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

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SUMMARY

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*, encoding 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.

Keywords

ErbB; erbb3; HER3; melanophore; metamorphosis; stem cell; zebrafish

INTRODUCTION

The generation of adult form remains an enduring problem in developmental biology. An interesting system for studying the genetic and cellular mechanisms underlying adult morphology is the larva-to-adult transformation, or metamorphosis, of many amphibians and fishes (Moran, 1994; Parichy, 1998; Webb, 1999; Brown and Cai, 2007). In zebrafish, *Danio rerio*, metamorphosis includes dramatic changes in which an early larval morphology is transformed into that of the adult. During this time, many traits are modified including the digestive, excretory, and sensory systems, the central and peripheral nervous systems, 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., 2008).

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One particularly accessible trait that changes during metamorphosis is the pigment pattern (Kelsh, 2004). The zebrafish early larval pigment pattern develops in the embryo from pigment cells, or chromatophores, that are derived from neural crest cells. This embryonic/early larval pigment pattern is completed by 5 days post-fertilization (dpf) and includes several stripes of black melanophores with yellow xanthophores scattered widely over the flank. This pattern persists until the onset of metamorphosis (~14 dpf) when melanophores begin to differentiate outside of the embryonic/early larval stripes. During the next two weeks, new adult stripes begin to form as some metamorphic melanophores migrate to sites of adult stripe formation (between the embryonic/early larval stripes) and other melanophores differentiate already at these sites. The result is a juvenile pigment pattern with two “primary” stripes that include melanophores, bordering an “interstripe” that includes xanthophores; iridophores are found throughout the flank.

Embryonic/early larval chromatophores and metamorphic chromatophores might be expected to have commonalities as well as differences in their genetic requirements. For example, several mutants lack chromatophore types or pigments both before and after metamorphosis [e.g., *nacre*, *csf1r*, *golden* (Lister et al., 1999; Parichy et al., 2000b; Lamason et al., 2005)]. Others exhibit defects in the adult but not in the embryo [e.g., *leopard*, *jaguar/obelix*, *ednrb1* (Parichy et al., 2000a; Iwashita et al., 2006; Watanabe et al., 2006)]. Mutants in this latter class are particularly interesting because they identify genes uniquely required for the establishment, maintenance, or differentiation of latent precursors that contribute to adult form. Included among these mutants are two with similar phenotypes, *puma* and *picasso*, each having a grossly normal embryonic/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*, encoding 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* (Stein and Staros, 2006). Ligands for ErbB receptors include EGF as well as neuregulins (NRGs) 1, 2 and 3. ErbB receptors have several roles during development, including critical functions in glial morphogenesis (Lyons et al., 2005; Britsch, 2007), and their misregulation is associated with a variety of cancers (Citri and Yarden, 2006; Linggi and Carpenter, 2006; Breuleux, 2007; Bublil and Yarden, 2007; Sergina and Moasser, 2007). The receptors form dimers with individual monomers having varying activities and ligand specificities: for instance, ErbB3 lacks endogenous kinase activity while ErbB2 lacks its own high affinity ligand. Whereas several heterodimers are possible, only a subset seem 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 as well (Soltoff et al., 1994; Frolov et al., 2007; Poumay, 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 by genetic mosaic analyses that *erbb3b* functions both autonomously and non-autonomously to the metamorphic melanophore lineage. Using pharmacological inhibition and morpholino knockdown, we also identify the temporal requirements for ErbB signals in adult pigment pattern metamorphosis. We demonstrate a major critical period during embryonic neural crest cell migration, at least two weeks before the larva-to-adult transformation, indicating a novel role for ErbB signals in establishing latent precursors that will subsequently contribute to the adult pigment pattern. Using sensitized genetic backgrounds, we also find cryptic requirements for ErbB signals during pigment pattern metamorphosis, suggesting redundant functions with other pathways at this later stage. Our study thus provides new

insights into the development of adult form and the genetic requirements for trait expression before and after metamorphosis.

MATERIALS AND METHODS

Fish stocks

Fish were maintained at 26−28 °C, 14L:10D according to standard methods (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. Pilot studies confirmed that treating embryos with 3 μM of either drug phenocopied an excess neuromast defect of *erbb3b* mutants (data not shown) (Lyons et al., 2005), whereas lower doses were less effective and higher doses caused lethality of embryos, larvae, or both. Fish were treated with either drug in 10% Hanks solution. To facilitate penetration into the tissues, 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 previously described splice-blocking morpholino against *erbb3b* [TGGGCTCGCAACTGGGTGGAAACAA; (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 of isolated cell types, 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) at room temperature with gentle agitiation (Clark et al., 1987). Cells were picked and transferred to wash medium (5% fetal bovine serum in PBS) for 10 min, 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, GGCGAAGGTGTTGAAGTAAT; erbb2, CACCGGAAGTTTACTCACCAA, GATCTCCAACATTTGACCAT; erbb3a, TGACTCCATCCACTACTGCTG, TTCTTCACCAGCACCTCTGTT; egfr, CCGTTGGTGTGTGTTTTGAG, GCTTTTCAGGAGGGAGACTTTC; dct, ACCTGTGACCAATGAGGAGATT, TACAACACCAACACGATCAACA; β-actin, GTTTTCCCCTCCATTGTT, GGTGTTGAAGGTCTCGAACA; erbb4, CTGCTGCTCAACTGGTGTGT, CCAGTGCCATCACAGCTTCT.

For genotyping of the $picasso^{wp.r2e2}$ mutant allele, we amplified genomic DNA (pcs-wpr2e2*: TTGGTTACCATTGTGGTTGTTT, TCTTCATGGTAGCTCAGAAACATC) from

individual embryos and digested the resulting 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.

All sequencing reactions were performed with ABI Big Dye 3.1 and resolved on ABI 3100 genetic analyzers.

In situ hybridization

Analyses of mRNA distributions in embryos followed standard protocols (Parichy et al., 2000b). In situ hybridizations on larvae followed (Elizondo et al., 2005), but used overnight incubations for hybridizations and antibodies (detailed protocol available on-line, 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 prcessed as above to determine genotypes retrospectively.

Immunohistochemistry

Trunks of 12 dpf larvae were fixed in 4% PFA in PBS for 6 hrs at room temperature with gentle agitation then permeabilized by washing overnight in deionized water at room temperature. Specimens were equilibrated in PDTX (PBS containing 1% DMSO and 0.3% Triton X) three times for 30 min each, then blocked with 5% goat serum in PDTX for 4 hours at room temperature. Larvae were incubated overnight at 4°C with primary antibody mAB16A11 (Marusich et al., 1994; Henion et al., 1996) against the HuC/D antigen (1:200 in blocking solution), washed extensively in PDTX, incubated overnight at 4°C 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 HR cameras using Zeiss Axiovision 3 and corrected for contrast and color balance when necessary in Adobe Photoshop CS3.

All 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 of interest 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 cofactor yielded qualitatively equivalent results (data not shown). Analyses of melanophore densities were treated as multifactorial analyses of variance or covariance with replicates as blocks. Residuals in all analyses were examined for normality and homoscedasticity. Least squares means (correcting for size, replicate variation, or both) are presented in figures below, with significant differences assessed post hoc by Tukey-Kramer comparisons of all means 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 for qualitative degree of stripe disruption. Breaks in stripes were considered present when ≤3 melanophores were present over a defined anterior–posterior length, as scaled by hpa (above): stripes exhibiting breaks no wider than 0.5 hpa were scored “0”; breaks between 0.5−1 hpa were scored “1”; breaks greater than 1 hpa were scored “2”.

Dorsal and ventral stripes were scored individually, and resulting scores were summed to generate a "stripe break score" ranging from 0−4. To test for differences among treatment groups, we compared ordinal scores using both non-parametric Wilcoxon tests as well as contingency table analyses. Both methods yielded equivalent results (data not shown); 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, however, *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 formed. To assess how pattern formation in this region differs from the mid-trunk, we recorded daily images from additional fish. In the posterior of both wild-type and *picasso* mutants, there were greater numbers of persisting embryonic/early larval melanophores as compared to the mid-trunk (Fig. 2). Concomitantly, *picasso* mutants exhibited more differentiating metamorphic melanophores in this region as compared to more anteriorly, perhaps reflecting community effects on melanophore survival (Parichy et al., 2000b; Parichy et al., 2003).

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 (data not shown; all of these alleles are recessive and homozygous viable, though weaker than wild-type)(Talbot, personal communication)(Lyons et al., 2005). Sequencing *erbb3b* cDNAs revealed 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 detected *erbb3b* expression in glia by in situ hybridization (Fig. 4A), consistent with the earlier embryonic expression in these cells. To see if *erbb3b* might be expressed in other tissues at levels below the threshold of detection by in situ hybridization, we used RT-PCR on cDNAs isolated from metamorphic melanophores, as well as juvenile caudal fin, which comprises melanophores, melanophore precursors, dermal bone, skin, vasculature, and other cell types. We detected *erbb3b* transcripts in both isolated melanophores and in juvenile fin (Fig. 4C). We also detected the *erbb3b* paralogue, *erbb3a*, in glia and in fin, though not in metamorphic melanophores (Fig. 4B,C). Since ErbB receptors act as heterodimers, we tested if other erbb genes are expressed in metamorphic melanophores, where they might provide heterodimerization partners for erbb3b (Fig. 4C): *erbb2* was expressed in metamorphic melanophores and in fin; *egfr* was not expressed in metamorphic melanophores, though it was expressed in fin; and we could not detect *erbb4* in melanophores or in fin (data not shown). *erbb2* and *egfr* also are widely expressed in zebrafish embryos (Goishi et al., 2003; Lyons et al., 2005)

To determine what steps in metamorphic melanophore development require *erbb3b*, we examined molecular marker expression (Fig. 5A–F). *picasso* mutant larvae 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, similar to embryonic stages (Lyons et al., 2005), 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 (Figs. 6A,B) but often developed few if any metamorphic melanophores in the mid-trunk (Figs. 6A,C), similar to *picasso* mutants (Figs. 1, 2). 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* in promoting adult pigment pattern development.

Given that metamorphic melanophores express both *erbb3b* and *erbb2*, we sought to further test the role of ErbB signaling within this lineage. We reasoned that intrinsic differences between wild-type and *erbb3b* mutant melanophores could be further revealed as differences in their abilities to populate the flank in a background lacking endogenous melanophores. We therefore transplanted wild-type or *picasso* mutant cells to *nacre*^{w2} mutant hosts, which lack their own melanophores owing to a mutation in *mitfa*, a transcription factor required 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 during the larva-to-adult transformation 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). These results suggest autonomous differences in the development of *picasso* mutant metamorphic melanophores compared to the wild-type. Interestingly, metamorphic melanophores failed to develop in *picasso* → *nacre* chimeras even in the furthest anterior and posterior regions of the flank, where metamorphic melanophores normally develop in *picasso* mutants; this difference 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 both autonomously and non-autonomously during metamorphic melanophore development.

ErbB activity is required during embryogenesis for metamorphic melanophore development

The adult pigment pattern defect of *picasso* mutant larvae could reflect *erbb3b* activities early or late in metamorphic melanophore development. For example, *erbb3b* could function early 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 differentiation into metamorphic melanophores. To distinguish among these possibilities, we blocked ErbB signaling at stages ranging from embryo to metamorphosing larva using two 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 an excess neuromast defect that phenocopies an excess neuromast defect

of *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), inhibitor effects presumably reflect abrogation of signals associated with erbb3b:erbb2, erbb3:egfr or other heterodimers (see Introduction). By contrast, potential activities of these receptors that are independent of the kinase function should not be affected.

Wild-type embryos treated with AG1478 developed embryonic/early larval pigment patterns indistinguishable from wild-type and *picasso* mutants. When these same fish reached metamorphosis, however, they were markedly deficient for metamorphic melanophores: both 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 embryos with a structurally distinct ErbB inhibitor, PD158780, yielded identical results (Fig. 8 and data not shown).

To test if this embryonic requirement for ErbB signaling is unique to zebrafish, we examined two more species (Quigley et al., 2004; Quigley et al., 2005). We chose *D. albolineatus* because its more uniform pigment pattern (Fig. 7F) might depend on mechanisms different than the stripes of zebrafish (Mills et al., 2007). *Danio albolineatus* embryos developed gross 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 relatively refractory to perturbation. Consistent with this prediction, *D. nigrofasciatus* embryos treated with AG1478 developed adult pigment pattern defects (Fig. 7I), but these defects were less severe than those we observed in zebrafish or *D. albolineatus*.

In mammalian systems in vitro, AG1478 is highly selective for EGFR-dependent signals and shows lesser effectiveness 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). The specificity of these inhibitors for zebrafish, in vivo, is not known. While the similarity of adult pigment patterns between drug-treated fish and the *picasso* mutant is consistent with abrogation of erbb3b-dependent signals, we would expect these inihibitors to affect signaling through other ErbB receptors as well, particularly since 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, we should see an enhancement of the *picasso* phenotype. When we treated sibling *picasso*$^{wp.r2e2}$ and *picasso*$^{wp.r2e2}$/+ embryos with AG1478 for the first 4 d of development, homozygotes unexpectedly developed edema and died by 7 dpf. The simplest explanation for this result is that a single functional allele of *erbb3b* is sufficient to protect against lethality due to AG1478 kinase-inhibition. This implies that *erbb3b* has activities that are independent of kinase activity (which is itself presumably mediated by erbb3b:erbb2 or erbb3b:egfr heterodimers). Consistent with this idea are results from several studies that have revealed kinase-independent activities of various 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 with ErbB inhibitors 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 the drug effects we observed on adult pigment patterns were largely or exclusively mediated by abrogation of signals that depend on *erbb3b*.

Inhibitor effects support a model in which ErbB signals are required in embryos for adult pigment pattern formation. To further test the role of *erbb3b* specifically, we sought an independent means of blocking *erbb3b* activity. We reasoned that the limited perdurance of morpholino oligonucleotides (3−5 d) should allow us to knock-down *erbb3b* activity at early stages, while permitting later activity during metamorphosis (Nasevicius and Ekker, 2000; Mellgren and Johnson, 2004). We therefore injected single cell embryos with a morpholino oligonucleotide against *erbb3b* (Lyons et al., 2005) and raised these embryos 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: (i) embryonic knockdown of *erbb3b* by morpholino injection; and (ii) treating embryos with either of two pharmacological inhibitors that are specific to *erbb3b*-dependent signals (as evidenced by their failure to enhance the pigment pattern phenotype of an *erbb3b* presumptive null allele).

Adult pigment pattern requirement for ErbB activity during neural crest migration

The critical period for ErbB signaling elucidated above includes the time of neural crest migration. To further test for a coincidence with neural crest development, we treated embryos with ErbB inhibitors for shorter intervals. Preliminary analyses with wild-type revealed extensive variation in the severity of defects among individuals, perhaps due to stochastic differences in the extent of pattern regulation during metamorphosis (Parichy and Turner, 2003b; Yamaguchi et al., 2007). We therefore used a sensitized background, the *kit*^{b5} mutant, to reveal the early critical period more precisely. *kit* mutants lose embryonic/early larval melanophores and subsequently develop late metamorphic melanophores already in stripes. A defect in pattern regulation is indicated by a failure to regenerate normal fin pigment patterns after fin amputation (Johnson et al., 1995; Parichy et al., 1999; Rawls and Johnson, 2000).

We treated *kit* mutant embryos with ErbB inhibitors beginning between 8 hpf and 70 hpf for periods of 2 h to 26 h depending on the time of initiation. Such analyses across multiple independent experiments revealed peak sensitivities for adult pigment pattern formation between ~14−22 hpf, with affected individuals developing stripe defects reminiscent of *picasso* mutants (AG1478: Fig. 10A–C,E,F; PD158780: data not shown). Since adult pigment patterns were comparatively refractory to treatments after ~22 hpf, we asked whether extending the duration of treatment at later stages would enhance the frequency or severity of adult pattern defects. Treating embryos between 26−48 hpf did not significantly 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 further tested whether ErbB signals might have reiterated activities by treating individual embryos with inhibitors at two different periods. When early treatments (8−11 hpf) were combined with later treatments (beginning ≥22 hpf), we observed more severe melanophore deficiencies in the adult pigment pattern, with defects extending further anteriorly and posteriorly than in the *picasso* mutant. Remarkably, treatments ≥8 h apart often resulted in spatially separated melanophore-deficient patches (e.g., Fig. 10D). The increased severity of these defects compared to those of embryos treated just once suggests early and late functions

for ErbB signals: defects arising from early abrogation of ErbB kinase activity can presumably be regulated, provided ErbB function is allowed subsequently.

The major critical period for ErbB signals defined above (~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 sought to explore the role of *erbb3b* specifically in the early patterning of neural crest-derived cells using molecular markers. In comparison with wild-type siblings, *picasso* mutant embryos 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 (between neural tube or notochord and somites) and were instead found further ventrally (Fig. 11A). We observed a similar patterning defect for cells expressing *mitfa* (Fig. 11B), identifying putative melanophore and xanthophore precursors (Lister et al., 1999; Parichy et al., 2000b). By contrast, we did not find clear defects in the distributions of committed dct^{+} melanoblasts (Fig. 11C) or cells in the lateral migratory pathway (between epidermis and somites), consistent with the normal patterning of *picasso* mutant embryonic/early larval melanophores. Finally, given the defects in ventromedial migrating cells described above, as well as defects in the peripheral nervous system of mammalian *erbb3* and *erbb2* mutants (Britsch et al. 1998; Britsch, 2007), we examined dorsal root and sympathetic ganglia by immunohistochemistry. We observed gross reductions in the numbers of dorsal root and sympathetic ganglia in *picasso* mutant larvae at 12 dpf (Fig. 11D), consistent with the findings of Honjo et al. (submitted). These data reveal an *erbb3b*-dependence of neural crest morphogenesis that correlates with the early *erbb3b*-dependence of adult pigment pattern formation.

Sensitized genetic backgrounds reveal requirements for ErbB signals during metamorphosis

The preceding experiments demonstrated a critical period for ErbB signaling during embryogenesis. During later development, metamorphic melanophores express both *erbb3b* and *erbb2* (Fig. 4C), but wild-type larvae treated with pharmacological inhibitors of ErbB signaling during metamorphosis failed to exhibit adult pigment pattern defects (Fig. 7C,E). Resistance at these stages could indicate redundancies between ErbB signals and other pathways, regulation in cell behaviors, poor penetration into tissues, or higher thresholds of inhibition for the salient processes, as compared to embryonic stages. Given these possibilities, we sought to further test roles for ErbB signals during metamorphosis. Since higher doses of inhibitors were lethal and so could not be tested for the durations required, we re-tested the ErbB-dependence of pigment pattern formation during metamorphosis using sensitized backgrounds: *kit* mutant and $csf1r^{j4e1}$ mutant *D. rerio*; phenotypically wild-type, doubly heterozygous *kit*/+; *csf1r*/+ mutant *D. rerio*; and *D. albolineatus*. We chose these zebrafish mutants because previous analyses identified temporally and genetically distinct populations of metamorphic melanophores that are revealed by the *kit* and *csf1r* mutant phenotypes (Johnson et al., 1995;Parichy et al., 1999,2000b). *kit* mutants are missing early metamorphic melanophores, but retain late metamorphic melanophores. Conversely, *csf1r* mutants retain early metamorphic melanophores, but are missing late metamorphic melanophores. Comparing further deficits in residual melanophores should therefore indicate whether one or the other metamorphic melanophore population exhibits a greater requirement for ErbB signaling. Finally, we also examined *D. albolineatus* because of the differences in melanophore development in this species compared to zebrafish, including fewer melanophores overall, increased frequency of melanophore death, and reduced melanophore migration (Quigley et al., 2005;Mills et al., 2007).

In each sensitized 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. 7E; 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 for erbb3b-dependent signals, 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 of severities observed for fish doubly mutant for *kit* and *picasso*$^{wp.r2e2}$ (data not shown). These data support a model in which ErbB signals are essential during embryogenesis but also function redundantly with other pathways during metamorphosis to promote adult pigment pattern development.

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 embryonic/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 pigment pattern metamorphosis. Genetic mosaic analyses further suggest complex modes by which ErbB signals promote adult pigment pattern formation, with effects that are both autonomous and non-autonomous to the metamorphic melanophore lineage.

The requirement for ErbB signals by glia 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 for pigment cell development in vivo.

Our analyses indicate that adult pigment pattern formation exhibits an early critical period for ErbB signaling. This conclusion is supported by effects of ErbB inhibitors on both wild-type and sensitized genetic backgrounds. While the specificities of pharmacological inhibitors are difficult to know with certainty, several lines of evidence suggest that effects in this study reflect inhibition of ErbB-dependent signals. First, we observed the same phenotypes with two inhibitors that are structurally distinct. Second, induced pigment pattern defects phenocopy mutants for *erbb3b* null alleles. Third, inhibitors failed to enhance pigment pattern defects of these *erbb3b* null alleles. Fourth, qualitatively similar pigment pattern defects resulted from knockdown of *erbb3b* via zygotic morpholino injection. While more definitive evidence will require the generation of conditional alleles that are beyond the scope of this study, our results do point to a model in which ErbB signals—depending in part on erbb3b—play an essential role during embryogenesis to promote much later adult pigment pattern formation.

This early critical period contrasts markedly with other genes involved in adult pigment pattern formation. For instance, analyses of temperature-sensitive *csf1r* and *puma* mutant alleles indicate critical periods during pigment pattern metamorphosis (Parichy and Turner, 2003a; Parichy et al., 2003). Likewise, studies of a temperature-sensitive *kit* allele during regeneration of the fin pigment pattern indicate a role during pattern formation, rather than prior to this time

(Rawls and Johnson, 2001). The critical period for ErbB signals also may be earlier than that in mouse for Ednrb, which is required during melanoblast migration to the dermis, rather than during earlier neural crest dispersal (Shin et al., 1999). Nevertheless, even our analyses in a sensitized genetic background can suggest only a range of times: both drugs act very rapidly and in a quickly reversible manner (Fry et al., 1997; Lenferink et al., 2001; Levitzki and Mishani, 2006), but we do not know how long it takes for effective concentrations to reach beneath the epidermis, or to be cleared after embryos are transferred to inhibitor-free solution. The peak sensitivity observed for embryos treated between 14−22 hpf may thus indicate somewhat later critical periods in promoting adult pigment pattern formation, presumably during neural crest migration. Although we cannot absolutely exclude effects on earlier steps of neural crest specification, these appear somewhat less likely as treated fish did not exhibit gross defects typical of mutants affecting such processes (Nguyen et al., 1998; Dutton et al., 2001).

We can envisage at least two complementary modes by which embryonic ErbB signaling contributes to later metamorphic melanophore development. In the first mode, these signals would act autonomously to promote the early establishment of a precursor population that will subsequently generate metamorphic melanophores. This activity could be specific to metamorphic melanophores, if fate-restricted precursors exist this early in development, but the activity could equally well affect a broader range of neural crest derivatives. For example, both pigment cells and glia are thought to share a bipotent embryonic precursor (Dutton et al., 2001; Dupin and Le Douarin, 2003; Dupin et al., 2003), both can be generated by multipotent adult neural crest-derived stem cells in the skin (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 could have a direct role in establishing such precursors. Or, ErbB signals could serve to expand the population of multipotent neural crest cells, with regulation allowing for some neural crest derivatives to form apparently normally (Milos and Dingle, 1978; Raible and Eisen, 1994; Vaglia and Hall, 2000; Yang and Johnson, 2006). Such regulation could explain why the embryonic/early larval pigment pattern is normal in *erbb3b* mutants: if multipotent cells are allocated to fill a defined number of “embryonic/early larval niches”, before the filling of “metamorphic niches”, a depleted total number of cells could leave metamorphic niches vacant.

A second mode by which early ErbB signals could promote later adult pigment pattern formation is through activity that is non-autonomous to the metamorphic melanophore lineage. This could occur either if ErbB-expressing cells provide trophic support to metamorphic melanophore precursors, or if they contribute otherwise to a microenvironment where these precursors reside. Such interactions could be identical to, or in addition 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 raise the possibility that peripheral nerves or ganglia serve as niches for metamorphic melanophore precursors. Consistent with this idea is the early mispatterning of neural crest-derived cells that would form ganglia (this study); subsequent defects in dorsal root ganglia, sympathethetic ganglia, and other peripheral nerves, particularly at the axial levels with later pigment pattern defects (this study, Figs. 5A, 11D; Honjo et al., submitted); and the presence in other model organisms of multipotent neural crest-derived cells in peripheral nerves or ganglia that are able to produce melanocytes and other cell types (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). A more direct test of this idea in zebrafish will require both ablating these structures, which has not yet been possible owing to regeneration (Reyes et al., 2004) (unpublished data), and identifying very early markers of the metamorphic melanophore lineage.

Beyond the embryonic critical period for ErbB signals, our data also suggest a role during metamorphosis as treating sensitized backgrounds with either of two ErbB inhibitors resulted in melanophore deficiencies, albeit of differing magnitudes. As at embryonic stages, ErbB signals may act autonomously to metamorphic melanophores, consistent with their expression of *erbb3b* and *erbb2*. Non-autonomous roles also could be manifested if interactions among melanophores promote the survival, proliferation, or differentiation of these cells, consistent with community effects in other mutant backgrounds (Parichy et al., 2000b; Parichy and Turner, 2003b).

Together, our data support a model in which ErbB signals, mediated at least in part through erbb3b, are required during embryogenesis to establish a population of latent precursors that will subsequently generate metamorphic melanophores. Later, during metamorphosis, ErbB signals contribute to melanophore development but appear to be partly or entirely redundant with other pathways. Furthermore, our analyses show that both early-appearing, *kit*-dependent metamorphic melanophores, and later-appearing *kit*-independent (*csf1r*-dependent) metamorphic melanophores require ErbB signals. We speculate that *erbb3b* both promotes the development of latent precursors intrinsically and also is required extrinsically in forming a niche where they will reside until recruited to differentiate at metamorphosis.

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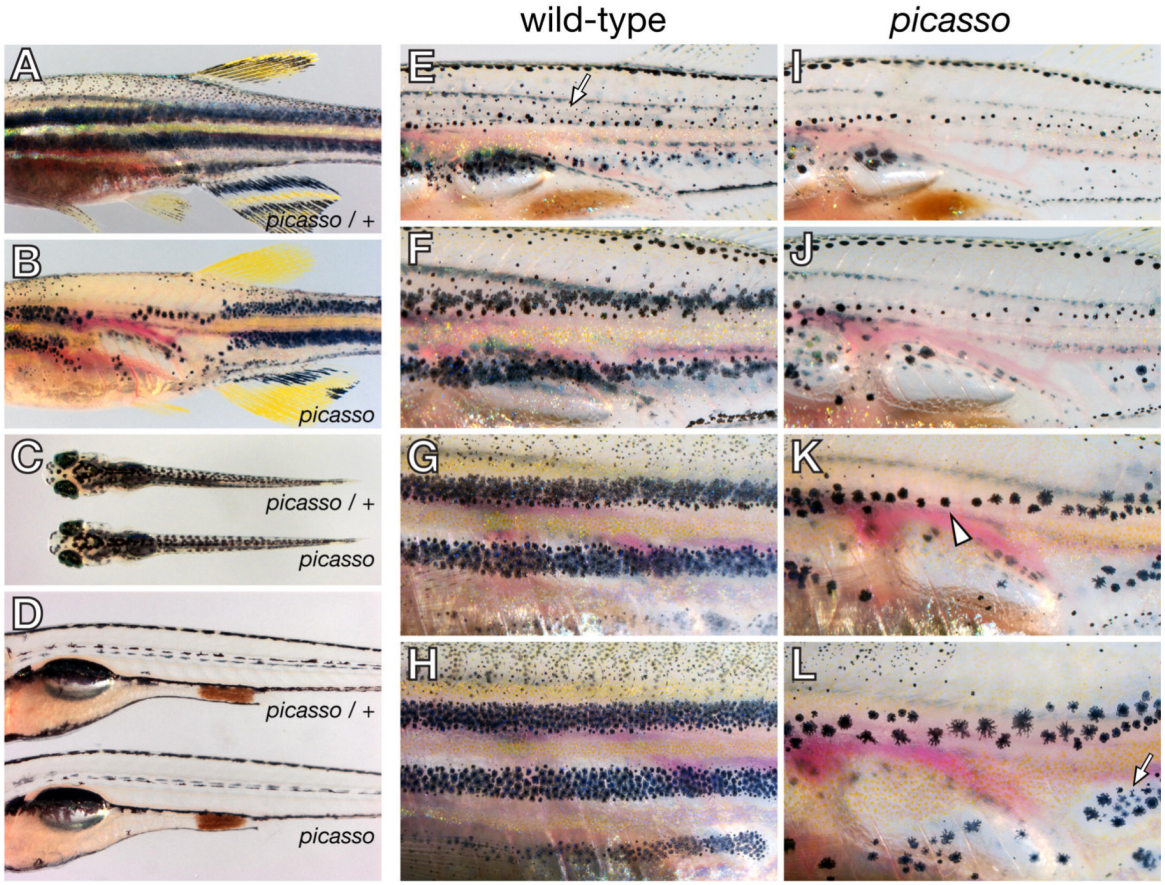

Fig. 1. Defective adult pigment pattern but normal embryonic/early larval pigment pattern of *picasso* mutants

(**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 revealing normal development of initially dispersed metamorphic melanophores that organize into stripes (arrow, E), as well as metamorphic melanophores that develop 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) persist into the adult. (E,I) 17 dpf. (F,J) 23 dpf. (G,K) 31 dpf. (H,L) 40 dpf.

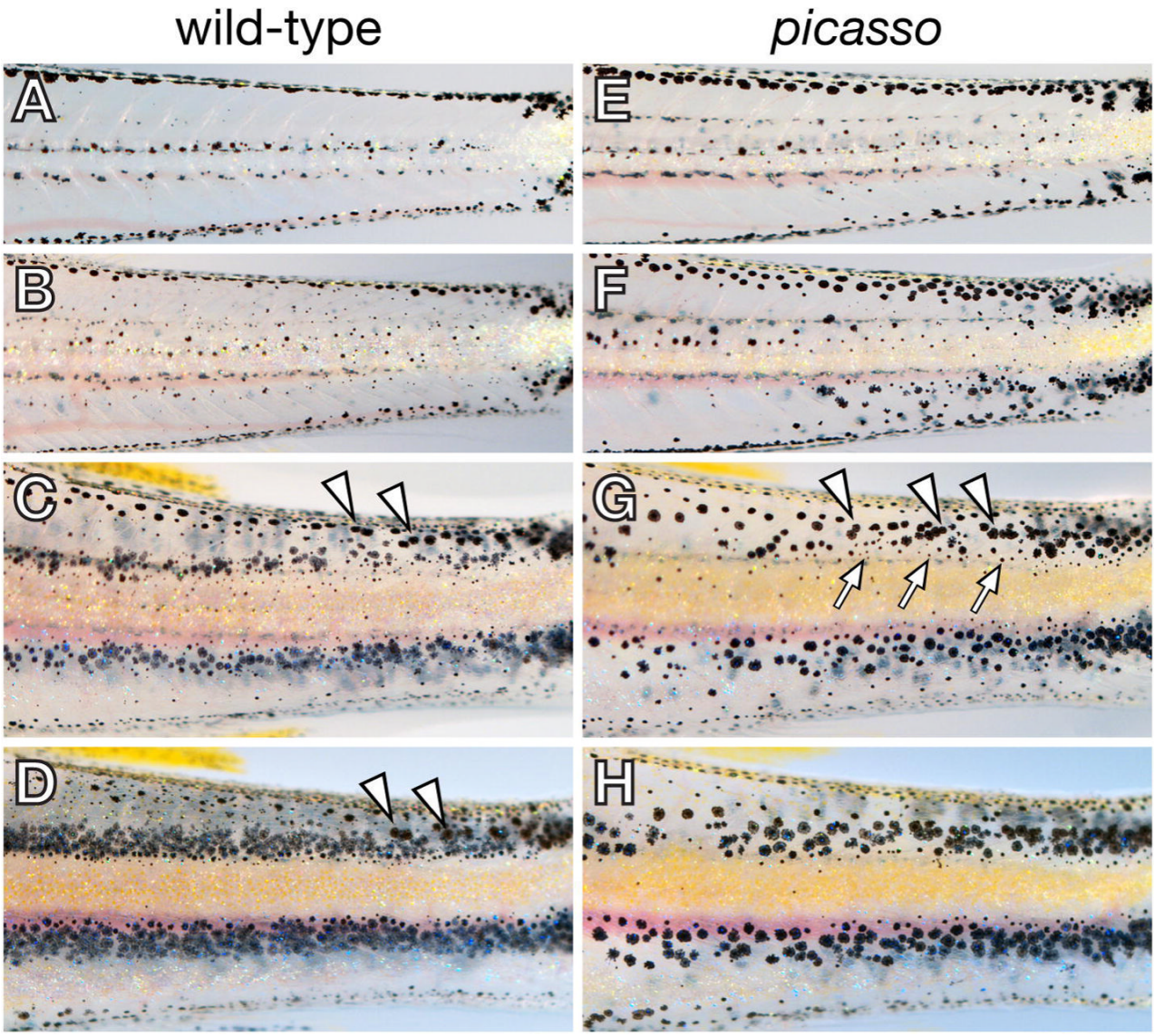

Fig. 2. Regulative posterior adult stripe formation in the *picasso* mutant

Adult pigment pattern development on the posterior trunk of wild-type (**A–D**) and *picasso* mutant (**E–H**) larvae. Shown are individual, representative larvae of each genotype. (**A–D**) In wild-type, most dorsal early larval melanophores remained dorsally, though a very few were incorporated into the adult dorsal primary stripe in its posterior region (arrowheads in C,D). (**E–H**) In *picasso* mutants, more embryonic/early larval melanophores (e.g., arrowheads in G) were incorporated into an incomplete dorsal primary stripe. Additional metamorphic melanophores differentiated (e.g., arrows in G) where persisting embryonic/early larval melanophores contributed to the adult stripe. Thus, residual adult stripes in the posterior of *picasso* mutants resulted from an increased contribution of embryonic/early larval melanophores, as well as increased numbers of metamorphic melanophores compared to the mid-trunk. (**A,E**) 14 dpf. (**B,F**) 16 dpf. (**C,G**) 20 dpf. (**D,H**) 24 dpf.

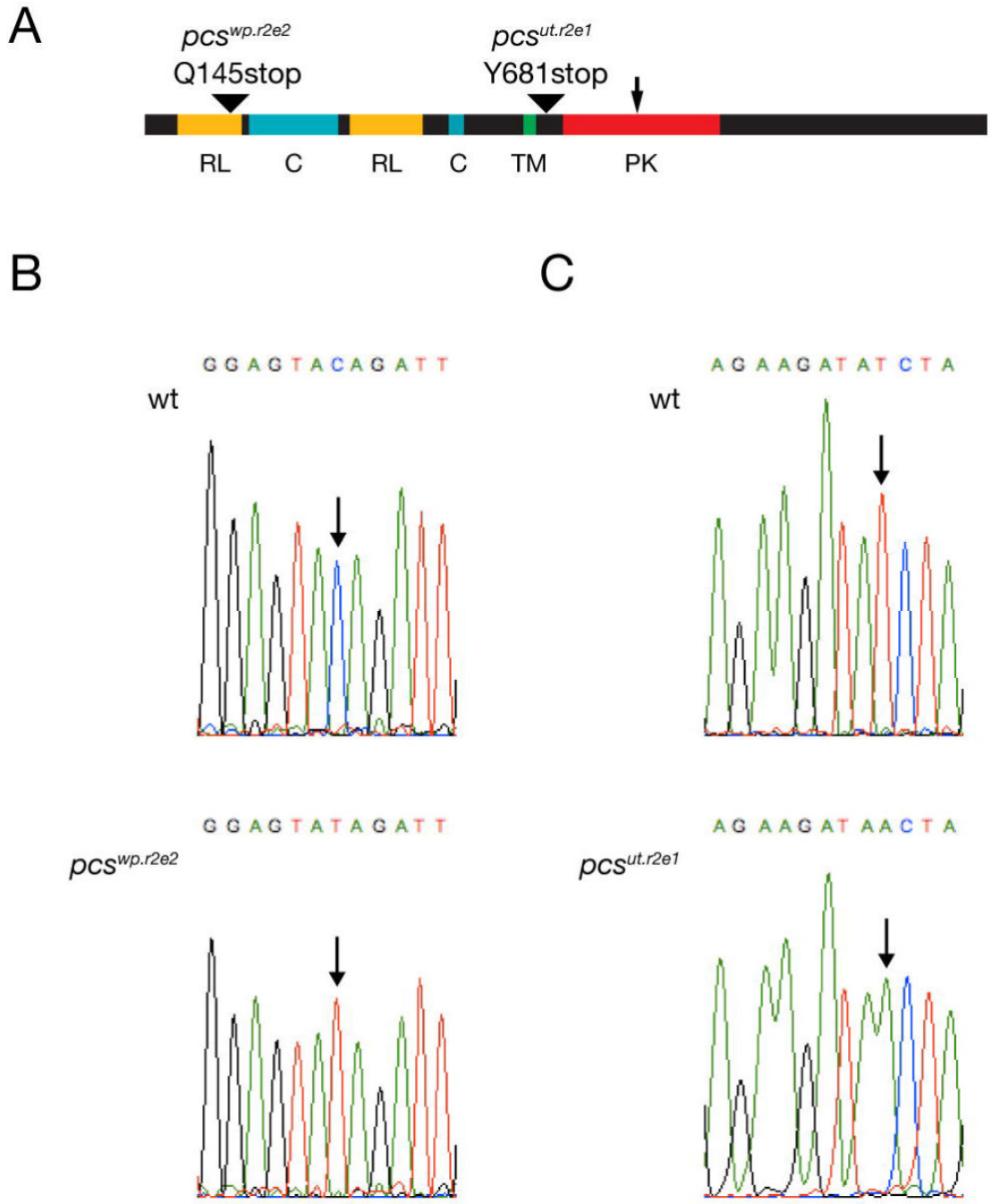

Fig. 3. *picasso* is allelic to *erbb3*

(**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 in $pcs^{ut.r4e1}$ (T2043A: Y681stop).

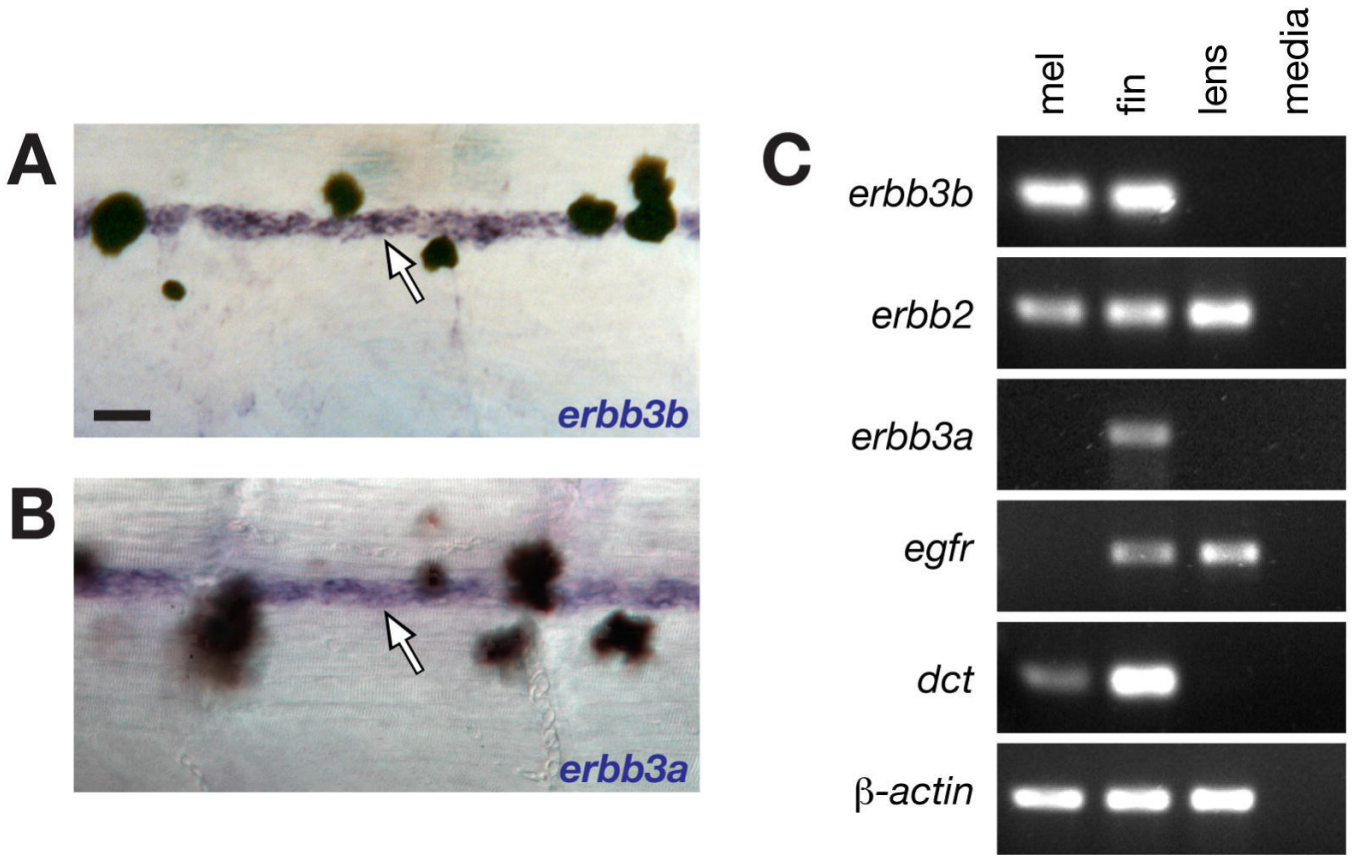

Fig. 4. ErbB gene expression in metamorphosing larvae

(**A,B**) erbb3 loci are expressed in glia during zebrafish metamorphosis (~18 dpf). Arrows, glial cells surrounding midbody lateral line. (**A**) *erbb3b*. (**B**) *erbb3a*. (**C**) RT-PCR reveals metamorphic melanophore expression of *erbb3b* and *erbb2*, but not *erbb3a* or *egfr*. All four ErbB genes are expressed in adult fin, comprising chromatophores and their precursors, skin, bone, nerve, and other cell types. 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 to fully differentiated melanophores (mel). Scale bar: in A, 0.5 mm for A,B.

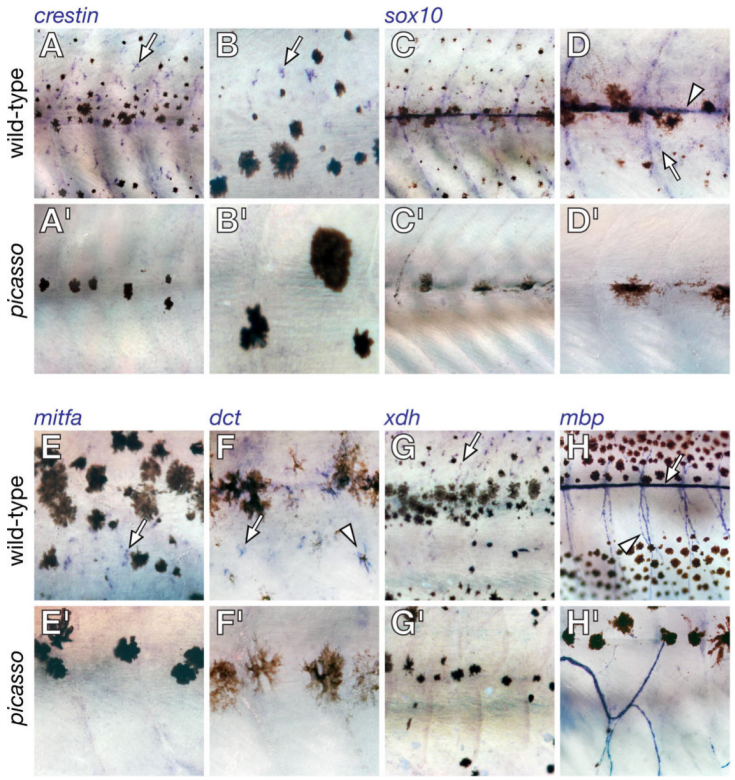

Fig. 5. Metamorphic deficiencies for early and late markers of neural crest-derived lineages in *picasso* mutant larvae

Shown are corresponding regions of the mid-trunk for wild-type (above) and *picasso* mutant larvae at middle stages of metamorphosis (~18 dpf). Note that individual melanophores are much more spread in *picasso* mutants, which is typical of mutants with reduced densities of melanophores and their precursors (Parichy and Turner, 2003b; Parichy et al., 2003). (**A,A'**) *crestin* marks most or all neural crest-derived cells in embryos (Luo et al., 2001) and identifies a dispersed population of cells in the hypodermis of metamorphosing larvae (arrow) that may be pigment cell precursors. *crestin*$^+$ cells were dramatically fewer in *picasso* mutants. (**B,B'**) Higher magnification image of *crestin*$^+$ cells in wild-type showing typical melanoblast morphology, 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 to wild-type. (**D,D'**) Higher magnification showing *sox10*$^+$ glia along midbody lateral line (arrowhead) and individual *sox10*+ cells (arrow) in the hypodermis 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 are numerous in the hypodermis of wild-type but not *picasso* larvae. (**F,F'**) *dct* identifies melanophore precursors (arrow) and melanophores (arrowhead)(Kelsh et al., 2000); *dct*$^+$ cells are reduced or absent in *picasso* mutants. (**G,G'**) *xanthine dehydrogenase* (*xdh*) encodes an enzyme in the pteridine synthesis pathway of xanthophores (Parichy et al., 2000b). *xdh*$^+$ cells (arrow) are numerous in wild-type but transiently reduced in number in *picasso*. (**H,H'**) *myelin basic protein* (*mbp*) marks mature glia in the peripheral nervous sytem (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) of wild-type larvae. Although *mbp*$^+$ cells are present in *picasso*, they are fewer in number and their associated nerves are misrouted.

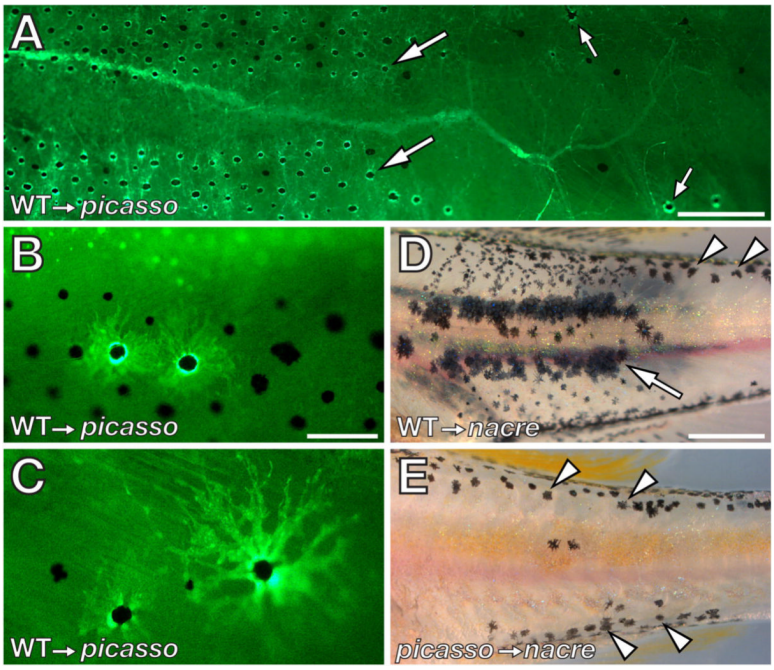

Fig. 6. Autonomous and non-autonomous roles for *erbb3b* in pigment pattern metamorphosis

(**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 exhibits misrouting as well, perhaps owing to defects in glial-dependent fasciculation (Gilmour et al. 2002; Lyons et al., 2005). (**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.

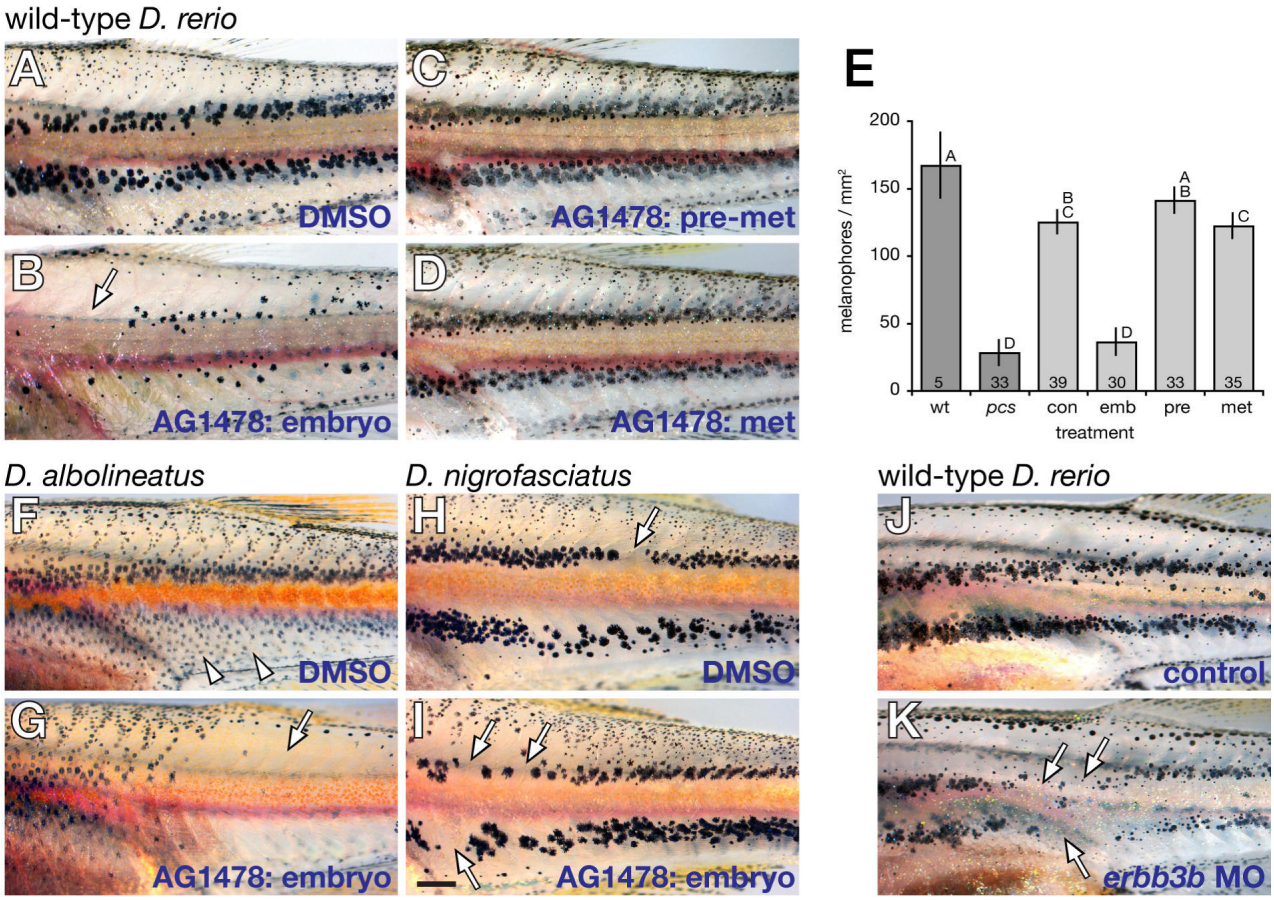

Fig. 7. Embryonic requirement for ErbB signaling in pigment pattern metamorphosis

(**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) shows adult pigment pattern resembling severely affected *picasso* mutants. (**C**) Individual treated with AG1478 during the pre-metamorphic, early larval period (5−14 dpf) exhibits a pigment pattern indistinguishable from controls. (**D**) An individual treated with AG1478 throughout metamorphosis (15−28 dpf) also is indistinguishable from controls. (**E**) Metamorphic melanophore densities in the mid-trunk (means ± 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), and similar defects between *picasso* mutants (*pcs*) and fish treated with AG1478 as embryos (emb). Letters above bars indicate means that are not significantly different from one another by post hoc Tukey-Kramer comparisons. Numbers within bars are samples sizes. (**F, G**) *D. albolineatus* normally develop a more uniform melanophore pattern of metamorphic melanophores (arrowheads in F) compared to *D. rerio*, and exhibit a severe melanophore deficiency when embryos are 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 has relatively subtle effects (arrows in I). (**J, K**) Embryonic morpholino knockdown of *erbb3b* results in adult melanophore deficiencies (K) compared to 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.

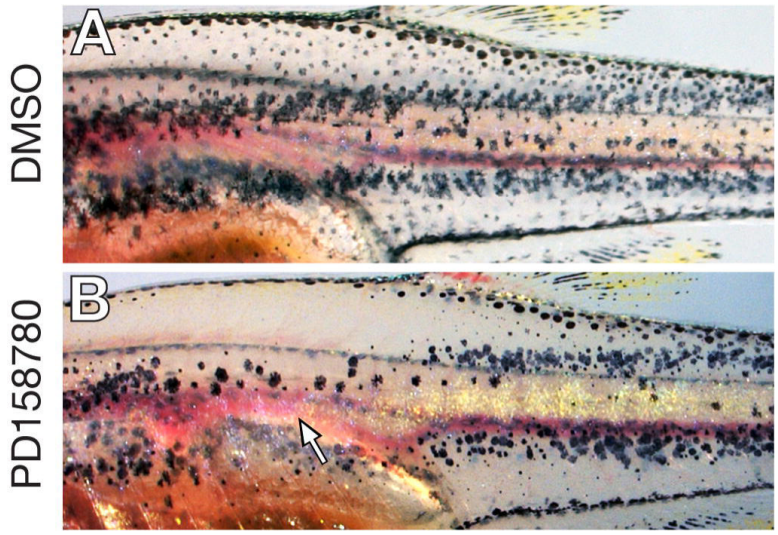

Fig 8. Treatment of embryos with ErbB inhibitor PD158780 results in adult pigment pattern defect similar to *erbb3b* null alleles

(**A**) Control treated with DMSO alone. (**B**) Representative individual treated with PD158780 for 2 dpf. Arrow, region deficient for metamorphic melanophores.

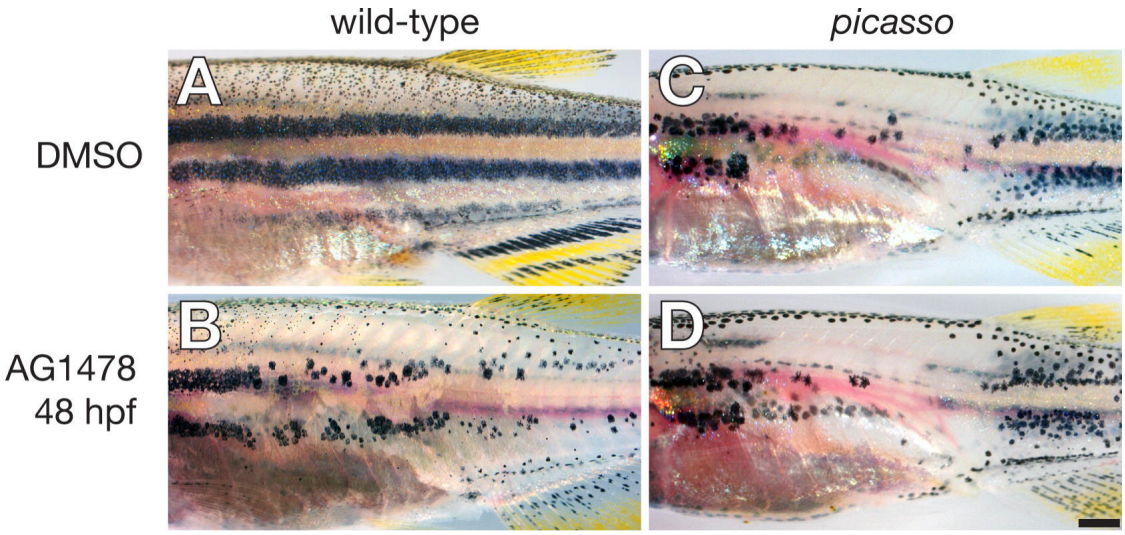

Fig 9. ErbB inhibitor treatment for 48 hpf does not enhance the *picasso* null phenotype

(**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.

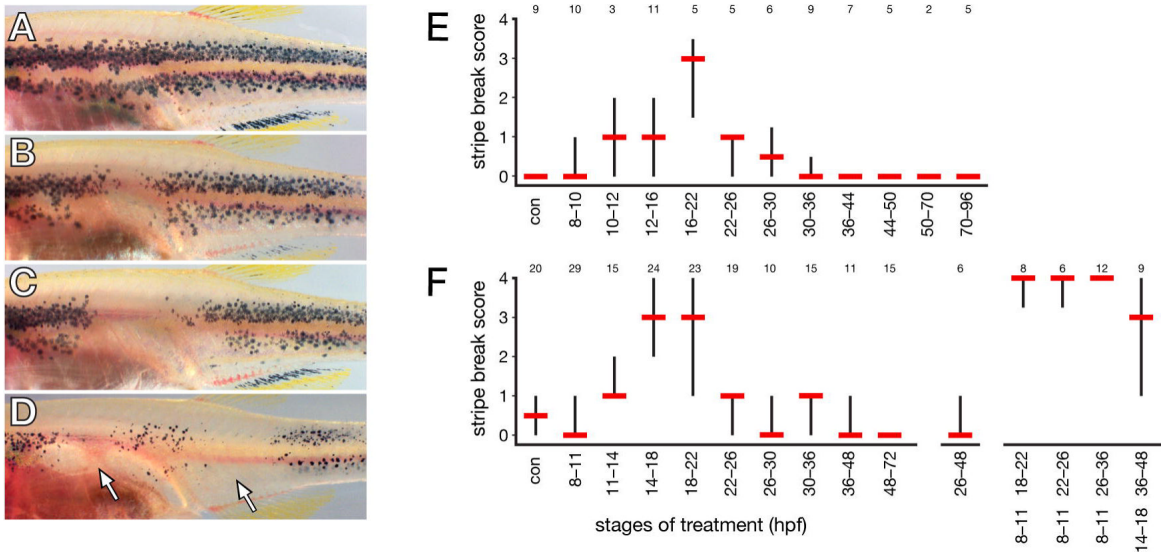

Fig. 10. *kit* mutant reveals critical period for ErbB activity during neural crest migration

Embryos were reared to juvenile stages after treatment with AG1478 as embryos. (**A**) Normal *kit* mutant pigment pattern after AG1478 treatment between 26−30 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 14−18 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 in kit mutant treated between 8−11 hpf then again between 26−30 hpf. (**E,F**) Quantitation of stripe break defects in *kit* mutants treated with AG1478 at various stages of embryonic development 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 16−22 hpf, with lesser defects apparent between 8−36 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 occurring between ~14−22 hpf, with lesser defects before and after this period ($\chi^2 = 115.6$, d.f.=14, $P<0.0001$). More severe defects are generated with repeated treatments (right).

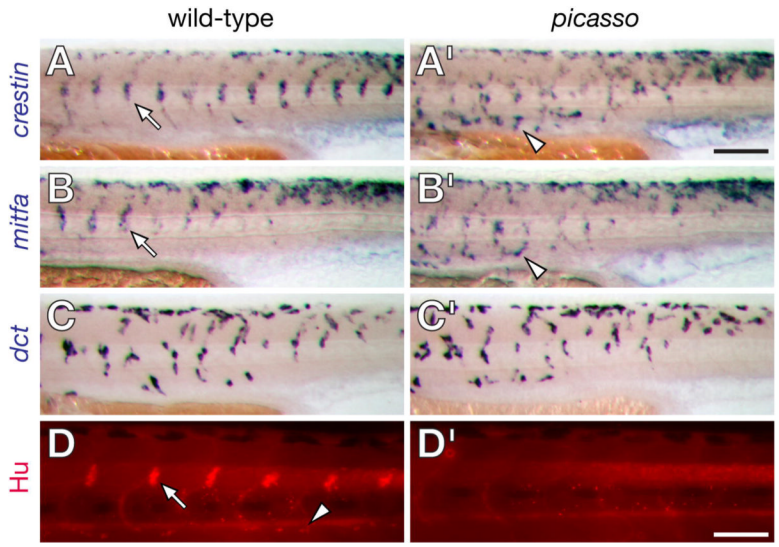

Fig. 11. *picasso* mutant embryos have defects in neural crest morphogenesis

(**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'.

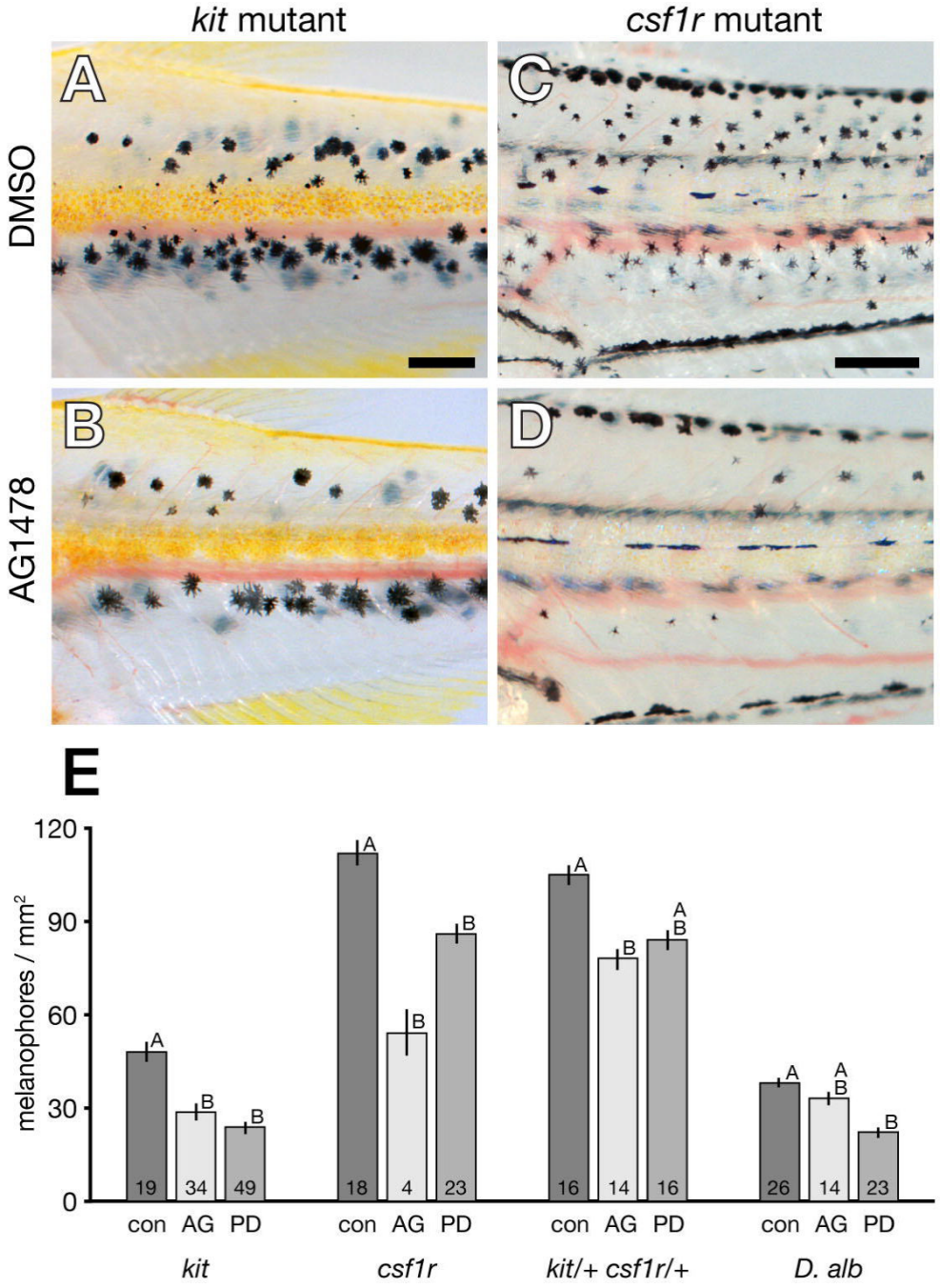

Fig. 12. Cryptic ErbB requirements during metamorphosis revealed by sensitized genetic backgrounds

(**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 to 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, as determined by post hoc Tukey-Kramer comparisons of means. 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.