BCDO2 acts as a carotenoid scavenger and gatekeeper for the mitochondrial apoptotic pathway

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SUMMARY
Carotenoids and their metabolites are widespread and exert key biological functions in living organisms. In vertebrates, the carotenoid oxygenase BCMO1 converts carotenoids such as β,β-carotene to retinoids, which are required for embryonic pattern formation and cell differentiation. Vertebrate genomes encode a structurally related protein named BCDO2 but its physiological function remains undefined. Here, we show that BCDO2 is expressed as an oxidative stress-regulated protein during zebrafish development. Targeted knockdown of this mitochondrial enzyme resulted in anemia at larval stages. Marker gene analysis and staining for hemoglobin revealed that erythropoiesis was not impaired but that erythrocytes underwent apoptosis in BCDO2-deficient larvae. To define the mechanism of this defect, we have analyzed the role of BCDO2 in human cell lines. We found that carotenoids caused oxidative stress in mitochondria that eventually led to cytochrome c release, proteolytic activation of caspase 3 and PARP1, and execution of the apoptotic pathway. Moreover, BCDO2 prevented this induction of the apoptotic pathway by carotenoids. Thus, our study identifying BCDO2 as a crucial protective component against oxidative stress establishes this enzyme as mitochondrial carotenoid scavenger and a gatekeeper of the intrinsic apoptotic pathway.

KEY WORDS: Carotenoids, Zebrafish, Mitochondria, BCDO2 (BCO2), Reactive oxygen species, Apoptosis

INTRODUCTION
Animals, including humans, metabolize carotenoids to apocarotenoids (such as retinaldehyde, the chromophore of G-protein-coupled receptors that mediate phototransduction) (Palczewski, 2006) and to all-trans-retinoic acid, a hormone-like compound that is crucial for processes as diverse as embryonic development, tissue homeostasis and immunity (Chambon, 1996; Agace, 2008). For carotenoid metabolism, vertebrate genomes encode two types of carotenoid-cleaving enzymes annotated as β-carotene-15,15'-monooxygenase (BCMO1) and β-carotene-9',10'-dioxygenase (BCDO2, also named BCO2 and CMO2) (von Lintig, 2010). BCMO1 is the key enzyme for vitamin A production (Hessel et al., 2007) and converts a limited number of proretinoid carotenoids, such as β,β-carotene (BC), to retinaldehyde by symmetric cleavage at position C15,C15 (Paik et al., 2001; Redmond et al., 2001; Lindqvist and Andersson, 2002). The second carotenoid-cleavage enzyme, BCDO2, displays broad substrate specificity towards various dietary carotenoids and catalyzes an eccentric oxidative cleavage at position C9',C10', resulting in the production of apocarotenoids different from retinoids (Hu et al., 2006; Mein et al., 2010; Amengual et al., 2011). There is also a marked difference in the subcellular localization of these two vertebrate carotenoid-oxygenases. BCMO1 is a cytoplasmic protein (Lindqvist and Andersson, 2002), whereas BCDO2 localizes to mitochondria (Amengual et al., 2011).

Though the biochemical function of BCDO2 as carotenoid cleavage enzyme is now well established, its physiological role is controversial. On the one hand, several studies propose that BCDO2 catalyzes the first step in pathways for the production of biologically active apocarotenoid signaling molecules (Lian et al., 2007; Ford et al., 2011). Such compounds are widespread in living organisms, e.g. in plants, and regulate important physiological responses (Moise et al., 2005). On the other hand, studies in Bcdo2 knockout mice implicate BCDO2 in carotenoid catabolism and homeostasis. When challenged with artificial diets, carotenoids accumulated in mutant animals and induced oxidative stress (Amengual et al., 2011). Vertebrates show significant differences in carotenoid metabolism and functions. Rodents such as mice display low to undetectable levels of these compounds in blood and tissues (Hessel et al., 2007; Amengual et al., 2011), indicating that they have developed mechanisms to prevent carotenoid accumulation. Indeed, recent research revealed that intestinal carotenoid absorption is under negative-feedback regulation by vitamin A (Lobo et al., 2010b) and that non-proretinoid carotenoids are rapidly metabolized by BCDO2 (Ford et al., 2010; Amengual et al., 2011). By contrast, many mammals, including humans, and oviparous vertebrates, such as birds and fish, have significant levels of carotenoids in blood and tissues. These carotenoids exert important physiological functions as colorants, antioxidants and filters of phototoxic blue light in the eyes, and are involved in the immune response (Bone et al., 2000; Blount et al., 2003; Krinsky et al., 2003). Thus, the issue arises as to whether BCDO2 functions are conserved between rodents and other members of the vertebrate kingdom.

To study carotenoid metabolism in such vertebrates, lower primates, gerbils and ferrets were used as models in several studies (Lee et al., 1999). But a major flaw in the use of these animals for research is the lack of manageable and cost-efficient protocols for their genetic manipulation. To overcome this problem and to further elucidate the role of BCDO2 in carotenoid metabolism, we took advantage of the zebrafish model (Danio rerio) and human
cell lines, both systems being highly suited for genetic and pharmacological manipulation (Lieschke and Currie, 2007). More importantly for this study, carotenoids are crucial for zebrafish development and the egg yolk contains significant amounts of these compounds that must be mobilized and distributed during embryogenesis (Lampert et al., 2003).

MATERIALS AND METHODS

Materials

All chemicals, unless stated otherwise, were purchased from Sigma-Aldrich (St Louis, MO). Canthaxanthin and β,β-carotene (BC) were purchased from Wild (Germany).

Test for enzymatic activity

Full-length zebrafish bcdo2 cDNA (GenBank Accession Number AJ290391.1) was cloned into the expression vector pTRChis (Invitrogen, Carlsbad, CA). The plasmid was transfected into an E. coli strain capable of synthesizing BC and assays were performed as described previously (von Lintig and Vogt, 2000). For tests of enzymatic activity, murine BCDO2 was expressed as a recombinant protein in E. coli analyzed with canthaxanthin (Wild, Germany) and 4-oxo-N-(4-hydroxyphenyl)-all-trans-retinamide (4-oxo-4HPR) (Research Chemicals, Toronto, Canada) as previously described (Amengual et al., 2011).

Zebrafish strains and maintenance

Zebrafish (strain AB/TL) were bred and maintained under standard conditions at 28.5°C. Morphological features were used to determine the stage of the embryos in hours (hpl) or days (dpi) post fertilization. Embryos used for in situ hybridization were raised in the presence of 200 μM 1-phenyl-2-thiourea (PTU).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed according to published protocols (Isken et al., 2008). Bcdo2 was cloned into the vector pCRII-TOPO (Invitrogen, Grand Island, NY), and antisense RNA probes were synthesized as outlined by the manufacturer (Roche Applied Sciences, Indianapolis, IN). Additional RNA probes used for in situ hybridization experiments were for gata1, gata2, and bhae3.

Injections of morpholino oligonucleotides

For targeted knockdown of the BCDO2 protein, antisense morpholino oligonucleotides (MO) (GeneTools) were designed that targeted bases –9 to –16 of bcdo2 (bcdo2-MO1, 5′-TGTAGACAATGACTTGGTGTTATG-3′) and the splice acceptor site of exon 2/intron 3 (E2I3-MO2, 5′-TAGGTGAGCTCTCTACTGTCTTC-3′) of bcdo2 mRNA. For controls, the standard morpholino oligonucleotides (GeneTools) were used (control-MO: 5′-GTATTGTGGATTTCAGTACAGATGT-3′). The injected volume was –3 nl, corresponding to 5.1 ng of MO per embryo.

Treatments and staining of embryos

4-oxo-4HPR was prepared from stocks in dimethyl sulfoxide and applied to achieve a 1 μM concentration in egg water. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed with the In Situ Cell Death Detection Kit, TMR Red (Roche Applied Sciences, Indianapolis, IN). o-Dianisidine staining of zebrafish embryos was performed according to published protocols (Isken et al., 2008).

Cell lines and culture

COS7 monkey kidney cells, HepG2 human liver carcinoma cells, HeK293 human embryonic kidney cells and NIH-3T3 mouse embryo fibroblasts were maintained in high-glucose DMEM, whereas human breast carcinoma MDA231, T47D and BT549 cells were maintained in RPMI media, supplemented with 10% fetal bovine serum (FBS). Cells were cultured in a 37°C humidified CO2 incubator. Cytochrome c and COX IV endogenous protein co-localization studies and treatment with carotenoids were performed as previously described (Amengual et al., 2011).

RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

RNA was isolated from zebrafish embryos (± indicated treatments) and cultured cells with the Trizol reagent (Invitrogen, Grand Island, NY), and purified with the RNaseasy system (Qiagen, Valencia, CA). Quantitative real time PCR (Q-RTPCR) was carried out with Taqman chemistry, namely TaqMan Gene Expression Master Mix and Assays on Demand probes (ABI) for zebrafish bcdo2 (Dr01344457_m1, gpx1 (Dr03071768_m1), catalase (Dr03090949_m1) and hbb3 (Dr03090427_m1).

Histological methods

Measurement of mitochondrial membrane potential (ΔΨm) in HepG2 and T47D cells after carotenoid treatment was carried out as outlined by the manufacturer (ImmunoChemistry Technologies, Bloomington, MN). Staining for reactive oxygen species (ROS) was carried out with the Image-it LIVE ROS kit (Molecular Probes, Invitrogen). Chromatin condensation in cell lines was evaluated by using the DNA vital dye, Hoechst 33342. All experiments were carried out in duplicate and repeated three times. About 50-60 cells from 10-12 fields were analyzed per experiment.

Small-interfering RNA preparation and transfection

bcdo2 small-interfering RNA [FlexiTube siRNA Premix: Qiagen (Valencia, CA)], a target-specific 19 nucleotide siRNA set (pre-validated pool of four individual siRNAs) designed to knock down bcdo2 expression, was used to silence Bcdo2 expression. Cells in the exponential phase of growth were seeded in six-well plates at a concentration of 5 × 10^5 cells/well. Twenty-four hours later they were transfected with siRNA specific for bcdo2 and non-targeting siRNA at a final concentration of 100 nM according to the manufacturer’s protocol. Silencing of bcdo2 was examined 72 hours after transfection.

Microscopy

Fluorescent images were obtained by using a Zeiss LSM 510 UV Meta confocal microscope with an HCX Plan 63×/1.4 oil-immersion objective lens. Images were acquired with Zeiss confocal software, version 2.0, or with an Axioplan 2 fluorescence microscope (GFP filter set: excitation 473, emission 520; Samrock, Rochester, NY) with a Zeiss AxioCam MR camera at 10× magnification.

Statistical analyses

Results are presented as mean±s.d. and the number of experiments is indicated in the figure legends. All determinations for each experiment were performed at least in triplicate. Statistical significance was assessed by using the two-tailed Student’s t-test.

RESULTS

Zebrafish BCDO2 biochemistry and cellular localization

We previously cloned a bcdo2 cDNA (annotated as β-carotene-15,15′-dioxygenase 2, like bcdo2l) from this fish and showed that zebrafish egg yolk contains carotenoids (Kiefer et al., 2001; Lampert et al., 2003). To determine the subcellular localization of the enzyme, we expressed zebrafish BCDO2 as V5-tagged protein in COS7 cells. Immunostaining and confocal imaging revealed that BCDO2 was distributed in cells in a pattern resembling that expected for mitochondria (Fig. 1A). Therefore, we performed co-immunostaining for cytochrome c oxidase IV (COXIV), a mitochondrial marker protein. Merged images showed that BCDO2 colocalized with COXIV, suggesting a mitochondrial localization for this enzyme (Fig. 1A). We next expressed zebrafish BCDO2 in an E. coli strain capable of synthesizing BC. We have previously established this system to assess the activity of carotenoid oxygenases from various organisms (von Lintig and Vogt, 2000) and BC is the major carotenoid in the zebrafish egg yolk (Lampert et al., 2003). HPLC analyses of lipid extracts from this E. coli strain showed that an apocarotenoid product was formed upon expression of zebrafish BCDO2 that evidenced a similar retention
time and spectral characteristics to the β-10′-apocarotenal standard (Fig. 1B). Thus, we demonstrated that the zebrafish bcdo2 gene encoded a mitochondrial carotenoid-9′,10′-oxygenase.

**bcdo2** is expressed in the developing zebrafish

Using whole-mount in situ hybridization, we next analyzed the expression pattern of bcdo2 during development (Fig. 2). bcdo2 mRNA expression became detectable during segmentation in ventral cell layers of the head and trunk (Fig. 2A). At 24 hours post fertilization (hpf), bcdo2 mRNA expression in ventral cell layers became more restricted to anterior regions of the embryo (Fig. 2B,C). Beginning at 32 hpf, bcdo2 mRNA became detectable in the pericardial region (Fig. 2D,E). At 48 hpf, cells of the atrium and the ventricle also expressed bcdo2 as confirmed in cross-sections (Fig. 2F-J). At 3 and 4 days post fertilization (dpf), liver and gut cells also expressed bcdo2 (Fig. 2J-L).

**Knock down of bcdo2 causes anemia**

To address the requirement of bcdo2 for development, we disrupted its function by targeted gene knock down with antisense morpholino oligonucleotides (MO) (Nasevicius and Ekker, 2000). MO1 was directed against the 5′-region of bcdo2 mRNA and MO2 was directed against the splice acceptor site of exon 2 (supplementary material Fig. S1A). After injection of the MO1 into eggs at the one-cell stage, we confirmed efficient targeted knockdown of BCDO2 by immunoblot analysis with a polyclonal antiserum raised against murine BCDO2 (Fig. 3G) (Amengual et al., 2011). By RT-PCR analysis, we also demonstrated that MO2 interfered with bcdo2 mRNA splicing (supplementary material Fig. S1B,C). Morphants showed no gross patterning defects or obvious malformations of organ systems such as the eyes and brain. Under closer microscopic inspection, we noted that the blood in Bcdo2 morphants was no longer red-colored as in control embryos (Fig. 3A-D; supplementary material Fig. S1D). In 3 dpf MO1-treated morphants, the filling of the heart with blood cells was reduced (Fig. 3E,F).

**BCDO2 deficiency induces blood cell apoptosis at larval stages**

To investigate whether BCDO2 is required for erythropoiesis, we analyzed the expression pattern of the hematopoietic transcription factors gata1 and gata2. Genetic disruption of gata1 results in a reduction of mature erythrocytes as demonstrated in mouse and zebrafish mutants (Pevny et al., 1991). However, the expression of both these marker genes was unaltered in zebrafish morphants when compared with controls (Fig. 4A-F), indicating that primitive erythropoiesis and the proliferation erythroid progenitor cells were not affected. Furthermore, embryonic α-globin (hbae3), a late marker of erythrocyte differentiation, appeared to be expressed in morphants at 2 dpf (Fig. 4M,N). We next performed staining for hemoglobin by using o-Dianisidine with staged embryos. This analysis revealed that erythrocytes were present in morphants and controls at 2 dpf (Fig. 4G,H). But at 3 and 4 dpf, this picture changed such that a strong reduction of o-Dianisidine stainable cells was noted in morphants (Fig. 4I-L). We next performed staining for the marker of erythrocyte differentiation, appeared to be expressed in morphants at 2 dpf (Fig. 4G,H). But at 3 and 4 dpf, this picture changed such that a strong reduction of o-Dianisidine stainable cells was noted in morphants (Fig. 4I-L). Blood smears of 3 dpf larvae showed that erythrocytes of morphants exhibited fragmented nuclei, indicating that these cells had undergone apoptosis (Fig. 4O,P). To verify this assumption, we then performed TUNEL staining with 2 dpf bcdo2 morphants and wild-type siblings. Importantly, this staining indicated a large number of TUNEL-positive blood cells in morphants that accumulated in the region where the caudal hematopoietic tissue develops (Murayama et al., 2006; Jin et al., 2007) (Fig. 4Q,R). This region is colonized by hematopoietic stem cells at this developmental time point and provides a niche for blood cell differentiation (Ellett and Lieschke, 2010; Stachura and Traver, 2011). The large amount of TUNEL-positive cells in this region indicated that these differentiating blood cells were most sensitive to apoptosis in BCDO2 deficiency.

Previously, it has been shown that PTU can exacerbate anemia in fish larvae (Yu et al., 2010). Hence, we also raised bcdo2 morphants in the absence of this compound. At 3 dpf, bcdo2 morphants showed reduced staining for hemoglobin and
erythrocytes with fragmented nuclei (supplementary material Fig. S2A,C). Additionally, q-RT-PCR analysis revealed decreased hemoglobin B (hbbe3) mRNA levels in bcdo2 morphants when compared with controls (supplementary material Fig. S2B). Thus, we concluded that knockdown of BCDO2 resulted in apoptosis of blood cells in the zebrafish larva.

### 4-oxo-4HPR inhibits BCDO2 and recapitulates the blood phenotype bcdo2 morphants

We have previously shown that the synthetic retinoid fenretinide (4HPR) can inhibit the activity of BCMO1 (Lobo et al., 2010a). BCMO1 accepts carotenoid substrates with a non-modified \( \beta \)-ionone-ring site that is present in 4HPR (Lindqvist and Andersson, 2002). By contrast, BCDO2 displays broad substrate specificity by converting carotenoids with different ring modifications, including ring hydroxylation (Amengual et al., 2011). Thus, we wondered whether the 4HPR derivative 4-oxo-4HPR could inhibit BCDO2 activity. Murine BCDO2 was used for this analysis because this enzyme displayed high enzymatic activity in our in vitro test system (Fig. 5A). Thus, we incubated protein extracts of recombinant BCDO2 with canthaxanthin in the presence of increasing concentrations of 4-oxo-4HPR (Fig. 5B-D). Canthaxanthin, like 4-oxo-4HPR, possesses a 4-oxo-substituted \( \beta \)-ionone ring (Fig. 5C) and is an abundant carotenoid in zebrafish egg yolk (Lampert et al., 2003). BCDO2 efficiently converted canthaxanthin to cleavage products with spectral characteristics of 4-oxo-10'-apocarotenal and rosafluene (Fig. 5B,C) but the conversion rate for canthaxanthin significantly decreased in the presence of increasing amounts of 4-oxo-4HPR (Fig. 5D).

To analyze the effect of 4-oxo-4HPR in vivo, we administered this substance to 2 dpf zebrafish embryos and stained them the next day with o-dianisidine. When compared with the vehicle-only-treated control embryos, 4-oxo-4HPR-treated embryos developed anemia (Fig. 6A). Thus, 4-oxo-4HPR recapitulated the blood phenotype of bcdo2 morphants, probably by inhibiting BCDO2 activity.

Our previous studies in mice indicated that BCDO2 protects cells against oxidative stress (Amengual et al., 2011). Thus, we next analyzed gene expression of glutathione peroxidase 1 and catalase, which protect erythrocytes against oxidative stress in the fish (Yu et al., 2010). Quantitative real-time PCR with isolated blood cells showed a 3.5- to 7.0-fold increase in mRNA expression levels of these enzymes in 4-oxo-4HPR-treated fish relative to controls (Fig. 6B).

Our whole-mount in situ hybridization experiments did not provide clear evidence that bcdo2 was expressed in blood cells (Fig. 2). Thus, we used a more sensitive RT-PCR approach for bcdo2 expression with isolated blood cells. This analysis revealed that Bcdo2 was expressed in the blood (supplementary material Fig. S3A). We then asked whether bcdo2 expression is also responsive to oxidative stress. To accomplish this, we challenged 2 dpf larvae raised in the absence of PTU with egg water containing 0.03\% H\(_2\)O\(_2\). After, 24 hours we isolated protein and RNA from these fish and determined BCDO2 protein and mRNA levels. In treated larvae, bcdo2 expression was increased at the protein and mRNA levels when compared with control siblings (Fig. 6C,D). Additionally, q-RT-PCR analysis of isolated blood cells revealed a 3.3- to 4.1-fold increase of bcdo2 expression in H\(_2\)O\(_2\)-treated larvae (supplementary material Fig. S3B). Besides bcdo2, the zebrafish genome encodes a putative paralog (annotated as bcdo2b) with 59\% pairwise amino acid sequence identity. We performed RT-PCR analysis for both bcdo2 genes with 2 and 3 dpf embryos. bcdo2b was not expressed (supplementary material Fig. S3A), thus excluding the possibility that it exerts a developmental role similar to bcdo2.

### Carotenoids elicit ROS production and decrease \( \Delta \Psi \text{m} \) in a BCDO2-dependent manner

Our analysis in zebrafish indicated that knockdown of BCDO2 resulted in oxidative stress that damaged cardiac tissue and induced apoptosis in red blood cells. To mechanistically relate BCDO2 to apoptosis, we decided to take advantage of in vitro cell culture systems. Therefore, we screened several human cell lines for BCDO2 expression by immunoblot analysis (Fig. 7A). This analysis revealed that, with exception of HepG2 cells, all tested cell
lines expressed this enzyme when cultured under standard conditions. We then chose T47D cells and HepG2 cells and treated them with BC or canthaxanthin. After 2 hours of incubation, the carboxy-H2DCFDA dye was added to detect reactive oxygen species (ROS). In the presence of reactive oxygen species (ROS), this reduced fluorescein compound becomes oxidized and emits a bright green fluorescence. Microscopy showed that treated HepG2 cells showed strong green fluorescence, whereas T47D cells showed no such fluorescence (Fig. 7B).

ROS can result from impairment of mitochondrial respiration. To study the effects of carotenoids on this process, we analyzed mitochondrial membrane potential ($\Delta \Psi_m$) of both cell lines with the JC-1 dye (Reers et al., 1991). When mitochondria have a normal $\Delta \Psi_m$, this dye crosses the membrane and forms J-aggregates that appear red under UV light. By contrast, the dye remains in its monomeric form in the cytoplasm and emits a green fluorescence when mitochondrial respiration is impaired. Thus, vehicle-treated HepG2 cells showed red fluorescence for intact mitochondria, whereas carotenoid-treated HepG2 cells showed strong green fluorescence (Fig. 7C). Conversely, after carotenoid-treatment, T47D cells retained their $\Delta \Psi_m$ potential, indicated by strong red JC-1 staining in the mitochondria (Fig. 7C). Hence, we concluded that carotenoids can cause ROS production and reduce $\Delta \Psi_m$ in HepG2 cells, whereas these effects were absent in T47D cells, which express endogenous BCDO2.

**Carotenoids induce the intrinsic apoptotic pathway in HepG2 cells**

$\Delta \Psi_m$ is an important indicator of mitochondrial health and loss of $\Delta \Psi_m$ can lead to the formation of the mitochondrial permeability transition pore (MPTP) and release of cytochrome c into the cytoplasm (Liu et al., 1996). Thus, we determined the localization of cytochrome c in HepG2 cells by immunocytochemistry and confocal imaging (Fig. 7D). In non-treated HepG2 cells, staining for cytochrome c merged with the staining of COXIV, an intrinsic...
mitochondrial marker protein. As expected, in cells treated with 
H2O2, which induces oxidative stress, staining for cytochrome c 
and COXIV did not merge, indicative of cytochrome c release into 
the cytoplasm. Importantly, cytochrome c release also was seen in 
HepG2 cells treated with BC. To demonstrate directly that this 
process involves MPTP formation, we treated cells with 
cyclosporine A, a well-described blocker of MPTP (Pastorino et al., 
1998). Indeed, cyclosporine A prevented cytochrome c release in 
cells treated with BC as well as in cells treated with H2O2 (Fig. 
7D).

Cytochrome c release to the cytoplasm results in the activation 
of the caspase cascade (Samali et al., 1999). During the execution-
phase of apoptosis, pro-caspase 3 undergoes proteolytic processing 
at conserved aspartic residues to produce two subunits, large (19 
kDa) and small (17 kDa), that dimerize to form the active enzyme 
(Hui et al., 2004). In fact, only pro-caspase 3 was detected by 
immunoblotting in control HepG2 cells treated with BC (Fig. 
7D).

Biochemically active caspase 3 has been found to be responsible 
for chromatin condensation and DNA fragmentation, which are 
hallmarks of apoptosis (Zhuang et al., 2007). Using the DNA 
vital dye Hoechst 33342, we evaluated the effects of different 
carotenoids and retinoids on chromatin condensation. Blue 
fluorescent Hoechst 33342 brightly stains the condensed 
chromatin of apoptotic cells and more dimly stains the normal 
chromatin of live cells. After treatment with these compounds, 
HepG2 cells showed a marked increase in blue fluorescence 
(supplementary material Fig. S4A), whereas T47D cells did not. 
Cumulatively, these results suggest that through cytochrome c 
release and activation of caspase 3 and PARP1, carotenoids 
induced apoptosis in HepG2 cells, whereas T47D cells did not 
undergo such effects.

Fig. 5. BCDO2 converts substrates with 4-oxo-ionone ring sites. Recombinant murine BCDO2 was expressed in E. coli BL21 cells for 6 hours in 
the presence or absence of 0.5 mM IPTG. (A) 40 mg of protein of non-induced and induced cells were subjected to SDS-PAGE and immunoblot 
analysis. SDS-PAGE was stained with Coomassie Blue and BCDO2 was detected by an anti-His6 antiserum in immunoblot analysis. (B) HPLC profiles 
at 360 nm of a lipid extract from in vitro tests for BCDO2 enzymatic activity. The reaction was carried out with 20 μM canthaxanthin at 28°C for 10 
seconds (solid trace) and 10 minutes (dotted trace). Spectral characteristics of peak 1 (canthaxanthin), peak 2 (4-oxo-β-10′-apocarotenal), peak 3 
(rosafluene) are given above the HPLC traces. (C) Reaction sequence of canthaxanthin cleavage catalyzed by BCDO2. (D) Canthaxanthin cleavage by 
BCDO2 is inhibited by 4-oxo-fenretinide (4-oxo-4HPR). Recombinant murine BCDO2 was incubated in the presence of increasing concentrations of 
canthaxanthin in the absence and presence of 4-oxo-4HPR (0 to 5 μM). Reactions were run for 5 minutes at 28°C. In the graph, substrate cleavage 
(pmole) is plotted versus substrate concentration (μM). Diamonds, no 4-oxo-4HPR; squares, 0.5 μM no 4-oxo-4HPR; triangles, 2 μM no 4-oxo-4HPR; 
crosses, 5 μM 4-oxo-4HPR. Experiments represent data from three independent experiments. Error bars indicate s.e.m.
BCDO2 dictates apoptotic responses to carotenoids and synthetic retinoids in human cancer cells

To finally demonstrate that protection of T47D cells and susceptibility of HepG2 cells against carotenoid-induced apoptosis is strictly dependent on BCDO2 function, we performed a genetics-based experiment. Thus, we treated T47D cells with siRNA directed against bcdo2 or transiently transfected HepG2 cells with a plasmid encoding murine bcdo2. HepG2 cells transiently expressing BCDO2 were then treated with carotenoids and assessed for hallmarks of the intrinsic apoptotic pathway as described above. Our analysis showed a reduction of the carboxy-H2DCFDA dye fluorescence for HepG2 cells expressing bcdo2, when compared with non-transfected HepG2 cells, when challenged with different carotenoids and retinoids (Fig. 8C). Similarly, HepG2 cells expressing BCDO2 showed normally polarized mitochondria membranes, as indicated by strong red fluorescence after JC-1 dye staining (supplementary material Fig. S4B). Finally, immunoblot analyses for cleaved caspase-3 demonstrated that this mediator of apoptosis remained relatively unchanged after treatment of BCDO2-expressing HepG2 cells with carotenoids (Fig. 8D). Conversely, we expected that knockdown of Bcdo2 by siRNA treatment in T47D cells would enhance ROS production upon carotenoid treatment. This was evidenced by the increase in H2DCFDA staining when compared with control siRNA untreated cells (Fig. 8E). Interestingly, in T47D cells Bcdo2 expression was induced by treatment with carotenoids, whereas the Bcdo2-specific siRNA decreased Bcdo2 mRNA levels, as demonstrated by qRT-PCR (supplementary material Fig. S5A,B).

N-acetyl-L-cysteine protects zebrafish erythrocytes from 4-oxo-4HPR-induced apoptosis

Our analyses of human cell lines provided a mechanistic explanation of how carotenoids can induce apoptosis. To translate this finding into the zebrafish model, we determined whether endogenous ROS and apoptosis induction could be abrogated with N-acetyl-L-cysteine (NAC). NAC is an antioxidant with both catalase-like and glutathione reductase-like activities (Yu et al., 2010). Therefore, we treated 2 dpf larvae with 4-oxo-4HPR that can recapitulate the blood phenotype of bcdo2 morphants in the presence and absence of 5 mM NAC. Then 24 and 48 hours post-treatment, we stained larvae with o-Dianisidine. In 4-oxo-4HPR-only-treated fish we observed a marked reduction in hemoglobin...
BCDO2 in embryonic development

**Fig. 7. Carotenoids induce mitochondrial oxidative stress and trigger cytochrome c release in a BCDO2-dependent manner.**

(A) Immunoblot analysis for BCDO2 protein expression in various human cell lines. Note that the BCDO2 antiserum detects an additional protein with higher molecular weight in some cell lines. (B) HepG2 cells (panels 1-3) were incubated in the presence of vehicle (panel 1), 1 μM BC (panel 2) and 1 μM canthaxanthin (panel 3). T47D cells (panels 4-6) were incubated in the presence of t-butyl hydroperoxide (panel 4), 1 μM BC (panel 5) and 1 μM canthaxanthin (panel 6). After a 2-hour incubation, the non-fluorescent carboxy-H2DCFDA dye was added, which (in the presence of reactive oxygen species) emits bright green fluorescence. Data were obtained from three independent experiments. Representative images were obtained with a fluorescence microscope at 20× magnification. (C) HepG2 cells (panels 1-3) were incubated in the presence of vehicle (panel 1), 1 μM BC (panel 2) and 1 μM canthaxanthin (panel 3). T47D cells (panels 4-6) were incubated in the presence of the membrane-depolarizing agent carbonylcyanide-m-chlorophenylhydrazone (panel 4), 1 μM BC (panel 5) and 1 μM canthaxanthin (panel 6). After a 2-hour incubation, the mitochondrial membrane potential was assessed by JC-1 staining. Red fluorescence of JC-1 aggregates is indicative of intact mitochondria (panel 1) and green fluorescence of JC-1 monomers dispensed in the cytoplasm indicates mitochondria with depolarized membrane potentials (panel 4). Data were obtained from three independent experiments. Representative images were taken under a confocal microscope at 20× magnification. (D) HeLa cells (rows 1-5) were incubated in the presence of vehicle (row 1), 0.03% hydrogen peroxide (H2O2) (rows 2 and 4) and 1 μM BC (rows 3 and 5). Cells in panels 4 and 5 were pre-treated with cyclosporine A. Localization of cytochrome c and the mitochondrial marker protein COXIV was determined by confocal microscopy upon immunostaining at 63× magnification. Cytochrome c, red fluorescence; COXIV, green fluorescence; colocalization of cytochrome c and COXIV, yellow to orange fluorescence.

**DISCUSSION**

This study demonstrates a crucial role for the mitochondrial protein BCDO2 in protecting cells against carotenoid-induced oxidative stress and apoptosis (for current model see Fig. 9). We provide evidence in both the zebrafish model and human cell lines that this protective role is directly related to the presence of this oxidative stress inducible mitochondrial protein. Our findings indicate that vertebrates essentially require a mitochondrial carotenoid oxygenase to avoid the detrimental side reactions of carotenoids during embryonic development.

A pathway for the catabolism of carotenoids

BCDO2 belongs to a family of structurally related enzymes that includes RPE65 and BCMO1 (von Lintig et al., 2010). In zebrafish, RPE65a plays a crucial role for ocular vitamin A metabolism (Schonthaler et al., 2007) and BCMO1 is required for retinoic acid production in local tissue compartments (Lampert et al., 2003). BCDO2 was first characterized as a BC and lycopene-metabolizing enzyme (Kiefer et al., 2001). Later, it was shown that this protein also catalyzes oxidative cleavage of carotenoids with 3-hydroxy-ionone rings such as zeaxanthin and lutein (Mein et al., 2011; Amengual et al., 2011). We have demonstrated here that BCDO2 even cleaves canthaxanthin with 4-oxo-substituted ring sites. In this reaction, BCDO2 subsequently removes both ring ends of the carotenoid substrate by cleavage, ultimately resulting in the production of two ionone molecules and the C14-dialdehyde rosaluen.

Several studies have suggested that both BCDO2 cleavage products can influence the activities of cell signaling pathways and molecular targets (Mein et al., 2008; Ziouzenkova and Plutzky, 2008). However, this appears problematic because such a broad substrate specificity of BCDO2 would result in the production of numerous metabolites with little specificity. By contrast, BCMO1 displays narrow substrate specificity by exclusively promoting the production of vitamin A (Lindqvist and Andersson, 2002; Maeda et al., 2011). Thus, we propose that BCDO2 is primarily required for carotenoid catabolism. The introduction of oxygen into a parent carotenoid by BCDO2 generates apocarotenoids that can be further metabolized by endogenous enzymes. A mitochondrial retinol dehydrogenase, RDH13, reduces the aldehyde groups of carotenoid-cleavage products to the corresponding alcohols has recently been identified (Belyaeva et al., 2008). A concerted action of BCDO2 with RDH13 would also prevent side reactions of reactive aldehyde groups of the primary cleavage products (Siems
et al., 2002). The apocarotenols then could undergo α-oxidation for further catabolism, as has been described for phytol a structurally related plant isoprenoid (van den Brink and Wanders, 2006).

**A BCDO2-dependent pathway for apoptosis induction**

An important question is why do vertebrates require a pathway for carotenoid catabolism in mitochondria? Carotenoids are lipids that possess an extended polyene chromophore and can undergo redox and radical reactions (El-Agamey et al., 2004). Mitochondria are membrane-rich structures that bear oxygen-dependent redox systems. We have previously demonstrated in Bcdo2−/− mice that abnormal carotenoid level reduces mitochondrial respiration and induces oxidative stress in tissues (Cuperus et al., 2010; Amengual et al., 2011). It is well established that oxidative damage to mitochondria can induce apoptosis of cells (Roberson et al., 1997). Indeed, we found that the susceptibility of cells to induction of apoptosis by carotenoids was largely determined by BCDO2. Thus, HepG2 cells devoid of endogenous BCDO2 were highly susceptible to carotenoid-induced apoptosis, as evidenced by a decrease in mitochondrial membrane potential, activation of caspase 3, cytochrome c leakage from the mitochondria, changes in cellular morphology and eventually chromatin condensation. We also showed that cyclosporine A, which blocks the mitochondrial transition pore, prevented this carotenoid-induced process in HepG2 cells. Moreover, engineered expression of recombinant BCDO2 prevented carotenoid-induced apoptosis of these cells. In a similar fashion, T47D cells expressing endogenous BCDO2 were largely protected against apoptotic responses induced by carotenoids, whereas silencing of bcdo2 with siRNA made these cells susceptible to apoptosis induction.

The mitochondrial protective role of BCDO2 also was strikingly seen in the developing zebrafish. Knock down of BCDO2 resulted in anemia. Blood cells are highly susceptible to oxidative stress in both zebrafish and mouse models (Yu et al., 2010). Our findings in these fish larvae indicate that mitochondria must be protected...
against even low levels of endogenous carotenoids to avoid oxidative stress. A puzzling question is why blood cells are especially vulnerable in BCDO2-deficiency of the fish larva. We think it is unlikely that the putative paralog encoded by the bcdo2b gene can protect other tissues against such impairment because this gene was not transcribed during relevant embryonic stages. An explanation for this phenomenon could be that carotenoids are transported in the blood. Thus, blood cells are more exposed to these compounds than other cell types and tissues. The latter assumption may also explain that defects in bcdo2 morphants occur relatively late in development after a functional circulation has been established. In agreement with a mitochondrial protective role, oxidative stress regulated genes were induced in the blood of larvae treated with the BCDO2 inhibitor 4-oxo-4HPR. Conversely, treatment with the antioxidant NAC prevented anemia in 4-oxo-4HPR-treated fish. Furthermore, we also provided evidence that bcdo2 is induced in response to oxidative stress. This finding categorizes bcdo2 as an oxidative stress responsive gene. In the future, it might be worth identifying oxidative stress regulated signaling pathways that target the Bcdo2 promoter.

**General implications for vertebrate carotenoid biology**

Our findings establish BCDO2 as a crucial component for zebrafish development. Thus, the question arises as to whether bcdo2 plays a similar role in other vertebrates. In chicken, a promoter mutation specifically alters bcdo2 expression in skin but other tissues are not affected (Eriksson et al., 2008). Studies in gold finches indicate that carotenoids can cause adverse health effects when large amounts of these dietary compounds are acquired for the coloration of feathers during molting of male animals (Huggins et al., 2010). In cattle, a putative loss-of-function mutation in bcdo2 alters BC and retinoid levels in blood and tissues (Berry et al., 2009; Tian et al., 2010). Although one study reports that this mutation has no adverse effects, a second study that analyzed larger populations of different cattle varieties provides evidence that mutations in bcdo2 are partially recessive. Bcdo2<sup>−/−</sup> mice show impaired mitochondrial function and increased activity of cell signaling pathways related to oxidative stress when challenged with artificial carotenoid rich diets. However, these same Bcdo2<sup>−/−</sup> mice develop normally when raised on vitamin A-rich diets. Notably, intestinal carotenoid absorption is greatly attenuated by dietary retinoids (Lobo et al., 2010b). This characteristic explains why Bcdo2 deficiency does not result in similar effects during mouse embryogenesis (Amengual et al., 2011). A recent study shows that Bcdo2 is expressed in the mouse embryo and provided a protocol by which mouse embryos can be supplied with carotenoids (Kim et al., 2011). It will be interesting to employ this protocol to further study the effects of carotenoids on embryonic development in a mammalian animal model.

In humans, genetic polymorphisms in BCMO1 alter carotenoid and retinoid metabolism (Lindqvist et al., 2007; Leung et al., 2008; Ferrucci et al., 2009), but no mutations of BCDO2 have been described. Clinical studies implicate carotenoids as Janus-faced micronutrients associated with beneficial and adverse health effects. In populations at risk of lung disease, such as smokers, supplementation with BC increased the incidence of cardiovascular disease and lung cancer in large clinical trials (Omenn et al., 1996). Conversely, carotenoids such as lycopene have been associated with chemo-preventive effects against certain forms of cancer (Clinton, 1998). Our findings suggest that both results are associated with mitochondrial BCDO2. Putative cancer-preventing effects might be related to the induction of apoptosis in malignant cells by carotenoids. The reported detrimental consequences might be caused by mitochondrial oxidative stress that induces survival pathways and cell proliferation. It will be fascinating to further explore the role of BCDO2 in various aspects of carotenoid function in health and disease.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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**Fig. 9. Schematic overview of the role for mitochondrial BCDO2 in cells.** (A) Cells that express endogenous or recombinant BCDO2 can degrade carotenoids to apocarotenoids. The primary cleavage products can be further metabolized by RDH13 to the corresponding alcohols. These apocarotenoid alcohols might be then further catabolized by peroxisomal α-oxidation. (B) Carotenoids accumulate in the mitochondria in cells which lacking BCDO2 expression. As shown in Bcdo2<sup>−/−</sup> mice, this accumulation interferes with respiration and as shown in this study can induce membrane depolarization and ROS production. These events can cause cytochrome c release and activation of the caspase cascade that eventually leads to apoptosis.
References


endogenous transforming growth factor beta in PC-3 prostate cancer cells. Cell Growth Differ. 8, 101-111.


