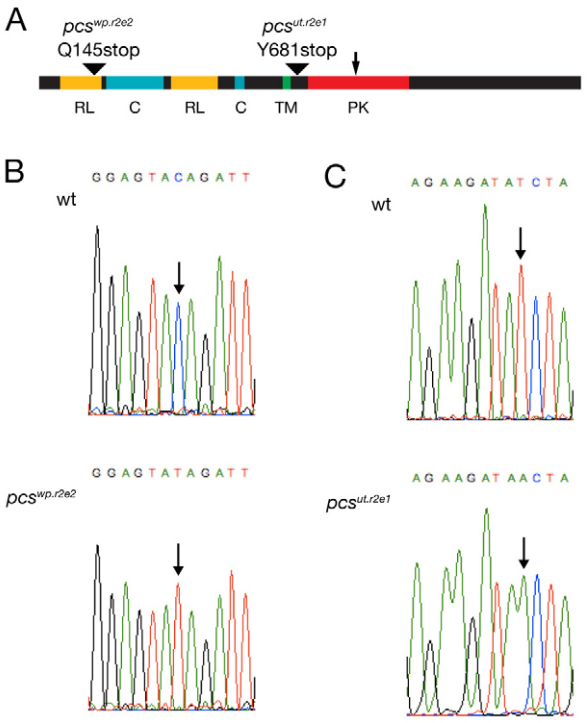

Fig. 3. *picasso* is allelic to *erbb3b*. (**A**) Schematic of *erbb3b* cDNA showing *picasso* lesions. RL, receptor L (ligand-binding) domains; C, furin-like cysteine-rich domains; PK, protein kinase domain; TM, transmembrane domain; arrow, N835 interruption to kinase domain characteristic of erbb3. (**B**) Premature stop codon in $pcs^{wp.r2e2}$ (C433T: Q145stop). (**C**) Premature stop codon (arrow) in $pcs^{ut.r4e1}$ (T2043A: Y681stop), as shown versus wild type.

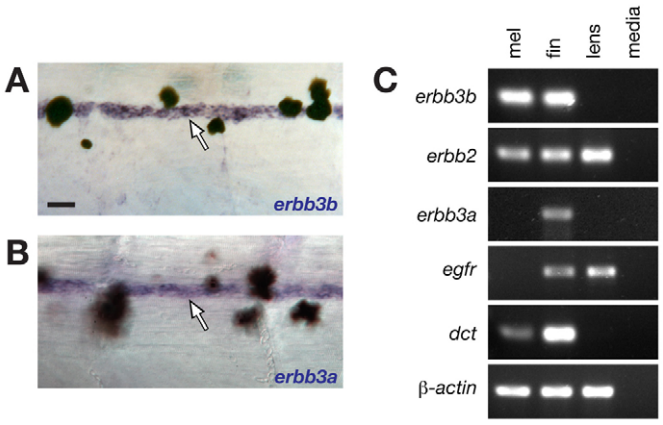

Fig. 4. ErbB gene expression in metamorphosing larvae. (**A**,**B**) erbb3 loci were expressed in glia (~18 dpf). Arrows, glial cells surrounding midbody lateral line. (A) *erbb3b*. (B) *erbb3a*. (**C**) RT-PCR revealed metamorphic melanophore expression of *erbb3b* and *erbb2*, but not *erbb3a* or *egfr*. All four ErbB genes were expressed in adult fin. mel, melanophore; *dct*, *dopachrome tautomerase* (encoding a melanin synthesis enzyme expressed by melanophores and their precursors). *dct* is more strongly expressed in melanoblasts (present in fin) compared with melanophores (mel). Scale bar: in A, 0.5 mm for A,B.

To determine what steps in metamorphic melanophore development require *erbb3b*, we examined molecular markers (Fig. 5A-F). *picasso* mutants were deficient during metamorphosis for cells expressing early neural crest markers (*crestin*, *sox10*), as well as early and late markers of the melanophore lineage (*mitfa*, *dct*). *picasso* mutants also had transiently fewer cells expressing xanthophore lineage markers (*xdh*, *csf1r*) and fewer *myelin basic protein*$^{+}$ (*mbp*$^{+}$) glia (Fig. 5G,H and data not shown).

erbb3b functions autonomously and non-autonomously to the metamorphic melanophore lineage

erbb3b might promote adult pigment pattern formation by acting autonomously to the metamorphic melanophore lineage, but also could have non-autonomous effects if, for example, *erbb3b*-dependent cells provide signals required by metamorphic melanophores or their precursors. To test these possibilities, we constructed genetic mosaics by transplanting cells between blastula stage embryos.

If *erbb3b* acts autonomously to the metamorphic melanophore lineage, then wild-type melanophores should develop in *picasso* mutants and these cells should form wild-type stripes. If *erbb3b* acts non-autonomously, then wild-type melanophores should develop where *picasso* mutant melanophores develop (anteriorly and posteriorly), but not where *picasso* mutant melanophores are absent (mid-trunk) (Fig. 1B). Wild-type (β-actin::EGFP^{+}) → *picasso* chimeras developed wild-type metamorphic melanophores at high density anteriorly and posteriorly (Fig. 6A,B), but often developed few if any metamorphic melanophores in the mid-trunk (Fig. 6A,C), like *picasso* mutants. In reciprocal *picasso* (β-actin::EGFP^{+}) → wild-type chimeras, we never found donor *picasso* mutant metamorphic melanophores in the adult pigment pattern. These findings suggest both non-autonomous and autonomous roles for *erbb3b*.

Given that metamorphic melanophores express *erbb3b* and *erbb2*, differences between wild-type and *picasso* mutant melanophores could be further revealed as differences in their abilities to populate a flank lacking melanophores. We therefore transplanted wild-type or *picasso* mutant cells to $nacre^{w2}$ mutant hosts, which lack their own melanophores because of a mutation in *mitfa*, which functions cell-autonomously in melanophore specification (Lister et al., 1999; Parichy and Turner, 2003a). In wild-type → *nacre* chimeras, embryonic/early larval melanophores often developed, and metamorphic melanophores differentiated to form patches of stripes (Fig. 6D). In *picasso* → *nacre* chimeras, embryonic/early larval melanophores developed about as often, but metamorphic melanophores did not appear and, instead, embryonic/early larval melanophores persisted into the adult (Fig. 6E). Interestingly, metamorphic melanophores failed to develop in *picasso* → *nacre* chimeras, even anteriorly and posteriorly; this difference from *picasso* mutants may arise because the melanophore-free *nacre* background would preclude community effects from contributing to pattern regulation in these regions (Fig. 2) (Parichy et al., 2000b; Parichy and Turner, 2003b). Together, genetic mosaic analyses indicate that ErbB signals are required autonomously and non-autonomously during metamorphic melanophore development.

ErbB activity is required in the embryo for metamorphic melanophore development

The adult pigment pattern of *picasso* mutants could reflect *erbb3b* activities early or late. For example, *erbb3b* could function in the embryo to establish a population of precursors that differentiates at metamorphosis. Or *erbb3b* could act later in maintaining or expanding such a population, or still later, during their differentiation into metamorphic melanophores. To distinguish among these

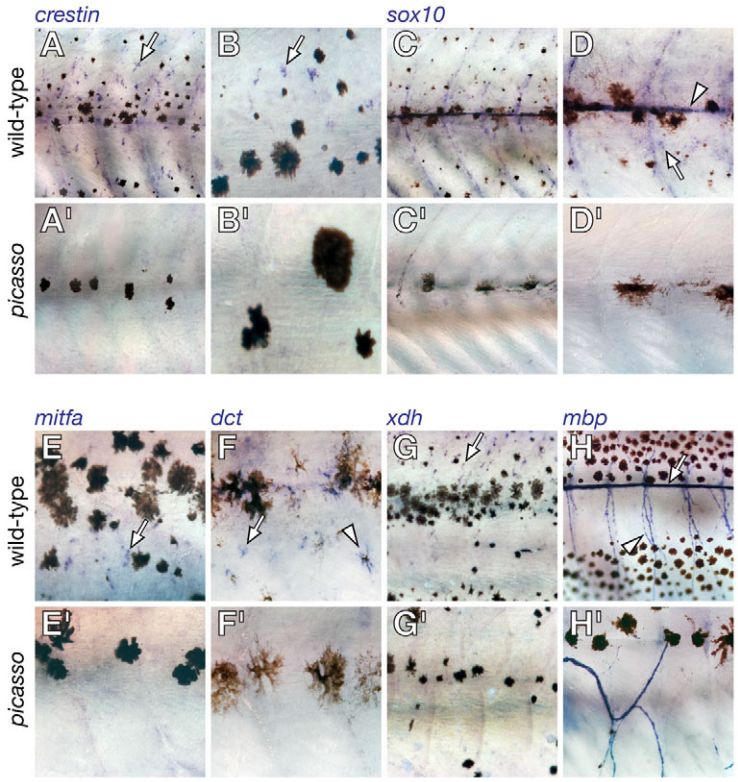

Fig. 5. Metamorphic deficiencies for early and late markers of neural crest-derived lineages in *picasso* mutant larvae. Shown are corresponding regions of the mid-trunk for wild type (above) and *picasso* mutants during mid-metamorphosis (~18 dpf). Individual melanophores are more spread in *picasso* mutants, typical of reduced melanophore densities (Parichy and Turner, 2003b; Parichy et al., 2003). (**A**,**A′**) *crestin* marks neural crest-derived cells in embryos (Luo et al., 2001) and identifies dispersed cells in the hypodermis of metamorphosing larvae (arrow). *crestin*$^+$ cells were dramatically fewer in *picasso* mutants. (**B**,**B′**) Higher magnification image of *crestin*$^+$ cells in wild type, and their absence in *picasso*. (**C**,**C′**) *sox10* marks non-ectomesenchymal neural crest-derived cells in embryos, including pigment cell and glial precursors (Dutton et al., 2001; Gilmour et al., 2002), and identifies comparable populations in metamorphosing larvae (Parichy et al., 2003). *sox10*$^+$ cells were fewer in *picasso* compared with wild type. (**D**,**D′**) Higher magnification showing *sox10*$^+$ cells along the lateral line (arrowhead) and in the hypodermis (arrow) of wild type, but not *picasso*. (**E**,**E′**) *mitfa* marks melanophore and xanthophore precursors in embryos and is essential for melanoblast specification (Lister et al., 1999; Parichy et al., 2000b). *mitfa*$^+$ cells (arrow) were numerous in wild type but not in *picasso*. (**F**,**F′**) *dct* identifies melanophore precursors (arrow) and melanophores (arrowhead) (Kelsh et al., 2000); *dct*$^+$ cells were reduced or absent in *picasso*. (**G**,**G′**) Xanthine dehydrogenase (*xdh*) encodes an enzyme in the pteridine synthesis pathway of xanthophores (Parichy et al., 2000b). *xdh*$^+$ cells (arrow) were numerous in wild type but transiently fewer in *picasso*. (**H**,**H′**) Myelin basic protein (*mbp*) marks mature glia in the peripheral nervous system (Brosamle and Halpern, 2002; Lyons et al., 2005) and *mbp*$^+$ cells line the midbody lateral line (arrow) as well as ascending and descending nerve fibers (arrowhead). Although *mbp*$^+$ cells were present in *picasso*, they were fewer in number and their associated nerves were misrouted.

possibilities, we blocked ErbB signaling using pharmacological inhibitors AG1478 (Levitzki and Gazit, 1995; Lyons et al., 2005; Levitzki and Mishani, 2006) and PD158780 (Fry et al., 1997; Rewcastle et al., 1998; Frohnert et al., 2003). Preliminary analyses showed that treating wild-type embryos with either AG1478 or PD158780 resulted in excess neuromasts that phenocopy *erbb3b* mutants (data not shown) (Lyons et al., 2005). As both drugs inhibit kinase activity by interfering with ATP-binding sites, and wild-type Erbb3 already has impaired or absent kinase activity (Guy et al., 1994), inhibitors presumably suppress signals originating with erbb3b:erbb2, erbb3:egfr or other heterodimers. Functions of these receptors that are independent of kinase activity should not be affected.

Wild-type embryos treated with AG1478 developed normal early larval pigment patterns. When these same fish metamorphosed, however, their pigment patterns and melanophore densities were indistinguishable from *picasso* mutants (Fig. 7B,E). By contrast, fish treated with AG1478 during the pre-metamorphic (early larval) period or during metamorphosis, developed adult pigment patterns and melanophore densities indistinguishable from controls (Fig. 7A,C-E; but see below). Treatment of wild-type fish with PD158780 yielded identical results (Fig. 8 and data not shown).

To test whether the embryonic requirement for ErbB signaling is unique to zebrafish, we examined two more species. We chose *D. albolineatus* because its more uniform pigment pattern (Fig. 7F) might depend on mechanisms different than zebrafish (Quigley et al., 2005; Mills et al., 2007). *Danio albolineatus* embryos developed defects in metamorphic melanophores similar to *D. rerio* when treated with AG1478 (Fig. 7G) or PD158780 (data not shown). We also examined *D. nigrofasciatus* (Fig. 7H), in which few metamorphic melanophores develop and, instead, most embryonic/early larval melanophores persist and reorganize to form adult stripes (Quigley et al., 2004). If AG1478 effects are limited to metamorphic melanophores, then the *D. nigrofasciatus* pigment pattern should be refractory to perturbation. Consistent with this

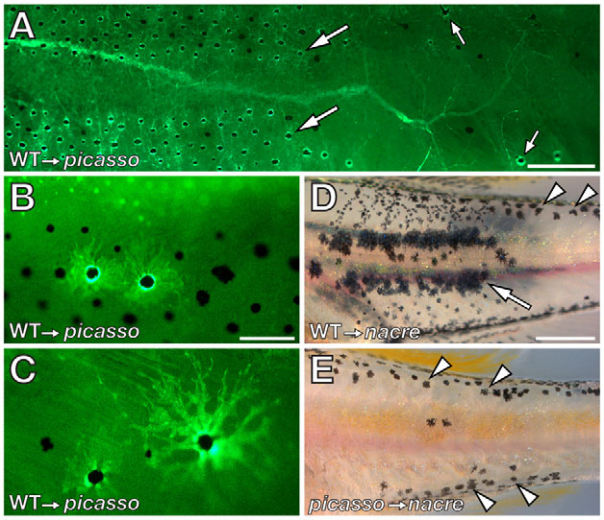

Fig. 6. Autonomous and non-autonomous roles for *erbb3b* in pigment pattern metamorphosis. (**A**) Wild-type → *picasso* chimeras frequently developed wild-type melanophores in stripes at high density anteriorly (arrows, left) but at lower density in the mid-trunk (small arrows, right; 75% of chimeras developed donor melanophores; chimeras with donor cells and total reared: *n*=24, 64, respectively). A wild-type midbody lateral line is misrouted as well. (**B**) Melanophores at high density anteriorly that are either donor-derived ($EGFP^+$) or host-derived ($EGFP^-$). (**C**) Melanophores in the mid-trunk are more spread, which is typical at low density. In reciprocal *picasso* → wild-type chimeras, we did not observe donor metamorphic melanophores (*n*=7, 50). (**D**) Wild-type → *nacre* chimeras developed patches of donor-derived metamorphic melanophores that populated stripes (arrow) and scales (84% of chimeras developed metamorphic melanophores; *n*=75, 155). Persisting embryonic/early larval melanophores (arrowheads) are identifiable by location, large size and browner color (Quigley et al., 2004). (**E**) *picasso* → *nacre* chimeras developed melanophores (arrowheads), but did not develop metamorphic melanophores [79% of chimeras developed embryonic/early larval melanophores or fin melanophores (not shown); *n*=58, 195]. Donor cells in all chimera combinations contributed at similar frequencies to other derivatives, including muscle, epidermis, eye and neurons of the lateral line. Scale bars: in A, 500 μm; in B, 200 μm for B,C; in D, 1 mm for D,E.

prediction, *D. nigrofasciatus* embryos treated with AG1478 developed adult pigment pattern defects (Fig. 7I) less severe than those of zebrafish or *D. albolineatus*.

In mammalian systems, AG1478 is highly selective for EGFR-dependent signals and is less effective against other ErbB receptors (Levitzki and Gazit, 1995), whereas PD158780 is highly effective against all ErbB family members (Fry et al., 1997; Frohnert et al., 2003; Stonecypher et al., 2005). Specificities in zebrafish are not known. Although the similarity of adult pigment patterns between drug-treated fish and the *picasso* mutant is consistent with the suppression of erbb3b-dependent signals, we would expect these inhibitors to affect signaling through other ErbB receptors as well, particularly as protein tyrosine kinase domains are highly conserved between zebrafish and human orthologues (e.g. domain-specific identities, similarities: Erbb2, 84%, 92%; Egfr, 87%, 95%; Erbb3, 75%, 87%). We therefore repeated these experiments on *picasso* mutants: if signals independent of *erbb3b* are inhibited, then the severity of the *picasso* phenotype should be enhanced. When we treated sibling $picasso^{wp.r2e2}$ and $picasso^{wp.r2e2}$/+ embryos with AG1478 for the first 4 days of development, homozygotes unexpectedly developed edema and died by 7 dpf. Thus, a single copy of *erbb3b* protects against AG1478-dependent lethality, implying that *erbb3b* has functions that are independent of kinase activity (that, itself, presumably originates with erbb3b:erbb2 or erbb3b:egfr heterodimers). Consistent with this idea are several studies that have revealed kinase-independent activities of receptor tyrosine kinases, including Erbb3 (Offterdinger et al., 2002; Rawls and Johnson, 2003; Massie and Mills, 2006; Hsu and Hung, 2007).

Given the preceding results, we treated embryos for shorter periods: wild-type embryos treated for only 2 dpf developed pigment patterns similar to wild-type embryos treated for 4 dpf (Fig. 9A,B); moreover, both $picasso^{wp.r2e2}$ and $picasso^{wp.r2e2}$/+ embryos treated for 2 dpf survived and developed pigment patterns indistinguishable from untreated $picasso^{wp.r2e2}$ controls (Fig. 9C,D; log-transformed melanophore densities: $F_{1,25}$=2.46, P=0.13). We observed identical outcomes with PD158780 (data not shown). By comparison with the $picasso^{wp.r2e2}$ null phenotype, these data suggest that inhibitors affect adult pigment patterns largely or exclusively by suppressing erbb3b-dependent signals.

These data indicate that ErbB signals are required in embryos for adult pigment pattern formation. To further test this conclusion, we sought an independent means of blocking *erbb3b* activity. We reasoned that the limited perdurance of morpholino oligonucleotides (3-5 days) should allow us to knock-down *erbb3b* in the embryo, while permitting later activity at metamorphosis (Nasevicius and Ekker, 2000; Mellgren and Johnson, 2004). We therefore injected embryos with a morpholino oligonucleotide against *erbb3b* (Lyons et al., 2005) and raised them into adults. Morpholino-injected fish showed defects qualitatively similar to *picasso* mutants (Fig. 7K).

Overall then, two independent lines of evidence show that *erbb3b* is required early for much later adult pigment pattern development. Specifically, the *erbb3b* mutant adult pigment pattern phenotype can be phenocopied in wild-type fish by: (1) embryonic knockdown of *erbb3b* via morpholino injection; and (2) treating embryos with either of two pharmacological inhibitors that, in this context, are specific to *erbb3b*-dependent signals.

Adult pigment pattern requirement for ErbB activity during neural crest migration

The critical period for ErbB signals includes neural crest migration. To further test this coincidence, we treated embryos with ErbB inhibitors for shorter intervals. Preliminary analyses with wild-type yielded extensive variability in defect severity, perhaps owing to stochastic differences in pattern regulation (Parichy and Turner, 2003b; Yamaguchi et al., 2007). We therefore used a sensitized background, kit^{b5}, to delineate the critical period more precisely. *kit* mutants lose embryonic/early larval melanophores, subsequently develop metamorphic melanophores already in stripes, and also have defects in pattern regeneration (Johnson et al., 1995; Parichy et al., 1999; Rawls and Johnson, 2000; Yang and Johnson, 2006).

We treated *kit* mutant embryos beginning between 8 hpf and 70 hpf for periods of 2-26 hours. Such analyses across multiple independent experiments revealed peak sensitivities for adult pigment pattern formation between ~14 and 22 hpf, with affected individuals developing stripe defects reminiscent of *picasso* mutants (AG1478: Fig. 10A-C,E,F; PD158780: data not shown). As adult pigment patterns were comparatively refractory to treatments after ~22 hpf, we asked whether longer treatments at

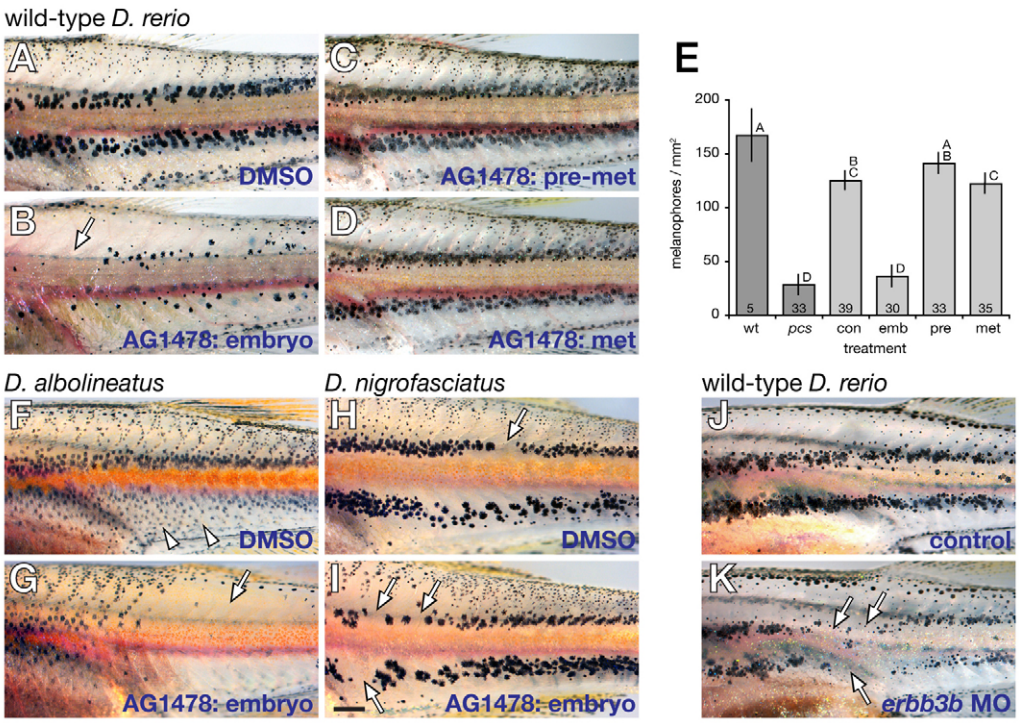

Fig. 7. Embryonic requirement for ErbB signaling in pigment pattern metamorphosis. (**A**) Control wild-type zebrafish treated from embryonic through juvenile stages with DMSO alone. (**B**) Wild-type treated with AG1478 during embryonic stages (70% epiboly – 4 dpf) showed adult pigment patterns resembling severely affected *picasso* mutants (arrow). (**C**,**D**) Individuals treated with AG1478 during the pre-metamorphic, early larval period (5-14 dpf, C) or throughout metamorphosis (15-28 dpf, D) exhibited pigment patterns indistinguishable from controls. (**E**) Metamorphic melanophore densities in the mid-trunk (mean±95% confidence intervals) showing similarities between wild-type (untreated), control (con, DMSO-treated) and fish treated with AG1478 during pre-metamorphosis (pre) and metamorphosis (met), as well as similar defects between *picasso* mutants (*pcs*) and fish treated with AG1478 as embryos (emb). Letters above bars indicate means that are not significantly different (P>0.05). Numbers within bars are samples sizes. (**F**,**G**) *D. albolineatus* normally develop a more uniform melanophore pattern of metamorphic melanophores (arrowheads in F) compared with *D. rerio*, and exhibited a severe melanophore deficiency when embryos were treated with AG1478 (G). (**H**,**I**) In *D. nigrofasciatus*, adult stripes largely comprise persisting embryonic/early larval melanophores with occasional gaps (arrow in H), and embryonic treatment with AG1478 had relatively subtle effects (arrows in I). (**J**,**K**) Embryonic morpholino knockdown of *erbb3b* resulted in adult melanophore deficiencies (K) compared with controls (J), providing independent evidence for an early *erbb3b*-dependence of adult pigment pattern formation. Scale bar: in I, 0.5 mm for A-D,F-I,J,K.

later stages would enhance adult pattern defects. Treating embryos between 26 and 48 hpf did not alter later phenotypes (Fig. 10F), consistent with an earlier critical period. Finally, because *erbb3b*, *erbb2* and *egfr* are expressed as early as 8-11 hpf [(Goishi et al., 2003; Thisse and Thisse, 2004; Lyons et al., 2005); data not shown], we tested whether ErbB signals have reiterated activities by treating embryos twice. When early treatments (8-11 hpf) were combined with later treatments (beginning at 22 hpf and later), we observed more severe melanophore deficiencies in the adult. Remarkably, treatments at least 8 hours apart often resulted in spatially separated melanophore-deficient patches (e.g. Fig. 10D). The increased severity of these defects suggests early and late functions even in the embryo: defects arising from early ErbB kinase inhibition can presumably be regulated so long as ErbB function is allowed later.

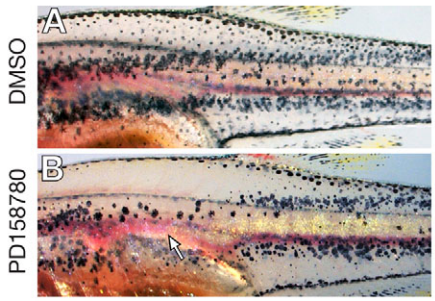

Fig. 8. Treatment of embryos with ErbB inhibitor PD158780 results in adult pigment pattern defect similar to *erbb3b* null alleles. (**A**) Treated with DMSO alone. (**B**) Treated with PD158780 for 2 dpf. Arrow, region deficient for metamorphic melanophores.

The major critical period for ErbB signals (~14–22 hpf) corresponds approximately to the time when neural crest cells are migrating at the axial levels affected in the *picasso* mutant (Raible et al., 1992; Vaglia and Hall, 2000). Therefore, we explored the role of *erbb3b* in the early patterning of neural crest-derived cells. In comparison with wild-type siblings, *picasso* mutants at 26 hpf had similar numbers of cells expressing the pan-neural crest marker *crestin*, but these cells did not localize at sites of ganglion formation in the medial migratory pathway and were instead found further ventrally (Fig. 11A). We observed a similar defect for cells expressing *mitfa* (Fig. 11B). By contrast, we did not find clear defects in the distributions of *dct*$^{+}$ melanoblasts (Fig. 11C) or cells in the lateral migratory pathway, consistent with the normal patterning of *picasso* mutant embryonic/early larval melanophores. Finally, given the defects in ventromedial migrating cells, and defects in the peripheral nervous system of mammalian *ErbB3* and *ErbB2* mutants (Britsch et al., 1998; Britsch, 2007), we examined ganglion development. We observed gross reductions in the numbers of dorsal root and sympathetic ganglia in *picasso* mutant larvae at 12 dpf (Fig. 11D). These data reveal an *erbb3b*-dependence of neural crest morphogenesis that correlates with the early *erbb3b*-dependence of adult pigment pattern formation.

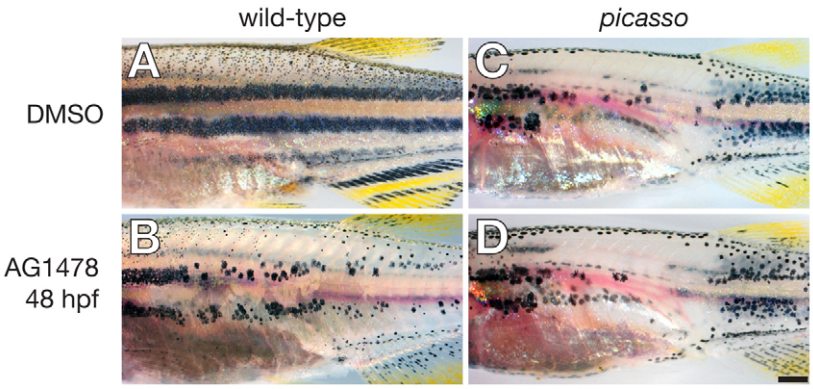

Fig. 9. ErbB inhibitor treatment for 48 hpf does not enhance the *picasso* null phenotype. (**A**) Wild-type control treated with DMSO. (**B**) Wild-type treated with AG1478 exhibits a severe pigment pattern defect. (**C**) *picasso* mutant control treated with DMSO. (**D**) *picasso* mutant treated with AG1478 has a pigment pattern defect indistinguishable from that of the control. Scale bar: in D, 0.5 mm for A-D.

Sensitized genetic backgrounds reveal requirements for ErbB signals during metamorphosis

The preceding experiments demonstrated a critical period for ErbB signaling in embryos. During later development, metamorphic melanophores express both *erbb3b* and *erbb2* (Fig. 4C), but inhibitor treatments during metamorphosis had no effect on the adult pigment pattern (Fig. 7C-E). This suggests redundancies with other pathways, regulation in cell behaviors, poor penetration into tissues or higher thresholds for inhibition. Given these possibilities, we sought to further test roles for ErbB signals during metamorphosis. As higher doses were lethal, we re-tested the inhibitors on sensitized mutant backgrounds: *kit*, *csf1r*^{j4e1} and *kit*/+ *csf1r*/+. We chose these because they reveal distinct populations of metamorphic melanophores (Johnson et al., 1995; Parichy et al., 1999; Parichy et al., 2000b): *kit* mutants lack early metamorphic melanophores, but retain late metamorphic melanophores; *csf1r* mutants retain early metamorphic melanophores, but are missing late metamorphic melanophores. Comparing further deficits should therefore indicate whether one or the other population has a greater requirement for ErbB signaling. Finally, we also examined *D. albolineatus* because of the differences in melanophore development in this species compared with zebrafish.

In each background, we observed moderate to severe reductions in metamorphic melanophore numbers upon treatment with AG1478 or PD158780 during metamorphosis (Fig. 12; 19-58% fewer melanophores than corresponding controls), changes that were considerably more severe than observed for wild-type larvae (Fig. 7D,E; 3% fewer melanophores than controls). Furthermore, *kit* mutant and *csf1r* mutant zebrafish exhibited similar reductions in residual melanophore numbers, suggesting that ErbB signals are required by precursors to both early and late metamorphic melanophores. Consistent with the specificity of these effects, the *picasso*$^{wp.r2e2}$ mutant pigment pattern defect was not enhanced under these conditions and *kit* mutants treated with AG1478 exhibited pigment patterns that fell within the range observed for fish doubly

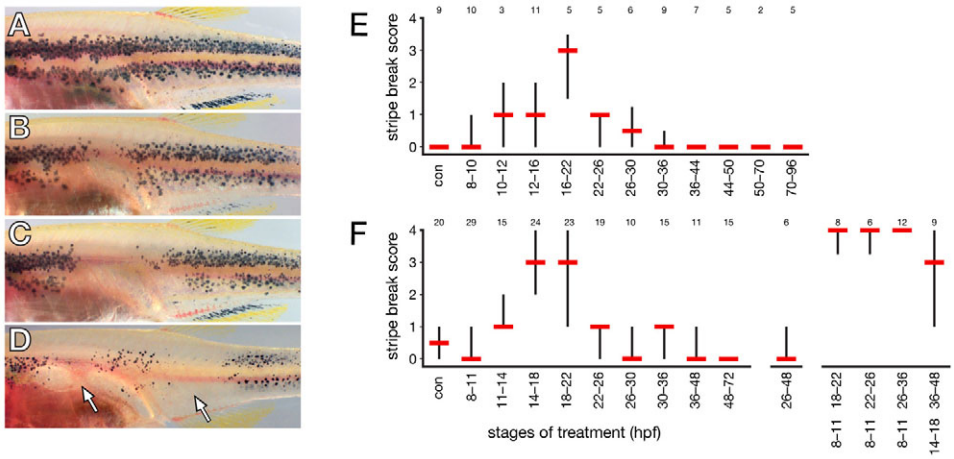

Fig. 10. *kit* mutant reveals critical period for ErbB activity during neural crest migration. Embryos were reared to juvenile stages after treatment with AG1478 as embryos. (**A**) Normal *kit* mutant pigment pattern after AG1478 treatment between 26 and 30 hpf (stripe break score=0; for details see Materials and methods). *kit* mutant adults exhibit about half as many stripe melanophores as wild-type fish and completely lack melanophores over the dorsum and on scales (Johnson et al., 1995). (**B**,**C**) Treatment with AG1478 between 14 and 18 hpf results in pattern defects that are moderate (B, stripe break score=3) to severe (C, stripe break score=4). (**D**) Distinct melanophore-free patches (arrows) in *kit* mutant treated between 8 and 11 hpf then again between 26 and 30 hpf. (**E**,**F**) Quantitation of stripe break defects in *kit* mutants treated with AG1478 in two separate experiments. Shown are median stripe break scores (red bars) and interquartile ranges (50% of scores, black vertical bars). Numbers above each treatment stage are sample sizes of adult fish analyzed. con, DMSO-treated controls. (E) Initial experiment reveals peak sensitivity between 16 and 22 hpf, with lesser defects extending between 8 and 36 hpf (test of differences in median locations across all treatments, Wilcoxon test χ^2 approximation=38.6, d.f.=11, P<0.0001). (F) Second experiment confirms peak sensitivity between ~14 and 22 hpf (χ^2=115.6, d.f.=14, P<0.0001). More severe defects are generated with repeated treatments (right).

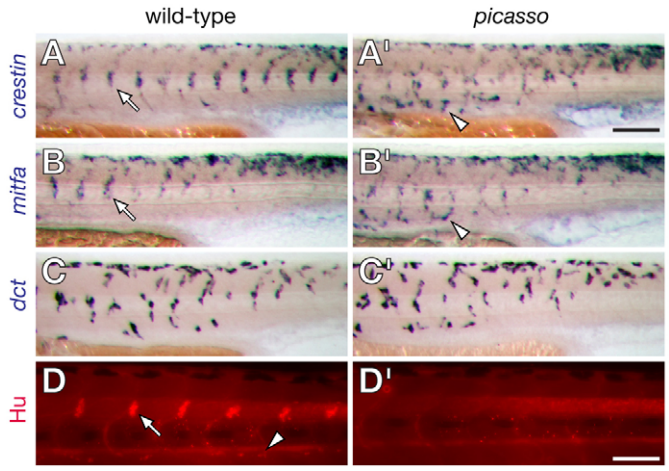

Fig. 11. *picasso* mutant embryos have defects in neural crest morphogenesis. (**A**,**A′**) *crestin*$^{+}$ cells form segmentally arranged clusters prior to ganglion formation in wild-type embryos (e.g. arrow in A) but appear to migrate past their normal target sites in *picasso* mutant embryos (e.g. arrowhead in A′). (**B**,**B′**) *mitfa*$^{+}$ cells in wild-type exhibit some segmental patterning and a defect in *picasso* mutants similar to that of *crestin*$^{+}$ cells. (**C**,**C′**) *dct*$^{+}$ melanoblast distributions do not differ consistently between wild-type and *picasso* mutant embryos. (**D**,**D′**) Anti-Hu immunoreactivity shows dorsal root ganglia (e.g. arrow in D) and sympathetic ganglia (e.g. arrowhead in D) in wild-type larvae but their absence in *picasso* mutants. Scale bar: in A′, 800 μM for A-C′; in D′, 600 μM for D-D′.

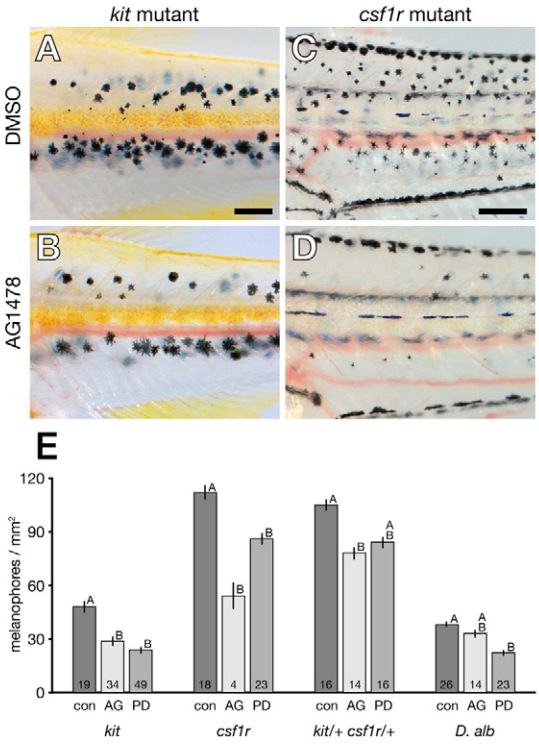

Fig. 12. Cryptic ErbB requirements during metamorphosis revealed by sensitized genetic backgrounds. (**A**) *kit* mutants (reared with DMSO) develop fewer metamorphic melanophores than wild type. (**B**) *kit* mutants reared during metamorphosis with AG1478 have an additional metamorphic melanophore deficiency. (**C**) *csf1r* mutants have similar numbers of melanophores to wild type through the middle metamorphic stage shown (Parichy et al., 2000b). (**D**) *csfr1r* mutants reared in AG1478 during metamorphosis exhibit a sharp reduction in melanophore numbers, as well as increased larval mortality (not shown). (**E**) Melanophore densities (±1 s.e.) are moderately or significantly reduced in sensitized backgrounds when treated with AG1478 (AG) or PD158780 (PD) compared with DMSO-only controls (con). Shared letters above bars indicate treatments that are not significantly different from one another (P>0.05) within each genetic background. Treatment sample sizes are shown at the base of each bar. Scale bar: in A, 300 μM for A,B; in C, 300 μM for C,D.

mutant for *kit* and *picasso*$^{wp.r2e2}$ (data not shown). These data support a model in which ErbB signals are essential in the embryo but also function redundantly with other pathways during metamorphosis.

DISCUSSION

We have identified a crucial role for ErbB signaling in *Danio* adult pigment pattern development. While *picasso* mutants homozygous for *erbb3b* null alleles exhibit normal early larval pigment patterns, they are grossly deficient for metamorphic melanophores of the adult pigment pattern. Unexpectedly, metamorphic melanophore precursors require ErbB signals during neural crest development, ~2 weeks before they begin to differentiate, and also exhibit a cryptic requirement for ErbB signals during metamorphosis. Genetic mosaic analyses further suggest complex modes by which ErbB signals promote adult pigment pattern formation.

A glial requirement for ErbB signals is well documented (Riethmacher et al., 1997; Britsch et al., 1998; Lyons et al., 2005; Pogoda et al., 2006; Britsch, 2007), but roles in pigment cell development have remained obscure. Normal human melanocytes express EGFR, ERBB2, ERBB3 and ERBB4, and stimulation with ligand promotes migration in vitro (Gordon-Thomson et al., 2001; Stove et al., 2003; Gordon-Thomson et al., 2005; Mirmohammadsadegh et al., 2005). ErbB receptors also are expressed in melanoma cells, and are associated with melanoma progression in a teleost model (Wellbrock et al., 2002; Gomez et al., 2004) and with human melanoma proliferation in vitro (Stove et al., 2003; Gordon-Thomson et al., 2005; Funes et al., 2006). Our finding that the *picasso* mutant phenotype results from lesions in *erbb3b* provides the first evidence that ErbB signals are required to promote normal pigment cell and pigment pattern development in vivo [although Fitch et al. (Fitch et al., 2003) describe melanocytosis resulting from EGFR overexpression in skin].

This study indicates that adult pigment pattern formation requires ErbB signaling in the embryo. Our analyses used pharmacological inhibitors that several lines evidence suggest are specific to ErbB-dependent signals. First, we observed the same phenotypes with two inhibitors. Second, pigment pattern defects phenocopied *erbb3b*-null alleles. Third, inhibitors failed to enhance defects of these null alleles. Fourth, similar defects resulted from morpholino-knockdown of *erbb3b*. Our results thus point to a model in which ErbB signals – depending in part on erbb3b – play an essential embryonic role in promoting much later adult pigment pattern formation.

This early critical period contrasts with other genes. For example, *csf1r* and *puma* are required during pigment pattern metamorphosis (Parichy and Turner, 2003a; Parichy et al., 2003) and *kit* is required during pattern formation in the fin (Rawls and Johnson, 2001). The critical period for ErbB signals in zebrafish also may be earlier than for Ednrb of mouse (Shin et al., 1999). Nevertheless, our analyses can suggest only a range of times: both drugs act rapidly and are

quickly reversible (Fry et al., 1997; Lenferink et al., 2001; Levitzki and Mishani, 2006), but we do not know how long it takes for concentration changes in solution to reach beneath the epidermis. The peak sensitivity observed for embryos treated between 14-22 hpf may therefore indicate somewhat later critical periods, presumably during neural crest migration.

We can envisage at least two complementary models in which embryonic ErbB signals contribute to later metamorphic melanophore development. In the first model, these signals act autonomously to establish precursors of metamorphic melanophores. This activity could be specific to metamorphic melanophores or could apply to a broader range of neural crest derivatives. For example, both pigment cells and glia share a common precursor (Dutton et al., 2001; Dupin and Le Douarin, 2003; Dupin et al., 2003), both can be generated by adult neural crest-derived stem cells (Sieber-Blum et al., 2004; Amoh et al., 2005; Wong et al., 2006), and both are affected by the *erbb3b* mutation and by ErbB inhibitor treatments. ErbB signals also may expand a precursor population. If so, then incomplete regulation could explain why the early larval pigment pattern is normal in *picasso* mutants: if precursors are allocated to fill a defined number of 'embryonic/early larval niches' before filling 'metamorphic niches', a depleted total number of cells could leave metamorphic niches vacant.

In a second model, ErbB signals promote adult pigment pattern formation non-autonomously to the metamorphic melanophore lineage. This could occur if ErbB-expressing cells provide trophic support to metamorphic melanophore precursors or contribute otherwise to a micro-environment where these precursors reside. Such interactions would be analogous to the non-autonomous mechanisms by which ErbB signals in glia promote neuronal survival and nerve integrity (Riethmacher et al., 1997; Chen et al., 2003; Sharghi-Namini et al., 2006). These observations also raise the possibility that peripheral nerves or ganglia serve as niches for metamorphic melanophore precursors. Consistent with this idea are the defects in ganglion development seen here and in the accompanying study (Honjo et al., 2008), and the presence of cells in peripheral nerves or ganglia of other organisms that are able to produce melanocytes and other cell neural crest derivatives (Nichols et al., 1977; Nichols and Weston, 1977; Ciment et al., 1986; Nataf and Le Douarin, 2000; Rizvi et al., 2002; Joseph et al., 2004).

Beyond the embryo, our data also indicate a role for ErbB signals during metamorphosis. At this stage, ErbB signals are likely to act autonomously to metamorphic melanophores, given their expression of *erbb3b* and *erbb2*, but also could have non-autonomous effects if interactions among melanophores promote the survival, proliferation or differentiation of these cells (Parichy et al., 2000b; Parichy and Turner, 2003b).

In conclusion, this study supports a model in which ErbB signals, which are mediated in part through *erbb3b*, are required in the embryo to establish latent precursors that will subsequently generate metamorphic melanophores. Later, during metamorphosis, ErbB signals contribute to melanophore development but are partly or entirely redundant with other pathways. We speculate that *erbb3b* promotes the development of latent precursors intrinsically, and also is required extrinsically to form a niche where these cells reside until recruited at metamorphosis.

Thanks to members of the Parichy laboratory for helpful discussions and for assistance with fish rearing, to Will Talbot's laboratory for complementation testing of *picasso* against *erbb3b*, to Melissa Harris for comments on the manuscript, to Dave Raible's laboratory and especially Hillary McGraw for discussions and advice, and to Judith Eisen and Yasuko Honjo for sharing data prior to publication. Supported by NIH R01 GM62182 and NSF IOB 0541733 to D.M.P.

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