

# Mutational Analysis of *Endothelin Receptor b1* (*rose*) during Neural Crest and Pigment Pattern Development in the Zebrafish *Danio rerio*

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Pigment patterns of fishes are a tractable system for studying the genetic and cellular bases for postembryonic phenotypes. In the zebrafish *Danio rerio*, neural crest-derived pigment cells generate different pigment patterns during different phases of the life cycle. Whereas early larvae exhibit simple stripes of melanocytes and silver iridophores in a background of yellow xanthophores, this pigment pattern is transformed at metamorphosis into that of the adult, comprising a series of dark melanocyte and iridophore stripes, alternating with light stripes of iridophores and xanthophores. Although several genes have been identified in *D. rerio* that contribute to the development of both early larval and adult pigment patterns, comparatively little is known about genes that are essential for pattern formation during just one or the other life cycle phase. In this study, we identify the gene responsible for the *rose* mutant phenotype in *D. rerio*. *rose* mutants have wild-type early larval pigment patterns, but fail to develop normal numbers of melanocytes and iridophores during pigment pattern metamorphosis and exhibit a disrupted pattern of these cells. We show that *rose* corresponds to *endothelin receptor b1* (*ednrb1*), an orthologue of amniote *Ednrb* genes that have long been studied for their roles in neural crest and pigment cell development. Furthermore, we demonstrate that *D. rerio ednrb1* is expressed both during pigment pattern metamorphosis and during embryogenesis, and cells of melanocyte, iridophore, and xanthophore lineages all express this gene. These analyses suggest a phylogenetic conservation of roles for *Ednrb* signaling in the development of amniote and teleost pigment cell precursors. As murine *Ednrb* is essential for the development of all neural crest derived melanocytes, and *D. rerio ednrb1* is required only by a subset of adult melanocytes and iridophores, these analyses also reveal variation among vertebrates in the cellular requirements for *Ednrb* signaling, and suggest alternative models for the cellular and genetic bases of pigment pattern metamorphosis in *D. rerio*. © 2000 Academic Press

## INTRODUCTION

The mechanisms responsible for patterning phenotypes of adult vertebrates remain largely unknown. Yet, identifying the genes and cell behaviors underlying adult form will be essential for a deeper understanding of normal development, syndromes in which development has gone awry, and the identification of clinical strategies for correcting these

problems. A useful system in which to assess later patterning mechanisms is the complex life cycle, in which individuals undergo a metamorphosis some time during their development (Moran, 1994). In a biomedical context, metamorphosis is an outstanding model for postembryonic and fetal development in mammals (Tata, 1993). In a comparative context, a metamorphosis is present in the majority of animals and is most often thought to “decouple” traits expressed at different stages, thereby permitting independent adaptations to different environments and selective regimes (Haldane, 1932; Ebenman, 1992; Moran, 1994). Among vertebrates, both amphibians and fishes undergo a metamorphosis affecting numerous traits, including the

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skin, body shape, appendages, and behavior (Webb, 1999; Shi, 2000). Nevertheless, the extent of trait interdependence across stages and how uncoupling might be achieved remain largely unknown (Parichy, 1998).

One trait that undergoes extensive changes at metamorphosis is the pattern formed by neural crest-derived pigment cells in vertebrates. Pigment patterns of fishes and amphibians result from the spatial arrangements of three principal classes of pigment cells: black melanocytes (melanophores); silver iridophores; and yellow xanthophores (Bagnara, 1998; Reedy *et al.*, 1998). In the zebrafish *Danio rerio*, these cells form very different patterns during different life cycle phases (Kirschbaum, 1975; Milos and Dingle, 1978a; Milos *et al.*, 1983; Johnson *et al.*, 1995b; Kelsh *et al.*, 1996; Parichy *et al.*, 2000). In embryos, neural crest cells differentiate as all three classes of pigment cells to generate an early larval pigment pattern, comprising a series of melanocyte and iridophore stripes, with xanthophores widely distributed over the flank. This pattern remains largely unchanged during early larval development. At ~14 days, however, metamorphic changes in the pigment pattern begin to be manifested. First, beginning between 14 and 21 days, early metamorphic (EM) melanocytes arise dispersed over the flank in regions not previously occupied by these cells (previously referred to as early stripe melanocytes; Johnson *et al.*, 1995b). These cells gradually coalesce at prospective sites of the first adult stripes, dorsal and ventral to the horizontal myoseptum, and simultaneously, early larval melanocytes in this region begin to die. Subsequently, beginning between 21 and 28 days, additional, late metamorphic (LM) melanocytes differentiate already in the position of adult stripes (referred to as late stripe melanocytes; Johnson *et al.*, 1995b). By 30 days, the adult pigment pattern has been established, initially comprising two stripes of melanocytes with overlying iridophores, as well as lighter interstripe regions of xanthophores and iridophores, with additional stripes arising as the fish grow (Fig. 1A).

Recent studies of *D. rerio* mutants have started to identify genes required for pigment cell development and pigment pattern formation (Kelsh *et al.*, 1996). For example, specification of early larval and adult melanocytes depends on *nacre* (Lister *et al.*, 1999), an orthologue of the transcription factor-encoding locus *mitf*, which also is essential for melanocyte specification in mammals (Opdecamp *et al.*, 1997). Early larval and adult pigment patterns also depend on the activities of the ancestrally paralogous genes *kit* and *fms*, each encoding receptor tyrosine kinases expressed by pigment cell precursors (Parichy *et al.*, 1999, 2000). Whereas *kit* is essential for development of early larval melanocytes and EM melanocytes, *fms* is essential for development of early larval xanthophores and LM melanocytes. Three loci thus have been characterized molecularly that are essential for both early larval and adult pigment pattern development.

In addition to mutations affecting pigment patterns at

both life cycle phases in *D. rerio*, other mutations identify genes that are essential for the pigment pattern in early larvae or adults, but not both. For example, *shady* mutants lack early larval iridophores, but subsequently develop wild-type adult pigment patterns (Kelsh *et al.*, 1996; Haffter *et al.*, 1996; S.S.L. and R.N.K., unpublished data). Conversely, *leopard* mutants have wild-type early larval pigment patterns, but develop spots rather than stripes as adults (Kirschbaum, 1975; Johnson *et al.*, 1995b). It is not known how activities of such genes are partitioned across life cycle stages. For example, these loci could represent novel genes, not previously implicated in amniote pigment pattern development, with life cycle stage-specific expression and requirements. Indeed, one such mutant, *hagoromo*, which perturbs adult but not early larval stripe development, has been identified as a *D. rerio* orthologue of *Dactylin*, a gene not previously suspected to have a role in pigment pattern formation (Kawakami *et al.*, 2000). Alternatively, genes with life cycle stage-specific effects could represent orthologues of previously identified genes acting in known genetic pathways. In this event, stage-specific effects presumably would reflect differential regulation of such loci across life cycle phases, specific features of the genetic background (e.g., genetic redundancy and pleiotropic relationships, which may differ from amniotes), or the presence of cryptic cellular populations with differing genetic requirements. Distinguishing between these possibilities will be an essential step in understanding morphological reorganization at metamorphosis in *D. rerio*, and the genetic and cellular bases for postembryonic development in vertebrates more generally.

One mutant in which visible pigment pattern defects are limited to just a single stage of the life cycle is *rose*. At early larval stages, *rose* mutants exhibit pigment patterns indistinguishable from those of wild-type (Johnson *et al.*, 1995b). During pigment pattern metamorphosis, *rose* mutants also experience an increase in melanocyte number initially similar to that observed in wild-type larvae between days 14 and 21. During later metamorphosis, however, *rose* mutants develop fewer melanocytes than wild-type between days 21 and 28. Adult *rose* mutants exhibit only half as many stripe melanocytes as wild-type adults; although these cells are arranged in stripes dorsally, only spots are formed ventrally (Figs. 1B–1D). Consistent with the stages at which phenotypic effects are manifested, genetic analyses suggest that *rose* is essential for the development of LM melanocytes, but not EM melanocytes (Johnson *et al.*, 1995b; Parichy *et al.*, 2000). Fish doubly mutant for *rose* and either *fms* or *leopard* (which ablate LM melanocytes) do not have more severe melanocyte deficits than single mutants. In contrast, fish doubly mutant for *rose* and *kit* (which ablates EM melanocytes), lack virtually all adult melanocytes, indicating additive effects of the two mutations. *rose* mutant adults—but not early larvae—also exhibit a severe deficit of iridophores.

Previous analyses mapped the *rose* mutation to the cen-

tromere of linkage group 1, in the vicinity of *endothelin receptor b1* (*ednrb1*; Johnson et al., 1995a, 1996; Postlethwait et al., 1998). In amniotes, endothelins were initially identified for physiological roles in vasoconstriction (Yanagisawa et al., 1988). These 21-amino acid peptides are the ligands for seven pass G-protein-coupled endothelin receptors, and several endothelins and endothelin receptors now have been identified in amniotes (Arai et al., 1990; Sakurai et al., 1990; Hall et al., 1999; Gether, 2000). An unexpected role for endothelin signaling in neural crest development was discovered when mouse knock-outs of *Endothelin receptor B* (*Ednrb*) and *endothelin-3* (*edn3*) were found to lack virtually all melanocytes, and to exhibit aganglionic megacolon due to a failure in the development of neural crest-derived enteric neurons (Baynash et al., 1994; Hosoda et al., 1994). Genetic analysis further indicated that *Ednrb* and *edn3* correspond to *piebald lethal* (*s<sup>l</sup>*) and *lethal spotting* (*ls*), respectively, two mouse mutants long studied for their defects in neural crest development (Little, 1915; Mayer and Maltby, 1964; Mayer, 1965, 1967; Pavan and Tilghman, 1994; also see Gariepy et al., 1996; Shin et al., 1997). Subsequent analyses have identified roles for endothelin receptor signaling in the proliferation and differentiation of neural crest-derived cells including melanocytes and their precursors, and in promoting the dendricity of fully differentiated melanocytes (Hara et al., 1995; Swope et al., 1995; Lahav et al., 1996, 1998; Reid et al., 1996; Imokawa et al., 1996; Yoshida et al., 1996; Stone et al., 1997; Opdecamp et al., 1998; Sviderskaya et al., 1998). Moreover, a critical period for *Ednrb* activity in mouse has been identified during early development, corresponding to the period of neural crest cell dispersal: ablation of *Ednrb* signaling during or prior to this time results in partial or complete absence of melanocytes, whereas ablation of *Ednrb* signaling after this time does not result in pigment pattern defects (Shin et al., 1999). Together, these analyses of *Ednrb* function in amniotes suggest *D. rerio ednrb1* as a good candidate for correspondence to *rose*.

Here, we test the correspondence of *rose* and *ednrb1*, and identify cell populations that express *ednrb1* in *D. rerio*. We identify three *ednrb1* lesions in three independent *rose* alleles, strongly supporting the correspondence of *rose* and *ednrb1*. Furthermore, we demonstrate that *ednrb1* is expressed both during pigment pattern metamorphosis, when pigment pattern defects are first manifested, and, unexpectedly, during embryogenesis as well. Finally, we show that *ednrb1* is expressed by melanocytes and their precursors, as well as xanthophore precursors, iridophores, and iridophore precursors. These findings reveal an essential role for *ednrb1* signaling during pigment pattern development in *D. rerio*, as in amniotes, although in contrast to amniotes, *ednrb1* is essential for the development of only a subset of adult pigment cells.

## MATERIALS AND METHODS

### Fish Rearing and Mutant Alleles

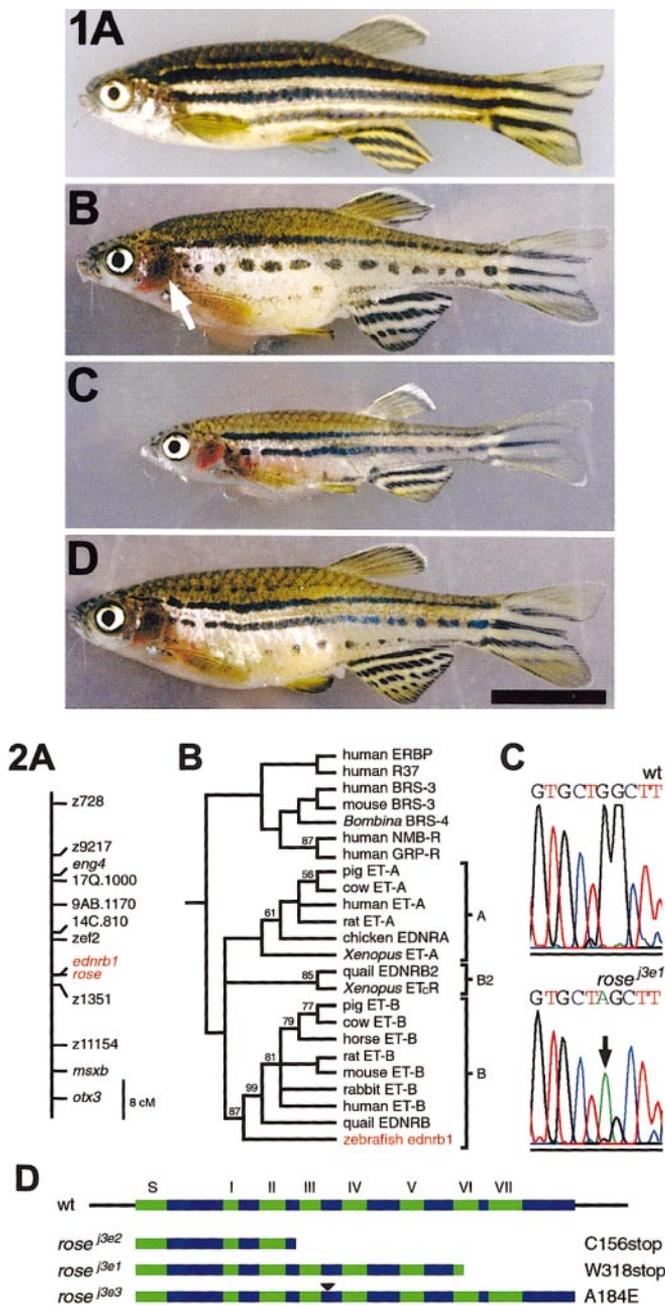
Rearing of *D. rerio* followed Westerfield (1993). All mutant *rose* alleles analyzed are recessive. *rose<sup>b140</sup>* was isolated as a spontaneous allele in the AB background, and was provided initially by C. Walker. *rose<sup>j3e1</sup>*, *rose<sup>j3e2</sup>*, and *rose<sup>j3e3</sup>* are ethylnitrosourea-induced alleles in the SJD genetic background and were identified by noncomplementation of *rose<sup>b140</sup>*. *ednrb1* expression was also assessed in embryos segregating a mutation in *endothelin-1* (kindly provided by C. Miller and C. Kimmel; Miller et al., 2000), and embryos homozygous for *fms<sup>4blue</sup>* (Parichy et al., 2000) and *shady<sup>ly82</sup>* (Kelsh et al., 1996).

### Cloning, Sequencing, and Phylogenetic Analysis

To clone a *D. rerio ednrb1* cDNA, we used degenerate reverse transcriptase PCR to isolate an internal fragment, and then isolated 5' and 3' ends of this transcript by rapid amplification of cDNA ends (RACE) using a commercially available kit and a RACE cDNA library derived from 24-h wild-type embryos (Clontech, Palo Alto, CA). We then used these end sequences to clone and sequence a full-length *D. rerio ednrb1* cDNA (Genbank Accession No. AF275636). To test the correspondence of *ednrb1* and *rose*, we compared sequences of PCR-amplified exons from genomic DNA isolated from wild-type and *rose* mutant backgrounds. To amplify exon sequences, we first designed primers to amplify across the location of introns predicted by comparison with amniote *ednrb* sequences. We sequenced introns 1–5 in their entirety, but were unable to amplify intron 6. We then used sequence from introns 1–5, as well as 5' and 3' untranslated regions to amplify entire coding sequences from *ednrb1* exons 1–6. For exon 7, we designed a 15-nucleotide forward primer immediately 3' to intron 6; our analyses of mutant *ednrb1* sequences exclude these 15 nucleotides from consideration. All primer sequences are available upon request. Sequencing was performed using AmpliTaq FS or BigDye (Perkin Elmer, Norwalk, CT) dye terminator chemistry on an ABI-377 automated sequencer, and four to eight individuals were sequenced for each genetic background. Nucleotide and protein sequences were compared against existing databases by BLAST and phylogenetic analyses were performed using PAUP 4.0.0d55 (Swofford, 1999), after inspecting sequences aligned with the Pileup program of the GCG (Genetics Computer Group, Madison, WI) software package.

### In Situ Hybridization

Methods for riboprobe preparation and *in situ* hybridization of embryos have been described (Jowett and Yan, 1996), and employed digoxigenin- and fluorescein-labeled riboprobes, alkaline phosphatase-conjugated anti-digoxigenin or anti-fluorescein Fab fragments, and NBT/BCIP (Roche, Indianapolis, IN) or FastRed (Sigma, St. Louis, MO) substrates. Hybridizations were carried out for 12–24 h at 68–72°C, followed by stringency washes at the same temperatures. Larvae older than 5 days were fixed in 4% paraformaldehyde, 5% DMSO in phosphate-buffered saline, and were cut transversely after 30 min to facilitate penetration of reagents. All sectioning was performed after embryos or larvae were stained as whole mounts. For *ednrb1*, *kit* (Parichy et al., 1999), *fms* (Parichy et al., 2000), and *dopachrome tautomerase* (*dct*, Kelsh et al., 2000) we used full-length cDNAs as templates for riboprobe generation



**FIG. 1.** Wild-type and *rose* mutant adult *D. rerio*. (A) Wild-type adults exhibit several dark stripes comprising melanocytes and iridophores, with intervening light stripes comprising xanthophores and iridophores. (B–D) *rose* mutant adults have fewer melanocytes than wild-type, and these cells form stripes dorsally but spots ventrally. *rose* mutants also exhibit an iridophore defect, which is especially noticeable lateral to the gills (arrow) and over the abdomen. (B) *rose*<sup>b140</sup> adults exhibit a pigment pattern defect but are both viable and fertile as homozygotes. (C) *rose*<sup>j3e1</sup> adults exhibit a similar pigment pattern defect to *rose*<sup>b140</sup>, but are severely runted. Shown is an individual comparable in age to that shown in (B). *rose*<sup>j3e1</sup> adults that attain larger sizes exhibit only a single dorsal

with either T3 or T7 polymerases (Promega). We also used a 540-bp fragment of *D. rerio* xanthine dehydrogenase (*xdh*) for riboprobe synthesis [this gene is also identified as *D. rerio* expressed sequence tag (EST) fc18g02; Accession Nos. AI657925, AI641077], and *D. rerio* ESTs to generate a 1200-nucleotide riboprobe for an endothelin-1-like transcript (fa95f03; AI331947, AI330487), and a ~4-kb riboprobe for the receptor tyrosine kinase *c-ret* (fc36a03; AI793987). A cDNA encoding the microphthalmia-associated transcription factor, *mitf/nacre*, was provided by D. Raible and J. Lister (Lister *et al.*, 1999).

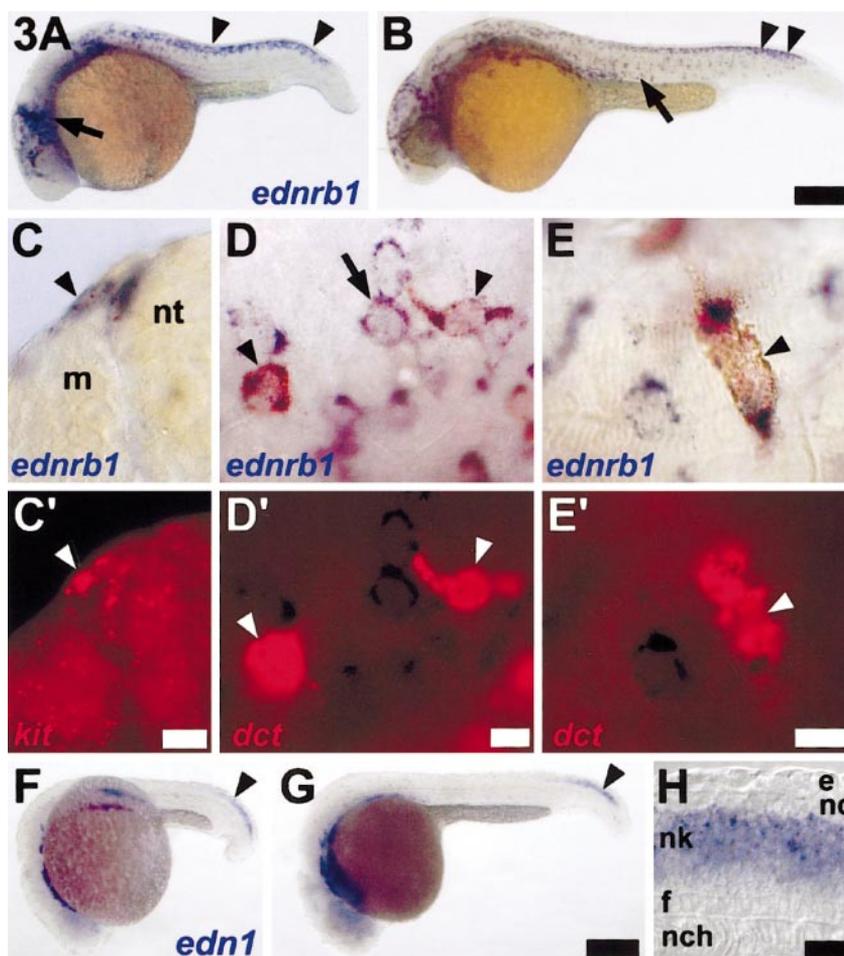
## RESULTS

### *rose* Corresponds to a *D. rerio* Orthologue of Endothelin Receptor *b*

Previous studies mapped *rose* near *ednrb1* (Postlethwait *et al.*, 1998); we further mapped *rose* meiotically within ~0.1 cM of *ednrb1* (Fig. 2A). As *Ednrb* mutants in amniotes exhibit melanocyte deficiencies (see Introduction), we asked whether *D. rerio* *ednrb1* might correspond to an *ednrb* orthologue, and whether mutations within *D. rerio*

melanocyte stripe and a fragmented ventral melanocyte stripe, as in *rose*<sup>b140</sup>. (D) *rose*<sup>j3e3</sup> mutant adults have a marginally less severe melanocyte defect, typically having one additional melanocyte stripe compared to *rose*<sup>b140</sup> and *rose*<sup>j3e1</sup>. Scale bar: 1 cm.

**FIG. 2.** *rose* corresponds to a *D. rerio* orthologue of endothelin receptor *B*. (A) Meiotic mapping places *rose* within 0.1 cM of *ednrb1* (B) Phylogenetic analyses reveal three clades of endothelin receptor-encoding genes (A, B2, B) and demonstrate that *ednrb1* is more closely related to amniote *ednrb* genes than either *ednra* or *EDRNB2/ETcR* genes. Shown is a phylogenetic reconstruction (50% majority rule consensus tree) based on analyses of amino acid sequences (413 informative characters); a similar topology was obtained from analyses of nucleotide sequences (665 informative characters; data not shown). Bootstrap support for all clades is 100% (1000 replicates) except where indicated by numbers above branches. Corresponding sequence gi numbers are (top to bottom): 7521407; 4885323; 416726; 6226779; 1705504; 128383; 232185; 1246127; 119605; 345834; 7549758; 2961105; 507894; 3036823; 539483; 544259; 108668; 6226861; 2144084; 6681269; 7331300; 119622; 1429269; 9858055 ERBP, endothelin receptor type-B-like protein; R37, G-protein-coupled receptor 37; BRS, bombesin receptor subtype; NMB-R, neuromedin-B receptor; GRP-R, gastrin-releasing peptide receptor. (C) Sequence analysis of *ednrb1* exons reveals a G → A transversion (arrow) in *rose*<sup>j3e1</sup> predicted to generate a premature stop codon in the sixth transmembrane of *Ednrb1*. (D) *ednrb1* lesions are present in multiple *rose* mutant alleles. Shown are *ednrb1* open reading frames in wild-type and each of three *rose* mutant backgrounds. Premature stop codons were identified in *rose*<sup>j3e1</sup> and *rose*<sup>j3e2</sup>, each predicted to result in a null allele. A missense substitution was detected in the phenotypically weaker allele, *rose*<sup>j3e3</sup>. Green, predicted signal or transmembrane domains; dark gray, untranslated regions in wild-type cDNA. No lesions were detected in exons amplified from *rose*<sup>b140</sup>, *rose*<sup>j3e988</sup>, or *rose*<sup>j3e1527</sup> mutant backgrounds; the nature of these mutations remains unresolved.



**FIG. 3.** *ednrb1* is expressed by cells of the neural crest–melanocyte lineage and is temporally and spatially correlated with *endothelin-1* expression in the neural tube. (A) *ednrb1*<sup>+</sup> (blue) cells are abundant over the anterior head, at the midbrain–hindbrain boundary (arrow), within the trunk premigratory neural crest (arrowhead), and in trunk neural crest migratory pathways at 22 h. (B) *ednrb1*<sup>+</sup> cells are more widely scattered over the anterior trunk (arrow) but are still present in the premigratory neural crest in the posterior trunk (arrowhead) at 27 h. (C–E) Two-color *in situ* hybridization reveals *ednrb1* expression by melanocytes and their precursors. (C,C') Corresponding brightfield and epifluorescence views revealing *ednrb1*<sup>+</sup> cells (blue, C) adjacent to the neural tube (nt) at the entrance to dorsolateral (arrow head) and ventrolateral neural crest migratory pathways. Both cells coexpress the melanocyte lineage marker, *kit* (red, C'). Shown is a 12- $\mu$ m cryosection through the posterior trunk of a wild-type embryo. (D,D') Corresponding images of a whole mount 24-h embryo showing cells coexpressing *ednrb1*<sup>+</sup> (blue, D) and *dct* (red, D'; arrowheads) near the midbrain–hindbrain boundary. Adjacent cells express only *ednrb1* (e.g., arrow). (E,E') During later development (here, 28 h), *ednrb1* continues to be expressed (blue, E) by lightly melanized melanocytes (arrowhead) that coexpress *dct* (red, E'). (F–H) *endothelin-1* is expressed in the posterior trunk and tail developing neural tube. (F) *endothelin-1* (*edn1*) expression is evident in the neural keel posteriorly (arrowhead) as well as in lateral mesoderm and in the vicinity of branchial arches anteriorly, at 20 h. (G) *endothelin-1* continues to be expressed in the neural keel of the developing post-anal tail (arrowhead), here shown at 24 h. (H) A longitudinal optical section through the midline posterior trunk of a whole mount 22-h embryo reveals *endothelin-1* expression within the middle of the neural keel (nk), although not in the floorplate (f) or dorsal-most layer of neural keel cells (presumptive neural crest, nc). e, epidermis; nch, notochord. Scale bars: (A,B) 400  $\mu$ m; (C,C') 10  $\mu$ m; (D,D') 10  $\mu$ m; (E,E') 10  $\mu$ m; (F,G) 70  $\mu$ m; (H) 20  $\mu$ m.

*ednrb1* might be responsible for the *rose* phenotype. We cloned a full-length *ednrb1* cDNA, including a 1278-nucleotide open reading frame predicted to encode 426 amino acids. Comparisons of deduced amino acid sequences reveals greater similarity of *D. rerio* *ednrb1* to amniote Ednrb proteins than amniote Ednra proteins (e.g.,

71 and 65% amino acid similarity to human ET-B and ET-A, respectively). Additionally, *D. rerio* *ednrb1* is more similar to quail EDNRB than the recently identified, melanocyte lineage-specific quail EDNRB2 (87 and 68% amino acid similarity, respectively). Figure 2B presents a maximum parsimony phylogenetic reconstruction of *endothelin*

receptor-like genes in vertebrates and reveals a clade of *ednrb* orthologues, *ednra* orthologues, and a clade consisting of quail *EDNRB2* and *Xenopus ET<sub>C</sub>R*. These three distinct clades were evident in maximum parsimony and distance-based UPGMA reconstructions from nucleotide and amino acid sequences, as well as reconstructions based on maximum likelihood analyses of nucleotide sequences (data not shown). Together, these data indicate that *D. rerio ednrb1* is more closely related to amniote *ednrb* genes than either *ednra*, *EDNRB2* (quail), or *ET<sub>C</sub>R* (*Xenopus*), and thus can be considered a *D. rerio* orthologue of *ednrb*.

To test the correspondence of *rose* and *ednrb1*, we amplified *ednrb1* coding sequences from genomic DNA isolated from wild-type and *rose* mutant backgrounds. This analysis revealed *ednrb* lesions in three *rose* alleles (Figs. 2C, 2D). In both *rose*<sup>*3e1*</sup> and *rose*<sup>*3e2*</sup>, premature stop codons are likely to result in null alleles (Gether, 2000). In *rose*<sup>*3e3*</sup>, a nonconservative Ala → Glu substitution is present at a site that is otherwise invariant in all endothelin receptors in Fig. 2B, suggesting that this substitution is likely to be functionally significant. Consistent with these inferences, *rose*<sup>*3e1*</sup> homozygotes are severely runted (suggesting additional, nonpigmentary roles for *ednrb1*) whereas *rose*<sup>*3e3*</sup> homozygotes are vigorous and often exhibit less severe pigment pattern defects compared to either *rose*<sup>*3e1*</sup> or *rose*<sup>*b140*</sup> (Fig. 1). We did not identify a lesion within coding sequences of the spontaneous allele *rose*<sup>*b140*</sup> although homozygous *rose*<sup>*b140*</sup> exhibit greatly reduced levels of *ednrb1* mRNA compared to wild-type (data not shown). Although the nature of this mutation remains unresolved, the pigment pattern defect in these fish is similar to that of *rose*<sup>*3e1*</sup>. Together, these data strongly suggest that *rose* corresponds to a zebrafish orthologue of *ednrb*.

### ***ednrb1* is Expressed by the Neural Crest—Melanocyte Lineage**

Although *ednrb1* mutant *D. rerio* do not exhibit defects in the early larval pigment pattern, *ednrb* genes of murine and avian embryos are expressed by premigratory and early migrating neural crest cells (Nataf *et al.*, 1996; Brand *et al.*, 1998; Southard-Smith *et al.*, 1998; Lecoin *et al.*, 1998). To test whether *D. rerio* neural crest cells similarly express *ednrb1*, we examined the distribution of *ednrb1*+ cells by *in situ* hybridization during embryogenesis. By 18 h, *ednrb1*+ cells are present in the position of premigratory neural crest cells along the trunk dorsal neural tube. Subsequently, *ednrb1*+ cells are present in neural crest migratory pathways during the stages of neural crest cell migration, consistent with a neural crest origin: *ednrb1*+ cells are visible in a ventromedial neural crest migratory pathway (between somites and neural tube or notochord) several hours before they are visible in a dorsolateral neural crest migratory pathway (between somites and epidermis), concordant with the relative time of neural crest cell dispersal into these two pathways (Raible and Eisen, 1994). More anteriorly, *ednrb1*+ cells are abundant during these stages

at sites of neural crest origin both at the midbrain–hindbrain boundary and immediately posterior to the otic vesicles (Schilling and Kimmel, 1994). Relatively few *ednrb1*+ cells are observed over the hindbrain.

Since *Ednrb* promotes the development of melanocytes during embryonic stages in amniotes (Pavan and Tilghman, 1994; Shin *et al.*, 1999), we asked whether cells in the melanocyte lineage express *ednrb1* in *D. rerio*. To test this possibility, we used two-color *in situ* hybridization to stain simultaneously for *ednrb1* and three different markers of the melanocyte lineage: *kit*, which encodes a type III receptor tyrosine kinase required for normal melanocyte migration and survival (MacKenzie *et al.*, 1997; Parichy *et al.*, 1999); *mitf*, which encodes a basic helix–loop–helix–zipper transcription factor essential for melanocyte specification (Opdecamp *et al.*, 1997; Lister *et al.*, 1999); and *dct*, which encodes an enzyme in the melanin synthesis pathway (Steel *et al.*, 1992; Kelsh *et al.*, 2000). These analyses reveal cells that coexpress *ednrb1* and *kit* (Fig. 3C), *ednrb1* and *mitf* (data not shown), and *ednrb1* and *dct* (Fig. 3D) as early as 24 h. During later development through ~48 h, *ednrb1* expression is also detectable in lightly melanized melanocytes that coexpress *dct* (Fig. 3E). Thus, *ednrb1* is expressed by cells in the neural crest–melanocyte lineage in the zebrafish embryo.

### **Coordinate Expression of *ednrb1* and *endothelin-1***

*Ednrb*-type receptors exhibit affinity for endothelin-1, endothelin-2, and endothelin-3 ligands (Sakurai *et al.*, 1990; Takasuka *et al.*, 1994). *In vivo*, avian *endothelin-3* and amniote *endothelin-1* and *endothelin-3* are expressed at sites consistent with roles in promoting melanocyte development via *Ednrb*-mediated signal transduction, and *in vitro*, endothelin-3 and endothelin-1 are equally effective at promoting melanocyte development (Swope *et al.*, 1995; Reid *et al.*, 1996; but see Brand *et al.*, 1998). In *D. rerio*, we identified an EST similar to that of *endothelin-1* genes of amniotes. Phylogenetic analyses using this nucleotide sequence and the exclusively mammalian *endothelin* sequences available in GenBank tend to place the *D. rerio* sequence as the basal-most member in a clade of *endothelin-1* orthologues (60% of 1000 bootstrap replicates using parsimony based reconstruction from 220 informative characters). A UPGMA phenogram similarly clusters the *D. rerio* EST with mammalian *endothelin-1* genes (data not shown). This EST is thus likely to represent a *D. rerio* *endothelin-1* orthologue.

To test a potential role for *D. rerio* *endothelin-1* in signaling to *ednrb1*+ neural crest cells, we examined the expression of *endothelin-1* during embryonic stages. As early as 12 h, we observe *endothelin-1* expression within the mid-trunk neural keel (data not shown), in addition to other sites of expression reported previously (Miller *et al.*, 2000). During subsequent development through at least 32 h, *endothelin-1* expression gradually diminishes anteriorly and increases posteriorly, and at a given axial level is

detectable slightly before *ednrb1* expression in overlying neural crest cells (Figs. 3F–3H). The expression of *D. rerio endothelin-1* thus differs from that in amniotes, in which endothelins presumed to have roles in neural crest development are expressed by epidermis or mesoderm lining neural crest migratory pathways (Brand et al., 1998; Nataf et al., 1998a,b). The spatial and temporal patterns of *ednrb1* and *endothelin-1* expression are consistent with a role for endothelin-1-mediated signaling during neural crest development in *D. rerio* (particularly since endothelin precursors must first be processed proteolytically to generate active endothelin peptides; Baynash et al., 1994). Nevertheless, molecular genotyping of embryos and early larvae segregating an *endothelin-1* mutation did not reveal gross alterations in *ednrb1* expression or numbers of differentiated pigment cells in homozygous *endothelin-1* mutants compared to heterozygous or wild-type individuals (data not shown; also see Miller et al., 2000). Thus, *endothelin-1* may promote—but is not essential for—the development of *ednrb1*+ neural crest cells.

### ***ednrb1* Is Expressed by Xanthophore Precursors and Iridophores**

*Ednrb* signaling in amniotes promotes the development of multiple neural crest-derived cell types (Hosoda et al., 1994; Stone et al., 1997), raising the possibility that *ednrb1* might contribute to the development of other neural crest-derived cell types in *D. rerio* as well. Ectothermic vertebrates have two additional major classes of neural crest-derived pigment cells, yellow xanthophores, and silver iridophores, which derive their coloration from pteridine pigments and purine-containing reflecting platelets, respectively (Bagnara, 1998; Reedy et al., 1998). Thus, we asked whether *ednrb1* is expressed by these nonmelanocyte lineages. To test whether *ednrb1* is expressed by xanthophores or their precursors, we used two-color *in situ* hybridization with probes for *ednrb1* and *fms*, which is expressed by cells in the xanthophore lineage (Parichy et al., 2000). We find numerous cells that coexpress *ednrb1* and *fms* (Fig. 4A). To further test whether these are xanthophore precursors, we examined the distribution of *ednrb1*+ cells in *fms* mutant embryos, in which xanthophore precursors fail to disperse from the neural crest (Parichy et al., 2000). In contrast to wild-type, *fms* mutant embryos exhibit abnormal accumulations of *ednrb1*+ cells at the midbrain–hindbrain boundary, where many xanthophore precursors originate (Fig. 4C). Further posteriorly, between 28 and 48 h of development, *fms* mutant embryos also lack unmelanized *ednrb1*+ cells that are present between melanocyte stripes in wild-type embryos, and *fms* mutants exhibit greater numbers of *ednrb1*+ cells in a nondispersed position along the dorsal myotomes (Fig. 4D). Finally, since *fms* may be expressed by precursors to other pigment cell classes as well, we also tested for coexpression of *ednrb1* and *xdh*, which encodes an enzyme involved in the synthesis of pteridine pigments contained by xanthophores (Reaume et al., 1991; Ziegler et

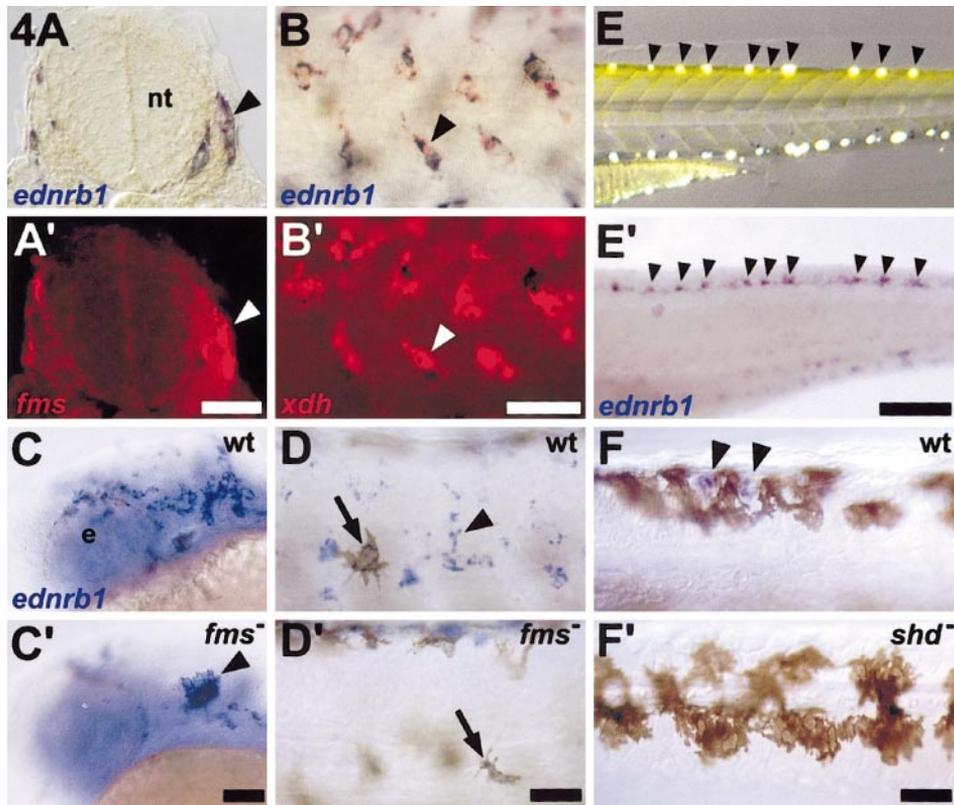
al., 2000). Two-color *in situ* hybridization reveals numerous cells expressing both *ednrb1* and *xdh* (Fig. 4B). Together, these data indicate that *ednrb1* is expressed by xanthophore precursors in the zebrafish embryo.

The iridophore defect in adult *ednrb1* mutant *D. rerio* raised the possibility that *ednrb1* might be expressed by iridophores or their precursors as well. Iridophores lose their reflecting platelets during processing for *in situ* hybridization and molecular markers specific to iridophores have not been described. To test whether iridophores express *ednrb1*, we thus compared the positions of iridophores prior to fixation with the positions of *ednrb1*+ cells in embryos processed individually for *in situ* hybridization ( $N = 15$ ). Figure 4E shows the correspondence of iridophores and *ednrb1*+ cells in a 3-day wild-type larva (a stage when *ednrb1* expression is diminished in melanocytes and xanthophore precursors). Thus, differentiated iridophores express *ednrb1*. As an independent test of this inference, we also compared *ednrb1* expression between wild-type and *shady* mutant embryos and larvae, which lack iridophores but not other pigment cell classes prior to 3 weeks (Kelsh et al., 1996; S.S.L. and R.N.K., unpublished data). In contrast to wild-type, *shady* mutants lack unmelanized cells expressing high levels of *ednrb1* along the dorsal midline and within other larval stripes where iridophores are found at 72 h (Fig. 4F). An absence of unmelanized *ednrb1*+ cells in *shady* embryos also is observed at 48 h, a stage at which most differentiated iridophores have not yet become visible (data not shown). Together, these data indicate that *ednrb1* is expressed by iridophores and their precursors during normal pigment pattern development in *D. rerio*.

Although amniote *Ednrbs* also promote the development of neural crest-derived enteric neurons (Hosoda et al., 1994), we do not observe coexpression of *ednrb1* and the neuronal marker *c-ret* (Bisgrove et al., 1997; Kelsh and Eisen, 1999) at any of 7 time points examined between 19 and 72 h; however, we do not exclude the possibility of coexpression at levels below the threshold of detection (D.M.P. and J.F.R., unpublished data).

### ***ednrb1* Is Expressed during Pigment Pattern Metamorphosis**

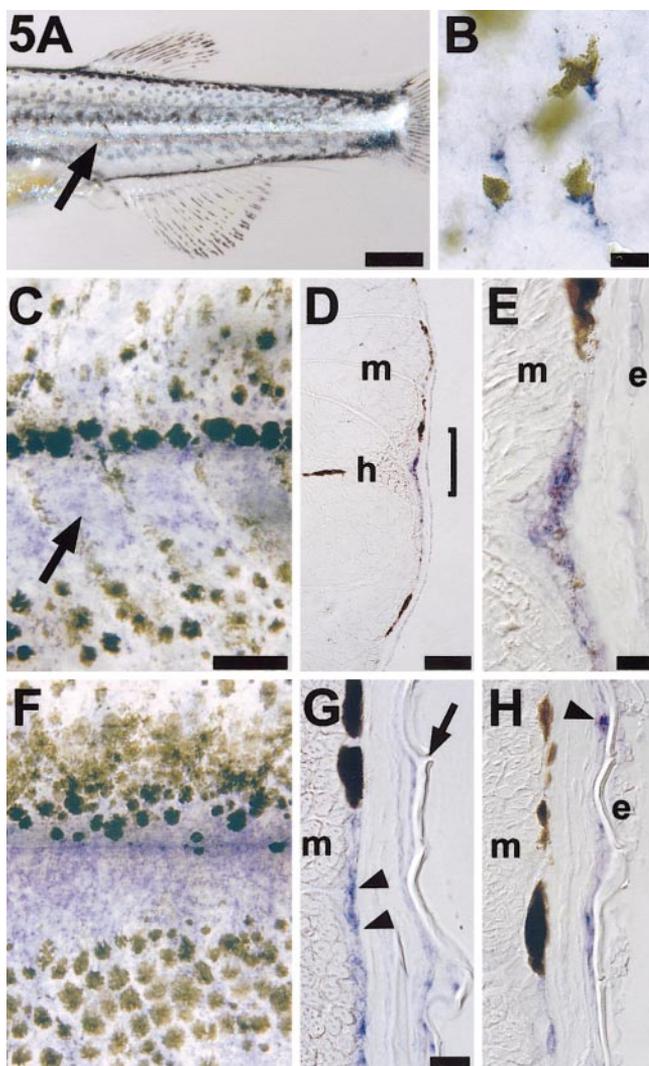
Despite the absence of any gross pigment pattern defects in *ednrb1* mutant embryos, *ednrb1* mutant adults exhibit a severe deficit of iridophores and a severe reduction in melanocyte number compared to wild-type (Fig. 1; Johnson et al., 1995b; Parichy et al., 2000). This could reflect a failure in the development of precursors that normally would arise due to *Ednrb* signaling at embryonic stages. Or, the pigment pattern defects arising during the development of the adult pigment pattern could reflect a later requirement for *ednrb1*. To investigate this possibility, we tested whether *ednrb1* is expressed by cells in melanocyte or iridophore lineages during pigment pattern metamorphosis. *In situ* hybridization during early stages of pigment pattern



**FIG. 4.** *ednrb1* is expressed by nonmelanocytic neural crest-derived pigment cell lineages during embryonic pigment pattern development. (A–D) Molecular marker and mutant analyses reveal *ednrb1* expression by xanthophore precursors. (A,A') Corresponding brightfield and epifluorescence views revealing *ednrb1*+ cells (blue, A) that coexpress *fms* (red, A'; e.g., arrowhead). Shown is a 12- $\mu$ m cryosection through the anterior trunk of a 24-h embryo. (B,B') *ednrb1*+ cells (blue, B) also coexpress the xanthophore marker *xdh* (red, B'; e.g., arrowhead). Shown are cells in the dorsolateral neural crest migratory pathway in the middle trunk region of a 24-h embryo. (C,C') Comparison of *ednrb1* expression in wild-type (C) and *fms* mutant (C') embryos reveals an abnormal accumulation of *ednrb1*+ cells at the midbrain–hindbrain junction (arrowhead). Shown are the heads of 26-h embryos. e, eye. (D,D') In the midtrunk at 32 h, unmelanized *ednrb1*+ cells are present over the flank, between developing melanocyte stripes in wild-type embryos (D; e.g., arrowhead), but unmelanized cells are not present in this region in *fms* mutants. In contrast, *ednrb1*+ melanocytes are present in both wild-type and *fms* mutants (arrows). (E, F) *ednrb1* is expressed by differentiated iridophores. (E) Individual iridophores (arrowheads) can be observed along the dorsal myotomes as highly reflective cells under oblique illumination in the posterior trunk of a living 72-h larva that has been treated with phenylthiourea to inhibit melanin synthesis. Yellow coloration in the dorsal trunk is due to xanthophore pigmentation. (E') In the same individual processed for *in situ* hybridization, *ednrb1*+ cells (arrowheads) are present along the dorsal myotome in a pattern coincident with iridophores observed prior to histological preparation. Additional iridophores in ventral regions of the larva stain as well, but are out of the focal plane in (E'). In comparison with *ednrb1* expression by iridophores, *ednrb1* expression by xanthophore and melanocyte lineages is dramatically reduced at these stages and cannot be seen here. (F,F') Comparison of *ednrb1* expression between wild-type (F) and *shady* mutant (F') embryos also supports the inference that *ednrb1* is expressed by iridophores. (F) At 72 h in wild-type, unmelanized cells along the dorsal myotomes exhibit robust *ednrb1* expression (arrowheads). (F') In *shady* mutants, which lack iridophores, *ednrb1* expression by unmelanized cells in the corresponding region is not observed. Scale bars: (A,A') 60  $\mu$ m; (B,B') 40  $\mu$ m; (C,C') 40  $\mu$ m; (D,D') 100  $\mu$ m; (E,E') 60  $\mu$ m; (F,F') 50  $\mu$ m.

metamorphosis (14–21 days) reveals a horizontal stripe of *ednrb1*+ cells coincident with the position of the first adult iridophore stripe, suggesting that these cells are iridophores or their precursors (Figs. 5A, 5C–5E). Additionally, scattered *ednrb1*+ cells are present elsewhere over the flank and some of these are lightly melanized, indicating that *ednrb1* also is expressed by the melanocyte lineage during metamorphosis (Fig. 5B). During later stages (22–30 days), *ednrb1* continues to be expressed by densely packed unmelanized

cells between melanocyte stripes, as well as less densely packed cells that are widely dispersed in the skin (Fig. 5F). Analysis of *ednrb1* expression in cryosections during these stages indicates that dense staining for *ednrb1* occurs adjacent to melanocyte stripes, and these cells occupy a similar plane to stripe melanocytes, immediately superficial to the myotomes (Fig. 5G). In contrast, dispersed *ednrb1*+ cells occur more superficially in the dermis, and are frequently associated with the inner surface of dermal



**FIG. 5.** *ednrb1* is reexpressed during pigment pattern metamorphosis. (A) A wild-type metamorphosing larva exhibits a bright stripe of iridophores (arrow) immediately ventral to the horizontal myoseptum and between the first developing dorsal and ventral melanocyte stripes. Shown is the posterior trunk of an 18-day larva. (B) Lightly melanized melanocytes express *ednrb1* during pigment pattern metamorphosis. Shown are *ednrb1*<sup>+</sup> (blue) melanocytes in flat-mounted skin from a 20 day larva. An additional dark melanocyte can be seen deeper within the larva and out of the plane of focus. (C) *ednrb1* is strongly expressed by cells coincident with the developing first iridophore stripe (arrow) immediately ventral to the horizontal myoseptum. Shown is a 21-day larva. (D) A 12- $\mu$ m cryosection through the trunk of a larva at the stage in (A). Blue *ednrb1*<sup>+</sup> cells can be seen superficial to the myotome (m) and beneath the epidermis near the horizontal myoseptum (h). (E) Higher magnification view of the bracketed region in (D), showing *ednrb1*<sup>+</sup> cells between the myotome (m) and epidermis (e). Spatial resolution among these cells does not allow distinguishing whether faint melanin deposition is associated with *ednrb1*<sup>+</sup> cells or intermingled cells that may not express *ednrb1*. (F) During later stages of pigment pattern metamorphosis (here, 28 days), *ednrb1* expression is maintained between melanocyte stripes and also is

scales (Fig. 5H). Many of these unmelanized *ednrb1*<sup>+</sup> cells are likely to be iridophores that contribute to the zebrafish adult pigment pattern or melanocyte precursors. Together, these data indicate that *ednrb1* is expressed both during embryogenesis and during pigment pattern metamorphosis by cells of the melanocyte and iridophore lineages.

## DISCUSSION

The results of this study identify roles for an *endothelin receptor B* orthologue during pigment pattern development in *D. rerio*. These findings have implications for our understanding of roles for endothelin receptors during pigment pattern development in amniotes and teleosts, and suggest two alternative models for the molecular and cellular bases of pigment pattern metamorphosis in *D. rerio*.

### *ednrb1* Is Essential for Pigment Pattern Development in *D. rerio*

Studies of amniotes have implicated *ednrb*-like genes in promoting the development of cells in the neural crest-melanocyte lineage (e.g., Shin *et al.*, 1999). Our finding of three independent *ednrb1* lesions in three different *rose* alleles strongly suggests a correspondence of *rose* and *ednrb1* in *D. rerio*, and, in turn, reveals a phylogenetic conservation of endothelin receptor-mediated signaling across amniote and teleost lineages. These findings thus extend previous studies demonstrating conserved genetic pathways during the development of neural crest and pigment cells across even widely divergent groups of vertebrates (e.g., Lahav *et al.*, 1994; Liem *et al.*, 1995; Wehrle-Haller and Weston, 1995; Opdecamp *et al.*, 1997; Lister *et al.*, 1999; Parichy *et al.*, 1999; Nguyen *et al.*, 2000). These observations lend further credence to the notion that studies of the genetically tractable organism *D. rerio* may provide insights relevant not only to normal amniote development but also human disease syndromes (Kelsh and Eisen, 1999; Dooley and Zon, 2000). Moreover, as an additional genome duplication in teleosts appears to have furnished paralogous genes with, in some instances, the partitioning of gene activities (Force *et al.*, 1999; de Martino *et al.*, 2000), *D. rerio* mutants also may exhibit fewer pleiotropic effects than mutants of orthologous amniote loci, facilitating studies of defects associated with particular cell types or organ systems. For example, *D. rerio kit* mutants

evident at lower levels within melanocyte stripes. (G) Cryosection of a similarly staged larva reveals *ednrb1*<sup>+</sup> cells (arrowheads) in the same plane as melanocytes that contribute to dark horizontal stripes. Arrow, dermal scale. (H) Unmelanized *ednrb1*<sup>+</sup> cells (arrowhead) also are evident superficial to stripe melanocytes, associated with the inner surface of scales. Scale bars: (A) 400  $\mu$ m; (B) 10  $\mu$ m; (C,F) 100  $\mu$ m; (D) 100  $\mu$ m; (E) 10  $\mu$ m; (G,H) 20  $\mu$ m.

have defects in melanocyte morphogenesis, but lack the pleiotropic effects on hematopoiesis and gametogenesis that complicate studies of Kit signaling during development of the amniote neural crest–melanocyte lineage (Parichy *et al.*, 1999). Similarly, *Ednrb* mutations in mouse and human are associated with aganglionic megacolon as well as piebaldism, and null alleles are lethal (Puffenberger *et al.*, 1994; Jackson, 1997; Spritz, 1998). Studies of roles in pigment cell morphogenesis and differentiation during later stages of development are thus complicated by neonatal lethality due to defects in the enteric nervous system. Our results show that in *D. rerio*, however, even *ednrb1* presumptive null mutants (such as *ednrb<sup>3se1</sup>*) are viable and survive through adult stages (perhaps reflecting the existence of additional *ednrb* homologues; see below), and this will permit a fuller assessment of roles for *Ednrb*-mediated signaling in the development of pigment cell lineages.

Our identification of roles for *ednrb1* in pigment pattern development in *D. rerio* suggests a conservation of molecular mechanisms for pigment cell development across vertebrates. Yet, our analyses also reveal interspecific variation in the timing of endothelin receptor expression. Several endothelin receptor-encoding loci have been identified in other taxa, including *ednra* and *ednrb* orthologues of mammals and birds, and more recently, a second *ednrb*-like gene, *EDNRB2*, of quail, and *ET<sub>C</sub>R*, of *Xenopus laevis*, encoding an endothelin-3-specific receptor. Phylogenetic analyses of endothelin receptor genes revealed a clade consisting of *ednrb* orthologues, including *D. rerio ednrb1*, and a separate clade comprising quail *EDNRB2* and *Xenopus ET<sub>C</sub>R*. In *D. rerio*, we observe *ednrb1* expression by early migrating neural crest cells as well as melanoblasts and early differentiated melanocytes in both dorsolateral and ventromedial migratory pathways. In contrast, amniote *ednrb* orthologues are expressed by early migrating neural crest cells and do not appear to be expressed by melanocyte precursors that have entered the dorsolateral neural crest migratory pathway (although *dct+* melanoblasts express *Ednrb* *in vitro*; Nataf *et al.*, 1996; Southard-Smith *et al.*, 1998; Opdecamp *et al.*, 1998; Shin *et al.*, 1999), whereas avian *EDNRB2* is expressed by melanoblasts migrating in the dorsolateral neural crest migratory pathway as well as differentiated melanocytes, and *ET<sub>C</sub>R* is expressed by differentiated *X. laevis* melanophores (Karne *et al.*, 1993; Lecoin *et al.*, 1998). Thus, *ednrb1* expression during early development in *D. rerio* has characteristics of both *ednrb*- and *EDNRB2*-class genes in amniotes.

### ***ednrb1* Mutant Phenotype Suggests Alternative Models for Pigment Pattern Development**

Our finding that *rose* corresponds to *ednrb1* and our analyses of *ednrb1* expression relative to other loci suggest potential roles for this gene in the development of neural crest-derived cell lineages, and also provide insights into the mechanisms of pigment pattern metamorphosis. The appearance of a mutant phenotype only during the devel-

opment of the adult pigment pattern raised the possibility that the *rose* locus might be expressed only during metamorphosis. The embryonic and early larval expression of *ednrb1* by cells of melanocyte, iridophore, and xanthophore lineages is thus somewhat unexpected. Since *ednrb1* mutant early larvae do not exhibit gross pigment pattern defects, these observations point to a possible nonessential role in the development of early larval pigment cells. For example, genetic redundancy could allow a defect in *ednrb1* signaling to be compensated for by the activity of a paralogous locus (see below). Or, morphogenetic redundancy could allow compensation via other genetic pathways; e.g., a loss of *ednrb1* signaling that normally stimulates proliferation might be overcome by enhanced kit signaling that also stimulates proliferation (Reid *et al.*, 1996; Kelsh *et al.*, 2000; Rhim *et al.*, 2000). Alternatively, *ednrb1* mRNA expression in these cells during embryogenesis may be gratuitous (Cavener, 1989) and may not reflect the potential for *ednrb1*-mediated signaling (e.g., if post-transcriptional processing does not occur, or if *ednrb1* is required by neural crest cells only prior to the expression of lineage-specific molecular markers). In either event, these findings contrast with studies of murine embryos, in which *Ednrb* is required by virtually all melanocyte precursors such that a failure of *Ednrb* signaling results in a near complete absence of melanocytes and melanoblasts (Hosoda *et al.*, 1994). Moreover, the requirement for murine *Ednrb* strictly during neural crest migration (Shin *et al.*, 1999) and the immediate manifestation of defects in *Ednrb* mutants (Pavan and Tilghman, 1994) differ from *D. rerio*, in which the *ednrb1* mutant phenotype arises only late in development (although our study cannot address the temporal requirement for *ednrb1* activity in *D. rerio*).

In contrast, the melanocyte and iridophore defects in adult *ednrb1* mutants reveals an essential role for this gene in adult pigment pattern development. This finding prompts two related questions: (i) how does *ednrb1* promote the development of these pigment cells? and (ii) how are these effects partitioned across life cycle stages to influence the adult phenotype, but not the early larval phenotype? Below, we suggest two models for the genetic and cellular bases of *ednrb1* activity during pigment pattern development in *D. rerio*. Although we focus on melanocytes for simplicity, neither model specifies the differentiative potential of individual *ednrb1+* cells. These cells could be competent to differentiate as both melanocytes and iridophores, or cells could be committed to only one or the other cell lineage.

In the first model, *ednrb1* is essential for promoting the development of only one of two or three distinct populations of pigment cell precursors. In this model, *ednrb1* is not required by precursors that generate the early larval pigment pattern, or by precursors to EM melanocytes (14–21 days), but is required by precursors to LM melanocytes (21–28 days). One way in which such genetically distinct populations of precursors might arise is through partially redundant gene activity. Specifically, if an *ednrb1*

paralogue is present in *D. rerio* and expressed by early larval and EM melanocyte precursors, such a locus could allow the development of these cells even when *ednrb1* activity is abolished. In turn, a failure of LM melanocyte precursors to express such a paralogue could explain the *ednrb1*-dependence of this population. Two lines of evidence are consistent with this model. First, the identification of “*EDNRB2*-class” genes in other vertebrates (Fig. 2B) and an apparent additional round of genome duplication in teleosts (Amores et al., 1998) each raise the possibility that other *Ednrb*-like genes may be present in *D. rerio*. Second, our analysis of the ancestrally paralogous genes *kit* and *fms* (Rousset et al., 1995) indicates distinct roles for these loci in promoting early larval and EM melanocytes (*kit*), or LM melanocytes (*fms*; Parichy et al., 2000).

In a second model, *ednrb1* is essential for the development of a single population of pigment cell precursor that is recruited to differentiate at different times during the life cycle. In this model, *ednrb1* contributes to expanding a single precursor population such that, during normal development, these cells are sufficiently abundant to supply differentiated pigment cells both during early larval pigment pattern formation and during the entire period of pigment pattern metamorphosis. Thus, a single *ednrb1*-dependent population contributes to early larval melanocytes, EM melanocytes, and LM melanocytes. This model postulates that in *ednrb1* mutants, which develop early larval and EM melanocytes but not LM melanocytes, only enough precursors are generated to supply melanocytes during embryonic development and the first phase of pigment pattern metamorphosis; the supply is exhausted by the second phase of pigment pattern metamorphosis. Should an *ednrb1* paralogue exist, its activity presumably would be insufficient to fully expand this single population of precursors when *ednrb1*-mediated signaling is lost. Isolation of new molecular markers for pigment cell precursors as well as screening for additional *ednrb* orthologues and analyses of temporal requirements for their activities will help to distinguish between these models.

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