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nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate

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SUMMARY

We report the isolation and identification of a new mutation affecting pigment cell fate in the zebrafish neural crest. Homozygous *nacre* (*nac^{w2}*) mutants lack melanophores throughout development but have increased numbers of iridophores. The non-crest-derived retinal pigment epithelium is normal, suggesting that the mutation does not affect pigment synthesis per se. Expression of early melanoblast markers is absent in *nacre* mutants and transplant experiments suggested a cell-autonomous function in melanophores. We show that *nac^{w2}* is a mutation in a zebrafish gene encoding a basic helix-loop-helix/leucine zipper transcription factor related to *microphthalmia* (*Mitf*), a gene known to be required for development of eye and crest pigment cells in the mouse. Transient expression of the wild-type *nacre* gene restored melanophore development in *nacre*$^{-/-}$ embryos. Furthermore, misexpression of *nacre* induced the formation of ectopic melanized cells and caused defects in eye development in wild-type and mutant embryos. These results demonstrate that melanophore development in fish and mammals shares a dependence on the *nacre/Mitf* transcription factor, but that proper development of the retinal pigment epithelium in the fish is not *nacre*-dependent, suggesting an evolutionary divergence in the function of this gene.

Key words: Zebrafish, *Danio rerio*, Mitf, *microphthalmia*, Melanocyte, Neural crest, Pigmentation, *nacre*

INTRODUCTION

The neural crest is a population of cells that segregate from the neural tube and migrate throughout the periphery of the vertebrate embryo to differentiate as a variety of cell types, including neurons and glia of the peripheral and enteric nervous systems, head and neck cartilage, and pigment cells (Le Douarin, 1982). While it is widely accepted that cells in the premigratory crest are initially multipotent and become more fate-restricted with time (Henion and Weston, 1997; Le Douarin et al., 1994), the mechanisms by which these diverse cell types are specified are not well understood. One approach to this question is the analysis of genetic systems in which mutations affecting particular neural crest derivatives may be identified. For example, a subset of the 91 mutations described to date that alter mouse coat color (Mouse Genome Database, May 1999) are known to affect the specification, survival or differentiation of melanocytes. Cloning of such loci has identified complementary growth factor/receptor combinations (Baynash et al., 1994; Fleischman, 1993; Hosoda et al., 1994) as well as transcription factors (Epstein et al., 1991; Southard-Smith et al., 1998) that are integral to these processes.

Mutations in the mouse *microphthalmia* locus encoding the basic helix-loop-helix/leucine zipper protein Mitf (Microphthalmia-associated transcription factor) show allele-specific combinations of defects in coat color, eye development, osteoclasts and mast cells (Hodgkinson et al., 1993; Moore, 1995). *Mitf* has been shown to be necessary for specification and/or survival of melanocytes (Opdecamp et al., 1997). Moreover, misexpression of *Mitf* in cultured mouse fibroblasts results in their adoption of melanocyte-specific traits (Tachibana et al., 1996), suggesting that *Mitf* may be sufficient to directly promote pigment cell fate.

In recent years, the zebrafish *Danio rerio* has emerged as an attractive embryological and genetic model for numerous aspects of vertebrate development, including formation of the neural crest and its derivatives (Raible and Eisen, 1994; Raible et al., 1992; Schilling and Kimmel, 1994). In contrast to the single pigment cell type of mammals, zebrafish neural crest gives rise to three distinct types of chromatophores. Melanophores first appear at approximately 24 hours postfertilization (hpf) in the dorsolateral trunk and head (Kimmel et al., 1995). These cells synthesize melanin even as they are migrating. Xanthophores containing pteridine pigments are first evident at approximately 42 hpf as pale yellow coloration on the dorsal aspect of the head. Around this same time iridophores, which contain reflecting platelets composed of purines, are first observable around the choroid

of the eye and later along the dorsal midline of the tail. Proliferation of each cell type continues and, by 6 days postfertilization, the embryonic pigment pattern is established: four horizontal melanophore stripes, three with associated iridophores and xanthophores covering the dorsal third of the embryo along its length.

Loci involved in pigmentation were among the first mutations identified in zebrafish (Chakrabarti et al., 1983; Streisinger et al., 1981, 1986) and constitute the largest single group of mutations isolated in the large-scale Tübingen screen (Kelsh et al., 1996). Kelsh and co-workers divided these 285 mutations comprising 94 loci into seven phenotypic classes based upon the processes of pigment cell development that appeared to be affected. Among the class with missing or reduced numbers of pigment cells, and therefore of potential relevance to neural crest cell fate specification, were mutants that lacked all three pigment cell types (*colourless*), or nearly all iridophores (*shady*) or xanthophores (*salz*, *pfeffer*). However, while two mutations caused a reduction in melanophore number (*sparse* and *sparse-like*), no mutations were identified that were completely missing melanophores alone.

In an effort to understand how cell fates are specified in the neural crest of the zebrafish, we have undertaken a screen to identify mutations that affect neural crest derivatives. In this paper, we describe the isolation and identification of a new recessive mutation affecting pigment cell fate. Fish homozygous for the *nacre* (*nac^{w2}*) mutation are missing melanophores throughout development and do not express early melanoblast markers, but undergo normal development of the pigmented epithelium of the retina, indicating that the defect is specific to the neural crest and not in pigment synthesis per se. Homozygous *nacre* larvae have more iridophores than wild type, suggesting that the specification or differentiation of these two pigment cell types may be coordinately regulated. We show here that the *nac^{w2}* allele is the result of a single base mutation in a zebrafish gene with homology to *Mitf*. The mutation is predicted to encode a truncated protein lacking the basic helix-loop-helix/leucine zipper motif. Misexpression of wild-type *nacre* not only restores melanophore development in homozygous mutant fish but induces the formation of ectopic pigmented cells in wild-type embryos. These results thus highlight the central importance of this transcription factor in the vertebrate melanophore lineage but also suggest divergence in its function during the evolution of fish and mammals.

MATERIALS AND METHODS

Fish culture and maintenance

Adult fish were maintained at 28.5°C on a 14 hour/10 hour light/dark cycle. For mutagenesis, adult AB males were treated with *N*-ethyl *N*-nitrosourea according to published methods (Solnica-Krezel et al., 1994) and outcrossed to wild-type females. These F_1 progeny were intercrossed and the *nacre* mutation was identified by intercrossing adults from one of the resulting F_2 families. For mapping, a homozygous *nac^{w2}* female was mated to a male of the WIK strain. Embryos were staged according to Kimmel et al. (1995).

Mosaic analysis

Wild-type embryos were labeled at the 1- to 2-cell stage by injection with lysinated rhodamine dextran and allowed to develop to late blastula stage. Approximately 50-100 cells were then transplanted to unlabeled host embryos (Ho and Kane, 1990) obtained from matings of *nac^{w2}/nac^{w2}* adults. Chimeric embryos were examined the next day for the presence of melanophores; positive embryos were viewed under fluorescence optics to assess the origin of the melanized cells.

Whole-mount in situ hybridization

Embryos were processed for whole-mount in situ hybridization as previously described (Thisse et al., 1993). Digoxigenin- and fluorescein-labeled riboprobes for *trp2* (R. N. Kelsh, personal communication), c-*kit* (Parichy et al., 1999), *fkd6* (Odenthal and Nusslein-Volhard, 1998) and the *Mitf*-related gene *3A.1* (see below) were synthesized with T7 RNA polymerase from templates linearized with *Eco*RI (*trp2* and *3A.1*), *Xba*I (c-*kit*), or *Bam*HI (*fkd6*). Coloration reactions with NBT/BCIP, INT/BCIP or Fast Red substrates were used to visualize hybridized probes.

Isolation of a zebrafish *Mitf*-related gene

Degenerate oligonucleotide primers were designed against conserved peptides PNSPMA and PDMRWNK of mouse and human Mitf and used in RT-PCR with first-strand cDNA prepared from 24 hour zebrafish embryos. The sequence of the primers was as follows: sense, 5′-GCNCCNAA(C/T)AG(C/T)CCNATGGC-3′; antisense, 5′-TT(A/G)TTCCANC(G/T)CAT(A/G)TCNGG-3′. PCR conditions were: 1 cycle 94°C 3 minutes, 56°C 30 seconds, 72°C 1 minute; 39 cycles 94°C 30 seconds, 56°C 30 seconds, 72°C 1 minute; 1 cycle 72°C 5 minutes. PCR products were purified by agarose gel electrophoresis and subcloned using the TA cloning system (Invitrogen), then sequenced. Clones of interest were used to screen approximately 10^7 p.f.u. of a 15-19 hpf zebrafish embryo λZAP cDNA library (Appel and Eisen, 1998). Inserts were recovered from purified plaques by phagemid rescue and then sequenced. Sequence data has been submitted to GenBank (accession number AF119220).

Mapping

A polymorphism was identified between the strains AB and the AB derivative C32 (Johnson et al., 1995a), which contain an *Afl*III site in the 3′ UTR of the *Mitf*-related gene *3A.1*, and the strains SJD (Johnson et al., 1995a) and WIK, which do not. Genotyping was performed by amplification with the following primers: forward 5′-GCCAACTAAATTTCATGAACC-3′; reverse 5′-AAATCAACTAAT-TGTTTACACG-3′, followed by digestion with *Afl*III and agarose gel electrophoresis. AB and C32 give a 192 bp product, SJD and WIK a 214 bp product. To assign *3A.1* to a linkage group, segregation of this marker was examined using a C32×SJD haploid mapping panel (Johnson et al., 1996). Linkage of this marker to *nacre* was assessed by genotyping individual diploid embryos from a cross of heterozygous *nac^{w2}*/WIK adults.

Plasmid construction and embryo injections

A *Xho*I-*Xho*I fragment of the *3A.1* cDNA was inserted into the pCS2-MT vector to create pCS2-MT3A.1, encoding 6 myc epitope tags followed by amino acids 5-412 of the zebrafish Mitf-related protein. Site-directed mutagenesis was performed on pCS2-MT3A.1 using a uracil-containing template (Kunkel et al., 1991) and the following oligonucleotide: 5′-ATATCAATGACAGATTTAAGGAGCTGGG-3′ (mismatch underlined) to make pCS2-MT3A.1-I219F. MT3A.1 and MT3A.1 mutant coding sequences were subcloned from pCS2 into the *Cla*I and *Apa*I sites of the zebrafish heat-shock promoter vector pzHSP70/4prom (Shoji et al., 1998) to create pHS-MT3A.1 and pHS-MT3A.1-I219F.

Embryos for injection were obtained from natural matings. Approximately 1 nl supercoiled plasmid DNA diluted to a concentration of 25 ng/μl in H_2O was introduced into 1- to 4-cell embryos using a gas-driven microinjection apparatus. Phenol red at 0.1% was included in some preparations. Dead and dying embryos were removed between

3 and 6 hours. Heat shock was administered by placing embryos in an incubator at 37°C for the indicated times. Embryos were inspected between 24 and 48 hours and scored for the presence of ectopic pigmented cells and visible defects. Similarly, capped RNAs were synthesized using the Maxiscript kit (Ambion) from pCS2-based templates linearized with *Not*I, and diluted to 30 ng/μl for injection. GFP RNA was co-injected at 30 ng/μl. Injected embryos were screened at 8 hours for GFP expression and examined as above after 24 hours.

Photography

Adults were anesthetized with 0.003% MS222 (Sigma) and photographed on a dissecting microscope. Larvae were similarly anesthetized and mounted in agar. Fixed samples were mounted in 50-75% glycerol between bridged coverslips and photographed using a Zeiss Axioskop microscope. For histology, fixed embryos were embedded in Epon and sectioned at 2-3 μm with a Sorvall JB-4A microtome, then stained with methylene blue/azure II (Malicki et al., 1996). Images were scanned and adjusted in Adobe Photoshop for brightness/contrast and color balancing.

RESULTS

nacre mutants lack melanophores

A novel recessive mutation was identified in an ENU screen for genes affecting development of zebrafish neural crest derivatives, including pigment cells. Homozygotes for this mutation, which we have named *nacre* (*nac*^{w2}) (Fr., *mother of pearl*) lack melanophores throughout embryonic and larval development (Fig. 1A,B). Pigmentation of the retinal epithelium occurs in a manner indistinguishable from wild type, indicating that the melanin synthesis pathway is intact and overall eye morphology is unaffected. The absence of melanophores persists through adulthood (Fig. 2A), although a small percentage of adult fish display unpatterned patches or streaks of melanophores in one or more fins (Fig. 2B). Iridophores cover much of the ventral torso of the adult and a faint stripe of these cells is discernible dorsally (Fig. 2A). Xanthophore pigmentation in the adults is variable.

In contrast to the absence of melanophores, *nacre* embryos have an approximate 40% increase in the number of iridophores at day 3 (Fig. 1C). Xanthophore pigmentation is reduced slightly (Fig. 1D). Other neural crest derivatives, including the neurons of the dorsal root ganglia and enteric nervous system, are grossly normal in number, position and pattern in *nacre* mutants (data not shown). Likewise, craniofacial development is normal and *nac*^{w2} homozygotes have no noticeable reduction in fertility or lifespan compared to wild type. The *nac*^{w2} mutation therefore appears to specifically affect the development of pigment cells derived from the neural crest.

Transplants suggest that *nacre* behaves cell-autonomously

The melanophore phenotype in *nacre* could be the result of the absence of an intrinsic factor required for expression of melanophore fate or the lack of a growth or survival factor for melanoblasts. Mosaic analysis (Ho and Kane, 1990) was employed to distinguish between these two possibilities. Wild-type embryos were injected at the 1-cell stage with lineage tracer, allowed to develop to high blastula stage (approx. 2000 cells) and then 50-100 cells were transplanted to unlabeled isochronic *nacre* hosts. Melanized cells with characteristic pigment cell morphology were identifiable in these chimeras the next day, and such cells always contained the lineage tracer indicating their wild-type source (Fig. 3). Therefore, at least with regard to melanophores, *nacre* acts in a cell-autonomous fashion. Because of the large and variable number of cells transplanted, it was not possible to determine the cell autonomy of the iridophore phenotype in *nacre* mutant embryos using this approach.

Expression of melanoblast markers is absent in *nacre*

To determine the stage at which melanophore development is perturbed in *nacre* mutants, we examined the expression of melanoblast markers by whole mount in situ hybridization. In wild-type embryos, expression of the gene encoding tyrosinase-related protein-2 (Trp2) is detectable at least 4 hours

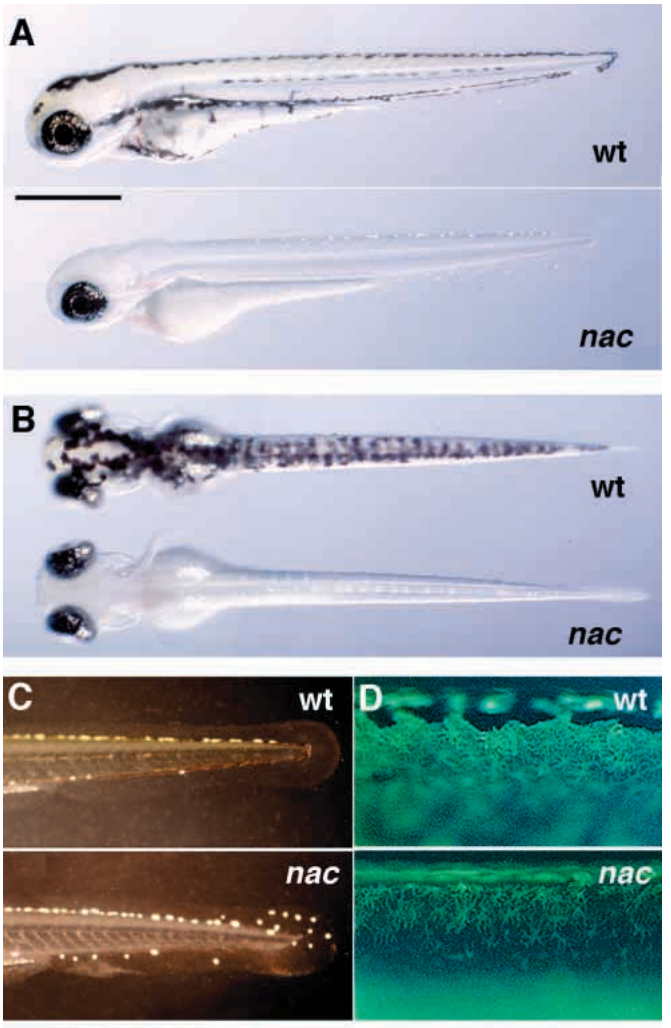

Fig. 1. The *nacre* mutant. Lateral (A) and dorsal (B) views of wild-type (top) and *nacre* (bottom) larvae at 3 days postfertilization. *nacre* homozygotes are missing all neural-crest-derived melanophores, but pigmentation of the eye is normal. (C) Tail iridophores, viewed with epi-illumination. *nacre* mutants (bottom) have increased numbers of iridophores, including many in the tail fin. (D) Xanthophore pigmentation, viewed under UV light, is slightly reduced in *nacre* mutants (bottom). All visible fluorescence is due to xanthophores, while the dark patches in the top panel are melanophores. Scale bar is approximately 500 μm in A-C, 100 μm in D.

before pigmentation is evident (R. N. Kelsh, personal communication, and data not shown). At 23 hpf, expression of *trp2* is almost entirely absent from the neural crest of *nacre* embryos but robust in the RPE (Fig. 4A). Similarly, expression of a zebrafish homolog of c-*kit* (Parichy et al., 1999), a receptor tyrosine kinase required for the survival and proliferation of mouse melanoblasts, is specifically lost from the neural crest (Fig. 4B), although a few faintly expressing cells are present. The absence of these markers suggests that *nacre* gene function is required at an early step in melanophore development in the zebrafish.

Isolation of a zebrafish gene related to *microphthalmia*

The melanophore phenotype of *nacre* is similar in some respects to that of mutations in the mouse *microphthalmia* (*Mitf*) locus, encoding the microphthalmia-associated transcription factor (Moore, 1995). Although most *Mitf* alleles also have effects on the retinal pigment epithelium, the genetics of the locus are rather complex, and at least one murine allele with a 'black-eyed white' phenotype has been described (Kreitner, 1957), where eye development is nearly normal but coat pigmentation is greatly reduced. Moreover, *Mitf* is the earliest melanoblast marker yet to be identified and has been implicated in the transcriptional regulation of *tyrosinase* and related genes (Yasumoto et al., 1997), as well as c-*kit* (Tsujimura et al., 1996). We therefore examined if a similar gene or genes were present in zebrafish by a degenerate PCR approach. Primers were designed against the conserved peptide PNSPMA in the amino terminus and the peptide PDMRWNK

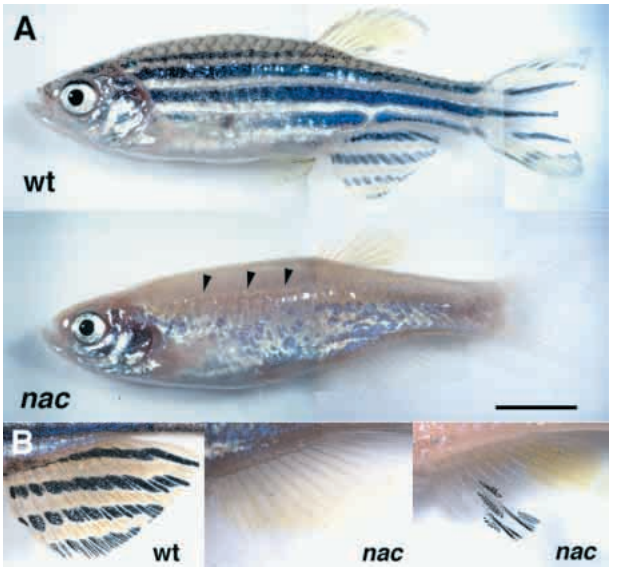

Fig. 2. Pigmentation in wild-type and *nacre* adults. (A) The melanophore defect persists throughout development. *nacre* adults display widespread iridophore pigmentation, primarily on the ventral torso, and variable xanthophore pigmentation, but no melanophore stripes. Arrowheads indicate a faint iridophore stripe.
(B) Melanophores are occasionally observed in adult fins. Shown here, anal fins of typical wild-type (left) and *nacre* (center) adults, and from a *nacre* animal with a patch of melanophores (right). Scale bar is approximately 500 mm in A, 250 mm in B.

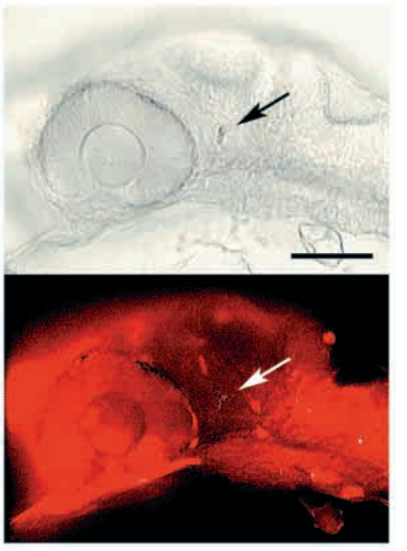

Fig. 3. Transplants suggest that *nacre* functions cell-autonomously. Cells from a wild-type embryo labeled with a lineage tracer were transplanted to a *nacre* host. The arrows indicate a pigmented cell with melanophore morphology (top panel, Nomarski optics) which contains the rhodamine-dextran lineage tracer (bottom panel, fluorescence) demonstrating its donor embryo origin. Scale bar, 100 μm.

in helix 2 of the HLH dimerization domain of the mouse and human Mitf proteins. These primers were used to amplify from 24 hpf cDNA and the products were subcloned and sequenced. One fragment bearing similarity to other vertebrate *Mitf*s was used to screen a 15-19 hpf cDNA library (Appel and Eisen, 1998).

The longest clone identified, designated clone 3A.1, was approximately 1.6 kilobases and contained 80 nucleotides upstream of a long open reading frame encoding a protein with a predicted length of 412 amino acids. (We refer to this gene hereafter simply as *3A.1*.) Comparison of this deduced amino acid sequence to Mitfs of other vertebrates (Fig. 5) revealed a

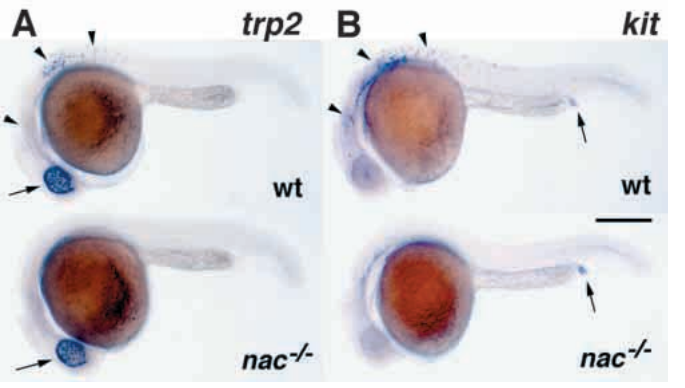

Fig. 4. Expression of melanoblast markers is absent from *nacre*$^{-/-}$ neural crest. Whole-mount in situ hybridization was performed on 23 hpf embryos with probes for *trp2*(A) and c-*kit* (B). Expression of *trp2* is strong in the eye of *nacre* mutants (arrow) but absent from the neural crest (arrowheads). Likewise, *nacre*$^{-/-}$ embryos show normal expression of the receptor tyrosine kinase c-*kit* in the intermediate cell mass (arrow) and elsewhere but little or no expression in presumptive melanoblasts (arrowheads). Scale bar, 250 μm.

high degree of conservation in the basic region and helix-loop-helix/leucine zipper motifs, required for DNA binding and dimerization, respectively, as well as significant homology throughout the rest of the protein, including a consensus site for phosphorylation by MAP kinase (Hemesath et al., 1998) and nearby transcriptional activation domain (Sato et al., 1997). The 5′ end of *3A.1* most closely matches the melanocyte-specific splice forms of mouse and human *Mitf* (Hodgkinson et al., 1993; Tachibana et al., 1994). Sequence corresponding to exon 3 of the human mRNA (Tassabehji et al., 1994) is absent from all the zebrafish cDNA clones. Interestingly, this sequence is also absent from some proportion of chicken (Mochii et al., 1998a) and human (Yasumoto et al., 1997) *Mitf* transcripts. The function of this region of the protein is not known. Conversely, the predicted zebrafish protein contains a stretch of amino acids just amino terminal to the basic region that is absent from the other Mitfs, at a position known to involve alternative splicing in the mouse to generate isoforms with distinct activities (Hemesath et al., 1994; Sato et al., 1997). 5′RACE and RT/PCR approaches with primers flanking the divergent regions of the zebrafish cDNA did not reveal any other splice forms expressed at 24 hpf (data not shown).

Expression of the *Mitf*-related gene during embryonic development

We analyzed the expression of the zebrafish *Mitf*-related gene *3A.1* in wild-type and *nacre* embryos by whole-mount in situ hybridization. During normal development, *3A.1* RNA is first detected at approximately the 16-somite stage (17 hpf) at the caudal margin of the eye primordium (data not shown). By the 18-somite stage, expression has spread in the eye and is detectable in head and trunk (Fig. 6A). Expression progresses in a general rostral-to-caudal fashion in the head and trunk, in cells of the premigratory neural crest and in migrating neural crest cells (Fig. 6B,C). Thus, expression of the *Mitf*-

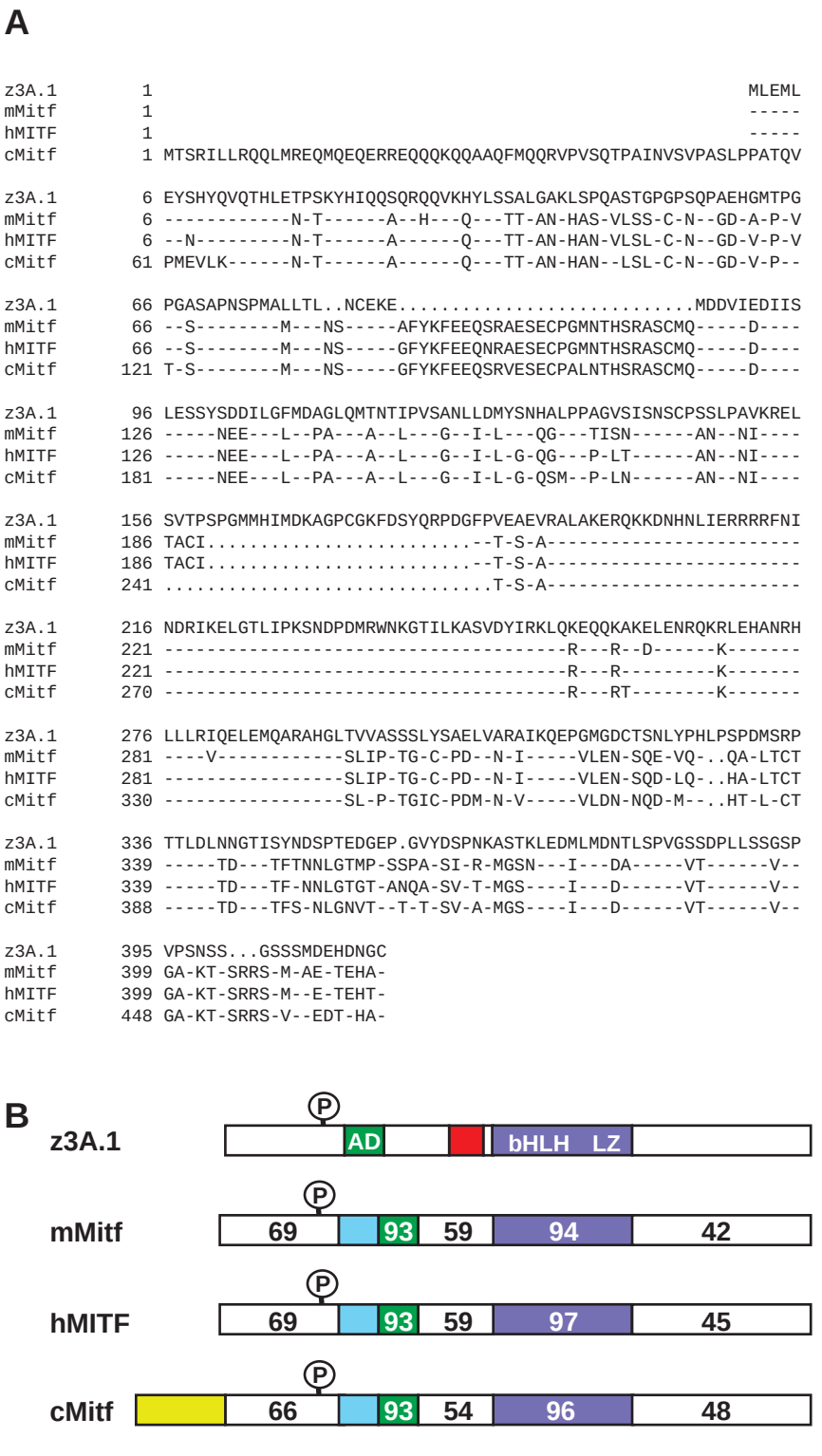

Fig. 5. Cloning of a zebrafish *Mitf*-related gene. (A) Alignment of predicted proteins. The deduced amino acid sequence of the zebrafish *Mitf*-related gene *3A.1* is shown aligned with those of mouse, human and chicken Mitfs. Amino acid identities with the zebrafish sequence are indicated by dashes and gaps are indicated by periods. GenBank accession numbers, top to bottom: AF119220, Z23066, NM_000248, D88363. (B) Schematic of conserved structure of Mitf-related proteins. Numbers indicate percentage amino acid identity of human, mouse, and chicken Mitfs to zebrafish 3A.1. All four proteins contain a consensus site for MAPK phosphorylation in the amino terminus (P), transcriptional activation domain (green), and basic helix-loop-helix/leucine zipper DNA-binding and dimerization domain (purple). The *3A.1* cDNA lacks an exon found in the other genes (encoding the domain in turquoise), which is also absent from some chicken and human Mitf transcripts. Conversely, 3A.1 contains a stretch of amino acids amino terminal to the bHLH/LZ domain not found in the other proteins (red). The melanocyte-specific isoforms are shown for mouse and human, while the single chicken isoform identified is highly expressed in the RPE and has a divergent amino terminus (yellow).

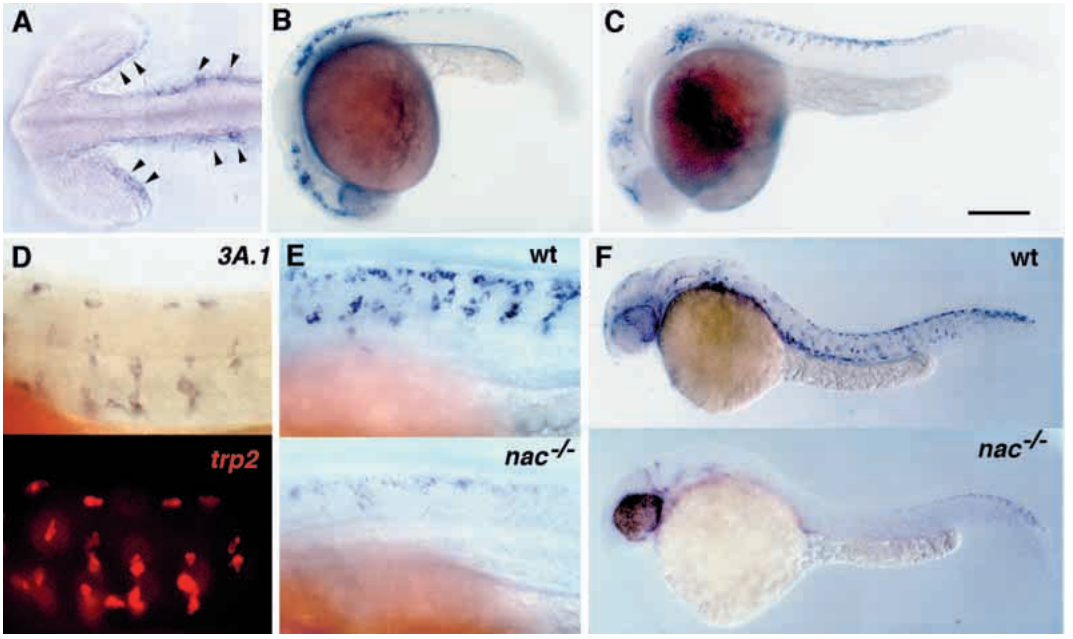

Fig. 6. Expression of the zebrafish *Mitf*-related gene during development. Whole-mount in situ hybridization was performed with an antisense probe to *3A.1*. (A) Expression (arrowheads) in the caudal margin of the eye and in head neural crest of an 18-somite (18 hpf) embryo. Expression is also detectable in a few cells in the trunk neural crest at this stage (not shown). *3A.1* expression expands in the eye and progresses in the head and trunk in a rostral to caudal manner as seen in 21 hpf (B) and 23 hpf (C) embryos. Migratory cells can clearly be seen by the latter timepoint. (D) *3A.1* (blue, top panel) and the melanoblast marker *trp2* (red, bottom panel) are coexpressed in migrating melanoblasts of 24 hpf embryos. (E) Expression of *3A.1* is reduced in *nacre* mutants. The top panel shows a closeup of the 23 hpf embryo from (C). *3A.1*-expressing cells can be seen on migratory paths at each somite level. Expression is much reduced and few cells have migrated away from the neural tube in a *nacre* mutant embryo at the same stage (bottom panel). (F) Reduced *3A.1* expression is still clearly evident at 30 hpf in a comparison of *albino* (top) and *nacre* (bottom) embryos. Scale bar: A, 100 μm; B,C, 200 μm; D,E, 50 μm; F, 250 μm.

related gene in the neural crest closely follows the timing of neural crest cell emigration (Raible et al., 1992). Double in situ hybridization experiments indicate that at 24 hpf the majority of *3A.1*$^{+}$ cells in the head and anterior trunk also express *trp2* (Fig. 6D), indicating that these cells are melanoblasts undergoing differentiation. The accumulation of pigment in embryos beyond 24 hours obscures the detection of gene expression in pigment cells, therefore *3A.1* expression was examined at later timepoints in *albino* and *golden* mutant backgrounds, which display an absence and reduction of melanin synthesis, respectively. Such analysis reveals that *3A.1* expression is downregulated in differentiated melanophores (data not shown). Expression of *3A.1* appears to be melanophore-specific through these early stages of development (data not shown). The spatial and temporal expression of this gene is thus analogous to that of mouse *microphthalmia*.

In contrast to the other melanoblast markers examined,

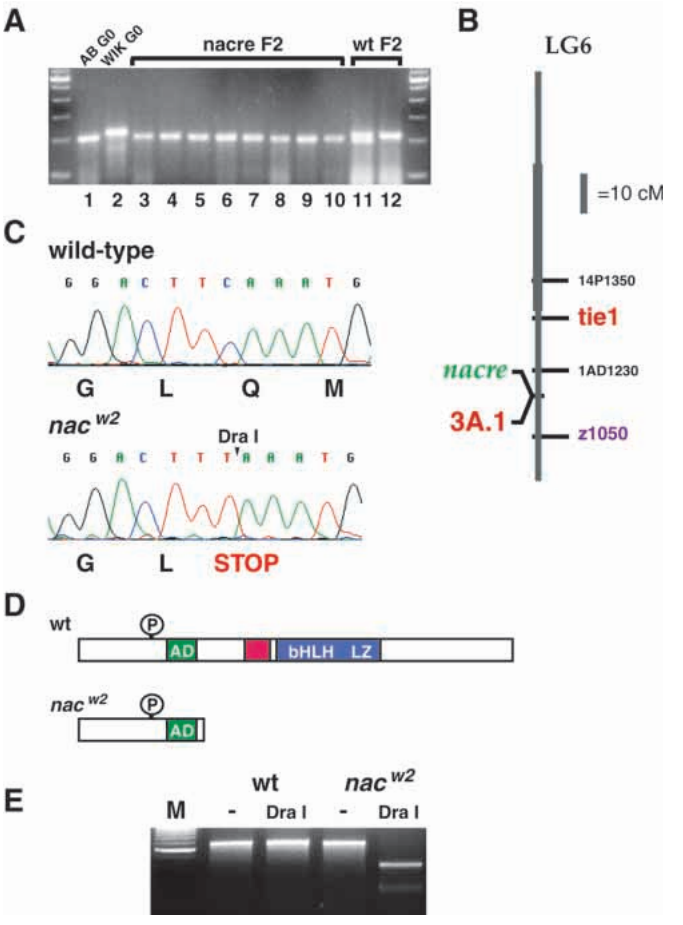

Fig. 7. *nac*^{w2} is a mutation in the zebrafish *Mitf*-related gene *3A.1*. (A) The zebrafish gene *3A.1* is closely linked to the *nacre* locus. PCR of genomic DNA from the AB and WIK strains with primers to the 3′UTR of *3A.1*, followed by digestion with *Afl*III, gives distinct products (lanes 1-2). In diploid embryos from a cross of hybrid AB(*nac*^{w2})/WIK parents, the *nacre* phenotype always segregates with the AB allele of *3A.1* (lanes 3-10). Wild-type embryos from the same cross are either heterozygous for this marker (lane 11) or homozygous for the WIK allele (lane 12). (B) *3A.1* and *nacre* map to the long arm of Linkage Group 6. (C) A single base substitution in *3A.1* is found in the *nac*^{w2} allele, creating a premature stop codon and resulting in a truncated protein (D). (E) The *nac*^{w2} mutation creates a *Dra*I restriction site (C) which is found in all *nacre* transcripts amplified by RT/PCR.

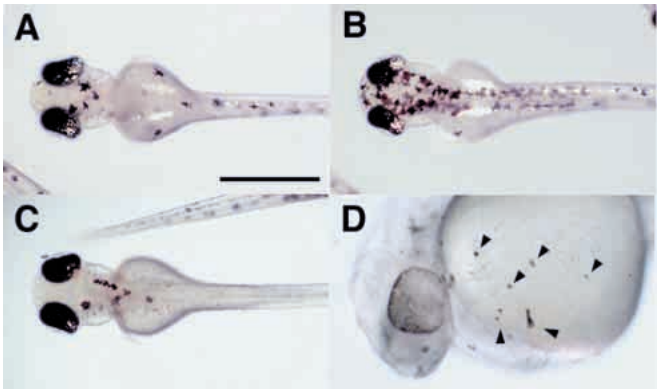

Fig. 8. Rescue of melanophore development by ectopic expression of wild-type *nacre*. Homozygous *nac*^{w2} embryos were injected with a construct bearing wild-type *nacre* under the control of the zebrafish heat-shock promoter. (A-C) dorsal views of representative examples are shown at approximately 72 hpf. Heat shock administered between 12 and 20 hpf restored melanophore development to varying degrees, ranging from quite extensive rescue (B) to just a few cells (C). Additionally, pigmented cells of unusual morphology were frequently observed in uncharacteristic locations, as seen here on the ventral yolk of a 27 hpf embryo (D, arrowheads). Scale bar, A-C, 500 μm; D, 200 μm.

expression of *3A.1* is reduced but present in neural crest cells of *nacre* homozygotes from the earliest time points examined (Fig. 6 and data not shown). At 23 hpf, *3A.1*$^{+}$ cells can be seen beginning to migrate down the middle of each somite in wild-type embryos, while in *nacre* embryos fewer *3A.1*-expressing cells are present and there is little migration away from the neural tube (Fig. 6E). At 30 hpf, *3A.1*$^{+}$ cells continue to be reduced in *nacre* embryos relative to *albino* control embryos (Fig. 6F), suggesting that decreased expression is not simply due to delayed onset.

nacre contains a premature stop codon in the *Mitf*-related gene *3A.1*

The *Mitf*-related gene *3A.1* was considered as a candidate for the *nacre* mutation based on its expression pattern and the phenotype of mouse *microphthalmia* mutants. Therefore, potential linkage between the two genes was investigated. *3A.1 was* mapped to linkage group 6 (LG6) by means of an *Afl*III restriction site polymorphism in the 3′ UTR using a haploid mapping panel (Johnson et al., 1996). The *nacre* phenotype always cosegregated with the AB allele of this marker in embryos obtained from crosses of F_1 hybrid AB(*nac*^{w2})/WIK fish (0 recombinants in 88 meiotic chromosomes), thus indicating tight linkage of *nacre* and *3A.1* (Fig. 7A,B).

RT/PCR with primers designed to amplify the entire *3A.1* coding sequence generated products of identical size from wild-type and *nac*^{w2} cDNA templates (data not shown). Upon subcloning and sequencing of the *nac*^{w2} product, a C-to-T transition was identified at position 417, which changes glutamine 113 to a stop codon (Fig. 7C). This base change also creates a *Dra*I restriction site, which is found in all *nac*^{w2} RT/PCR products but not in wild type (Fig. 7E). The presence of this mutation was confirmed in *nac*^{w2} genomic DNA (data not shown). In accordance with current zebrafish nomenclature standards (Westerfield, 1994), we have therefore reassigned the name *nacre* to the zebrafish *Mitf*-related gene *3A.1*. The predicted protein encoded by the *nac*^{w2} allele lacks 300 amino acids from the carboxy terminus, including the DNA-binding basic region and helix-loop-helix/leucine zipper dimerization domain (Fig. 7D), and as such would be presumed to represent a complete loss of function. It is noteworthy that the mutation occurs in an exon used commonly by the RPE- and melanocyte-

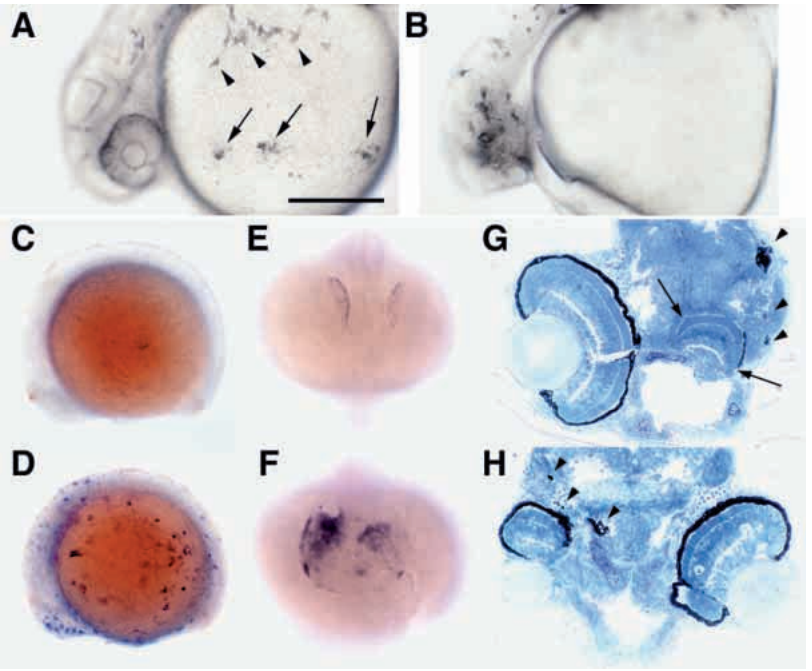

Fig. 9. Misexpression of *nacre* induces ectopic melanophores and disrupts eye development. (A,B) Wild-type embryos injected with nacre RNA at the 1- to 4-cell stage are shown at approximately 30 hpf. Embryos were frequently observed that displayed large patches of melanophores with abnormal morphology, as well as patches of similar cells in uncharacteristic locations such as the ventral yolk ball (A, arrows). Arrowheads indicate normal melanophores. Disorganization of one or both eyes in otherwise normal embryos was frequently observed (B). (C-F) Ectopic expression of *nacre* induces ectopic expression of *trp2*. Embryos were injected with pHS-MT3A.1-I219F (C) or pHS-MT3A.1 (D) and heat shocked from 10-12 hpf, then fixed at 14 hpf and processed for in situ hybridization with probes for melanophore marker *trp2* (blue) and the neural crest marker *fkd6* (orange). Widespread *trp2*-positive cells are observed in (D) prior to neural crest emigration. Injection of wild-type (F) but not mutant (E) *nacre* RNA expands the domain of *trp2* expression in the optic primordium of some embryos at 18 hpf. (G,H) Examples of disrupted eye morphologies in frontal sections of 72 hpf embryos. The embryo in G has one approximately normal eye on the left, and a second laminated structure with a small amount of RPE on the right (arrows). The eyes in the embryo in H are of reduced size and show disorganization and irregularities in the RPE. Arrowheads, ectopic pigment cells. Scale bar, A,B, 250 μm; C-F, 300 μm; G,H, 100 μm.

Table 1. Rescue of melanophore development in *nacre*$^{-/-}$ embryos by wild-type *nacre*

	Embryos with melanophores/total embryos	
Heat shock	Wild-type *nacre*	*nacI219F*
12-14 hpf	48/73	n.d.
18-20 hpf	107/142	0/97
none	81*/149	0/29

n.d., not determined.
*Fewer than 5 cells/embryos were observed without heat shock.

specific splice forms of other Mitfs (Amae et al., 1998), which contains an identified transactivation domain (Sato et al., 1997).

Expression of wild-type *nacre* rescues melanophore development in *nac*$^{-/-}$ embryos

The nature of the mutation in the *nac*^{w2} allele suggests that no functional Nacre protein is produced in *nac*^{w2} homozygotes. We therefore introduced the wild-type gene product back into *nac*$^{-/-}$ embryos to determine to what extent this was sufficient to restore melanophore development. Embryos were injected at the 1- to 4-cell stage with the plasmids pHS-MT3A.1, encoding wild-type nacre, or pHS-MT3A.1-I219F, encoding a predicted non-dimerizing (and therefore non-functional) mutant based on an allele of mouse *Mitf* (Steingrimsson et al., 1998), each under the control of the zebrafish heat-shock promoter (Shoji et al., 1998). Injected embryos were then shifted from 28.5°C to 37°C at various times after injection to activate expression of the transgene. As shown in Fig. 8 and Table 1, expression of wild-type *nacre* in this manner variably restored melanophore development. Rescue was observed in 66% of embryos heat-shocked from 12 to 14 hpf and 75% of embryos heat-shocked from 18 to 20 hpf. Rescue was also observed in 54% of injected embryos without heat shock, though typically fewer than five melanophores per embryo were present compared to 10-fold or greater numbers in heat-shocked embryos, likely reflecting leakiness of the promoter. In contrast, embryos injected with the mutant form of *nacre* showed no rescue with or without heat shock, although expression of the mutant protein was detectable by staining for the myc epitope tag (data not shown).

Misexpression of *nacre* induces ectopic pigment cells

In addition to melanophores with wild-type morphology, small non-dendritic pigmented cells, often in ectopic locations, were observed in most embryos displaying extensive rescue with pHS-MT3A.1 (Fig. 8D). Injection of wild-type embryos with this plasmid, with subsequent heat shock, also produced this phenotype (Table 2): pigment cells with abnormal morphology and/or location were observed in 75% of heat-shocked wild-type embryos injected with pHS-MT3A.1, while no such cells were observed in embryos injected with pHS-MT3A.1-I219F.

The effects of misexpressing *nacre* at much earlier stages of development (prior to formation of the neural crest) were assayed by introducing *nacre* RNA, along with RNA coding for GFP, into embryos at the 1- to 4-cell stage. Again ectopic pigment cells of unusual morphology were observed in 82% of *nacre* and 60% of wild-type-injected embryos (Fig. 9A,B; Table 2). Injection of RNA encoding the dimerization mutant of *nacre* did not induce the formation of pigmented cells.

Table 2. Pigment and eye phenotypes induced by *nacre* misexpression

Embryo genotype	DNA/RNA injected	Ectopic pigment	Eye defects
Wild type	*nac* DNA +HS‡	45/60	0/60
	nac DNA –HS	0/15	0/15
	I219F DNA +HS	0/92	0/92
	I219F DNA –HS	0/28	0/28
Wild type	*nac* mRNA	64/107	27/107
	I219F mRNA	0/18	0/18
nacre$^{-/-}$	*nac* mRNA	73*/89	23/89
	I219F mRNA	0/27	0/27

*Pigmented cells of wild-type morphology and location were generally not observed.
‡+HS: 1 hour heat shock at 37°C administered between 10 and 14 hpf; –HS, no treatment.

The results of these misexpression experiments suggest that, while a loss-of-function mutation in the zebrafish *microphthalmia*-related gene *nacre* leads to an absence of melanophores, ectopic expression of *nacre* may be sufficient to convert some embryonic cells to a melanophore fate. One possibility is that the abnormal melanophores arise from the aberrant migration of neural crest cells overexpressing *nacre*. However, misexpression of *nacre* by plasmid or RNA injection was found to induce widespread (mosaic) expression of *trp2* by the 10-somite stage (Fig. 9C,D and data not shown), several hours in advance of even the first migration of cells from the neural crest. *trp2* expression is found over all of the embryo, not just close to the neural tube or on neural crest migration pathways. Furthermore, pigment synthesis is clearly evident by 20 hpf in some embryos (data not shown), approximately 5 hours earlier that wild-type pigmentation is ordinarily observable. In contrast to experiments with the heat-shock cDNA construct described above, injection of *nacre* mRNA was not generally observed to rescue development of morphologically normal melanophores, even in embryos with extensive patches of abnormal melanophores. We presume the difference between these results is due to the time period when *nacre* mRNA is present: with mRNA injection, it is present from the earliest stages of development while, with heat shock, it is induced just before neural crest formation. Our results thus argue against a neural crest origin for these abnormal pigment cells and support the hypothesis that expression of *nacre* may be sufficient to activate a program of melanophore differentiation in cells outside of the neural crest.

Misexpression of *nacre* disrupts eye morphogenesis

Early misexpression of *nacre* by means of RNA injection also had profound effects on eye development. Gross eye defects, including reduction or absence of one or both eyes and disorganization of eye structures (Fig. 9B) were observed in 25% of wild-type and 26% of mutant embryos injected with *nacre* RNA. Expression of *trp2* was found to be greatly expanded in the optic primordium of a subset of embryos injected with *nacre* but not control mRNA (Fig. 9E,F). Plastic sections made from embryos with grossly malformed eyes often revealed discontinuity or absence of RPE, as well as reduction in size and

irregularities of shape of the eye as a whole, but lamination of the neural retina into morphologically recognizable cell layers was still generally observed (Fig. 9G,H). Other deficits were limited to alterations in CNS morphology associated with these eye defects. These gross eye phenotypes were not observed when RNA encoding the dimerization mutant was injected, even at much higher doses where cyclopia, an indicator of RNA toxicity, became evident (data not shown). These results indicate that, while *nacre* function is not required for proper development of the RPE, morphogenesis in the eye primordium is still sensitive to inappropriate expression of this regulator. Eye defects were never observed when *nacre* expression was induced during the segmentation period by heat shock (Table 2), suggesting that the period of sensitivity in the optic primordium is earlier than that for pigment cell induction and rescue of mutant melanoblasts.

DISCUSSION

nac^{w2} is a loss-of-function mutation in a zebrafish *Mitf*-related gene

In this study, we report the isolation and identification of a new zebrafish mutation that specifically disrupts development of neural-crest-derived melanophores (with a concomitant increase in iridophores) while leaving development of the pigmented layer of the retinal epithelium intact. We have demonstrated that this mutation lies in a gene with homology to *Mitf*, which we have named *nacre*. In addition to the large number of alleles known in the mouse *microphthalmia* locus, mutations in the *Mitf* gene have recently been identified in a variety of other species, including rat (Opdecamp et al., 1998), hamster (Hodgkinson et al., 1998) and quail (Mochii et al., 1998b) and are associated with Waardenburg syndrome type 2 in humans (Tassabehji et al., 1994). Like mouse *Mitf*, *nacre* appears to be required at the earliest stages of melanophore development. However, *nacre* is not absolutely required in all melanophores, as small patches of these cells are observed in some adults, typically in the fins. This may reflect differences in the stem cell populations from which melanophores at different stages of development are thought to be derived (Johnson et al., 1995b). In striking contrast to the *Mitf* genes of higher vertebrates, *nacre* appears to be dispensable for normal eye development, although it is expressed in the RPE and misexpression has functional consequences for eye development. While it is formally possible that a functional Nacre isoform is synthesized specifically in the RPE of *nac*^{w2} embryos, or in adult melanophores, the site of the lesion in an exon common to all known Mitf mRNAs, which encodes a demonstrated transcriptional activation domain, is strong evidence against this possibility. Furthermore, the *nac*^{w2} mutation creates a restriction site which is present in all *nacre* transcripts as detected by RT/PCR.

An alternative explanation, suggested by emerging findings from zebrafish genomics (Amores et al., 1998; Wittbrodt et al., 1998), is that a second *nacre/Mitf*-like gene exists in the zebrafish. In support of this, we have recently isolated a fragment of a second zebrafish cDNA with greater homology to *Mitf* than to any of the other members of the *MiT* family (J. A. L., unpublished results). We might predict that this gene will function redundantly with *nacre* in the RPE but be unable to compensate for the loss of *nacre* in the neural crest, perhaps as a result of incomplete overlap of expression patterns. A number of examples of such divergence in expression have been documented (Feldman et al., 1998; Nornes et al., 1998) and this has been proposed to be a general mechanism operating to preserve duplicated genes (Force et al., 1999). Intriguingly, a large-scale screen for mutations affecting development of the zebrafish retina identified three that disrupted both RPE and neural-crest-derived pigment cells (Malicki et al., 1996). The similarity between these mutants and the mouse *microphthalmia* phenotype was noted by the authors, who speculated that one of these might represent a zebrafish *Mitf* gene. Based on what we would expect to be a near complete loss of function in the *nac*^{w2} allele, we hypothesize that these mutations are in genes distinct from *nacre*. Mapping of these loci or complementation analysis may be required to resolve this issue.

nacre regulates pigment cell fate in zebrafish

Although the absence of melanophores is the most striking aspect of the *nacre* phenotype and the expression of *nacre* appears to be melanophore-specific, this cell type is not the only one affected: homozygous *nac*^{w2} embryos also show an increase of approximately 40% in the number of iridophores. This observation immediately suggests the possibility that melanoblasts may switch fate to become iridoblasts in the absence of functional Nacre protein. Supporting this idea, it has been shown that loss of Mitf can result in fate changes, as retinal pigment epithelial cells of the *silver* quail differentiate into neural retina as a result of diminished *Mitf* activity (Mochii et al., 1998b). The existence of a committed chromatophore stem cell has been postulated (Bagnara et al., 1979) and, furthermore, goldfish erythrophoroma cells (Matsumoto et al., 1989), as well as primary bullfrog pigment cells (Ide, 1978; Ide and Hama, 1976), will change pigment cell type under particular culture conditions. However, single cell labeling and lineage analysis have provided no evidence for a fate-restricted chromatophore precursor in zebrafish (Raible and Eisen, 1994).

An alternative explanation is that the increase in iridophores is a secondary effect of the absence of melanophores, e.g. that melanophores directly or indirectly restrict iridophore development and that the *nacre* phenotype therefore reflects a release of inhibition to iridophore proliferation or differentiation. Interactions between pigment cell types are known to be important in establishing adult pigment pattern (Johnson et al., 1995b) and may thus also be at work in embryonic pigment patterning. Although the fates of the cells that initiate expression of mRNA encoding the truncated protein in *nacre* mutants are not known, staining with the vital dye acridine orange, which marks apoptotic cells (Furutani-Seiki et al., 1996), does not reveal any differences in cell death between wild-type and *nacre* embryos during the period of neural crest emigration (J. A. L., unpublished results).

nacre and control of vertebrate pigment cell development

The identification of *nacre* as a *Mitf*-related gene in zebrafish demonstrates the conserved nature of the control of pigment cell fate within vertebrates. Moreover, the Nacre protein contains a potential MAP kinase phosphorylation site of the sort by which human Mitf activity has been shown to be regulated by c-Kit signalling (Hemesath et al., 1998),

suggesting that other relationships in this pathway may be conserved. As shown here, misexpression of *nacre* in zebrafish embryos induced the formation of ectopic pigment cells. While the exact source of these cells is not clear, two lines of evidence suggest that at least a portion of these cells do not derive from neural crest. First of all, misexpression of *nacre* by plasmid or RNA injection induces expression of the melanophore marker *trp2* in ectopic locations (such as the ventral yolk ball) prior to the onset of neural crest migration. Secondly, injection of nacre RNA into homozygous mutant embryos gives rise to exclusively abnormal melanophores, suggesting that their appearance is not a function of expression (and rescue) within the neural crest compartment. These in vivo experiments thus complement and extend earlier work done in cultured mouse fibroblasts (Tachibana et al., 1996), and support a role for Nacre/Mitf as a 'melanogenic' factor analogous to myogenic and neurogenic basic helix-loop-helix transcription factors such as MyoD (Weintraub et al., 1991) and NeuroD (Lee et al., 1995), respectively. Whether or not Nacre requires the cooperation of other factors to activate the melanophore differentiation program, and what the transcriptional targets might be, remains to be determined.

nacre/Mitf is the earliest melanoblast marker yet to be identified and a precise delineation of what factors regulate its expression will be a significant step toward understanding neural crest cell fate specification. While regulation of the *Mitf* promoter has been studied in the context of hormonal influences (Aberdam et al., 1998; Price et al., 1998), factors that control the initial activation of *Mitf* during embryogenesis are less well understood. Mutations in mouse *Pax3* have pleiotropic effects including a coat color phenotype and Pax3 has been shown to directly activate *Mitf* transcription in cultured cells (Watanabe et al., 1998). Recent work from our laboratory has identified Wnt signalling as a promoter of pigment cell fate in the zebrafish (Dorsky et al., 1998), and the possibility that *nacre* is a direct target of this pathway is currently being investigated.

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