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Provitamin A conversion to retinal via the β,β-carotene-15,15′-oxygenase (*bcox*) is essential for pattern formation and differentiation during zebrafish embryogenesis

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SUMMARY

The egg yolk of vertebrates contains carotenoids, which account for its characteristic yellow color in some species. Such plant-derived compounds, e.g. β-carotene, serve as the natural precursors (provitamins) of vitamin A, which is indispensable for chordate development. As egg yolk also contains stored vitamin A, carotenoids have so far been solely discussed as pigments for the coloration of the offspring. Based on our recent molecular identification of the enzyme catalyzing provitamin A conversion to vitamin A, we address a possible role of provitamin A during zebrafish (*Danio rerio*) development. We cloned the zebrafish gene encoding the vitamin A-forming enzyme, a β,β-carotene-15,15′-oxygenase. Analysis of its mRNA expression revealed that it is under complex spatial and temporal control during development. Targeted gene knockdown using the morpholino antisense oligonucleotide technique indicated a vital role of the provitamin A-converting enzyme. Morpholino-injected embryos developed a morphological phenotype that included severe malformation of the eyes, the craniofacial skeleton and pectoral fins, as well as reduced pigmentation. Analyses of gene expression changes in the morphants revealed that distinct retinoic acid-dependent developmental processes are impaired, such as patterning of the hindbrain and differentiation of hindbrain neurons, differentiation of neural crest derivatives (including the craniofacial skeleton), and the establishment of the ventral retina. Our data provide strong evidence that, for several developmental processes, retinoic acid generation depends on local de novo formation of retinal from provitamin A via the carotene oxygenase, revealing an unexpected, essential role for carotenoids in embryonic development.

Key words: Zebrafish, Carotenoid conversion, Vitamin A, β,β-carotene-15,15′-oxygenase, Retinoic acid, Neural crest, Craniofacial skeleton, Eyes, Pectoral fins

INTRODUCTION

Vitamin A deficiency (VAD) of the vertebrate embryo results in embryonic malformations, including hindbrain segmentation defects, neural crest cell death, the absence of posterior branchial arches, and abnormalities of facial structures, limb buds, eyes and somites (Wilson et al., 1953; Morriss-Kay and Sokolova, 1996; Maden et al., 1996; Maden et al., 2000; White et al., 1998; Dickman et al., 1997). Besides a structural requirement for vitamin A during the establishment of the visual apparatus, it functions as the precursor for retinoic acid (RA), a major embryonic signal that controls a wide range of developmental activities. RA is the ligand of two classes of nuclear receptors (RARs and RXRs) that influence transcriptional regulation of a multitude of target genes involved in biological processes as diverse as pattern formation, cell differentiation and control of metabolic activity (Giguére et al., 1987; Petkovich et al., 1987) (reviewed by Mangelsdorf and Evans, 1995).

Retinoid signaling depends on the availability of maternal sources for vitamin A and is controlled through the spatially and temporally regulated expression of enzymes involved in RA synthesis and catabolism (reviewed by Ross et al., 2000). Retinal dehydrogenases (Raldhs) have been clearly identified as the enzymatic activities catalyzing the final step in RA synthesis. Targeted inactivation of *Raldh2* in mouse, as well as mutations in *raldh2* in zebrafish, both generate phenotypes mimicking most features of an early onset embryonic VAD (Niederreither et al., 1999; Niederreither et al., 2000; Begemann et al., 2001; Grandel et al., 2002; Mic et al., 2002). During organogenesis, besides *raldh2*, two additional retinal dehydrogenases, *raldh1* and *raldh3*, are expressed at distinct sites where they may contribute to the generation of local RA signals (Li et al., 2000; Mic et al., 2002; Niederreither et al., 2002). It is assumed that retinal synthesis from retinol constitutes the upstream step of the RA-generating pathway. In the mammalian embryo, retinol is provided from the maternal circulation via the placenta, whereas avian, reptilian,

amphibian and fish embryos use retinoid stores in the egg yolk. A multitude of retinoid metabolizing enzymes were characterized for retinol to retinal conversion in vitro and can be assigned to two enzyme families: alcohol dehydrogenases (ADH) and short chain dehydrogenases/reductases (SDR) (for a review, see Duester, 2000). Although, so far, no genetic evidence exists that any of these enzymes may act in vivo upstream to the different types of Raldh, recent results suggest that Adh3, an ubiquitously expressed retinol-oxidizing enzyme, is needed for mouse development, as loss of its function leads to postnatal lethality (Molotkov et al., 2002).

Besides retinoids, provitamin A-carotenoids are present in the mammalian circulation and in the vertebrate egg yolk. The enzymatic conversion of these compounds results in the generation of retinal, the direct precursor of RA (Olson and Hayaishi, 1965; Goodman and Huang, 1965). A possible role of this alternative source for retinoids has not yet been addressed, as the molecular nature of this enzyme has remained elusive for a long time. Our recent research led to the molecular identification and functional characterization of the gene encoding the key enzyme in provitamin A conversion, a β,β-carotene-15,15′-oxygenase (*bcox*) in *Drosophila* (von Lintig and Vogt, 2000; von Lintig et al., 2001). Vertebrate orthologs were then identified based on sequence identity from various species, including human (Wyss et al., 2000; Redmond et al., 2001; Kiefer et al., 2001; Paik et al., 2001; Yan et al., 2001).

Building on this work, the present study aimed to elucidate the impact of provitamin A conversion on embryonic development in zebrafish (*Danio rerio*). We cloned the cDNA of the vitamin A-generating enzyme (*bcox*) and demonstrated the presence of its substrate β-carotene in zebrafish egg yolk by HPLC analyses. We then showed that *bcox* is expressed in clearly defined spatial compartments and translated into protein in the zebrafish embryo. To test whether there is an actual requirement for provitamin A conversion during embryonic development, we performed targeted gene knockdown experiments using morpholino antisense oligonucleotides (MO). The *bcox* morphants developed abnormalities of the craniofacial skeleton, pectoral fins and eyes, which are impairments well known from VAD embryos. Analyses of changes in gene expression in *bcox* morphants revealed specific defects in patterning and differentiation during several distinct RA-dependent developmental processes, thus providing the first evidence that carotenoids are indispensable as precursors for RA signaling in a vertebrate.

MATERIALS AND METHODS

Fish maintenance and strains

Zebrafish were bred and maintained under standard conditions at 28.5°C (Westerfield, 1994). Morphological features were used to determine the stage of the embryos in hours (hpf) or days (dpf) post-fertilization according to Kimmel et al. (Kimmel et al., 1995). Embryos used for in situ hybridization experiments were raised in the presence of 200 μM 1-phenyl-2-thiourea (Sigma) to inhibit pigmentation.

The zebrafish *mob*^{m819} mutant is a null allele of *ap2α* and details of the phenotype are available (J.H., A. Barrallo, A. K. Ettl, E. Knapik and W.D., unpublished).

Cartilage of zebrafish larvae was stained with Alcian Blue as described by Neuhauss et al. (Neuhauss et al., 1996). Microtome sections (3 μm) of plastic-embedded 5-day-old larvae were mounted on poly-L-lysine coated slides, air dried at 60°C, stained with Richardson's solution (1% azure, 1% Methylene Blue, 1% borax in deionized water), overlaid with DPX (Merck, Germany) and coverslipped.

Cloning and functional characterization of *bcox*

Total RNA from liver of adult zebrafish was isolated with the high pure RNA isolation kit (Roche Molecular Biochemicals) according to the manufacturer's protocol. For cloning a full-length cDNA encoding the putative zebrafish β-carotene-15,15′-oxgenase, RACE-PCRs were performed using a 5′/3′ RACE kit (Roche Molecular Biochemicals). For 3′-RACE, reverse transcription was performed with 500 ng of total RNA isolated from liver, an oligo-d(T)-anchor primer (5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV-3′) and Superscript reverse transcriptase II (Life Technologies). For PCR, the Expand PCR system (Roche Molecular Biochemicals), an anchor primer and a specific primer, 5′-GGTGCAGGGAACACTAATACG-3′derived from a published EST-fragment (GenBank Accession Number AW128477) were used. For 5′-RACE, reverse transcription was performed with 500 ng of total RNA from liver, a specific primer 5′-CTCGAATGGGTGCAGGGAACA-3′ derived from the EST fragment, and Superscript reverse transcriptase (Life Technologies, Inc.). For PCR, the Expand PCR system (Roche Molecular Biochemicals), the specific primer 5′-CTCGAATGGGTGCAG-GGAACA-3′ and an anchor primer were used. The PCR fragments were cloned in the vector pCR2.1 TOPO (Invitrogen) and subjected to sequence analyses. The full-length cDNA sequence of *bcox* was deposited with GenBank (Accession Number AJ290390).

The entire coding region of the *bcox* cDNA was amplified by RT-PCR. For reverse transcription 500 ng total RNA was incubated in the presence of a polydT_{17} primer and Superscript II (Life Technologies). For PCR, the Expand PCR system (Roche Molecular Biochemicals), and the primers 5′-ATGCAGTACGACTATGGCAAA-3′ and 5′-GCTGCTGCCTGGTATGAAGTA-3′ were used. The PCR product was ligated into the vector pCR-BluntII-TOPO (Invitrogen) and transformed in a β-carotene synthesizing and accumulating *E. coli* strain. Tests for enzymatic activity of the Bcox protein were performed as previously described (von Lintig and Vogt, 2000).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described by Hauptmann and Gerster (Hauptmann and Gerster, 1994). *bcox* was cloned into the vector pBluescript SK (Stratagene) and antisense and sense RNA probes were synthesized with the T7 RNA polymerase and T3 RNA polymerase, respectively. Additional RNA probes used for in situ hybridization experiments were *shh* (Krauss et al., 1993), *efna4* (Xu et al., 1996), *hoxb4a* (Prince et al., 1998), *fgf8* (Reifers et al., 1998), *myod* (Weinberg et al., 1996), *krx20* (*egr2* – Zebrafish Information Network) (Oxtoby and Jowett, 1993), *dlx2* (Akimenko et al., 1994), *pax6.1* (*pax6a* – Zebrafish Information Network) (Püschel et al., 1992), *pax2.1* (*pax2a* – Zebrafish Information Network) (Krauss et al., 1991), *ap2α* (*tfap2a* – Zebrafish Information Network) (J.H., A. Barrallo, A. K. Ettl, E. Knapik and W.D., unpublished) and *val* (Moens et al., 1998). The RNA probes were generated with the Dig RNA labeling kit (Roche Molecular Biochemicals) according to the manufacturer's protocol.

Injections of morpholino oligonucleotides and synthetic *bcox* mRNAs

For targeted knockdown of the *bcox* protein, two antisense morpholino oligonucleotides (GeneTools, LLC) were used that covered either nucleotides +1 to +25 (*bcox*-MO; 5′-TGTTTTTG-CCATAGTCGTACTGCAT-3′) or –24 to –1 (*bcox*-MOb; 5′-CCT-TTCAGATGCTTTCTTCAAGTTC-3′) of the *bcox* mRNA. For control injections, a standard control morpholino oligonucleotide [co-MO; 5′-CCTCTTACCTCAGTTACAATTTATA-3′ (Gene Tools,

LLC)] was used. The MOs were dissolved in 0.3× Danieau's solution [1× Danieau: 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6] to obtain a stock concentration of 1 mM (8.43 mg/ml). The stock solutions were diluted to 6, 3 and 0.5 mg/ml. The injected volume was ~3 nl, corresponding to 18, 9 and 1.5 ng morpholino oligonucleotide per egg, respectively. The *bcox* cDNA was cloned into the vector pCS2+ and RNA was synthesized in vitro using the mMESSAGEmMACHINE kit according to the manufacturer's protocol (Ambion Europe, UK). Co-injection of various amounts of the *bcox* mRNA was used to control the specificity of *bcox*-MO. For rescue experiments, various amounts of this modified *bcox* mRNA were co-injected with 9 ng of *bcox*-MO. In addition, the targeted knockdown of the *bcox* protein was monitored using western blot analyses.

Retinoic acid and citral treatments

bcox morphants and wild-type embryos were exposed to 10^{-9} M RA in egg water diluted from a stock of 3×10^{-1} M all-trans retinoic acid in dimethyl sulphoxide (DMSO) (Sigma, Germany). The start and duration of incubation are indicated for the respective experiments. Incubations were carried out in the dark and embryos were fixed at desired stages. At the three-somite stage, wild-type embryos were incubated in egg water containing 250 μM citral (Roth, Germany) for 1 hour. After treatment, embryos were washed three times for 10 minutes in egg water to remove citral and fixed at desired stages.

Western blot analysis

For western blot analyses we used a polyclonal serum raised against the mouse Bcox (Kiefer et al., 2001). We first verified the crossreaction of the serum with zebrafish Bcox expressed in *E. coli*. For western blot analyses with zebrafish embryos, four dechorionized embryos were directly homogenized in SDS lysis buffer (63 mM Tris HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 3.5% sodium dodecyl sulfate) and subjected to SDS-PAGE according to Westerfield (Westerfield, 1994). Alternatively, 100 dechorionized embryos were homogenized in 200 μl Tricine buffer (50 mM Tricine, 100 mM NaCl, pH 7.5) using a loose-fitting glass potter. Protein was harvested (50 μg) using StrataClean resin (Stratagene) and subjected to western blot analysis. Immunostaining was carried with the serum diluted 1:500 and the ECL system according to the manufacturer's protocol (Amersham Pharmacia Biotech).

HPLC analysis of carotenoids and retinoids of zebrafish embryos and larval eyes

For extraction of retinoids and carotenoids, freshly fertilized eggs were collected. For determination of the retinoid content the eggs were homogenized in 500 μl 2 M NH_2OH.

HPLC system 1

Extraction of retinoids and HPLC analysis were carried out as previously described (von Lintig and Vogt, 2000). For quantification of the molar amounts, peak integrals were scaled with defined amounts of reference substances. The reference substances all-trans and 13-cis retinol and retinal were purchased (Sigma, Germany); 11-*cis* retinal was isolated from dark adapted bovine eyes. Peak integrals were calculated using the 32 Karat software (Beckman Instruments). For determining the retinoid content of larval eyes, the eyes of dark-adapted larvae were removed by hand dissection with a scalpel under red safety light. Extraction of retinoids and HPLC analyses were carried out as previously described (von Lintig and Vogt, 2000).

HPLC system 2

For determining the carotenoid content, the eggs were homogenized in 33% ethanol PBS buffer (137 mM NaCl, 2.7 mM KCl, 7.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2) with a loose-fitting glass potter. Then 750 μl ethyl acetate/methyl acetate (8:1 v/v) was added and vigorously vortexed. After centrifugation at 5000 ***g***, the upper phase was collected. The extraction was repeated twice and the collected organic phases were dried under a stream of nitrogen and dissolved in HPLC solvent. Carotenoid analyses were performed on a Hypersil 3 μm column (Knauer, Germany) on a Beckman System Gold (Beckman Instruments) equipped with a multidiode array model 166 (Beckman Instruments). The HPLC solvent was n-hexane/ethyl acetate (81:19, v/v) containing 15 μl acetic acid per 100 ml solvent at a flow of 1 ml $minute^{-1}$. For quantification of the molar amounts, peak integrals were scaled with defined amounts of reference substances. The reference substances β-carotene, zeaxanthin and lutein were from Sigma; canthaxanthin and cryptoxanthin were from Roth (Germany). Echinenone was obtained from Roche (Switzerland). 4-Hydroxy-echinenone and isocryptoxanthin were obtained respectively by reduction of canthaxanthin or echinenone with $NaBH_4$. Peak integrals were calculated using the 32 Karat software (Beckman Instruments).

Photography

Live embryos and larvae where photographed in 2.5% methylcellulose/0.02% 3-aminobenzoic acid methylester (Sigma, Germany), stained whole-mount embryos in 100% glycerol on a dissecting microscope (Leica MZ FLIII) with an Axiocam (Zeiss). To assign tissue specificity of the *bcox* staining, the embryos were embedded in Technovit 7100 (Kulzer Histotechnik, Germany) and sectioned. Longitudinal and transversal cross sections of these preparations were analyzed by microscopy.

RESULTS

Cloning and functional characterization of *bcox*

For cloning the gene encoding β,β-carotene-15,15′-oxygenase in zebrafish, we searched the GenBank database and found an EST fragment (AW128477) of zebrafish origin with significant sequence identity with its mammalian homologues. For cloning the full-length cDNA we performed 3′- and 5′-RACE-PCRs. Sequence analyses revealed that the full-length *bcox* cDNA was 1774 bp in length and coded for a protein of 516 amino acid residues. The deduced amino acid sequence of the protein shared 55.7% identity with its human counterpart (Fig. 1A). Besides the overall amino acid sequence identity, we detected a pattern of six conserved histidine residues, which are most probably involved in the binding of the ferrous co-factor, and a conserved polyene chain oxygenase family motif EDDGVVLSXVVS (Redmond et al., 2001). Thus, we named this cDNA *bcox* (GenBank, AJ290390). To test Bcox for enzymatic activity, we cloned the cDNA into the expression vector pCR-BluntII-TOPO (Invitrogen). The vector construct was then transformed into an *E. coli* strain capable of synthesizing and accumulating β-carotene; we already successfully used this *E. coli* test system to assess enzymatic activity of carotene oxygenases from various species (von Lintig and Vogt, 2000; Kiefer et al., 2001). After co-expressing the Bcox protein in the β-carotene synthesizing *E. coli* strain, HPLC-analyses revealed the formation of retinal at the expense of β-carotene, as judged from the absorption spectra and retention times of the cleavage products compared to authentic standards. These analyses conclusively identified Bcox as a β,β-carotene-15,15′-oxgenase catalyzing the symmetric cleavage of provitamin A to produce retinal (Fig. 1B,C).

Analyses of the spatial and temporal expression patterns of *bcox* during zebrafish embryogenesis

We analyzed for *bcox* expression during development by

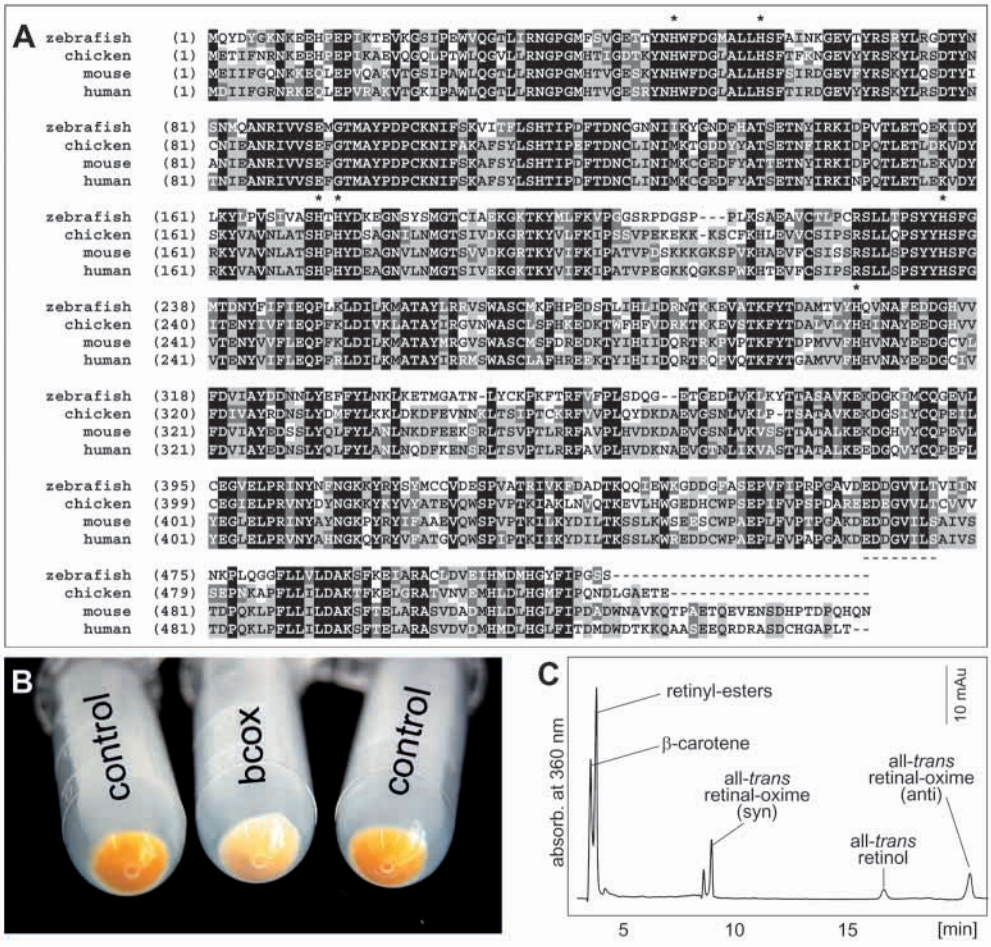


Fig. 1. (A) Linear alignment of the deduced amino sequence of β,β-carotene-15,15′-oxygenases of different vertebrate species. Identity is indicated in black and conserved amino acids are indicated in gray according to the PAM250 matrix. Conserved histidine residues probably involved in binding the co-factor Fe^{2+} are marked by asterisks. A putative polyene chain oxygenase family motif is underlined. Besides the zebrafish sequence, the following sequences were used: AAG15380 (human), NP067461 (mouse) and AJ271386 (chicken). (B) Color shift of *bcox*-expressing *E. coli*-strain caused by the cleavage of β-carotene to retinal. (C) HPLC profile at 360 nm of an extract derived from a β-carotene-synthesizing *E. coli* strain expressing *bcox* separated on HPLC-system 1. The composition of the individual retinoids is indicated in the figure.

whole-mount in situ hybridization. Strong *bcox* mRNA expression first became detectable during early segmentation stages. As shown for the 14-somite stage, specific expression was detected along the embryonic axis from the head to the trunk (Fig. 2A,B). In cross-sections (Fig. 2C-E), *bcox* was found to be expressed in ventral cell layers adjacent to the yolk, corresponding to mesendodermal and ventral mesodermal cells of the head and trunk (Warga and Nusslein-Volhard, 1999; Alexander and Stainier, 1999). Additionally, *bcox* mRNA expression was also detected in the head at the ventral side of the optic primordia at segmentation stages (Fig. 2F,G). At 1 dpf, *bcox* mRNA expression could be detected in individual cells in the head and trunk as well as in the fin buds (Fig. 2H,I). These cells resembled cranial and trunk neural crest both in their temporal occurrence and in their spatial distribution. To test whether these *bcox*-expressing cells were neural crest, we determined *bcox* mRNA expression in the zebrafish mutant *mont blanc*/*ap2α* (Neuhauss et al., 1996) (J.H., A. Barrallo, A. K. Ettl, E. Knapik and W.D., unpublished), which lacks most differentiated neural crest cells. In *mont blanc*/*ap2α* (*mob*^{m819}), *bcox* expression corresponding to trunk neural crest was mostly absent and it was reduced in cranial neural crest. *bcox* expressing cells were restricted to a more dorsal position (Fig. 2J,K), thus corresponding to the impairments in neural crest migration recently described for this mutant (Barrallo et al., 2003). At 2 dpf, *bcox* mRNA expression was still detected in cells around the eye in wild type, which may either be pigment epithelium cells or neural crest derived pigmented cells contributing to the xanthophores, iridophores and melanocytes covering the eye (Fig. 2L,M). In addition, strong expression persisted in the ventral pharyngeal region (Fig. 2L,N). Furthermore, expression was detected in the pectoral fin buds and in the otic vesicle. At 3 to 4 dpf, towards the end of embryogenesis, liver and gut cells strongly expressed *bcox* (Fig. 2O-Q).

To confirm the presence of the Bcox protein, we subjected protein extracts from 1-3 dpf embryos to immunoblot analysis. A single band of the expected molecular size (59 kDa, deduced from the *bcox* amino acid sequence) was stained (Fig. 2R). Thus, *bcox* is transcribed and translated into protein to catalyze carotenoid conversion to vitamin A during development in clearly defined spatial compartments of the zebrafish embryo.

Provitamin A carotenoids exist in the zebrafish egg

The egg yolk of lower vertebrates, such as amphibians and teleosts, generally contains large amounts of maternally derived vitamin A in the form of retinal bound via a labile Schiff-base linkage to vitellogenins (Plack et al., 1959; Seki et al., 1987; Costaridis et al., 1996; Irie and Seki, 2002). Until now, it has been assumed that these retinoid stores are exclusively used for embryonic retinoid metabolism (Costaridis et al., 1996). However, our results indicate that provitamin A cleavage to retinal may impact, in addition, embryonic retinoid metabolism. The prerequisite for this is the

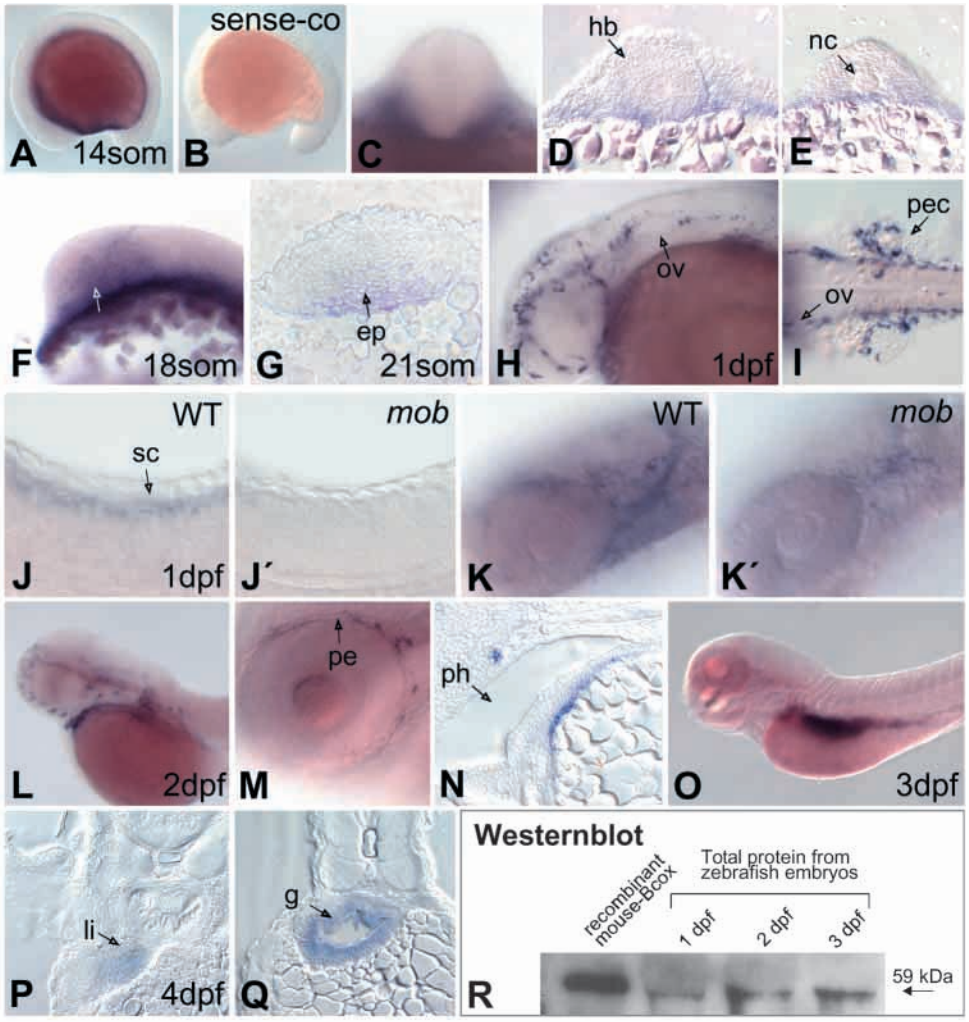


Fig. 2. Expression of *bcox* in zebrafish wild-type and *mob*^{m819} embryos and larvae. (A,B,F,H,J-M,O) Lateral views, anterior towards the left; (I) dorsal view, anterior towards the left; (C) optical section of a whole-mount embryo; (G,N) sagittal and (D,E,P,Q) transverse sections. At segmentation stages, *bcox* is expressed along the AP axis close to the yolk boundary (A, compare with B); representative 14-somite embryos are shown. (C) A view anterior to the trunk at the level of the anterior somites and (D,E) cross-sections show expression in the vegetalmost cell layers adjacent to the yolk, corresponding to mesendoderm and mesoderm. (F) *bcox* expression in the head mesendoderm and in the ventral part of the optic primordia (see arrow) in a 18-somite embryo. (G) Cross-section through the optic primordium of a 21-somite embryo. (H,I) At 1 dpf, expression is found in the pharyngeal region, in individual cells around the eye, in subcutaneous cells of the head and trunk, as well as in the pectoral fin buds. (J,J′) In the zebrafish mutant *mob*^{m819} (J′) *bcox* expression is absent in the trunk (compare with J), and (K′) highly reduced in the head (compare with K), reflecting the impairments in neural crest development of this mutant (Barrallo et al., 2003). (L-N) At 2 dpf, *bcox* expression is found in the pharyngeal region (N), in the fin buds (L) and in single cells around the eyes (M). (O-Q) In larvae (O), *bcox* expression is found in the liver (P) and the gut (Q). (R) Western blot analysis of Bcox protein levels in protein extracts of zebrafish embryos. A single band with a molecular mass of 59 kDa was detected. As a control, recombinant murine Bcox (64 kDa) was used. ep, eye primordium; g, gut; hb, hindbrain; li, liver; nc, notochord; ov, otic vesicle; pe, pigment epithelium; pec, pectoral fin bud; ph, pharynx; sc, subcutaneous cells.

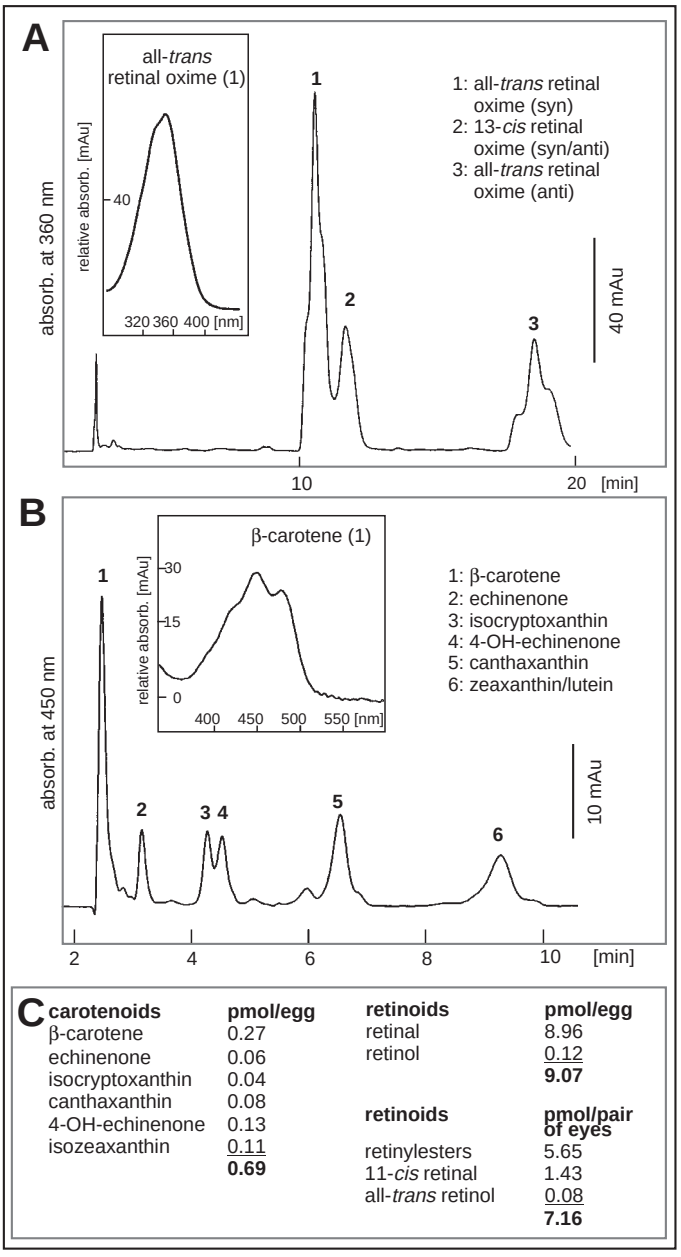
existence of provitamin A in the yolk, which has so far not been demonstrated in zebrafish. Therefore, we extracted lipophilic compounds from freshly fertilized eggs and subjected them to HPLC-analyses (Fig. 3A,B). In the egg extracts, we detected and identified various carotenoids by this analysis. As summarized in Fig. 3, the major carotenoid was β-carotene, but, additionally, significant amounts of C_4-substituted carotenoids (echinenone, isocryptoxanthin, canthaxanthin, 4-hydroxy-echinenone) and C_3-substituted carotenoids (lutein, zeaxanthin) were present, as judged by their retention times and spectral characteristics compared to authentic standards. As quantified by HPLC analyses, yolk retinal existed in substantial molar excess compared to carotenoids in a ratio of 100 to 6. Costaridis et al. (Costaridis et al., 1996) have already shown that embryonic RA levels are low compared with total egg yolk retinoid contents. To address the fate of these large amounts of yolk retinal, we analyzed the retinoid content of the larval eyes (4 dpf). Here, we recovered already ~80% of the total egg retinal as retinylesters and 11-cis-retinal with only traces of free retinol. Thus, the major destination of the huge amounts of yolk retinal is the visual system. Based on these analyses, we concluded that the yolk provitamin A content, even though low compared with retinal content, would be sufficient to contribute to embryonic RA signaling.

Morpholino-mediated inhibition of *bcox* translation results in embryonic malformations

Having identified all prerequisites for provitamin A conversion in the zebrafish embryo, we next tested for its actual requirement by performing targeted gene knockdown experiments using antisense morpholino oligonucleotides (Nasevicius and Ekker, 2000). We designed an MO spanning the region +1 to + 25 (*bcox*-MO) of the *bcox* mRNA, as predicted from its cDNA sequence, and injected increasing amounts (1.5, 9 or 18 ng) of *bcox*-MO or the same amounts of the co-MO. We confirmed the efficient targeted knockdown of Bcox translation by immunoblot analyses. When compared with controls, a strong reduction in Bcox levels was detected in protein extracts from embryos treated with 9 ng of *bcox*-MO (Fig. 4G). *bcox*-MO-injected larvae but not the controls developed malformations, which occurred in a dose-dependent

manner. The body axis of *bcox*-MO-injected larvae was curved and the eyes were much smaller (Fig. 4B-D). Morphants raised from eggs injected with larger amounts (9 and 18 ng) of *bcox*-MO developed in addition an edematically enlarged pericardial cavity (Fig. 4C,D). *bcox* morphants also developed a pronounced pharyngeal phenotype in that they lacked gill arches and tissue in the branchial region (Fig. 6A-D). Furthermore, the pectoral fins were reduced in size (Fig. 6F,G) and *bcox* morphants did not inflate the swim bladder. Additionally, the pigmentation was altered and particularly the iridophores were absent (Fig. 4E,F).

To test whether these malformations are a specific effect of Bcox deficiency, we tried to rescue the morphants phenotype by co-injection of various amounts of *bcox* mRNA with *bcox*-MO (9 ng). Co-injection of mRNA balanced the morphants phenotype as judged by visual inspection of the larvae and by Alcian Blue staining of the arch skeleton (see Fig. 6E). Furthermore, we repeated the experiments with a different MO spanning the region –24 to –1 of the *bcox* mRNA (*bcox*-MO2). Injection of *bcox*-MO2 caused similar malformations of the arch skeleton and eyes, but about twice the amount of *bcox*-



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carotenoids	pmol/egg
β-carotene	0.27
echinenone	0.06
isocryptoxanthin	0.04
canthaxanthin	0.08
4-OH-echinenone	0.13
isozeaxanthin	0.11
	0.69

retinoids	pmol/egg
retinal	8.96
retinol	0.12
	9.07

retinoids	pmol/pair of eyes
retinylesters	5.65
11-*cis* retinal	1.43
all-*trans* retinol	0.08
	7.16

Fig. 3. Carotenoid and retinoid contents of zebrafish eggs and larval eyes. (A) Retinoid composition of the zebrafish eggs. A HPLC profile at 360 nm of an extract isolated from 180 zebrafish eggs separated on HPLC system 1. (B) Carotenoid composition of zebrafish eggs. A HPLC-profile at 450 nm of an extract isolated from 165 eggs separated on HPLC system 2. (C) Carotenoid and retinoid contents of zebrafish eggs and larval eyes (4 dpf). The values give the means of at least two independent experiments. The individual retinoids and carotenoids were analyzed and quantified on HPLC systems 1 or 2, respectively. Molar amounts were calculated by equilibration of the HPLC systems with defined amounts of authentic reference substances.

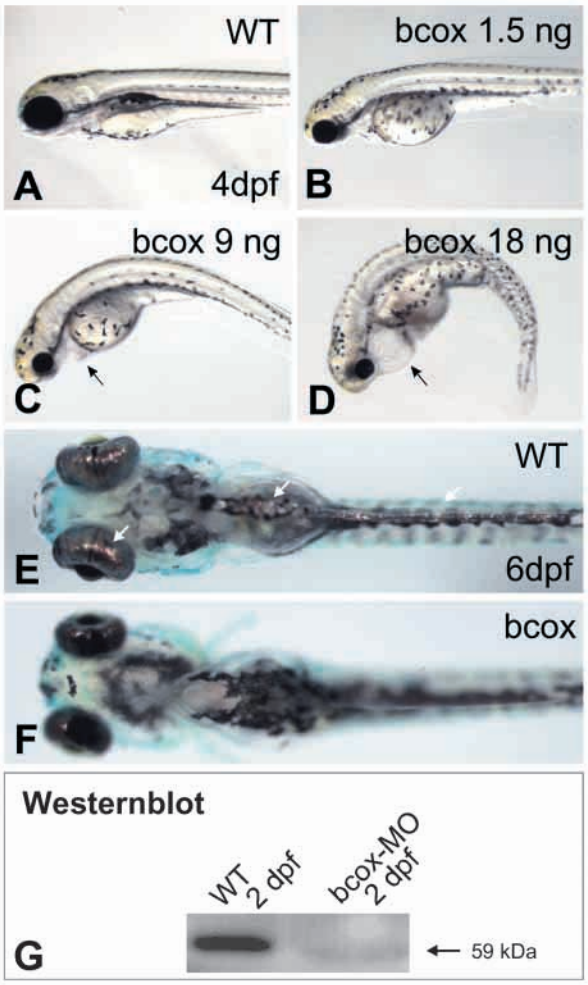

Fig. 4. Morphological phenotype of *bcox* morphants. (A-D) Lateral views of 4 dpf *bcox* morphants raised from eggs injected with 1.5 ng (B), 9 ng (C) and 18 ng (D) *bcox*-MO compared with a non-treated sibling (A). The *bcox*-MO treatment provoked malformations, i.e. a curved neck, smaller eyes and an edematically enlarged pericardial region (see arrows) occurred dose dependently in repeated independent experiments. (E,F) The pigmentation of *bcox* morphants (F) is altered when compared with controls (E). As indicated by arrows, the iridophores are lacking. (G) Western blot analysis of Bcox protein levels in protein extracts derived from 2-day-old *bcox*-MO-treated embryos and wild-type siblings.

MO2 was required, when compared with *bcox*-MO. To avoid potential artifacts associated with injection of large amounts of MOs, we decided to use 9 ng of *bcox*-MO in all further experiments.

Targeted gene knockdown of *bcox* interferes with hindbrain development

As evidenced in mouse, chicken and zebrafish embryos, RA signaling is crucial in patterning of caudal hindbrain and determining rhombomere identity (Maden et al., 1996; White et al., 2000; Niederreither et al., 1999; Niederreither et al., 2000; Dupé and Lumsden, 2001). *bcox* mRNA expression was found in mesendoderm and ventral mesoderm, suggesting that it may, in combination with *raldh2*, contribute to generating RA during segmentation stages. To distinguish individual hindbrain rhombomeres, we analyzed the expression of *krx20* in r3 and r5 and the Eph-related receptor tyrosine kinase *efna4* in r1, r3 and r5. We found that r1 to r5 had a normal positional identity in *bcox* morphants (Fig. 5A-D). We then investigated whether *bcox* morphants develop patterning defects in hindbrain rhombomeres posterior to r5, as has been described for zebrafish embryos mutant for *neckless* (*nls/raldh2*) or *no-fin* (*nof/raldh2*), which are both alleles of *raldh2* (Begemann et al., 2001; Grandel et al., 2002). For this purpose, we analyzed *valentino* (*val*) expression in r5 and r6, as well as expression of *hoxb4a*, which is expressed with an anterior boundary between r6 and r7. *val* appears to be normally expressed in r5 and r6 in *bcox* morphants (Fig. 5E,F). We also performed whole-mount in situ hybridization for combinations of *krx20* and *myod*, as well as *fgf8* and *val*, to analyze whether the caudal hindbrain was extended and posteriorized towards the somite boundary, as in zebrafish *raldh2* mutants. *bcox* morphants did not develop these features of *no-fin* and *neckless* mutants (Fig. 5G-J).

By contrast, *hoxb4a* expression in *bcox* morphants appeared reduced, with a less distinct anterior expression boundary, and did not extend as far caudally as in wild-type controls (Fig. 5K,L). To provide evidence that this altered *hoxb4a* expression is caused by RA insufficiencies, we included exogenous RA (10^{-9} M) in the

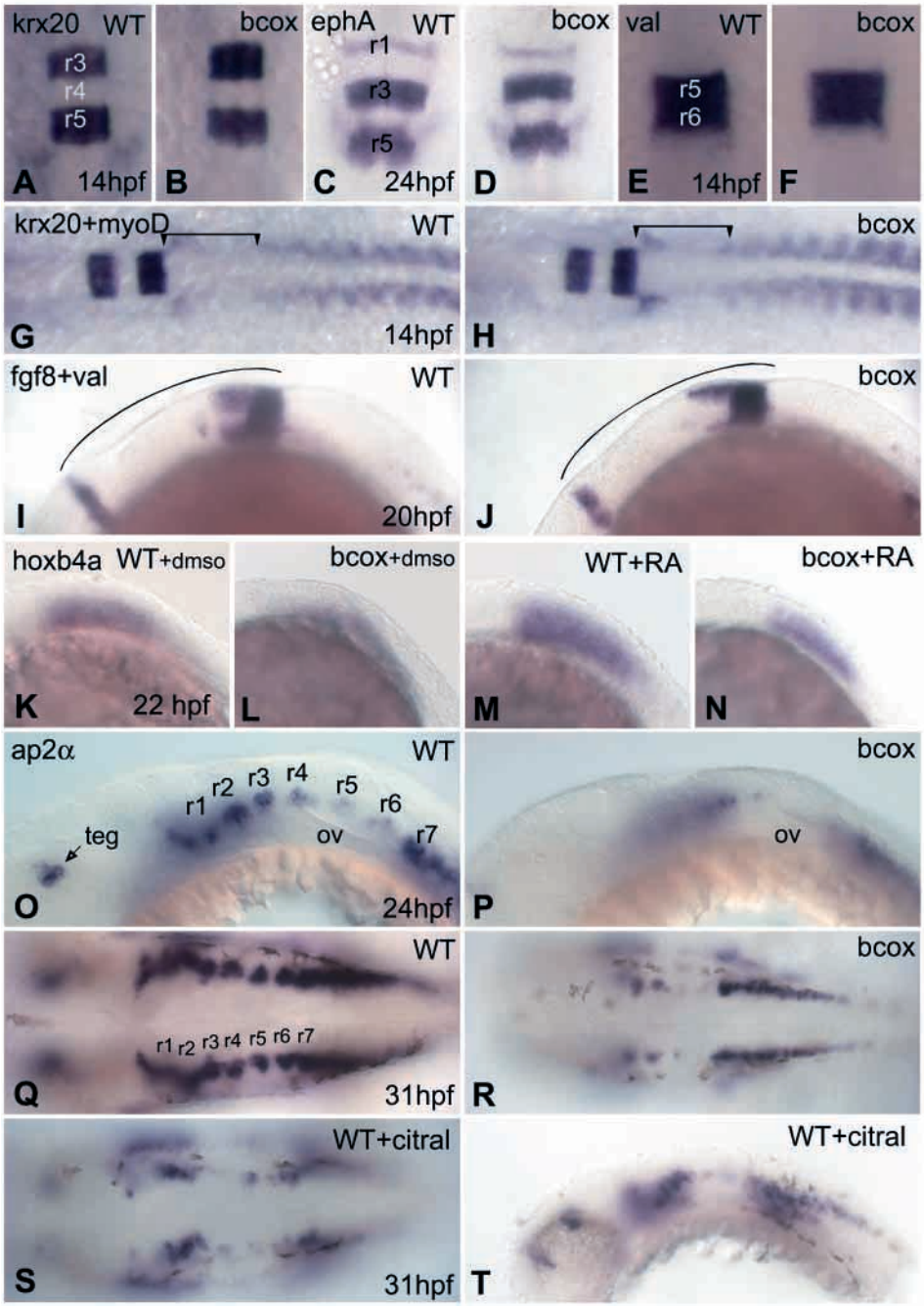

Fig. 5. Analyses of hindbrain development of *bcox* morphants. The expression patterns of *krx20* (A,B) *efna4* (C,D) and *val* (E,F) reveal no obvious alterations in the identity of rhombomeres 1 to 5 in *bcox* morphants when compared with controls (14 hpf/24 hpf, dorsal views). Whole-mount in situ hybridization for combinations of *krx20* and *myod* (G,H, 14 hpf, dorsal view), as well as *fgf8* and *val* (I,J, 20 hpf, lateral view) reveal no alterations in the distance between r5 and the first somite or between the midbrain hindbrain border and r5 (the bar and the curve give the distances found in wild-type embryos, respectively). (K,L, 22 hpf, lateral view) *hoxb4a* expression is reduced in *bcox* morphants (L) when compared with controls (L). (M,N, 22 hpf, lateral view) Treatment with 10^{-9} M RA restores *hoxb4a* expression in *bcox* morphants (N). (O,P, 24 hpf, lateral view; Q,R, 31 hpf, dorsal view) In *bcox* morphants (P,R), *ap2α* expression is reduced along the whole rostrocaudal expression domain in hindbrain and spinal cord, and is absent at the level of the otic vesicle. (S, dorsal, T, lateral) Citral treatment of wild-type embryos provoked similar changes in *ap2α* expression as found in *bcox* morphants. ov, otic vesicle; r, rhombomeres; teg, tegmentum.

incubation medium. The RA treatment restored *hoxb4a* expression in *bcox*-MO-injected embryos nearly to the level of non-injected siblings (Fig. 5M,N; *n*=25). During late somitogenesis and pharyngula stages, RA has been shown to be involved in specification of various derivatives of the hindbrain and branchial region in chicken embryos (Gale et al., 1996). To investigate potential later requirements for *bcox* in hindbrain development, we analyzed the expression of the transcription factor AP-2-alpha (*ap2α*), which is modulated by RA in the caudal hindbrain (J.H., A. Barrallo, A. K. Ettl, E. Knapik and W.D., unpublished). At 24 hpf, *ap2α* is expressed bilaterally in two stripes on each side in the hindbrain and anterior spinal cord of wild-type embryos. In *bcox* morphants, *ap2α* expression was reduced along the whole rostrocaudal expression domain in hindbrain and spinal cord, and was found to be completely or nearly absent at the level of r4 and r5 (Fig. 5O-R). *ap2α* expression was also reduced at the level of the tegmentum in *bcox* morphants (Fig. 5O,P). Similar impairments of *ap2α* expression in the hindbrain could be induced in wild-type embryos treated with the retinal dehydrogenase inhibitor citral, indicating that altered *ap2α* expression is indeed caused by RA insufficiency (Fig. 5S,T).

In summary, these analyses revealed that targeted knockdown of *bcox* did not interfere with RA-dependent specification of rhombomere identity during anteroposterior patterning of the neural tube. However, reduced *hoxb4a* expression as well as altered *ap2α* expression in the hindbrain suggested that a lack of *bcox* activity may interfere with later aspects of hindbrain development.

Impaired development of neural crest derivatives in the pharyngeal region of *bcox* morphants

For a more detailed analysis of the craniofacial and branchial arch phenotype, we stained the cartilage of the head skeleton

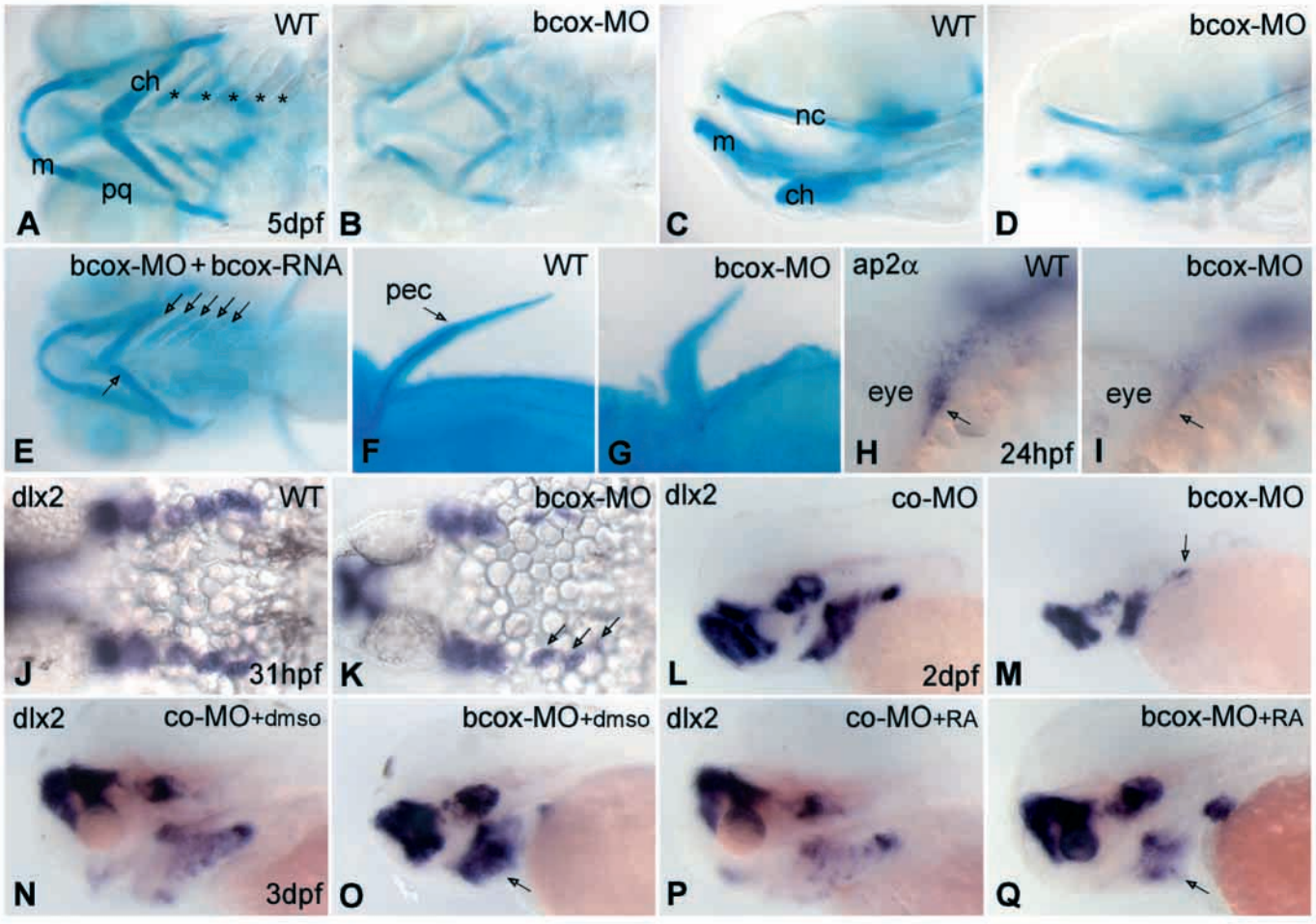

Fig. 6. Impairments of the development of the craniofacial skeleton in *bcox* morphants. (A-D) Alcian Blue staining of the cartilage of the craniofacial skeleton of *bcox* morphants (B,D) when compared with controls (A,C). The Meckel's cartilage and palatoquadrates are deformed, the ceratohyales project posteriorly, and the ceratobranchials are reduced or absent (see asterisks). (E) Co-injection of *bcox*-MO with *bcox* mRNA rescued the morphant phenotype (arrows indicates the restored cartilage). (F,G) The pectoral fins of *bcox* morphants are shortened and project laterally. (H,I) *ap2α* expression in the pharyngeal region at 24 hpf (lateral view) is reduced in cranial neural crest in *bcox* morphants when compared with controls (see arrows). At 31 hpf (J,K dorsal view), *dlx2* expression is reduced in the posterior arch primordia in *bcox* morphants (K, see arrows). At 2 dpf (L,M, lateral view), *dlx2* expression is altered in anterior arch primordia, whereas it is reduced in the posterior arch primordia in *bcox* morphants (M) when compared with controls (L; see arrow in M). At 3 dpf (N,O, lateral view), *dlx2* expression persisted in the pharyngeal region in *bcox* morphants (O, see arrow) whereas it is absent in controls (N). (P,Q, lateral view) Application of exogenous RA (10^{-7} M) results in a downregulation of *dlx2* expression in pharyngeal region of 3 dpf *bcox* morphants (Q, see arrow). ch, ceratohyal; m, Meckel's cartilage; nc, neurocranium; pec, pectoral fins; pq, palatoquadrate. In all pictures, anterior is towards the left.

with Alcian Blue. The first and second arches were present in the morphants but malformed and less chondrofied (Fig. 6A-D). The first arch was smaller and pinched in the middle and its anterior portions (Meckel's cartilages) were foreshortened and flattened in the front, while the palatoquadrates were shortened as well as flared posteriorly. As seen in lateral views, Meckel's cartilages projected ventrally. The hyoid arch was completely distorted and the ceratohyales projected posteriorly rather than anteriorly. From arches 3 to 7, the ceratobranchials were highly reduced or absent in all morphants. In contrast to elements of the arch skeleton, the neurocranium was properly formed in all *bcox* morphants analyzed (n=61). Alcian Blue staining also revealed that pectoral fins were present in *bcox* morphants but they were reduced in size and projected laterally rather than caudally (Fig. 6F,G).

As shown above, *bcox* expression was found in cranial neural crest. A direct role of RA signaling for neural crest cell differentiation and survival in the pharyngeal region has been revealed by the phenotypes of *rara*/*rarb* double mutants, as well as by *raldh2* mutants (Dupé et al., 1999; Begemann et al., 2001; Niederreither et al., 1999; Maden et al., 1996). We analyzed *ap2α* and *dlx2* expression to visualize cranial neural crest contributing to branchial arches in *bcox* morphants. At 24 hpf, *ap2α* expression was reduced in cranial neural crest in the pharyngeal region as compared with controls (Fig. 6H,I). At 31 hpf, *dlx2* mRNA expression was detected in all streams of hindbrain neural crest in both wild-type controls and *bcox*-MO-injected embryos, but it was highly reduced in the posterior arch primordia of *bcox* morphants (Fig. 6J,K). At 2 dpf, *dlx2* mRNA expression was detectable in both *bcox* and control morphants in mandibular and hyoid arch primordia, but its expression was mostly absent in the posterior arch primordia of *bcox*-MO-treated embryos (Fig. 6L,M). These alterations in *dlx2* expression indicated that targeted knockdown of Bcox interferes with the development of posterior cranial neural crest streams similar to what has also been reported for zebrafish *raldh2* mutants (Begemann et al., 2001; Grandel et al., 2001).

To pursue the further fate of pre-otic neural crest, we analyzed *dlx2* expression in 3 dpf larvae (Fig. 6N,O). In controls, *dlx2* mRNA expression was downregulated, consistent with the beginning of condensation and cartilage formation (Ellies et al., 1997). By contrast, in *bcox* morphants strong *dlx2* expression persisted in the pharyngeal region. To test whether this prolonged *dlx2* mRNA expression may be caused by a lack of RA, we applied exogenous RA at 2 dpf and analyzed *dlx2* expression 24 hours later. RA treatment resulted in a downregulation of *dlx2* mRNA expression (n=20), whereas in DMSO-treated *bcox* morphants (n=20) *dlx2* mRNA persisted in the pharyngeal region (Fig. 6N-Q).

To summarize, *bcox* morphants develop a severe branchial arch phenotype with altered morphology of the mandible and hyoid arches, as well as a loss of gill arches. *dlx2* expression was significantly altered in the neural crest, reflecting impairments in neural crest survival or differentiation particularly in the posterior arch primordia.

bcox is needed to establish the ventral retina

RA signaling has been shown to be crucial to establish the ventral part of the retina during early eye development in zebrafish (Marsh-Armstrong et al., 1994; Hyatt et al., 1996). We first investigated the eyes of *bcox* morphants using Nomarski optics. At 24 hpf, the borders of the presumptive ventral retina were less distinct and the choroid fissure was enlarged when compared with wild-type controls (data not shown). Therefore, we analyzed the expression of *pax6.1*, *pax2.1* and *shh*: *pax6.1* is expressed throughout the optic vesicles in all cells of the prospective retina, the retinal pigment epithelium and lens epithelium, whereas *pax2.1* expression is restricted to the optic stalk and retinal cells around the choroid fissure. These Pax gene expression territories are influenced by midline signaling via Shh (Macdonald et al., 1995).

At 30 hpf, analyses of *pax6.1* expression showed that the prospective retina was smaller and particularly reduced on the ventral side of the eyes (Fig. 7A,B,D,E), whereas the *pax2.1* expression domain was extended and governed the enlarged choroid fissure of *bcox* morphants (Fig. 7G,H). Analyses of *shh* expression revealed that it was not significantly altered in the forebrain of *bcox* morphants at early (14 hpf, Fig. 7I,J) or later embryonic stages (24 hpf, data not shown). We then tried to phenocopy the MO-provoked impairments by applying the Raldh inhibitor citral to the incubation medium. Similar to *bcox*-MO treatments, citral treatments of embryos resulted in a reduction of the prospective ventral retina that was mirrored by a diminished *pax6.1* expression domain (Fig. 7C,F).

To learn more about later developmental consequences, we also examined cross-sections through the larval eyes of *bcox* morphants. The global stratification of the retina was still observed, but the eyes were much smaller. Most strikingly, the outer segments of the photoreceptors appeared to be shortened and the cells less well stratified when compared with controls (Fig. 7K,L). Induction of a comparable retinal phenotype upon citral treatment has been described for zebrafish larvae (Marsh-Armstrong et al., 1994). We also determined the retinoid contents of the eyes of 4 dpf larvae by quantitative HPLC analyses and found a threefold reduction in *bcox* morphants, most probably reflecting the reduced number of photoreceptor cells (Fig. 7M).

DISCUSSION

The aim of this study was to elucidate whether provitamin A conversion, as an alternative to preformed supplies of maternally derived vitamin A, impacts embryonic retinoid metabolism and thus RA-dependent developmental processes in zebrafish. Based on our recent molecular identification of the β-carotene-15,15′-oxygenase (*bcox*), which catalyzes the conversion of provitamin A to retinal, we cloned its zebrafish homologue and report here on its expression patterns during embryogenesis. As judged by HPLC-analyses, provitamin A, the substrate of the enzyme, is stored in the zebrafish egg. Therefore, the components required for an endogenous provitamin A conversion are present in the zebrafish embryo. We performed a targeted gene knockdown of *bcox* with the antisense morpholino oligonucleotide technique and examined its phenotype. Detailed analyses of *bcox* morphants provide strong evidence that several RA-dependent developmental processes rely directly on tissue- or cell-specific provitamin A conversion and indicate a crucial functional role of carotenoids as precursors to RA in zebrafish development.

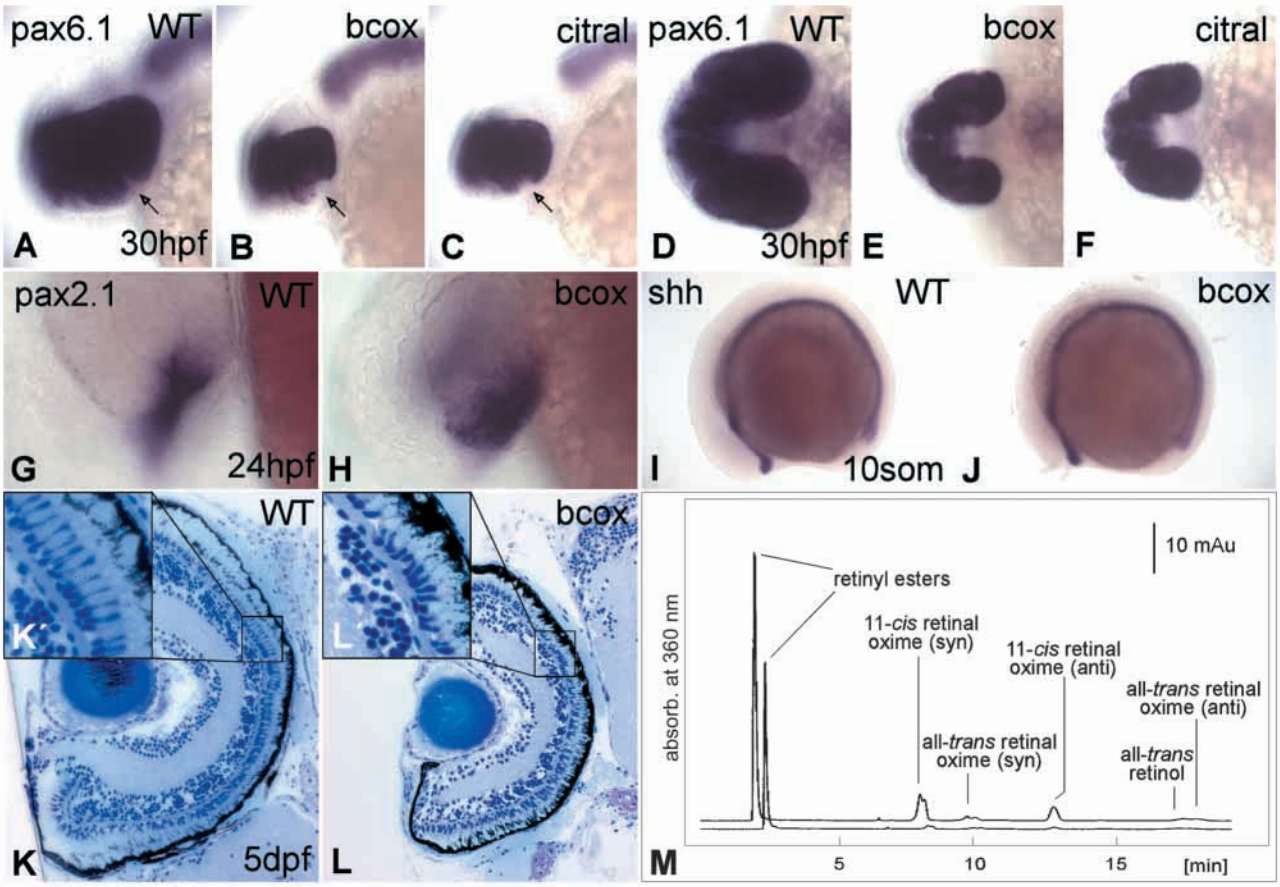

Fig. 7. Impairments of the eyes of *bcox* morphants. (A,D) *pax6.1* expression at 30 hpf in wild-type embryos, (B,E) *bcox* morphants and (C,F) citral-treated wild-type embryos (lateral and dorsal views). As indicated by arrows, *pax6.1* expression is particularly reduced in the ventral retina of *bcox* morphants and citral-treated wild-type embryos. (G,H) The *pax2.1* expression domain is enlarged in the optic stalk of *bcox* morphants (H) when compared with controls (G). (I,J) *shh* expression is not altered in *bcox* morphants (J) when compared with controls (I). (K,L) Cross-sections through the eyes of *bcox* morphants (L) and control siblings (K) at the same magnification. (K′,L′) The insets show a higher magnification of the outer layers of the retina. Note the shortened and less stratified outer segments of the photoreceptors in *bcox* morphants (L′). (M) Retinoid composition of the eyes of 4 dpf larvae of wild type (upper trace) when compared with *bcox* morphants (lower trace). The figure shows the profiles at 360 nm of extracts isolated from 80 eyes per trace and separated on HPLC system 1.

Both yolk-derived retinal and provitamin A are needed for hindbrain development

Raldh2 is already expressed during gastrulation and somitogenesis in mouse, chicken, *Xenopus* and zebrafish embryos. In several studies, it has been conclusively shown that Raldh2-dependent conversion of retinal to RA is required for anterioposterior patterning in the neural tube (for a review, see Gavalas, 2002). *bcox* is expressed in ventral cell layers adjacent to the yolk during segmentation, raising the possibility that it may contribute retinal and act in coordination with Raldh2 in RA-dependent hindbrain patterning. *bcox* morphants showed normal position and identity of hindbrain rhombomeres. This clearly excludes an early role of provitamin A conversion in RA-dependent anterioposterior patterning of the neural tube. By treatment with the RAR receptor pan-antagonist BMS493, Grandel et al. have shown that RA is required as early as the 30% epiboly stage for patterning of the hindbrain and spinal cord, as well as for the formation of pectoral fin buds in zebrafish (Grandel et al., 2002). In our analyses, *bcox* expression was not detectable prior to segmentation stages, thus strongly indicating that yolk-derived retinal is used for RA generation by Raldh2.

However, the phenotype of *bcox* morphants suggests a developmental role of provitamin A conversion during segmentation stages. Blocking RA signaling using BMS493 beginning at tail bud stage also failed to interfere with the patterning of hindbrain rhombomeres, but caused a diminished *hoxb4a* expression at the caudal hindbrain boundary and malformed pectoral fins (Grandel et al., 2002), impairments like those observed in *bcox* morphants. In addition, altered *ap2α* expression in the hindbrain in *bcox* morphants is also observed in the zebrafish *raldh2* mutant *neckless* (J.H., A. Barrallo, A. K. Ettl, E. Knapik and W.D., unpublished) or in our study of wild-type embryos treated with citral. This RA-dependent induction of *ap2α* expression in the caudal hindbrain is most probably required for the development of noradrenergic neurons in medulla oblongata and area postrema (J.H., A. Barrallo, A. K. Ettl, E. Knapik and W.D., unpublished). Thus, our data point to a biphasic contribution of different sources for retinal during zebrafish hindbrain development. At pre-segmentation stages, RA generation depends on preformed yolk retinal, which must be liberated from its vitellogenin-bound form in order to freely diffuse to

raldh2-expressing cells. At segmentation stages, the embryo relies at least in part on provitamin A and *bcox* function to provide precursors for RA generation. This need for ongoing provitamin A conversion to retinal for RA-dependent developmental processes is strongly evident from the phenotypes of *bcox* morphants affecting the eye, late differentiation in the hindbrain, as well as the development of branchial arches. To exert this role during embryonic development, yolk provitamin A first must be delivered to the site of action to become metabolically converted to retinal by *bcox* function. Recently, we identified a cell surface receptor (*ninaD*) that mediated the cellular uptake of carotenoids in *Drosophila* (Kiefer et al., 2002). Orthologs of *ninaD* also exist in vertebrates and may be involved in provitamin A metabolism in vertebrates as well.

Patterning defects in pharyngeal arches

bcox morphants develop defects in neural crest derivatives, such as diminished pigmentation and a malformed craniofacial skeleton. Our data indicate that *bcox* itself is expressed in cranial and trunk neural crest. So far, carotenoids and neural crest have been discussed solely with respect to neural crest-derived pigmentation (Kimler and Taylor, 2002). Our analyses suggest an additional role of carotenoids as precursors for retinoids for subsequent RA generation and signaling in neural crest contributing to the craniofacial skeleton. Several studies already showed that RA is indispensable for the development of caudal branchial arches in mouse, chicken and zebrafish (Niederreither et al., 1999; Maden et al., 1996; Wendling et al., 2000; Begemann et al., 2001). As discussed above, hindbrain segmentation was not altered in *bcox* morphants, thus excluding that defects in neural crest cell migration and branchial arches may be secondary to defects in anterioposterior hindbrain patterning. Emigration of all streams of hindbrain neural crest was also verified by the analyses of *dlx2* expression, showing that targeted knockdown of *bcox* may interfere with neural crest cells at later stages, affecting differentiation and potentially cell survival. Consistently, *dlx2* staining in posterior arch primordia was highly reduced already at 31 hpf and mostly abolished at 2 dpf in *bcox* morphants. Similar impairments have been reported in zebrafish *raldh2* mutants, indicating that *bcox* and *raldh2* may act sequentially in RA generation in caudal arch primordia (Begemann et al., 2001; Grandel et al., 2002). Interestingly, *raldh2* is expressed in the caudal part of branchial arch primordia but not in neural crest (Grandel et al., 2002). Therefore, retinoids synthesized via Bcox in neural crest cells may be delivered to *raldh2*-expressing cells. For example, a close association of migrating neural crest cells and *raldh2*-expressing mesodermal cells has been shown in chick embryos (Berggren et al., 1999). This *bcox*-dependent RA signal may then induce differentiation and prevent apoptosis in mesoderm/endoderm or neural crest or in all tissues. The transport of retinal, the initial product of β-carotene cleavage, between *bcox* and *raldh2*-expressing cells may additionally involve retinoid-oxidizing as well as retinoid-binding proteins. Even though so far we cannot exclude the expression of a different type of *raldh* in zebrafish neural crest, the loss of gill arches in *bcox* morphants and *raldh2* mutants supports a signaling mechanism in which *bcox* may locally supply retinal.

The altered morphology of the mandible and hyoid arch in *bcox* morphants indicate a putative role of *bcox* for the development of anterior arches. Here, in contrast to gill arches, *dlx2* expression was not abolished, but rather increased and its expression persisted at developmental time points when it was downregulated in controls at the beginning of condensation and cartilage formation (Ellies et al., 1997). This excludes a need of RA for the survival of pre-otic neural crest but, rather suggesting a role of *bcox* for the differentiation of these cells at later embryonic stages. The prolonged *dlx2* expression in anterior neural crest streams was downregulated by applying exogenous RA, thus substantiating that targeted knockdown of *bcox* interferes with RA-signaling here. As a result, the mandible and hyoid arch possess an altered morphology and are less chondrofied in *bcox* morphants than in controls. Malformations in first arch derived skeletal structures have been also reported for *Rara*/*Rarg* double null mice (Lohnes et al., 1994). In addition, effects of RA on chondrogenesis and synthesis of extra-cellular matrix macromolecules have been shown, i.e. in chicken craniofacial mesenchyme (Sakai and Langille, 1992). As *raldh2* is not expressed in anterior parts of the developing pharynx (Grandel et al., 2002), it must be supposed that a different type of *raldh* may act downstream of *bcox* in the pharyngeal region at later developmental time points. Besides branchial arches, neural crest-derived pigmentation was also impaired in *bcox* morphants, indicating that *bcox* may play a more general role in the development of cranial and trunk neural crest derivatives.

Impairments of the development of the ventral retina in *bcox* morphants

The eyes are known to be especially vulnerable against imbalances in retinoid levels during their development. Older studies already revealed that vitamin A deprivation of mammals during distinct developmental time windows results in microphthalmia of otherwise normal offspring (Warkany and Schraffenberger, 1946). Molecular analyses suggest a role of RA in patterning the dorsal-ventral axis of the eyes, as *Rara* and *Rarb* double mutant mice develop a reduced ventral retina and, as a consequence, microphthalmia (Kastner et al., 1994). A role of RA in specifying ventral characteristics of the retina has been also shown in zebrafish. Exogenous RA application results in proliferation of cells in the ventral region of the retina, leading to retina duplication (Hyatt et al., 1992; Hyatt et al., 1996), whereas inhibition of endogenous embryonic RA synthesis by citral results, by contrast, in strong reduction of the ventral retina (Marsh-Armstrong et al., 1994). A crucial process with respect to RA perturbations is the formation of the optic primordia (Hyatt et al., 1992). *bcox* is expressed at the ventral side of the primordia, beginning with early somite stages. *bcox* knockdown resulted in smaller eyes and on the cellular level in shortened and less stratified outer segments of the photoreceptors at larval stages. At 30 hpf, *bcox* morphants already exhibited morphological changes, i.e. a reduced ventral retina and an enlarged choroid fissure. At the molecular level, this was mirrored by reduced *pax6.1* expression in the presumptive retina. Comparable morphological changes and reduced *pax6.1* expression were also provoked by treatments with the Raldh inhibitor citral (this study) (Marsh-Armstrong et al., 1994), thus showing that targeted knockdown of *bcox* function phenocopies RA deficiency during eye development. A reciprocal regulation of *pax6* and *pax2* expression territories

in establishing the boundary between the optic cup and stalk has been previously shown in mice (Schwarz et al., 2000). The analysis of *pax2.1* expression revealed an enlarged expression domain in the eyes of *bcox* morphants. However, *pax2.1* expression remained restricted to the ventral part of the eyes (choroid fissure and optic stalk) and did not extend dorsally into the prospective neural retina, as has been described in zebrafish exposed to exogenous RA (Hyatt et al., 1996). This indicates that the expanded *pax2.1* expression domain is most probably a consequence of the loss of *pax6.1* expression in the ventral retina, rather than of a per se upregulated *pax2.1* expression. In addition, *shh* expression is normal, indicating no alterations in midline signaling in *bcox* morphants. A role for RA signaling in maintaining *shh* expression during forebrain development has recently been suggested for chicken embryos (Schneider et al., 2001).

Zymographic assays revealed that RA is first synthesized in the ventral retina beginning at the 12-somite stage in zebrafish, and that two different types of Raldh exist in the eyes (Marsh-Armstrong et al., 1994). In transgenic reporter zebrafish, a RA-responsive reporter gene also responds to RA first in the ventral retina and RA production in the dorsal retina occurs only later (Perz-Edwards et al., 2001). In zebrafish, *raldh2* expression is restricted to the dorsal retina (Grandel et al., 2002). In mice, a new type of Raldh, *Raldh3*, has been identified. It is expressed in the ventral retina during eye development and is most probably responsible for RA generation here (Mic et al., 2000; Li et al., 2000). The fact that zebrafish *raldh2* mutants show no obvious eye phenotype (Begemann et al., 2001; Grandel et al., 2002) indicates that a *Raldh3* orthologue might also exist in zebrafish that could act downstream of Bcox. Based on our data, loss of this putative *Raldh3* function might result in an eye phenotype comparable with the one described here for *bcox* morphants.

Conclusions

Our study reveals that *bcox* and provitamin A carotenoids play a vital role during zebrafish development. Comparable impairments as in *bcox* morphants have also been described in zebrafish *raldh2* mutants or were provoked by citral treatments of wild-type embryos (Begemann et al., 2001; Grandel et al., 2001; Marsh-Armstrong et al., 1994). Thus, our analyses identify the β,β-carotene-15,15′-oxygenase acting upstream of Raldhs and strongly suggest that provitamin A conversion is the prerequisite for RA-signaling in several distinct developmental processes in zebrafish embryos. The use of the non-toxic provitamin instead of preformed yolk retinal for RA-signaling processes may provide an additional control mechanism to finely balance retinoid levels at the cellular level in local tissue environments. We cannot yet unequivocally exclude the possibility that any of the ADH or SDR family members acts in between provitamin A conversion and RA generation. In zebrafish, application of the ADH1 inhibitor 4-methylprazole did not interfere with development (Costaridis et al., 1996). Additionally, *Adh1*- and *Adh4*-null mice show no impairments during development when sufficient vitamin A is available for the embryo (Deltour et al., 1997; Deltour et al., 1999). Recent results indicate that *Adh3*, a ubiquitously expressed retinol-oxidizing enzyme, is needed for mouse development (Molotkov et al., 2002). Interestingly, embryonic expression of a *bcox* homologue has been also reported in mice, indicating that a developmental function of provitamin A conversion may exist in higher vertebrates as well (Redmond et al., 2001). Thus, *Adh3* and *bcox* may both play a role during embryonic development by delivering retinal for certain aspects of retinoid signaling. Our findings in zebrafish promise to elucidate new aspects for provitamin A and carotene-oxygenases in vertebrate development.

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