THE ROLE OF BETA-CAROTENE AND ITS CLEAVAGE ENZYMES DURING VITAMIN A DEFICIENCY AND MAMMALIAN EMBRYONIC DEVELOPMENT

by

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Graduate Program in Nutritional Sciences

written under the direction of

Loredana Quadro

and approved by

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New Brunswick, New Jersey

MAY, 2014
ABSTRACT OF THE DISSERTATION

The role of beta-carotene and its cleavage enzymes during vitamin A deficiency and mammalian embryonic development

By ELIZABETH KUHN SPIEGLER

Dissertation Director:

Loredana Quadro

Vitamin A deficiency during pregnancy is a widespread health problem, which can detrimentally affect embryonic development. Since most of the world has limited access to preformed vitamin A (retinoids), it is important to understand whether the more abundant dietary precursor to vitamin A (β-carotene, bC) can adequately support embryonic development. bC is converted to vitamin A by its cleavage enzymes: the symmetric cleavage enzyme β-carotene 15,15'-oxygenase (CMO1) which generates retinaldehyde, and the asymmetric cleavage enzyme β-carotene 9',10'-oxygenase (CMO2), which generates β-apo-10'-carotenal. Both enzymes are expressed in mammalian embryos throughout development.

We studied bC metabolism in a mouse model of severe vitamin A deficiency – the Lrat/-Rbp/- mice, which cannot store retinol via lecithin:retinol acyltransferase (LRAT) or mobilize retinol from the liver via retinol-binding protein (RBP), and therefore produce highly malformed embryos when deprived of dietary vitamin A. We found that bC supplementation during a critical window of organ development rescued ~40% of
Lrat-/Rbp-/- embryos from symptoms of vitamin A deficiency, while supplementation after organ development did not improve the embryonic phenotype. This study indicated that bC is a good source of retinoids during pregnancy, but cannot fully support embryonic development even after prolonged administration.

We gained further insight into the ability to rescue embryos from vitamin A deficiency by studying Cmo1-/Rbp-/- mice and our novel Cmo1-/Rbp-/Cmo2/- strain. We found that spontaneous bC oxidation, rather than CMO2 activity, could generate small amounts of retinoids to improve the embryonic phenotype in the absence of CMO1. On the other hand, CMO2 deficiency on a background of vitamin A deficiency (Cmo2-/Rbp-/- mice) caused a severe embryonic phenotype that could not be rescued by bC despite the potential to generate retinaldehyde via CMO1. Cmo2-/Rbp-/- embryos were rescued following maternal supplementation with β-apo-10’-carotenal, the apocarotenoid normally generated from bC by CMO2 action, which also can be converted to retinoids by CMO1. We found that β-apo-10’-carotenal may influence mitochondrial energy homeostasis through a PKC signaling complex.

Overall, these studies showed that β-apo-10’-carotenal may be more effective than bC in supporting embryogenesis, and suggest that this apocarotenoid should be a recommended component of the human diet.
Acknowledgement

I am grateful to God for the blessing of so many people He has led into my life in the course of my doctoral studies. It has been a true privilege to work under the mentorship of my advisor, Loredana, whose passion for maternal-fetal nutrition was the reason I chose to attend Rutgers 6 years ago. I am so thankful for her example of passion, her commitment to making me stretch myself to be the best scientist I can be, and her tireless dedication to helping me plan, conduct, analyze, and understand my experiments. I am indebted to her for the countless hours she spent with me to discuss my research, and the time she spent reading and helping me revise this dissertation (as well as so many other writings and presentations throughout my time in her lab). My fondest memories of these days are of our puzzle-solving sessions as we exchanged ideas about the mysteries of carotenoid and retinoid metabolism. It is rare to find an advisor whose door is always open, and it was a treasure to find that in Loredana.

I have also been blessed with an amazing group of coworkers, who are better called friends. I learned so much about organization, diligence and perseverance from Lesley, and enjoyed the pleasure of her company and heartfelt conversations. Youn-kyung has been a great example of persistence and fearlessness at the bench, a patient teacher, and a good friend in the lab. I appreciated the companionship of Varsha and her thoughtful feedback on my experiments. I thank Alice, Rebeka, and Jasmin for their help with my experiments, and the enthusiasm they showed for learning new things; it was a privilege to mentor them. Brianna has been an amazing motivation and an exceptionally caring friend; I made it through many long nights in the lab with the reassurance of her company. I am so thankful to Michael for his true chivalry in our lab, his example of
quiet diligence, and the special help and kindness he offered especially as I was finishing this degree. Beatrice was an outstanding teacher and an intuitive scientist; it was a privilege to learn from her, and I am very grateful for her dedication to helping me obtain new evidence for this thesis.

I am also indebted to many fellow graduate students and post-docs who taught me, helped me, and cheered me on during these 6 years, especially Jushun Cheng, Mary Kiel, and Derek McMahon who taught me much about cell culture; Anna Dinh who was always willing to take time to help me; Yeonhee Park, Stylanios Fakas and Gil-Soo Han, who helped me greatly with isolating lipids; my fellow Nutritional Sciences students who were a privilege to have as classmates and friends; and my fellow TAs.

The faculty and staff at Rutgers were also wonderful teachers and mentors. I am grateful to my thesis committee – Dawn Brasaemle, Judy Storch, Paul Breslin, Dipak Sarkar, and Ulrich Hammerling – for their commitment to helping me through this process, and for all the time they spent reading and thinking about my thesis project. The materials and insights provided by Dr. Hammerling have been very helpful to us as we have tried to understand the function of CMO2, and his enthusiasm has been a source of motivation. I would also especially like to thank Wendie Cohick and Dan Hoffman for their words of wisdom at pivotal moments in my graduate career. I also thank the dedicated professors who taught my classes, and the members of the Rutgers Center for Lipid Research who provided useful feedback and a strong intellectual community.

Finally, I thank my family and friends for loving and encouraging me throughout these 6 years. I would not be at this point without the dedication of my parents to my education and up-bringing my whole life, and the sacrifices they made to help me reach
this point. I thank them and my dear friends for praying with and for me through the trials I faced. I especially thank my wonderful husband, who patiently followed me to New Jersey when I started this degree, listened to me as I related good times and bad, and helped me manage the stress of these challenges.

Above all, I thank God for the gift of life, the joy of exploring His creation, and every blessing He sent to make this degree possible when it seemed impossible by natural means. It is a testimony to His grace that I have arrived at this momentous occasion.

**Dedication**

This thesis is dedicated to the glory of God, who provided the strength, direction, and wisdom for its completion according to His sovereign timing and purposes.
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<tr>
<td>4HPR</td>
<td>4-hydroxyphenyl retinamide (Fenretinide)</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette, sub-family A, member 1</td>
</tr>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<tr>
<td>Akt</td>
<td>protein kinase B</td>
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<tr>
<td>ALCAT</td>
<td>lysocardiolipin acyltransferase</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
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<td>Apo10</td>
<td>β-apo-10′-carotenal</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>bC</td>
<td>β-carotene</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<td>CMO1</td>
<td>β-carotene 15-15′-oxygenase 1</td>
</tr>
<tr>
<td>CMO2</td>
<td>β-carotene 9′-10′-oxygenase 2</td>
</tr>
<tr>
<td>coA</td>
<td>co-enzyme A</td>
</tr>
<tr>
<td>CRBP</td>
<td>cellular retinol-binding protein</td>
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<tr>
<td>C_T</td>
<td>cycle threshold</td>
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<tr>
<td>CYP</td>
<td>cytochrome P450</td>
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<tr>
<td>DHRS3</td>
<td>dehydrogenase/reductase (SDR family) member 3</td>
</tr>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpc</td>
<td>days <em>post coitum</em></td>
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<tr>
<td>DUOX</td>
<td>dual oxidase</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GPX</td>
<td>glutathione peroxidase</td>
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<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
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<tr>
<td>HIF1-α</td>
<td>hypoxia-inducible factor 1-α</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
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<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>ISX</td>
<td>intestine-specific homeobox</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>LDLR</td>
<td>low-density lipoprotein receptor</td>
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<tr>
<td>LIPC</td>
<td>hepatic lipase</td>
</tr>
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<td>LPL</td>
<td>lipoprotein lipase</td>
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<tr>
<td>LRAT</td>
<td>lecithin:retinol acyltransferase</td>
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<tr>
<td>( L^{-/-} R^{-/-} )</td>
<td>( Lrat^{-/-} Rbp^{-/-} )</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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NPC1L1  NPC1 (Niemann-Pick disease, type C1, gene)-like 1
PAGE  polyacrylamide gel electrophoresis
PARP-1  poly-ADP ribose polymerase 1
PBS  phosphate-buffered saline
PKC  protein kinase C
PPAR  peroxisome proliferator-activated receptor
PRX  peroxiredoxin
qRT-PCR  quantitative real-time reverse transcription polymerase chain reaction
RA  retinoic acid
RAE  retinol activity equivalent
RAL  retinaldehyde
RALDH  retinaldehyde dehydrogenase
RAR  retinoic acid receptor
RBP  retinol-binding protein
RBPR2  retinol-binding protein 4 receptor 2
RXR  retinoid X receptor
RDH  retinol dehydrogenase
RE  retinyl ester
RNA  ribonucleic acid
ROH  retinol
ROS  reaction oxygen species
SD  standard deviation
SDR  short-chain dehydrogenases/reductases family

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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STRA6</td>
<td>stimulated by retinoic acid 6</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TTR</td>
<td>transthyretin</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Veh</td>
<td>vehicle</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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Chapter 1

Introduction and Literature Review

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1. Abstract

The requirement of the developing mammalian embryo for retinoic acid is well established. Retinoic acid, the active form of vitamin A, can be generated from retinol and retinyl esters obtained from food of animal origin, and from carotenoids, mainly β-carotene, from vegetables and fruits. The mammalian embryo relies on retinol, retinyl esters and β-carotene circulating in the maternal bloodstream for its supply of vitamin A. The maternal-fetal transfer of retinoids and carotenoids, as well as the metabolism of these compounds in the developing tissues are still poorly understood. The existing knowledge in this field has been summarized in this review in reference to our basic understanding of the transport and metabolism of retinoids and carotenoids in adult tissues. The need for future research on the metabolism of these essential lipophilic nutrients during development is highlighted.
Acquisition, storage & delivery of vitamin A

Dietary β-carotene and preformed vitamin A (mainly as retinyl ester, RE) are both converted to retinol (ROH) and re-esterified in enterocytes prior to packaging in chylomicrons. Chylomicrons reach the systemic circulation through the lymph ducts, and are hydrolyzed by Lipoprotein Lipase (LPL), leaving chylomicron remnants containing RE. Most remnants are taken up by the liver, which stores RE in stellate cells, or secretes ROH bound to retinol-binding protein (RBP) to meet the vitamin A needs of target tissues. ~25% of chylomicron remnants deliver RE directly to target cells. Within cells, vitamin A is converted to its biologically active form (retinoic acid, RA) to regulate gene transcription.
2. Vitamin A Metabolism

2.1. Absorption

Vitamin A is an essential nutrient that controls many crucial biological functions such as vision, reproduction, development, growth and immunity (Blomhoff et al., 2006). All retinoids (vitamin A and its derivatives) in animals are derived from the diet either as preformed vitamin A (retinyl esters, retinol and very small amounts of retinoic acid) from animal products or as carotenoids, mainly β-carotene, from vegetables and fruits (Sporn et al., 1994) (Fig. 1-1). Within the intestinal mucosa all retinol, regardless of its dietary origin, is re-esterified with long-chain fatty acids primarily by the action of the enzyme lecithin:retinol acyltransferase (LRAT), which is widely expressed in tissues (Batten et al., 2004; Liu and Gudas, 2005; O'Byrne et al., 2005). Together with other dietary lipids, the newly synthesized retinyl esters are packaged into chylomicrons and secreted into the lymphatic system (Goodman et al., 1966). Once in the general circulation, lipoprotein lipase (LPL), which is bound to the luminal surface of the vascular endothelium, catalyzes the lipolysis of triglycerides to generate free fatty acids and chylomicron remnants (Goldberg et al., 2009). After chylomicron remnants acquire apolipoprotein E, either in the plasma or in the space of Disse, approximately 75% of chylomicron remnant-retinyl ester is cleared by the liver, the major site of vitamin A storage and metabolism (Cooper, 1997). The remaining 25% is cleared by extrahepatic tissues (Goodman et al., 1965).

2.2. Transport, tissue uptake and metabolism
Once taken up by the hepatocytes, retinyl esters are hydrolyzed again to retinol (Goodman et al., 1965) to be transferred to stellate cells and then re-esterified by LRAT for storage (Matsuura et al., 1997). Alternatively, retinol can bind to its sole specific serum transport protein, retinol-binding protein (RBP), to be secreted into the bloodstream (Quadro et al., 2003; Soprano and Blaner, 1994). RBP is a 21 kDa protein with a single binding site for one molecule of all-trans-retinol. It is mainly, but not exclusively, synthesized within the hepatocytes (Quadro et al., 2003; Soprano and Blaner, 1994). RBP circulates in the blood as a 1:1 molar complex with another serum protein, transthyretin (TTR) (Monaco et al., 1995). The major function of RBP is to mobilize hepatic retinoid stores and deliver retinol to peripheral tissues (Quadro et al., 1999; Quadro et al., 2003), such as embryos (Quadro et al., 2004; Quadro et al., 2005). In the fasting circulation, retinol-RBP accounts for approximately 95-99% of all serum retinoids (Krasinski et al., 1990; Wilson et al., 1983). Upon vitamin A intake the concentration of retinoids in chylomicrons and chylomicron remnants can exceed that of plasma retinol (Kim et al., 2008). Blood levels of retinol-RBP in both humans and animals are maintained very constant, except in extreme cases of insufficient intake of vitamin A, protein, calories and zinc; or in response to hormonal factors, stress, and certain disease states (Biesalski et al., 1999; Soprano and Blaner, 1994; Yang et al., 2005).

The mechanisms that regulate the secretion of the complex retinol-RBP from the liver have yet to be fully elucidated. It should also be noted that even in the fasting state there are low concentrations of retinyl esters associated with circulating lipoproteins (in VLDL and LDL) and small amounts of circulating retinoic acid bound to albumin (De Leenheer et al., 1982).
Most tissues acquire vitamin A primarily from retinol-RBP. The peripheral uptake of vitamin A from the complex retinol-RBP seems to be mediated by STRA6, a large membrane protein that has been recently identified as the receptor for the circulating complex (Kawaguchi et al., 2007), at least in those tissues that do express Stra6. Notably, the concentrations of retinoids (retinol, retinyl esters, retinaldehyde) were diminished in the retinas of Stra6-/- mice (Ruiz et al., 2012), but not in other tissues (Berry et al., 2013). The precise molecular mechanism of this uptake still needs to be clarified. Emerging evidence supports the hypothesis that STRA6 may have other functions in addition to being the RBP receptor (Blaner, 2007). Berry and colleagues (Berry et al., 2011) recently reported that STRA6 functions as a cytokine receptor to transduce signaling by holo-RBP that can ultimately regulate insulin response, and also found that these functions were impaired in Stra6-/- mice (Berry et al., 2013). Additionally, in several human cell lines (bladder cancer, colon cancer, and normal fibroblast cells), Stra6 expression was dramatically induced (20- to 140-fold) by p53 or DNA damage perpetrated by UV radiation, oxidant exposure, or doxorubicin (Carrera et al., 2013). In renal and endothelial cells, increasing the ratio of apo-RBP (retinol-free) to holo-RBP (retinol-bound) elevated the expression of Stra6 and activated signaling through JAK2/STAT5, adenylate cyclase 6 (AC6), cAMP, and JNK1/p38, thus reducing the expression of cellular retinol-binding protein I (CRBP-I) and retinoic acid receptor alpha (RARα) and triggering apoptosis (Chen et al., 2012).

In vitro experiments (Isken et al., 2008) and observations made in the developing embryo (Kim et al., 2008) have suggested that STRA6 may also mediate the efflux of
retinol from the cell and thus act as a bi-directional transporter of retinol, with intracellular retinol concentration determining the polarity of transport. Recently, these studies were supported by further *in vitro* experiments confirming that STRA6 can facilitate both the influx of retinol (in conjunction with the intracellular carrier CRBP-I and the enzyme LRAT) and its efflux (in conjunction with CRBP-I and CRBP-II) (Kawaguchi et al., 2012). However, retinol efflux from cells occurred only in the presence of extracellular apo-RBP and was inhibited as holo-RBP concentration increased (Kawaguchi et al., 2012). Correspondingly, retinol efflux via STRA6 did not occur when cells were exposed to human plasma, where the concentration of holo-RBP exceeds that of apo-RBP (Kawaguchi et al., 2012). This study also revealed the STRA6 can mediate the exchange of retinol between holo-RBP and apo-RBP molecules (Kawaguchi et al., 2012).

*Str6* is expressed in a variety of tissues, including the placenta and embryo (Bouillet et al., 1997). Interestingly, in some tissues with high retinoid content, such as skin and liver, *Str6* is expressed at very low levels (Kawaguchi et al., 2007). Thus, other mechanisms of retinol uptake from the complex likely occur in these tissues. Spontaneous transfer of free retinol across the phospholipid bilayer is one of the possible mechanisms supported by experimental evidence (Noy and Xu, 1990a; Noy and Xu, 1990b; Noy and Xu, 1990c). However, a novel RBP receptor, “RBPR2”, was recently identified and is highly expressed in liver and intestine (Alapatt et al., 2013). While sharing <20% sequence homology, RBPR2 and STRA6 are structurally quite similar (Alapatt et al., 2013). It remains to be determined whether RBPR2 also functions in signal transduction.
Figure 1-2. Retinol is a metabolic precursor of active compounds.

Inside the cell, retinol (ROH), the vitamin A alcohol, is first oxidized and converted to retinaldehyde (RAL), which is known to be active in the visual cycle, even though recent data suggest that endogenous aldehydes can also control gene expression (Ziouzenkova et al., 2007a; Ziouzenkova et al., 2007b). Further oxidation of retinaldehyde (RAL) generates retinoic acid (RA), which acts as a ligand for specific nuclear receptors, such as RARs and RXRs, to regulate the transcription of a wide variety of target genes. Alternatively, retinol can also be converted to retinyl esters, which, inside the cells, represent the storage form of vitamin A. All these vitamin A derivatives are also called retinoids. The enzyme or class of enzymes catalyzing each reaction are abbreviated as: ADHs, alcohol dehydrogenases; RDHs, retinol dehydrogenases; RALDHs, retinal dehydrogenases; LRAT, lecithin:retinol acyltransferase; REHs, retinyl ester hydrolases.
Vitamin A exerts its activity through oxidized metabolites of retinol: retinaldehyde, involved in the visual cycle (von Lintig et al., 2010), and retinoic acid, which regulates the expression of many target genes through receptor-mediated events (Fig. 1-2) (Chen and Evans, 1995; Kastner et al., 1995; Kurokawa et al., 1995; Leblanc and Stunnenberg, 1995; Mangelsdorf et al., 1995; Pfahl and Chytil, 1996). Retinoic acid is the biologically active form of vitamin A and it functions as a ligand for specific nuclear receptors, retinoic acid receptors (RARs) or retinoid X receptors (RXRs), that regulate the transcription of numerous target genes, as homo- or hetero-dimers (Chen and Evans, 1995; Kastner et al., 1995; Kurokawa et al., 1995; Leblanc and Stunnenberg, 1995; Mangelsdorf et al., 1995; Pfahl and Chytil, 1996). More than 500 genes are known to be regulated by retinoic acid (Balmer and Blomhoff, 2002), a great number of which have been shown to control embryonic development (Mark et al., 2009). When retinoic acid signaling needs to be turned off, retinoic acid is degraded by members of the cytochrome P450 family of enzymes, such as CYP26A1, to produce more polar compounds, like 4-hydroxy or 4-oxo-retinoic acid, which are believed to be non-transcriptionally active (Abu-Abed et al., 2001). The mRNA levels of Cyp26a1 are induced by retinoic acid (reviewed in (Ross and Zolfaghari, 2011)).

Within cells, retinol is reversibly oxidized to retinaldehyde by enzymes including alcohol dehydrogenases (ADHs) of the medium-chain dehydrogenase/reductase (MDRs) superfamily, and retinol dehydrogenases (RDHs) of the short-chain dehydrogenase/reductase (SDRs) superfamily (Pares et al., 2008). Retinaldehyde is further oxidized to retinoic acid by the action of retinaldehyde dehydrogenases (RALDHs) (Duester, 2008). Being lipid molecules, retinoids must be bound to proteins
within cells. Several intracellular binding proteins for retinol, retinaldehyde and retinoic acid have been identified and extensively characterized. These include cellular retinol-binding proteins (CRBPI, II and III), cellular retinaldehyde-binding protein (CRALBP) and cellular retinoic acid-binding proteins (CRABPI and CRABPII). Each of these retinoid-binding proteins has a distinct expression pattern and plays a specific role in vitamin A transport and metabolism (reviewed in (Noy, 2000)).

Given the necessity of retinoids for diverse functions throughout the body, it is important for mammals to be able to store these lipid-soluble nutrients when vitamin A intake exceeds tissue requirements. In most tissues, LRAT serves this function, as evidenced by the dramatic depletion of retinyl esters in the livers, lungs, brains, eyes, and circulation of Lrat-/- mice (Batten et al., 2004; Liu and Gudas, 2005; O'Byrne et al., 2005). Many tissues of Lrat-/- mice also were found to have lower retinol levels than wild-type (WT) mice, although this effect seemed to depend on the vitamin A content of the diet (Liu and Gudas, 2005). In certain tissues, such as adipose, other acyltransferases seem to predominate over LRAT in the esterification of retinol, since retinyl ester levels increased in the adipose tissue of Lrat-/- mice (Liu and Gudas, 2005; O'Byrne et al., 2005). The severe retinoid deficiency of Lrat-/- mice was corroborated by the abolition of Cyp26a1 mRNA (Liu and Gudas, 2005) and stellate cell lipid droplets (O'Byrne et al., 2005) in the livers of these mice. When maintained on a vitamin A-deficient diet for one month, Lrat-/- mice had significantly reduced circulating retinol and RBP levels and 10-fold higher hepatic RBP levels than WT mice, indicating that Lrat-/- mice become vitamin A-deficient much more rapidly than WT. However, retinoic acid levels in liver, adipose tissue, and testes of Lrat-/- did not differ from WT. Although Lrat-/- mice were
largely unable to esterify retinol taken up by enterocytes, retinol and small amounts of retinyl esters were incorporated into their chylomicrons (O'Byrne et al., 2005).

2.3. *Retinoids and the mitochondrion*

Recently, novel functions for vitamin A have been uncovered, outside the well-known roles of retinoic acid in transcriptional regulation and retinaldehyde in the visual cycle. Specifically, Hammerling and colleagues reported that retinol could enhance mitochondrial oxidative phosphorylation in a PKCδ-dependent manner (Acin-Perez et al., 2010b). Retinol-treated mouse embryonic fibroblasts (MEFs) or liver mitochondrial fractions displayed greater oxygen consumption, ATP production, PKCδ activation (phosphorylation), and Pyruvate Dehydrogenase activity than untreated cells/fractions, and these effects were lost in PKCδ-deficient (and PKCδ retinol-binding domain-deficient) MEFs or cells treated with PKC inhibitors (Acin-Perez et al., 2010b). Retinol- and PKCδ-dependent ATP synthesis also depended on p66Shc, an adapter protein that complexes with PKCδ and cytochrome c (Acin-Perez et al., 2010a). Indeed, in MEFs with mutated p66Shc binding sites for PKCδ or cytochrome c, no retinol-dependent increase in ATP synthesis or oxygen consumption was observed (Acin-Perez et al., 2010a).

To explain these data, a model was proposed (Hoyos et al., 2012) wherein PKCδ chemically reduces cytochrome c by transferring an electron through the polyene chain of retinol, which itself is “docked” on p66Shc. (Indeed, a retinol derivative with an interrupted polyene chain could not enhance ATP synthesis (Acin-Perez et al., 2010a)).
In the process, PKCδ would be oxidized, which the authors proposed activates PKCδ signaling to increase flux through the Pyruvate Dehydrogenase Complex (PDHC) and citric acid cycle. As the initial reduction of cytochrome c by PKCδ would need to occur only during times of low electron flux through the electron transport chain, the resulting activation of PKCδ could signal the need to elevate flux through the oxidative phosphorylation pathway. However, this model contradicts the work of Giorgio and colleagues indicating that p66Shc is reduced by cytochrome c (Giorgio et al., 2005). Whether other retinoids or related compounds can bind and activate this signalosome remains to be determined.
3. Vitamin A and Development

3.1. Functions

Retinoids are crucial for the health of the mother, are required for maintenance of the placenta, and are absolutely essential for the developing embryo (Azaïs-Braesco and Pascal, 2000; Radhika et al., 2002). Indeed, retinoic acid, which is mainly synthesized within embryonic tissues, controls the expression of numerous key developmental target genes (Clagett-Dame and DeLuca, 2002), thus influencing the pattern formation of various organs, including hindbrain (Gould et al., 1998; Maden, 1999; Niederreither et al., 2000; White et al., 2000), spinal cord (Pierani et al., 1999; Sockanathan and Jessell, 1998), eye (Wagner et al., 2000), heart (Dersch and Zile, 1993; Niederreither et al., 2001), kidney (Batourina et al., 2001), lung (Malpel et al., 2000) and limb buds (Power et al., 1999; Stratford et al., 1999). Perturbations of the amount of retinoic acid available to the embryo lead to abnormal development. Animal model studies have established that severe maternal vitamin A deficiency results in early embryonic death (reviewed in (Clagett-Dame and DeLuca, 2002)). Less severe vitamin A deficiency, however, induces fetal developmental malformations. These have been described in several animal systems, and are known collectively as vitamin A deficiency syndrome (rat: (Thompson et al., 1964; Warkany and Schraffenberger, 1946; Wilson and Warkany, 1947; Wilson and Warkany, 1948; Wilson and Warkany, 1949), pig: (Hale, 1937), quail: (Dersch and Zile, 1993), and mouse: (Morriss-Kay and Wardt, 1999)). The features of the vitamin A deficiency syndrome include: cleft face, palate and lip; small or absent eye; abnormality of the urogenital system; abnormality of the heart and great vessels; and malformation of the forelimbs. RAR compound null mutant mice and RAR/RXR double mutants display
phenotypes that resemble those seen during gestational vitamin A deficiency (Kastner et al., 1997; Lohnes et al., 1994; Mendelsohn et al., 1994). Severe embryonic malformations have also been described in mutant mice lacking RDH10, the primary microsomal enzyme that catalyzes the oxidation of retinol to retinaldehyde in embryos (Farjo et al., 2011; Sandell et al., 2007); in mice lacking dehydrogenase/reductase superfamily member 3 (DHRS3), an enzyme that reduces retinaldehyde to retinol (Billings et al., 2013); in mice lacking RALDH2, an enzyme that controls the irreversible oxidation of retinaldehyde to retinoic acid (Niederreither et al., 1999); and in mice lacking CYP26A1, an enzyme that metabolizes retinoic acid into more polar hydroxylated and oxidized derivatives (Abu-Abed et al., 2001; Gottesman et al., 2001; Sakai et al., 2001). Complete postnatal lethality was observed in CrbpII-/- mice from dams fed a marginally vitamin A-deficient diet for the second half of gestation (Xueping et al., 2002). Retinoid excess during development also results in major embryonic defects which often overlap with those observed in retinoid deficiency (Cohlan, 1953; Mason, 1935; Morriss-Kay and Wardt, 1999; Takahashi et al., 1975; Wallingford and Underwood, 1986).
Figure 1-3. Maternal circulating forms of retinoids and carotenoids available to the developing embryo.

The mammalian embryo is entirely dependent on maternal circulating retinoids for its vitamin A supply. In the maternal bloodstream, we can identify two major retinoid forms: 1. retinol bound to RBP is the major form in the fasting state, when most of retinol is secreted into the circulation from the liver stores. 2. retinyl esters packaged in chylomicrons and their remnants may account for the majority of circulating retinoids upon dietary vitamin A intake. Although at lower concentrations, other forms of vitamin A circulate in the bloodstream, such as the vitamin A precursor β-carotene in chylomicrons and lipoprotein particles, and its metabolites (apocarotenoids). ROH, retinol; RE, retinyl ester; RA, retinoic acid; RBP, retinol-binding protein.
3.2. Sources of vitamin A for the embryo

Since there is no *de novo* fetal synthesis of vitamin A, to meet its requirement for retinoids, the developing mammalian embryo relies on circulating maternal vitamin A that reaches the embryo through the maternal-fetal barrier, i.e. placenta and yolk sac (Marceau et al., 2007). In the maternal bloodstream, two major retinoid forms can be identified: retinol bound to RBP is the major form in the fasting state (when most of retinol is secreted into the circulation from the liver stores), while retinyl esters packaged in chylomicrons and their remnants may account for the majority of circulating retinoids upon dietary vitamin A intake (Kim et al., 2008). Although at lower concentrations, other forms of vitamin A circulate in the bloodstream: 1. retinyl esters incorporated in lipoprotein particles (Goodman et al., 1965; Harrison and Hussain, 2001); 2. retinoic acid (both all-trans and 13-cis) in the fasting plasma of humans, rodents and cows (Blaner and Olson, 1994); 3. fully water soluble glucuronides of both retinol and retinoic acid (Barua et al., 1988; Barua and Olson, 1989); and 4. provitamin A carotenoids (Napoli and Race, 1988; Olson, 1989; von Lintig, 2010) (Fig. 1-3). Overall, the levels of these circulating retinoids reflect the maternal vitamin A status, which is determined by both their concentration within the stores and the recent dietary retinoid intake. Hence, alterations in maternal status and/or dietary vitamin A intake ultimately affect circulating retinoid levels and thus the amount of vitamin A available to cross the placenta towards the fetus (Wallingford and Underwood, 1987).
Figure 1-4. Pathways to apocarotenoid formation from β-carotene.

β-carotene (bC) is a symmetrical 40-carbon molecule containing a 30-carbon polyene chain (carbons counted from left to right, 1 to 15, then 15’ to 1’). β-carotene 15,15’-oxygenase (here indicated as BCO1) symmetrically cleaves bC at its 15,15’ double bond to generate two molecules of retinaldehyde (retinal). The molecule also contains four potential asymmetric cleavage sites (from left to right): cleavage at the 13’,14’ double bond yields β-apo-14’-carotenal and β-apo-13-carotenone; cleavage at the 11’,12’ double bond yields β-apo-12’-carotenal and β-ionylidene-acetaldehyde; cleavage at the 9’,10’ double bond yields β-apo-10’-carotenal and β-ionone; and cleavage at the 7’,8’ double bond yields β-apo-8’-carotenal and β-cyclocitral. Of these potential cleavage sites, only the 9’,10’ double bond is known to be enzymatically cleaved by β-carotene 9’,10’-oxygenase (here indicated as BCO2). However, all of the long-chain apocarotenoids have been detected in food and animal tissues (Barua and Olson, 2000; Fleshman et al., 2011). This figure was originally published in (Eroglu and Harrison, 2013). © the American Society for Biochemistry and Molecular Biology.
Recent evidence also has indicated that β-apocarotenoids (asymmetric cleavage products of β-carotene) are present in human and mouse diets and tissues (Fleshman et al., 2011; Ho et al., 2007; Shmarakov et al., 2010). These compounds may be generated enzymatically (see 4.1) or spontaneously (Hansen and Maret, 1988) (Fig. 1-4), and ultimately could generate retinoic acid following chain shortening (Wang et al., 1996) or enzymatic conversion (see 4.4, (Amengual et al., 2013)), providing another potential vitamin A source during development (Fig. 1-3). On the other hand, their independent ability to antagonize RARs could diminish vitamin A activity in target tissues (Eroglu et al., 2012).

3.3. Changes in endogenous retinoids during pregnancy and embryogenesis

Fetal acquisition of vitamin A remains relatively stable over a wide range of maternal dietary vitamin A intake, presumably as a result of maternal serum homeostatic mechanisms involving RBP (Ross and Gardner, 1994). Early studies from Satre and colleagues (Satre et al., 1992) examined changes in endogenous vitamin A levels in the maternal circulation and developing embryo over the course of normal pregnancy in mouse. They observed a large transient decrease in maternal plasma retinol levels and an apparent increase in mobilization from hepatic stores to the conceptus coinciding with the period of organogenesis (E9-14). Embryonic retinol levels increased with little or no change in retinyl ester and retinoic acid concentrations. Patterns of retinoid accumulation in embryonic liver indicated that the onset of vitamin A storage occurs by mid-organogenesis. In contrast, placental retinoid levels remained unchanged throughout
organogenesis. Analysis of the conceptus as a developmental unit revealed that during early organogenesis the majority of retinoid is contained in the placenta (8-fold more than in the embryo). However, by mid-organogenesis the retinoid content of the embryo exceeds that of the placenta. The data reported from mouse are in general agreement with those reported for humans and other species (Shah et al., 1987; Takahashi et al., 1977).

3.4. Transplacental transfer of retinol

The presence of measurable fetal hepatic vitamin A stores at birth indicates the efficiency of placental retinol transport during pregnancy (Ross and Gardner, 1994; Satre et al., 1992). It is clear that retinol crosses the placenta (Lorente and Miller, 1977), but, at the cellular level, the mechanism by which it is transferred to the fetus is poorly understood. Three possible mechanisms have been proposed: 1. direct transfer of retinol-RBP involving cellular uptake and release of the protein-ligand complex; 2. transfer of free retinol; and 3. cellular uptake of retinol by its specific receptor STRA6. It is noteworthy that the visceral endoderm of the yolk sac is a major site of RBP (Sapin et al., 1997; Soprano et al., 1986) and STRA6 (Kawaguchi et al., 2007) synthesis and of retinol uptake (Ward et al., 1997). Thus, it has been proposed that maternally-derived retinol passes from maternal blood circulating in the trophoblastic blood sinuses into the yolk sac cavity. There it comes into direct contact with the endodermal surface of the visceral yolk sac, where uptake occurs (Ward et al., 1997). Once within the endodermal cells, retinol must be transferred to the yolk sac vasculature for transport to the embryo, which would depend on RBP synthesized in the endodermal cells (Båvik et al., 1996). This RBP, located in the endoplasmic reticulum, binds and exports retinol (Ward et al., 1997).
The expression of RBP at later stages of gestation remains localized to the visceral endoderm of the yolk sac membranes. However, RBP expression also is detected (as early as 13.5 days post coitum (dpc)) at the junction of the uterine wall and placenta (decidua basalis) (Sapin et al., 1997). These data indicate that a second site for retinol uptake may correspond to the area that eventually forms the definitive placenta. However, active uptake by the yolk sac membranes may continue after formation of the definitive placenta. It is of note that maternal circulating RBP has been shown not to cross the maternal-fetal barrier (Quadro et al., 2004).

3.5. Transplacental transfer of other retinoids and their placental metabolism

Early studies investigated the ability of retinoids to pass through the placental barrier in humans, mice, rats and monkeys (Kraft et al., 1989; Satre et al., 1992; Tzimas et al., 1996; Ward and Morriss-Kay, 1995). Although retinyl palmitate is not transferred (Geelen, 1972), other retinoids (retinol; 13-cis, 9-cis, all-trans retinoic acid and their glycuronoconjugates) are transferred with specific kinetics (Nau et al., 1996). These studies suggest that the transfer of each retinoid from mother to embryo is promoted by a specific pathway. Furthermore, the placenta can metabolize some retinoids (retinol acetate can be oxidized by a placental lipoxygenase; (Datta and Kulkarni, 1996)) and thus may produce retinoids from maternally derived precursors (Dimenstein et al., 1996). The villous mesenchymal fibroblasts of the placenta also are able to esterify retinol to retinyl ester (Sapin et al., 2000). The placenta is suggested to serve as a site of vitamin A stores until the embryonic liver becomes functional. The placenta also has been proposed to buffer retinoid delivery, by releasing retinol to the fetus when maternal intake is deficient.
and by storing it to protect the embryo from a potential toxic excess of maternal retinoids. Vitamin A reserves also can be used within placental tissue (Sapin et al., 2000).

3.6. The retinol-RBP pathway versus the postprandial retinyl ester pathway

Mice lacking RBP (Rbp/-) are a very useful model to study the transfer of retinoids from mother to fetus (Quadro et al., 2003). Rbp/- mice are unable to efficiently mobilize their hepatic retinoids stores, and must continuously acquire vitamin A from the diet to support normal physiological processes, including embryonic development (Quadro et al., 2003;Quadro et al., 2004;Quadro et al., 2005). Indeed, when maintained on a vitamin A-sufficient diet (containing no less than 22 IU vitamin A/g of diet (Kim et al., 2011)), Rbp/- mice yield viable embryos that display only mild and transient cardiac embryonic developmental anomalies (Quadro et al., 1999;Wendler et al., 2003). The unremarkable phenotype of the Rbp/- embryos reflects the existence of an alternative pathway(s) of vitamin A delivery to the fetus. Indeed, in mice lacking retinol-RBP, high levels of retinyl esters incorporated in maternal circulating chylomicrons and/or VLDL particles provide the embryos with sufficient amounts of vitamin A to enable relatively normal embryonic development (Quadro et al., 2004;Wendler et al., 2003). However, the inability of the Rbp/- mice to mobilize efficiently the hepatic retinoid reserve makes them a good model to study embryonic vitamin A deficiency due to the limited amount of retinoids available to the developing embryo when the dams are maintained on a vitamin A-deficient diet. Under normal circumstances (normal maternal vitamin A status and presence of RBP), the retinol-RBP pathway is the primary contributor to fetal development, while the retinyl ester pathway is largely responsible for the accumulation
of fetal retinoid stores. This is especially true when maternal dietary vitamin A is limiting, as retinol would be released from the maternal liver bound to RBP to be delivered to fetus (Quadro et al., 2005). In contrast, fetal offspring from \textit{Rbp/-} dams maintained on a vitamin A-deficient diet display a wide range of embryonic vitamin A deficiency phenotypes, dependent upon the period of maternal dietary vitamin A deprivation. Moreover, early embryonic lethality is observed when \textit{Rbp/-} dams are maintained on a regimen of severe vitamin A deprivation (Quadro et al., 2005). In all cases, embryonic RBP rescues the embryos from early lethality (Quadro et al., 2005). Overall, \textit{Rbp/-} dams deprived of dietary vitamin A mimic the status of a vitamin A-deficient pregnant woman, thus representing a unique model system to analyze the metabolic links between maternal nutrition and developmental abnormalities.

### 3.7. Retinoid homeostasis in the developing tissues

The mechanisms through which the developing tissues maintain retinoid homeostasis despite the changes in the maternal vitamin A status have just recently been investigated. LRAT is a key enzyme in the generation of vitamin A stores (Batten et al., 2004; Liu and Gudas, 2005), and has been proposed to play a crucial role in maintaining retinoid homeostasis by diverting retinol away from its oxidative activation to retinoic acid in adult mammalian tissues (Ross, 2003). This action of LRAT has been proposed to be important especially under conditions of excessive retinoid intake (Ross, 2003). Using mice lacking both LRAT and RBP (\textit{Lrat/-Rbp/-}), our laboratory (Kim et al., 2008) showed that LRAT plays a crucial role in maintaining a tight regulation of retinoid levels during embryonic development and that this regulation is achieved by striking a balance
between retinyl ester synthesis and retinoic acid degradation via CYP26A1 activity. When dams were fed a vitamin A-excess diet, embryonic Cyp26a1 mRNA expression was up-regulated, not only in the absence of LRAT but also in WT mice (Kim et al., 2008), suggesting that there is a limit to the retinoid storage capability of the embryo, at least at 14.5 dpc.

In this model system, we found little evidence to support the notion that retinoic acid synthesis is regulated to counter the changes in maternal dietary vitamin A intake, as shown by the relatively constant gene expression levels of Raldh2 in the absence of LRAT or in wild type animals maintained on different regimens of dietary vitamin A intake (Kim et al., 2008). We also demonstrated that, similar to the embryonic tissues, an upregulation of retinoic acid catabolism is a compensatory mechanism that maintains retinoid homeostasis in the extraembryonic tissues (placenta) in the absence of LRAT for dams fed either the vitamin A-sufficient or -excess diet (Kim et al., 2008). In addition, the lack of a Raldh2 expression response to variations in maternal vitamin A intake in WT placenta confirmed that, also in extraembryonic tissues, regulation of retinoic acid homeostasis does not normally involve modulating the synthesis of RA, but rather its degradation (Kim et al., 2008). Only when severe maternal-fetal retinoid deficiency occurred, as observed for Lrat-/Rbp-/- dams maintained on the vitamin A-deficient diet, were placental expression levels of Raldh2 downregulated (Kim et al., 2008).

Interestingly, we also found that the expression levels of Stra6, the specific receptor for the complex retinol-RBP, were upregulated in response to maternal dietary vitamin A excess in WT embryos. Similarly, Stra6 mRNA levels were also elevated in Lrat-/Rbp-/- embryos compared to WT. This apparent discrepancy could be explained by
the hypothesis, put forward by Isken and colleagues (Isken et al., 2008) and recently supported by Kawaguchi and colleagues (Kawaguchi et al., 2012), that STRA6 acts as a bidirectional transporter of retinol. In this case, in times of excess retinoic acid, Stra6 expression would increase to drive retinol efflux and reduce the generation of potentially toxic levels of retinoic acid within the cell. Therefore, our data suggest an important role of STRA6 in maintaining retinoid homeostasis in the developing tissues. Furthermore, Stra6 was upregulated in the placenta of Lrat-/Rbp-/ mice maintained on the vitamin A-sufficient or -excess diets. However, in contrast to the embryo, vitamin A supplementation of WT dams did not upregulate the placental expression levels ofStra6, indicating that the placenta might have a larger retinoid storage capacity compared to the embryo and that the coordinated action of LRAT and CYP26A1 may be sufficient to maintain retinoid homeostasis in this organ (Kim et al., 2008).

Of note, in this report, it also was shown that the impairment of LRAT activity predisposed embryonic tissues to develop vitamin A deficiency (Kim et al., 2008).
4. Provitamin A Carotenoid (β-carotene) Metabolism

4.1. Absorption and transport

For the majority of the world population, provitamin A carotenoids are the main reliable source of vitamin A (Grune et al., 2010). Several provitamin A compounds, including β-carotene, α-carotene, β-cryptoxanthin, and as many as 50 other carotenoids, are found in the human diet mainly in orange, red, yellow, and leafy green vegetables and fruits (Grune et al., 2010; von Lintig, 2010). β-carotene (bC), the main provitamin A carotenoid, is absorbed by the intestine within mixed micelles. The intestinal absorption, solubility, transport and cleavage of bC are greatly impacted by the presence of bile salts, as well as the size of micelles (Erdman Jr et al., 1993). As a lipophilic molecule, bC is most readily absorbed in oil (Grune et al., 2010), and its absorption is influenced by many of the same factors as dietary lipids. bC is more readily absorbed from fruits than from vegetables, and its bioavailability in oil is generally greater than in plants (Grune et al., 2010). Polymorphisms in the genes encoding apolipoprotein B, hepatic lipase, lipoprotein lipase, and scavenger receptor class B member 1 (SR-B1) also have been shown to alter the intestinal uptake of bC and its distribution in body tissues (Lietz et al., 2010). Cholesterol and fatty acid transport proteins on both the apical side of enterocytes (SR-B1, NPC1L1, and CD36) and the basolateral side (ABCA1), are thought to facilitate the uptake of bC, as COS-7 cells took up bC following transfection with SR-B1 or CD36 (van Bennekum et al., 2005); Caco-2 cells simultaneously diminished bC uptake and
Figure 1-5. Cleavage of \( \beta \)-carotene and its conversion into retinoids.

When \( \beta \)-carotene is cleaved symmetrically by the action of the 15,15’-oxygenase enzyme (CMO1) two molecules of retinaldehyde (RAL) are produced. Retinaldehyde can then be oxidized to form retinoic acid (RA) or can be reduced to retinol (ROH), which is further converted into retinyl esters (RE), the storage form of vitamin A in tissues. *In vivo*, 95% of retinoids arising from \( \beta \)-carotene are produced by this pathway. However, \( \beta \)-carotene can also be cleaved asymmetrically by the action of the 9’,10’-oxygenase enzyme (CMO2) producing apocarotenals, which can be converted to retinaldehyde by chain shortening. Through this pathway, one molecule of \( \beta \)-carotene yields one molecule of retinaldehyde. Interestingly, Ziouzenkova and colleagues (Ziouzenkova et al., 2007a) first showed that apocarotenals may also function as ligands for PPAR and RXR receptors, inhibiting vitamin A signaling.
mRNA expression of Srb1, Npc1l1, and Abca1 following Ezetimibe treatment (During et al., 2005); and an antibody to SR-B1 reduced bC uptake by human retinal pigment epithelial cells (During et al., 2008).

Within the enterocytes, the first step of the conversion of bC to vitamin A is its symmetric cleavage by the cytosolic enzyme β-carotene 15-15'-oxygenase (CMO1), yielding two molecules of retinaldehyde (Redmond et al., 2001; von Lintig and Vogt, 2000; Wyss et al., 2000) (Fig. 1-5). A second carotenoid cleavage enzyme, β-carotene 9',10'-oxygenase (CMO2), has been cloned (Kiefer et al., 2001). CMO2 is a mitochondrial enzyme (Amengual et al., 2010) which cleaves bC asymmetrically generating apocarotenoids (predominantly β-apo-10’carotenol (Amengual et al., 2013)), which in turn can yield one molecule of retinaldehyde (Kiefer et al., 2001) (Fig. 1-5). Earlier studies attributed retinaldehyde formation from apocarotenals to the action of chain shortening enzymes (Wang et al., 1996), but recent evidence indicates that CMO1 can catalyze the conversion of β-apo-10’-carotenol to retinaldehyde (Amengual et al., 2013). Although both CMO1 and CMO2 are expressed in the intestine (Lindqvist and Andersson, 2004; Lindqvist et al., 2005), the contribution of the asymmetric cleavage enzyme has been considered minor compared to the role of CMO1 in the generation of retinoids from their provitamin A carotenoid precursors (Amengual et al., 2010; von Lintig, 2010). However, the recent evidence of “stepwise” retinaldehyde generation by concerted action of the two enzymes (see 4.4) suggests CMO2 may be more important to retinoid metabolism than was previously thought (Amengual et al., 2013).
Absorption, delivery & metabolism of intact \( \beta \)-carotene

In the intestine, dietary \( \beta \)-carotene (\( \beta \)C) can be absorbed intact via SR-B1, and incorporated into chylomicrons (CM). Intact \( \beta \)-carotene in chylomicrons (blue arrows) can follow several pathways, due to the exchange of core lipids among circulating lipoproteins (chylomicrons, LDL, and HDL). Lipoproteins containing \( \beta \)-carotene may be taken up by the liver through a number of receptors, potentially including LDLr, LRP, and LIPC (blue curves). \( \beta \)-carotene may be re-secreted from the liver in VLDL, which could deliver \( \beta \)-carotene to target cells directly via LPL (green arrow), or indirectly through the conversion of VLDL to IDL and LDL (purple arrows). Alternatively, \( \beta \)-carotene in lipoprotein particles can be taken up directly by target cells, potentially via
LDLr, SR-B1, or LPL (pink arrows). Within cells, β-carotene is converted to retinoids through central cleavage by CMO1, or eccentric cleavage by CMO2, ultimately generating retinoic acid (RA).
Whether bC is cleaved by CMO1 or CMO2, retinol dehydrogenases in the enterocytes convert retinaldehyde into retinol, which is then converted into retinyl ester mainly by the action of LRAT (O'Byrne et al., 2005). These esters are packaged into chylomicrons and secreted into the lymph ducts before ultimately reaching the systemic circulation (Goodman et al., 1966). The fate of the chylomicrons containing retinoids has been described in detail above (sections 2.1 and 2.2). In addition to the aforementioned fate of retinoids, recent work indicates that β-apo-10’-carotenol (e.g. generated by CMO2) can be released from hepatocytes bound to RBP, cross the plasma membrane via STRA6, and be esterified by LRAT (Amengual et al., 2013).

In humans, a significant fraction of the dietary bC (25-45%) is not cleaved in the intestine, but rather enters the circulation intact (Hickenbottom et al., 2002) (Fig. 1-6). The bioavailability of bC depends on a variety of conditions, including species of carotenoid (favoring all-trans isomers), dosage, food matrix, nutrient status of the host (including dietary intake of fat, fiber, vitamin A and other carotenoids), and host factors such as gender (favoring females), BMI and gut integrity (Grune et al., 2010). Mounting evidence suggests that polymorphisms in the genes encoding the carotenoid cleavage enzymes strongly contribute to the inter-individual differences in carotenoid bioconversion (Grune et al., 2010; Leung et al., 2009). As a result, among several double tracer studies, 27-45% of participants were found to be “poor converters” (Grune et al., 2010). This phenotype has been attributed, at least in part, to one of four prevalent single-nucleotide polymorphisms in CMO1; for instance, R267S variant T is found in 42% of
the Caucasian population, and is correlated with reduced bC cleavage in the intestine (Leung et al., 2009).

In addition to genetic factors, the vitamin A status of the individual strongly influences bC conversion, by a recently elucidated feedback mechanism. Initial studies in transgenic mice demonstrated that intestinal Cmo1 and Srb1 expression were elevated in mice lacking functional ISX, an intestine-specific homeobox transcription factor (Seino et al., 2008). In addition, Isx and Cmo1 expression were shown to depend on vitamin A status, with vitamin A deficiency reducing Isx mRNA levels and increasing Cmo1 levels (Seino et al., 2008). This work suggested that vitamin A production from bC increases when dietary vitamin A is limiting, by an ISX-dependent mechanism. More recent data have further clarified this mechanism: retinoic acid, the biologically active form of vitamin A, binds to retinoic acid receptors in the promoter of ISX to increase its expression. Then, ISX down-regulates the expression of Srb1 and Cmo1 (Lobo et al., 2010b). The ISX binding site in the Cmo1 promoter has been identified, and polymorphisms in this site correlate with serum bC levels in humans (Lobo et al., 2013).

The retinoic acid-dependence of the ISX regulatory pathway was confirmed by genetic and dietary manipulations. In bC-supplemented vitamin A-deficient Cmo1-/- mice, in which retinoic acid production was presumably negligible, Isx expression was diminished, whereas Srb1 expression and tissue bC levels increased. However, when Cmo1-/- mice were supplemented with dietary retinoids, Isx expression increased, while Srb1 expression and bC accumulation were reduced. Overall, these data demonstrate that when sufficient dietary vitamin A or provitamin A is converted to retinoic acid, the uptake and cleavage of bC (by SR-B1 and CMO1, respectively) are reduced, preventing
accumulation of toxic levels of retinoids (Lobo et al., 2010b). Other in vitro experiments suggest that human intestinal CMO1 expression is induced by thyroid hormone (triidothyronine) (Yamaguchi and Suruga, 2008).

The most recent data suggest that the bioefficacy of bC conversion to vitamin A in humans is 12:1 (Food and Nutrition Board. Institute of Medicine, 2001;Grune et al., 2010). However, it is important to note that the efficiency of intestinal bC cleavage varies greatly across species. Ferrets, gerbils, pre-ruminant calves, and some non-human primates are more similar to humans in their ability to absorb and metabolize bC, compared with other model animals such as mice and rats, which cleave almost all bC in the intestine rather than absorbing some of it in an intact form (Lee et al., 1999). Thus, careful discretion is needed in choosing the appropriate model system for studying bC metabolism. Several transgenic mouse models have facilitated bC research, and are discussed later in this chapter.

4.2. Tissue uptake and metabolism

bC circulating in lipoprotein particles in the fed state may be taken up by the liver or target tissues, such as lung, adipose tissue, muscle, corpus luteum, and adrenal gland (Fierce et al., 2008;Hessel et al., 2007;von Lintig et al., 2005). bC also can be re-secreted from the liver within VLDL and thus associate with LDL or even HDL (Erdman Jr et al., 1993). Therefore, tissue uptake of bC may occur by a variety of mechanisms, depending on the lipoprotein particle with which it is associated. As in the intestine, SR-B1 mediates the uptake of bC in RPE cell lines (During et al., 2008) and in the trunks, heads, and eyes of Drosophila melanogaster (Kiefer et al., 2002). The specific roles of other proteins
mediating the uptake of bC from lipoproteins in other tissues have not been investigated, but probably include LPL, SR-B1, and LDLR, all of which facilitate lipid absorption in extrahepatic tissues (Ishibashi et al., 1993; Rigotti et al., 1997; van Bennekum et al., 1999).

Following cellular uptake, bC may be stored in its intact form (Lakshman et al., 1989), or cleaved by CMO1 and/or CMO2. Both enzymes are expressed in liver and a variety of extrahepatic tissues including the human stomach, small intestine, pancreas, kidney, testis, adrenal gland, prostate, endometrium, skeletal muscle, and eye (Lindqvist and Andersson, 2004; Lindqvist et al., 2005), suggesting that dietary bC may be delivered to these tissues and be cleaved to provide a local vitamin A source (Napoli and Race, 1988; von Lintig et al., 2005; Wang et al., 1992). Certain human tissues express one carotenoid cleavage enzyme but not the other; for instance, only CMO1 was found in the colon, ovarian granulosa and theca, and skin epidermis, whereas only CMO2 was found in heart muscle fibers (Lindqvist et al., 2005).

Carotenoid cleavage is regulated not only at the level of the intestine, but also throughout the body. Cmo1 is a PPAR target gene; indeed, a PPARα/γ agonist increases hepatic Cmo1 expression (Boulanger et al., 2003). Accordingly, recent in vitro data demonstrated that Cmo1 expression is induced during adipogenesis, and that bC supplementation induces Cmo1 expression in inguinal white adipose tissue in a mouse model of vitamin A deficiency (Lobo et al., 2010a). In rats, lycopene feeding down-regulated Cmo1 expression in adrenal gland and kidney, which the investigators attributed to transcriptional activity of lycopene metabolites (Zaripheh et al., 2006).
Intestinal, hepatic, and testicular Cmo1 expression were found to increase in diabetic rats compared to controls (Takitani et al., 2008).

The regulation of Cmo2 expression has not been thoroughly investigated; however, one study reported an increase in hepatic Cmo2 mRNA following zeaxanthin feeding (Amengual et al., 2010). Nine weeks of lycopene supplementation led to a four-fold increase in Cmo2 in the lungs of ferrets (Hu et al., 2006). Interestingly, Cmo2 expression was shown to increase in bC-treated Cmo1-/ mouse liver (Shmarakov et al., 2010) and white adipose tissue (Amengual et al., 2011). Both hepatic Cmo1 and Cmo2 expression increased following chronic alcohol consumption in rats (Luvizotto et al., 2010). Overall, the tissue uptake and metabolism of bC depend on a variety of genetic, dietary, and health conditions, which warrant further study.

4.3. Lessons from Cmo1 knockout mice

Significant insight into the function of CMO1 has been gained from loss-of-function studies in mice. In 2007, Hessel and colleagues (Hessel et al., 2007) generated Cmo1-/ mice, and found that they accumulated large amounts of intact bC in serum, liver, adipose and intestine when the diet contained 1 mg/g bC but no preformed vitamin A. Correspondingly, vitamin A levels were reduced in several tissues known to express Cmo1 (lung, testis, uterus), confirming that this is the primary enzyme that generates retinoids from bC in adult mammalian tissues. Regardless of the diet, Cmo1-/ mice accumulated lipids in serum and liver, but especially on a high-fat diet. PPARγ target gene expression was elevated in Cmo1-/ visceral adipose, suggesting that bC conversion
to retinaldehyde and retinoic acid influences lipid metabolism through nuclear receptors bound to PPARγ (Hessel et al., 2007).

More recently, van Helden et al. (van Helden et al., 2010) discovered that markers of lung inflammation were elevated in female Cmo1-/- mice, which was ameliorated when supplemental bC was provided. These results suggested that bC cleavage by CMO2 could produce retinoic acid, which would modulate expression of inflammatory genes. Interestingly, retinol and retinyl ester formation increased to similar degrees in the lungs of WT and Cmo1-/- females supplemented with bC, questioning the role of CMO1 as the primary bC cleavage enzyme in all tissues (van Helden et al., 2010). Overall, CMO1 clearly has been indicated as the main bC cleavage enzyme in many adult tissues, with key roles in generating local vitamin A and modulating lipid metabolism. Although CMO1 is important for preventing lung inflammation, it appears that other mechanisms exist to ensure vitamin A homeostasis in lung and perhaps other tissues when preformed vitamin A is limiting in the diet.

4.4. Lessons from Cmo2 knockout mice

Although some in vitro studies have investigated the function of CMO2, until very recently the physiological role of CMO2 in mammals was unclear. In 2010, Amengual and colleagues (Amengual et al., 2010) showed that CMO2 cleaves a broad range of carotenoids, including not only bC, but also zeaxanthin, and lutein. The authors also generated Cmo2-/- mice, which appeared viable and fertile. When fed diets containing 50 mg/kg of zeaxanthin or lutein but no vitamin A, Cmo2-/- mice accumulated
these carotenoids (xanthophylls) and their metabolites in serum, heart, adipose, and liver. In the Cmo2/- liver, this led to the formation of 3-dehydrocarotenoids, their accumulation in mitochondria, a reduction in mitochondrial ADP-dependent (state 3) respiration, and up-regulation of protein levels of SOD2 (MnSOD), HIF1-α, phospho-AKT and phospho-MAPK. The latter two proteins were also up-regulated in Cmo2/- hearts. In HepG2 cells treated with the same dehydrocarotenoids detected in xanthophyll-fed Cmo2/- mice, ROS production was induced (as it also was with bC treatment) and mitochondrial membrane potential decreased. These results suggested that the accumulation of dehydrocarotenoids in mitochondria could directly affect the electron transport chain, thus triggering oxidative stress (Amengual et al., 2010).

Later work in zebrafish (Danio rerio) by the same group (Lobo et al., 2012b) showed that erythrocyte apoptosis and anemia occurred in larvae injected with antisense morpholino oligonucleotides against CMO2, or treated with a CMO2 inhibitor (4-oxo-4HPR). The targeted Cmo2 knockdown also caused mRNA levels of Glutathione Peroxidase 1 (Gpx1) and Catalase (Cat) to increase in isolated blood cells. Treatment of larvae with hydrogen peroxide induced Cmo2 mRNA expression in whole larvae and their blood cells, suggesting that Cmo2 is induced by oxidative stress. Indeed, antioxidant exposure (N-acetylcysteine) prevented erythrocyte anemia in 4-oxo-4HPR treated larvae. Human HepG2 cells, which do not express CMO2, generated ROS and depolarized their mitochondrial membranes following treatment with zeaxanthin, 3,3’-didehydrozeaxanthin, or bC, whereas these did not occur in a CMO2-expressing cell line (T47D, human breast carcinoma). Ultimately, the oxidative stress in carotenoid-treated
HepG2 cells led to cytochrome c release, pro-caspase 3 and PARP1 cleavage, and chromatin condensation, all hallmarks of the apoptotic pathway.

Overall, the above studies indicated that scavenging of carotenoids and their derivatives (dehydrocarotenoids) is an important function of CMO2 which protects mitochondria from oxidative stress. In spite of this progress, the function of CMO2 in mammalian retinoid metabolism has yet to be shown in vivo. New insights into its activity were recently provided by von Lintig and colleagues (Amengual et al., 2013). For many years, the generation of retinoids from asymmetrically-cleaved provitamin A carotenoids was attributed to the sequential activity of CMO2 (producing two apocarotenoids of different length) and chain shortening enzymes (reducing the chain length of the longer apocarotenoid until retinaldehyde is formed) (Wang et al., 1996). However, the recent evidence suggests that β-apo-10’-carotenol (the longer apocarotenoid generated by the 9’,10’ cleavage activity of CMO2) can be converted to retinaldehyde by CMO1 (Amengual et al., 2013). The molecular interaction of the two enzymes will be an interesting topic for future research, given their compartmental isolation within the cell.

The relative contributions of CMO1 and CMO2 to mammalian carotenoid metabolism also were clarified in this study (Amengual et al., 2013). bC supplementation of Cmo2/- and WT mice yielded similar bC concentrations in tissues, which were quite low compared to those of Cmo1/- or Cmo1/-Cmo2/- mice, reinforcing that bC cleavage is predominantly performed by CMO1 in vivo (Amengual et al., 2013). On the other hand, only bC-supplemented WT and Cmo1/- mouse livers accumulated β-apo-10’-
carotenol or its esters, whereas this apocarotenoid was undetectable in the CMO2-deficient strains, indicating that asymmetric bC cleavage by CMO2 occurs to a certain degree, however limited (Amengual et al., 2013).
5. Provitamin A Carotenoid β-carotene and Development

Being the most abundant vitamin A precursor in the human diet (Weber and Grune, 2012), βC is considered important during embryonic development. However, much of the current knowledge in this field pertains to the role of maternal circulating preformed retinoids. Only recently have the functions of provitamin A carotenoids begun to be elucidated.

5.1. Human studies

βC from the maternal circulation is able to reach the fetus, potentially via placental uptake of βC-containing chylomicrons, VLDL and LDL (Marceau et al., 2007). Several human studies have found significant correlations between βC levels in the maternal serum and umbilical cord, and reported that maternal serum βC levels were consistently higher than cord levels (Dimenstein et al., 1996; Karakilcik et al., 1996; Scaife et al., 2006; Yeum et al., 1998). Dimenstein and colleagues postulated that the dramatic difference in maternal and fetal circulating βC levels might reflect either enhanced metabolism of βC by the embryo, or else reduced placental uptake and transport of βC compared to other nutrients (Dimenstein et al., 1996). These authors observed that placental βC levels were nearly twice those of the maternal liver (Dimenstein et al., 1996). In a study of preterm rupture of fetal membranes, lower levels of βC were detected in amniotic fluid from women who had preterm ruptures (Barrett et al., 1994). In addition, fetal βC metabolism appears to depend on the maternal vitamin A status: Dimenstein and colleagues reported a correlation between maternal serum βC and
both cord serum and placental retinol levels, but only in women with subadequate serum retinol levels. On the other hand, maternal serum and placental bC levels were correlated only in women with adequate serum retinol (Dimenstein et al., 1996).

Although trials of vitamin A supplementation during pregnancy are more prevalent, several reports on bC supplementation have been published. Weekly oral supplementation with bC was shown to reduce maternal mortality during pregnancy by 49% in a large trial in Nepal (West Jr et al., 1999), although it did not significantly reduce maternal night blindness during pregnancy (Christian et al., 1998), and had no effect on fetal or early infant mortality (Katz et al., 2000). In a similar trial in Bangladesh, weekly oral bC treatment did not reduce maternal, fetal or neonatal mortality (West et al., 2011), nor did it improve birth size or gestational length (Christian et al., 2013). Another study investigated the role of maternal antioxidant status on birth weight, and found a significant correlation between birth weight and the combined intake of bC and vitamin E (Osorio et al., 2011). In Tanzania, combined bC and vitamin A supplementation had no effect on fetal deaths, birth weight or size, and maternal T-cell count among HIV-positive pregnant women (Fawzi et al., 1998). A small study of bC supplementation during the first month postpartum found no change in milk bC concentration in the treatment group, which the authors postulated was due to saturation of the milk with bC (Gossage et al., 2002). Simultaneous daily supplementation with zinc and bC during pregnancy was shown to improve plasma retinol levels in newborns and breast milk in Indonesia (Dijkhuizen et al., 2004). Maternal and neonatal serum retinol concentrations were improved when Indian pregnant women were supplemented with red palm oil as a source of bC (Radhika et al., 2003).
Considering the variety of outcomes of these bC supplementation trials depending on the population, more studies must be done to understand the role of maternal vitamin A status on the efficacy of bC supplements during pregnancy, to improve the design of supplementation programs.

5.2. Animal Studies

For ethical reasons, humans are not the most appropriate model to address questions related to the metabolism of bC in the developing tissues. Nevertheless, only a few studies on this topic have been conducted in animal models to date. In vitamin A-deficient gilts, weekly injection of bC and vitamin A prior to and during pregnancy increased maternal plasma vitamin A and bC, reduced embryonic mortality, and increased litter size, whereas dietary supplementation of these nutrients had no effect (Brief and Chew, 1985). In cows, uterine bC levels were shown to decline throughout the estrous cycle, while retinol levels increased. bC levels were unequally distributed between the horns of the bovine uterus, with ipsilateral levels (relative to the corpus luteum) exceeding contralateral levels (Costello et al., 2010). In Holstein cows, bC supplementation had no effect on reproductive performance at a dose of 400 mg/day in one study (Akordor et al., 1986), although another study found that milk production and pregnancy rate increased with the same dose of bC for <90 days (Aréchiga et al., 1998). bC may be more effective in increasing pregnancy rates in younger cows, according to one report (Ascarelli et al., 1985). Gestational bC supplementation increased bC concentrations in the colostrum and milk of mares and the serum of their foals (Kuhl et
al., 2012). Overall, it appears that bC supplementation of animals may improve reproduction to a certain extent.

5.3. Expression of Cmo1 and Cmo2 during development

Both CMO1 and CMO2 have been detected in the developing tissues, in numerous vertebrate species. In zebrafish, a vertebrate model organism, Lampert and colleagues identified Cmo1 transcripts during early segmentation in the cranial/neural crest and eye prior to embryonic day 2. In contrast, Cmo2 was detected in the embryonic heart only after day 2 (Lampert et al., 2003). Cmo1 expression was detected in chick embryos as early as 1.5 days of gestation, and bC was detected in the egg yolk of chicks at 5 days of gestation, when Cmo1 transcripts were still detectable (Mora et al., 2004). The human uterine endometrium and amniotic membranes express both Cmo1 and Cmo2 (Lindqvist and Andersson, 2004; Lindqvist et al., 2005; Marceau et al., 2007), although neither enzyme was detected in the human term placenta (Marceau et al., 2007).

In mice, Cmo1 expression was found in different developing tissues, depending on the gestational stage and analytical technique. Specifically, Paik and colleagues performed in situ hybridization during gestational days 7.5-8.5, and detected Cmo1 only in the maternal tissues surrounding the fetus (i.e. the uterus) (Paik et al., 2001). In contrast, Redmond and colleagues successfully detected embryonic Cmo1 during gestational days 7-15 by Northern blotting (Redmond et al., 2001). Our laboratory performed an extensive analysis of Cmo1 and Cmo2 expression levels in mouse embryos, placentas and yolk sacs from 6.5 to 14.5 dpc (Kim et al., 2011). Cmo1 was shown to have a very distinct pattern of mRNA expression levels during gestation that was mirrored by
that of the protein. Its expression was detected in the embryonic tissues from 8.5 dpc. In contrast, Cmo2 mRNA levels were steadier and less abundant compared to Cmo1 (Kim et al., 2011). In later studies, we discovered that placental mRNA levels of Cmo1 were elevated in marginally vitamin A-deficient pregnant mice (Lrat/-/Rbp-/) compared to WT controls (Wassef et al., 2013; Wassef et al., 2012). This work also demonstrated that embryonic Cmo1 mRNA levels at 14.5 dpc were diminished in Lrat/-/Rbp-/-mice compared to WT (Wassef et al., 2012), supporting the notion of molecular cross-talk between CMO1 and LRAT (see 5.4).

Overall, these data argue in favor of the hypothesis that maternally circulating bC can be delivered to the developing tissues and there cleaved to generate retinoic acid in situ. This local pathway of vitamin A synthesis could be important, for example, during times of insufficient maternal dietary vitamin A intake.

5.4. Transfer of β-carotene from mother to fetus and its metabolism in the developing tissues

Our laboratory has recently provided novel insights into the ability of the developing embryo to metabolize and use bC by investigating the function of CMO1 specifically in mouse embryonic tissues (Kim et al., 2011). Firstly, loss of CMO1 function studies in an established model of mouse embryonic vitamin A deficiency (the Rbp-/- animals), revealed that lack of CMO1 in the developing tissues further exacerbates the severity of vitamin A deficiency and thus the embryonic malformations of the Rbp-/- mice. Indeed, in addition to the Rbp-/- like phenotype (i.e. eye malformations and
peripheral edema), the embryos of the double-knockout mice \( (Cmo1^{-/-}Rbp^{-/-}) \) showed a certain percentage of cleft face and palate or exencephaly. The severe developmental defects of the double-knockout mice \( (Cmo1^{-/-}Rbp^{-/-}) \) on a vitamin A-deficient diet during pregnancy were accompanied by reduced levels of retinol, retinyl esters and retinoic acid and were due to the lack of CMO1 in the developing tissues, rather than to a more severe maternal vitamin A-deficient status. This study also showed that CMO1 deficiency manifests itself in an autosomal dominant fashion, but with different degrees of penetrance depending upon the gene copy number.

The double-knockout model used in this study was instrumental to unequivocally demonstrate in vivo the ability of intact bC circulating in the maternal bloodstream to cross the placenta toward the fetus, as well as the ability of embryonic CMO1 to generate locally, i.e. in the developing tissues, retinoids from bC. Indeed, \( Cmo1^{+/-}Rbp^{-/-} \) embryos from double-knockout dams deprived of vitamin A throughout gestation and supplemented with bC from 6.5 to 9.5 dpc, showed a reduced frequency of developmental defects compared to un-supplemented animals. Specifically, more than 60% of the embryos were phenotypically normal. Accordingly, the maternal supplementation also increased embryonic levels of retinol, retinyl esters and retinoic acid (Kim et al., 2011).
Figure 1-7. Mechanisms of retinoic acid homeostasis in embryos lacking CMO1.

Real time RT-PCR and statistical analyses for Lrat, Cyp26a1, Raldh2 and Stra6 were performed as described (Kim et al., 2008; Kim et al., 2011) by using Cmo1-/ and wild-type (WT) E14.5 embryos (n=7 embryos/genotype) from dams maintained on a vitamin A-sufficient diet (vitamin A=25-28 IU/g of diet). * indicates p<0.05. In Cmo1-/ embryos, showing reduced levels of Lrat mRNA, Raldh2, Cyp26a1 and Stra6 mRNA levels are significantly elevated. We interpret these data as an indication that, in spite of the reduced levels of retinyl esters (RE), increased synthesis and catabolism of retinoic acid (RA) as well as increased efflux of intracellular retinol (ROH), prevent retinoid toxicity and maintain normal levels of retinoic acid in Cmo1-/ embryos.
These studies also provided evidence for a potential novel role of CMO1, independent from its major known function of bC cleavage (Kim et al., 2011). Indeed, unless supplemented, bC was not present in the purified diet of our experimental animals. Nevertheless, CMO1 deficiency not only increased the severity of vitamin A deficiency-associated embryonic malformations, but also reduced Lrat mRNA expression and activity, thus decreasing retinyl ester levels in the developing tissues. Therefore, it seems that CMO1 may impact retinoid metabolism, specifically influencing retinyl ester formation, at least in the developing tissues. At the moment, the molecular mechanisms responsible for this effect have not been elucidated and are under investigation. Our most recent data indicate that CMO1 also affects the acyl CoA:retinol acyltransferase reaction in embryos, as several species of phospholipids, triglycerides and cholesteryl esters were reduced in Cmo1-/− embryos compared to WT, as were the mRNA levels of the acyltransferases Lcat, Acat1, and Dgat2 (Dixon et al., 2014).

Interestingly, despite the reduced levels of retinoids, embryos lacking CMO1 from dams on a vitamin A-sufficient diet did not show any gross morphological defects (and were also viable; (Kim et al., 2011)). In agreement, embryonic retinoic acid levels were similar between Cmo1-/− and WT animals (Kim et al., 2011). To investigate the mechanisms preventing the appearance of signs of retinoid toxicity in embryos lacking CMO1 (and hence with reduced levels of retinyl ester) on a vitamin A-sufficient diet, we measured by quantitative real-time RT-PCR analysis the expression levels of genes encoding other key enzymes and receptors that we have previously shown to be important in maintaining retinoid homeostasis in the developing tissues, namely Raldh2, Cyp26a1 and Stra6 (Kim et al., 2008). As shown in Figure 1-7, when Lrat expression is
severely impaired, mRNA levels of all the above-mentioned genes are upregulated. These data confirm that retinyl ester synthesis via LRAT, retinoic acid degradation via CYP26A1 activity, and possibly elimination of excess of retinol via the RBP-retinol specific receptor STRA6 contribute to maintaining retinoic acid homeostasis in the developing tissues. In contrast to our previous published data (Kim et al., 2008), in this model, the pathway of synthesis of retinoic acid mediated by RALDH2 also seems to contribute to the homeostatic mechanisms that maintain a tight regulation of retinoid levels during embryonic development. Since all these genes are transcriptionally regulated by retinoic acid (Kim et al., 2008), it has been proposed that increased mRNA levels of *Lrat* and *Cyp26a1* correspond to elevated levels of retinoic acid in tissues (Liu and Gudas, 2005; Liu et al., 2008; Ross, 2003). In contrast, our data (Fig. 1-7 and (Kim et al., 2011)) suggest that changes in the expression levels of the above-mentioned genes reflect a flux of retinoids aimed at maintaining homeostatic levels of tissue retinoic acid. The effectiveness of these regulatory mechanisms, and thus the tissue levels of retinoic acid, may depend upon numerous factors, including, for example, the whole-body vitamin A status and the dietary intake of vitamin A.

Overall, this work has provided novel insights into the role of CMO1 and bC metabolism during embryogenesis and has raised many intriguing questions. For instance, what is the mechanism(s) through which CMO1 regulates embryonic development and retinol esterification independent of bC cleavage? What mechanisms mediate and regulate bC uptake in placenta and its transfer to the developing tissues? Is *Cmo1* expression regulated in the developing tissues as it is in adult tissues, and if so, how? These are all key topics that require further studies and that will certainly expand
our knowledge about the role of a metabolic pathway that is crucial during embryonic development.
Figure 1-8. Complexes of the electron transport chain and ATP synthase.

The electron transport chain consists of four protein complexes embedded in the mitochondrial inner membrane (complexes I, II, III, and IV) and two intermediate electron carriers (ubiquinone [Q] and cytochrome c [Cyt c]). NADH generated in the citric acid cycle contributes electrons to complex I (NADH:ubiquinone oxidoreductase), which are subsequently transferred to ubiquinone, complex III (ubiquinol:cytochrome c oxidoreductase), cytochrome c, and complex IV (cytochrome c oxidase). Alternatively, succinate may be converted to fumarate by complex II (succinate dehydrogenase) in the
citric acid cycle. The electrons transferred to complex II in this process are subsequently transferred to ubiquinone, complex III and complex IV. Ultimately, complex IV transfers electrons to molecular oxygen (O₂), reducing it to water (H₂O). Complexes I, III and IV also pump protons (H⁺) from the mitochondrial matrix to the intermembrane space. Ultimately, these protons may be pumped back into the matrix by ATP synthase, fueling the production of ATP from ADP and inorganic phosphate (Pᵢ). (Vonck and Schäfer, 2009) Image available in the public domain at http://en.wikipedia.org/wiki/File:Mitochondrial_electron_transport_chain%E2%80%94Et_c4.svg
Figure 1-9. Pathways that elevate and reduce oxidative stress in cells.

Metabolic pathways in cells either contribute to or respond to the formation of reactive oxygen species. Superoxide (·O$_2^-$) is formed when a single electron (e$^-$), such as one that has leaked from the electron transport chain, reacts with molecular oxygen (O$_2$). Superoxide may react with nitric oxide (NO) that has been synthesized from L-arginine by the action of nitric oxide synthase (NOS), generating the highly reactive peroxynitrite (ONOO$^-$). The antioxidant enzyme superoxide dismutase (SOD) can convert superoxide to hydrogen peroxide (H$_2$O$_2$); however, the iron-dependent Fenton reaction can cause hydrogen peroxide to split, forming the hydroxyl radical (OH$^-$). Alternatively, hydrogen peroxide can be converted to water and molecular oxygen by catalase (CAT) or glutathione peroxidase (GPX). The oxidized glutathione (GSSG) generated by the latter
reaction can be converted to its reduced form (GSH) by glutathione reductase (GSR). Failure to balance the generation of reactive oxygen species with their conversion to stable products leads to oxidative stress, characterized by oxidative damage to DNA, protein, and lipids. Figure reprinted from (Al-Gubory et al., 2010), © 2010, with permission from Elsevier.
6. Oxidative stress

The aforementioned studies of adult Cmo2/- mice indicated that CMO2 may protect cells from oxidative stress by cleaving carotenoids taken up by mitochondria (Amengual et al., 2010; Lobo et al., 2012b). This function of CMO2 could be relevant to embryonic development, since CMO2 is expressed in embryos (Kim et al., 2011), and embryos are sensitive to changes in oxidative state (Al-Gubory et al., 2010). The reduction of oxygen to water during cellular respiration consists of three intermediate single-electron reduction steps, forming superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH•-) sequentially (Sies, 1997). Mammalian mitochondria produce much of this O$_2^-$ during electron transport (Fig. 1-8) via Complexes I and III in the intermembrane space and mitochondrial matrix (where the O$_2^-$ concentration is 5- to 10-fold higher than the cytosol or nucleus), and as much as 30% of H$_2$O$_2$ in the cytosol is generated in mitochondria (Ott et al., 2007). Two to 3% of electrons leak out of mitochondria from the electron transport chain (Chance et al., 1979; Myatt and Cui, 2004). Thus, while the aforementioned oxygen metabolites arise naturally in mitochondria during cellular respiration, electron leakage or excessive generation of these reactive oxygen species (ROS) can initiate oxidation of cellular macromolecules if the balance of oxidants cannot be restored by antioxidants (Sies, 1997). O$_2^-$ also reacts with nitric oxide (NO) to form the highly reactive peroxynitrite (ONOO-), which itself can damage lipids, DNA and proteins (Al-Gubory et al., 2010; Radi et al., 1991a; Radi et al., 1991b). Outside the mitochondrion, ROS may be generated by NADPH oxidase, Xanthine oxidase, Cyclooxygenases, Lipoxygenases, Monoxygenases, and CYP450s (Al-Gubory et al., 2010) (Fig. 1-9).
A variety of antioxidant defenses protect both cytoplasmic and mitochondrial cellular components from oxidative stress (Fig. 1-9). Superoxide dismutases (SODs) catalyze the conversion of $O_2\cdot$ into $H_2O_2$ and $O_2$ (Al-Gubory et al., 2010; McCord et al., 1971). Subsequently, $H_2O_2$ may be converted to water by glutathione peroxidases (GPXs) or peroxisomal catalase (CAT) (Al-Gubory et al., 2010; Mills, 1957). Glutathione reductase is needed to convert oxidized glutathione (GSSG) into its reduced form (GSH) (Al-Gubory et al., 2010; Rall and Lehninger, 1952). Oxidized protein thiol groups (disulfides) can be reduced by GSH-dependent glutaredoxins (GRXs), or by thioredoxins, which further interact with peroxiredoxins (PRXs) that reduce $H_2O_2$ and lipid peroxides (Ott et al., 2007). Mitochondria have their own supply of GSH, as well as specific isoforms of SOD, GPX, PRX, and GRXs (namely, SOD2 [and SOD1 in some cell types], GPX1 [some of it], GPX4, PRXIII, and GRX2) (Ott et al., 2007). GPX4 is expressed in embryos beginning at 8.0 dpc, and is considered a highly important selenoprotein because its expression is stably maintained even when selenium is limiting (Ufer and Wang, 2011). Interestingly, knockouts of the mitochondrial isoforms of antioxidant enzymes are typically lethal in mice, while knockouts of the cytoplasmic variants often are not lethal (Ott et al., 2007). Aside from enzymatic defenses, vitamins C and E, several carotenoids (e.g. bC and lycopene), and polyphenols also maintain cellular oxidative homeostasis (Al-Gubory et al., 2010).

Pregnancy is inherently a state of oxidative stress (Al-Gubory et al., 2010). In the first place, key steps in embryonic development require oxidative stress to trigger apoptosis, including blastulation, neuronal differentiation, and digit formation (Dennery, 2007). Further oxidative stress occurs during placental vascularization and
steroidogenesis (Al-Gubory et al., 2010), and placental mitochondrial mass and activity increase during gestation relative to the scavenging abilities of antioxidants (Myatt and Cui, 2004). Nevertheless, antioxidant activity is critical during development: all of the major antioxidant enzymes and vitamins are present in the placenta (Myatt and Cui, 2004), and provide the embryo with greater antioxidant defenses once the placental circulation is established (Dennery, 2007). As a general rule, cell proliferation depends on a very reduced state, while a moderately oxidized state promotes differentiation, and further oxidation leads to cell death (Dennery, 2010). Early pregnancy loss and pre-eclampsia are both characterized by elevated levels of markers of oxidative stress in placenta, highlighting the delicate balance between oxidants and antioxidants that must be maintained in a normal pregnancy (Myatt and Cui, 2004). The effects of carotenoid treatment and CMO2 expression on redox homeostasis in pregnant female mice and their embryos will be addressed in Chapter 3.
7. Specific Aims

In the following chapters, we present studies designed to assess the mechanisms that regulate the development of embryos from vitamin A-deficient mothers upon maternal bC supplementation, and to investigate the contribution of the asymmetric bC cleavage enzyme (CMO2) to embryonic development. Based on the published literature and previous studies in our lab, we sought to address two specific aims:

Aim 1: To investigate the effectiveness of bC supplementation at different gestational stages in supporting the development of embryos prone to severe vitamin A deficiency syndrome.

bC is the main vitamin A source for most of the human population, but its ability to rescue embryos from vitamin A deficiency syndrome in the absence of other retinoid sources is not known. Additionally, it has not been investigated whether genes involved in retinoid homeostasis respond to acute or prolonged bC treatment during the development of severely vitamin A-deficient embryos. We investigated these questions using a mouse model of severe vitamin A deficiency (Lrat/-Rbp/- pregnant females fed a vitamin A deficient diet), supplemented with bC in a prolonged treatment during the critical window of organ development, or in an acute treatment at mid-gestation.

Aim 2: To analyze the role of β-carotene 9’,10’-oxygenase (CMO2) during mammalian embryonic development.
The contribution of the symmetric bC cleavage enzyme (CMO1) to retinoid generation during embryogenesis was recently demonstrated by our lab. On the other hand, CMO2 has been characterized mainly as an enzyme protecting mitochondria from oxidative stress, and only recently as a partner of CMO1 in the “stepwise cleavage” of carotenoids to retinoids. Considering the critical need for retinoids to support embryonic development, and the ability of CMO2 to generate β-apo-10’-carotenal which subsequently can be converted to retinoids, we investigated whether CMO2 would function as a retinoid-generating enzyme in embryos, or as an oxidative stress-related enzyme as previously shown. To address this question, we used novel mouse strains, namely the Cmo1-/Rbp-/Cmo2-/ and the Cmo2-/Rbp-/ mice, and examined their embryos after feeding dams a vitamin A-deficient diet and supplementing with bC, retinaldehyde, or β-apo-10’-carotenal during pregnancy.
Chapter 2

Embryonic phenotype, β-carotene and retinoid metabolism upon maternal supplementation of β-carotene in a mouse model of severe vitamin A deficiency

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$^{1}$ These authors made equal contribution to this work
1. Abstract

We investigated the effect of β-carotene (bC) supplementation during pregnancy in a mouse model of severe vitamin A deficiency, i.e. Lrat-/-Rbp-/- dams maintained on a vitamin A-deficient diet during gestation. bC, a provitamin A carotenoid, can be enzymatically cleaved to form vitamin A for use by the developing embryo. We found that an acute supplementation (13.5 days post coitum, dpc) of bC to Lrat-/-Rbp-/- dams on a vitamin A-deficient diet activated transcriptional mechanisms in the developing tissues to maximize the utilization of bC provided to the dams. Nevertheless, these regulatory mechanisms were inefficient under this regimen, as the embryonic phenotype was not improved. We further investigated the effect of a repeated supplementation of bC during a crucial developmental period (6.5-9.5 dpc) on the above-mentioned mouse model. This treatment helped prevent the embryonic abnormalities, as 40% of the embryos showed a normal phenotype. In addition, analysis of retinoic acid-responsive genes, such as Cyp26a1 in these embryos suggested that bC cleavage results in the production of retinoic acid which then can be used by the embryo. Taken together, these in vivo studies show that bC can be used as a source of vitamin A for severely vitamin A-deficient mammalian embryos.
2. Introduction

Adequate maternal nutrition during pregnancy is critical for fetal health (Monk et al., 2013; Ramakrishnan et al., 2012). One such essential nutrient is vitamin A, of which maternal dietary intake must be carefully monitored, as deficient or excessive vitamin A intake can cause a range of birth defects (Clagett-Dame and Knutson, 2011). Furthermore, a poor pre-pregnancy maternal vitamin A status (i.e. low stores of the fat-soluble vitamin in body tissues) reduces the availability of vitamin A to be mobilized from the maternal liver to the fetus during times of inadequate dietary intake of this nutrient, and thus increases the risk of fetal vitamin A deficiency (Kim et al., 2008).

Vitamin A deficiency among women of child-bearing age is a serious global health problem. According to the World Health Organization, nearly 10 million pregnant women worldwide suffer from night blindness, and ~20 million have low serum retinol levels (World Health Organization, 2009). Even in more developed countries (e.g. UK), 30% of women between 19 and 34 years of age have reported vitamin A intakes below the recommended lower limit (Grune et al., 2010). In many countries where vitamin A deficiency is prevalent, there is limited access to preformed vitamin A (retinol, retinyl esters, and retinoic acid) from meat and dairy products. On the other hand, plant products containing the vitamin A precursors (provitamin A carotenoids such as β-carotene [bC]) are more abundant (Grune et al., 2010). Various human studies have reported neutral or positive maternal and fetal health outcomes upon vitamin A supplementation, but fewer studies have tested the effects of bC supplementation exclusively (Thorne-Lyman and Fawzi, 2012). The Hohenheim consensus conference of 2009 recommended that some
dietary vitamin A be obtained as bC (at least 6 mg/day in the case of low retinoid intake) (Grune et al., 2010), but it is unclear under what conditions bC alone can deliver adequate amounts of vitamin A. Thus, it is important to understand whether supplementation with carotenoids is sufficient to support normal embryogenesis, under conditions of both maternal dietary vitamin A deficiency and vitamin A-deficient status.

Ingested vitamin A (as retinyl esters) and bC (up to 45% in its intact form, with the remainder being converted to vitamin A (Hickenbottom et al., 2002)) are taken up by enterocytes, and enter the lymphatic system packaged in chylomicrons (Goodman et al., 1966). In the vascular endothelium, chylomicron hydrolysis by lipoprotein lipase (LPL) generates chylomicron remnants, which still contain bC and retinyl esters (Goldberg et al., 2009). The majority (~75%) of retinyl esters in chylomicron remnants is taken up by the liver (Goodman et al., 1965) and hydrolyzed to retinol (Harrison et al., 1995), either to be stored in stellate cells after re-esterification by lecithin:retinol acyltransferase (LRAT) (MacDonald and Ong, 1988; Matsuura et al., 1997), or to be re-secreted bound to retinol-binding protein (RBP) (Quadro et al., 1999). The remaining 25% of retinyl esters from chylomicron remnants are taken up by peripheral tissues, including the placenta (Goodman et al., 1965). In addition, exchange of bC and vitamin A may occur among lipoprotein particles (HDL, LDL, VLDL) in the bloodstream (Zilversmit et al., 1982).

Tissue vitamin A needs are met either by uptake of retinoids and their precursors from circulating lipoproteins, or by uptake of RBP-retinol via STRA6, the cell-surface receptor for holo-RBP (Kawaguchi et al., 2007). Most cells, including those of the placenta and embryo (Kim et al., 2008), are capable of esterifying retinol via LRAT for storage (Zolfaghari and Ross, 2000). Alternatively, cellular retinol can be reversibly
oxidized to retinaldehyde via retinol dehydrogenases (e.g. RDH10 (Sandell et al., 2007; Sandell et al., 2012)). Retinaldehyde also can be generated by bC cleavage. Central cleavage by β-carotene 15,15’-oxygenase (CMO1) generates two molecules of retinaldehyde (von Lintig and Vogt, 2000), while eccentric cleavage by β-carotene 9’,10’-oxygenase (CMO2) (Kiefer et al., 2001) followed by chain shortening (Wang et al., 1996) or “stepwise cleavage” (Amengual et al., 2013) of β-apo-carotenoids ultimately can generate one molecule of retinaldehyde. The latter is irreversibly oxidized by retinaldehyde dehydrogenases (e.g. RALDH2) to retinoic acid (Niederreither et al., 1997), the biologically active vitamin A metabolite that regulates the transcription of hundreds of genes (Niederreither and Dollé, 2008). Retinoic acid can be converted to non-transcriptionally active metabolites by CYP26A1 (Niederreither et al., 2002).

Previous studies in mice have shown that maternal circulating bC can be taken up by embryos via the placenta, to support a large degree of normal embryogenesis in the absence of other vitamin A sources (Kim et al., 2011). However, prior work has not demonstrated to what extent bC can contribute to embryonic vitamin A needs when the pregnant mother is vitamin A-deficient (i.e. by status). Our lab has generated and described a model of marginal vitamin A deficiency, the Lrat/-Rbp/- strain (L-/R-/-). While phenotypically normal on a vitamin A-sufficient diet, these mice rapidly become vitamin A-deficient and generate highly malformed embryos when deprived of dietary vitamin A, due to their inability to store retinoids via LRAT or mobilize retinol via RBP (Kim et al., 2008). Recently, we showed that on a vitamin A-sufficient diet, placental bC uptake was regulated by different mechanisms in L-/R-/- and wild-type (WT) mice, due
to the marginal vitamin A-deficient status of the \(L-/R-/\) dams (Wassef et al., 2012). These results indicated that the maternal vitamin A status affects bC uptake and metabolism in the developing tissues.

In the present study, we use WT and \(L-/R-/\) mice to investigate the effects of dietary vitamin A deficiency or a vitamin A-deficient tissue status, respectively, on the uptake and processing of bC by maternal and embryonic tissues as well as the extent to which such vitamin A-deficient tissues can generate retinoids from bC to support normal embryogenesis. Even severely vitamin A-deficient developing tissues respond to bC supplementation by maximizing proper utilization of the provitamin A as a source of retinoids to support embryogenesis. However, improvement of the embryonic malformations can be achieved only when bC is provided to the dams at early stages of development. This regimen is also effective at improving the maternal vitamin A status.
3. Materials and Methods

3.1. Knockout Mice, Nutritional manipulation, and β-carotene supplementation

Wild-type (WT) and Lrat-/Rbp-/ (L-/R-/) double-knockout mice (Kim et al., 2008; Wassef et al., 2012) were used in the current study. All mice had a mixed genetic background (C57BL/6 x Sv/129), with the L-/R-/ being a model of vitamin A deficiency (Kim et al., 2008). Throughout the study, both water and diet were consumed *ad libitum*, and mice were maintained on a 12 hour light/dark cycle from 7 a.m. to 7 p.m. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Garber et al., 2010) and were approved by the Rutgers University Institutional Committee on Animal Care.

Prior to nutritional manipulation, all mice were maintained on a non-purified vitamin A-sufficient diet (18 IU/g, Prolab Isopro RMH3000 5p75) but nearly devoid of bC (trace to 1.2 μg/g). At 3 months of age, WT and L-/R-/ females were mated with their respective males, and the presence of a vaginal plug was established as 0.5 days *post coitum* (dpc). Henceforward, dams were fed a purified vitamin A-deficient diet (Research Diets, <0.2 IU/g vitamin A, 0 μg/g bC) until the time of sacrifice (14.5 dpc). Solutions of bC or its Vehicle (Veh) were prepared as previously described (Wassef et al., 2012). Briefly, 50 mg bC (Type II, Sigma Aldrich) was mixed into 5 mL Veh (ethanol : Cremophor : PBS, 1:11:18) by vortexing, and the concentration of the resulting solution was determined by spectrophotometry at 450 nm. Due to poor solubility of bC, the final concentration varied from 2-5 mg/mL.
For the acute bC supplementation study, WT and $L/-R/-$ pregnant dams were randomly assigned to the Veh or bC treatment groups, and were injected with 250 $\mu$L of the assigned solution intraperitoneally (IP) at 13.5 dpc. For the repeated bC supplementation study, $L/-R/-$ pregnant dams were randomly assigned to be given an IP injection of Veh or bC daily from 6.5-9.5 dpc. The resulting dose of one injection of bC given to the pregnant dams was ~40 $\mu$g/g body weight. Regardless of treatment, all dams were sacrificed at 14.5 dpc by CO$_2$ inhalation between 9:30 and 11:30 a.m. Serum and tissues (livers, placentas, and embryos) were collected, frozen, and stored at -80 °C until further processing.

3.2. HPLC

Retinoid (retinol and retinyl esters) and bC concentrations in maternal serum, liver, placenta and embryo were measured by reversed-phase HPLC analysis as described previously (Kim et al., 2008; Kim and Quadro, 2010).

3.3. RNA Extraction, cDNA synthesis, and Quantitative Real-time RT-PCR

RNA Extraction, cDNA synthesis, and quantitative real-time RT-PCR (qRT-PCR) were performed on embryos and placentas as previously described (Kim et al., 2008). Primer sequences were as published for $\beta$-Actin, Stra6, Raldh2, Cyp26a1 (Kim et al., 2008), Cmo1 (Wassef et al., 2012), Cmo2 (Shmarakov et al., 2010), and Rdh10 (Romand et al., 2008). Changes in mRNA expression were analyzed by the $\Delta\Delta$CT method.
3.4. Statistical analysis

Statistical analysis was performed using SPSS statistical software (IBM SPSS Statistics, version 16). Normal distribution of data was assessed by the Shapiro-Wilk test. Normally distributed data were analyzed by Student’s t test for comparisons of two groups, or by two-way ANOVA and post hoc analysis (least significant difference, LSD, for groups with equal variance; Tamhane’s analysis for groups with unequal variance) for comparisons of genotype and treatment effects, followed by Student’s t test. Data that were not normally distributed were analyzed by the Mann-Whitney U test for comparisons of two groups, or by the Kruskal-Wallis test followed by Mann-Whitney U test for comparisons of three or more groups. P < 0.05 was considered significant. Data are presented as mean ± standard deviation (SD).
Table 2-1. Phenotype distribution of $L^{-/-}R^{-/-}$ embryos from dams fed a vitamin A-deficient diet, supplemented with Veh or bC at 13.5 dpc

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n [embryos, (dams)]</th>
<th>Percent Resorbed</th>
<th>Normal</th>
<th>Eye and/or Edema</th>
<th>Cleft</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh 13.5</td>
<td>20 (7)</td>
<td>56% (25/45)</td>
<td>0%</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>bC 13.5</td>
<td>31 (10)</td>
<td>54% (37/69)</td>
<td>0%</td>
<td>13%</td>
<td>87%</td>
</tr>
</tbody>
</table>

$L^{-/-}R^{-/-}$ dams were fed a vitamin A-deficient diet from 0.5-14.5 dpc. Seven dams were injected with Veh at 13.5 dpc (Veh 13.5), and 10 dams were injected with bC at 13.5 dpc (bC 13.5). Percentage of resorbed embryos was calculated from the total number of implantations in the uterus.
Figure 2-1. Effects of β-carotene supplementation on gross morphology of L-/R-/- embryos from dams fed a vitamin A-deficient diet from 0.5-14.5 dpc.

Embryos (14.5 dpc) from Lrat-/Rbp-/- (L-/R-/-) dams fed a vitamin A-deficient diet during pregnancy (0.5-14.5 dpc) were analyzed following a single dose at 13.5 dpc (C-D) or multiple doses daily at 6.5-9.5 dpc (E) of bC, compared with Veh (A-B). Embryonic defects include malformed eye (e), peripheral edema (p), shortened forelimbs (f), and abnormal midfacial region (mf).
Table 2-2. Serum and tissue β-carotene levels (14.5 dpc) of WT and L-/R-/ dams fed a Vitamin A-deficient diet from 0.5-14.5 dpc, and injected with bC at 13.5 or 6.5-9.5 dpc

<table>
<thead>
<tr>
<th></th>
<th>Serum μg/dL</th>
<th>Liver μg/g</th>
<th>Placenta μg/g</th>
<th>Embryo μg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT, bC 13.5</td>
<td>541 ± 129</td>
<td>57 ± 27</td>
<td>1.53 ± 0.42</td>
<td>0.019 ± 0.010</td>
</tr>
<tr>
<td>L-/R/-, bC 13.5</td>
<td>624 ± 502</td>
<td>117 ± 43</td>
<td>1.81 ± 0.60</td>
<td>0.018 ± 0.009</td>
</tr>
<tr>
<td>L-/R/-, bC 6.5-9.5</td>
<td>1.37 ± 0.68*</td>
<td>389 ± 131*</td>
<td>2.59 ± 0.76</td>
<td>0.008 ± 0.007</td>
</tr>
</tbody>
</table>

WT and L-/R/- dams were fed a vitamin A-deficient diet from 0.5-14.5 dpc. Dams were injected with bC at 13.5 dpc (bC 13.5), or daily from 6.5-9.5 dpc (bC 6.5-9.5). Data presented as mean ± SD. Statistical comparisons were made between WT and L-/R/- bC 13.5, and between L-/R/- 13.5 and L-/R/- 6.5-9.5. Statistical analysis for WT vs. L-/R/- 13.5 data by Student’s t test [serum (equal variance not assumed), placenta, embryo], or Mann-Whitney U test (liver). Statistical analysis for L-/R/- 13.5 vs. 6.5-9.5 by Student’s t test. Sample size, n=3-11 dams/group; Placental and Embryonic levels include more than one embryo from each litter. *, p<0.05 vs. L-/R/- bC 13.5.
4. Results

We previously showed that the placental uptake of bC is enhanced in an established model of marginal vitamin A deficiency, the L-/R/- mice fed a vitamin A-sufficient diet (Kim et al., 2008; Wassef et al., 2012). Here we employed the same strain to understand whether a severe maternal vitamin A-deficient status would affect the uptake and metabolism of bC in the developing tissues. Indeed, on a vitamin A-deficient diet, L-/R/- mice rapidly become severely vitamin A-deficient, whereas the retinoid stores of WT mice are not readily depleted (Kim et al., 2008).

4.1. Acute maternal supplementation with bC at 13.5 dpc

We injected ~40 µg/g bC or Veh at 13.5 dpc into WT and L-/R/- dams fed a vitamin A-deficient diet from the onset of gestation, and analyzed their embryos at 14.5 dpc. A single injection of Veh or bC at 13.5 dpc did not support normal embryogenesis in the L-/R/- group, which generated severely malformed embryos with a large percentage of resorptions (Table 2-1). Embryonic defects at 14.5 dpc included malformed eyes, peripheral edema, cleft face/palate, and/or shortened forelimbs (Fig. 2-1A-D). Under the same dietary regimen and treatment, WT embryos developed normally (data not shown).

HPLC analysis revealed that bC-supplemented WT and L-/R/- dams had similar concentrations of bC in maternal serum, liver, placenta and embryo (Table 2-2). L-/R/- liver bC displayed a non-significant tendency to increase compared to WT (p=0.065).
Table 2-3. Tissue Retinol levels (14.5 dpc) of WT and L-/R-/- dams fed a Vitamin A-deficient diet from 0.5-14.5 dpc, and injected with Veh or bC at 13.5 dpc

<table>
<thead>
<tr>
<th></th>
<th>Liver μg/g</th>
<th>Placenta ng/g</th>
<th>Embryo ng/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT, Veh 13.5</td>
<td>2.4 ± 0.6</td>
<td>166 ± 44</td>
<td>81.0 ± 13.0</td>
</tr>
<tr>
<td>WT, bC 13.5</td>
<td>1.8 ± 0.4</td>
<td>112 ± 30#</td>
<td>74.1 ± 9.3</td>
</tr>
<tr>
<td>L-/R-/-, Veh 13.5</td>
<td>0.6 ± 0.2*</td>
<td>22 ± 2*</td>
<td>2.7 ± 1.9*</td>
</tr>
<tr>
<td>L-/R-/-, bC 13.5</td>
<td>0.9 ± 0.4*#</td>
<td>79 ± 22*</td>
<td>7.2 ± 7.2*</td>
</tr>
</tbody>
</table>

WT and L-/R-/- dams were fed a vitamin A-deficient diet from 0.5-14.5 dpc. Dams were injected with Vehicle (Veh 13.5) or β-carotene at 13.5 dpc (bC 13.5). Data presented as mean ± SD. Sample size: liver, n=5-8 dams/group; multiple placentas and embryos per dam (n=1-5 dams/group) were analyzed for n=3-12 placentas or embryos/group. Statistical analysis by two-way ANOVA + LSD/Tamhane’s post hoc, followed by Student’s t test (liver + embryo) or by Kruskal-Wallis + Mann-Whitney U test (placenta). *, $p<0.05$ vs. WT; #, $p<0.05$ vs. Veh.
Figure 2-2. Placental and embryonic mRNA expression levels of genes involved in β-carotene cleavage and retinoid homeostasis following β-carotene supplementation at 13.5 dpc.

qRT-PCR analysis was performed using mRNA from 14.5 dpc placentas (A) and embryos (B) from wild-type (WT) and Lrat⁻/⁻-Rbp⁻/⁻ (L⁻/-R⁻/-) dams treated at 13.5 dpc with Vehicle (Veh 13.5) or β-carotene (bC 13.5). Tissues of WT Veh 13.5 were set as calibrator at 1. Data are presented as mean ± SD fold of WT Veh 13.5. Sample size, n=5-10 placentas or embryos/group (from 3-5 dams/group). Statistical analysis was performed by two-way ANOVA with genotype and treatment as factors, followed by LSD or Tamhane’s post hoc analysis. Individual comparisons then were made by Student’s t test. *, p<0.05 vs. WT; #, p<0.05 vs. Veh.
Further HPLC analysis indicated that hepatic, placental, and embryonic retinol levels were significantly reduced in L-/-R-/- mice compared to WT, regardless of the treatment given at 13.5 dpc (Table 2-3). Hepatic retinol levels increased modestly but significantly following bC supplementation of L-/-R-/- dams, even though they did not reach the WT level. Placental retinol levels were significantly reduced upon bC injection of WT dams compared to Veh, whereas a trend towards an increase (p=0.057) was observed upon bC injection of L-/-R-/- dams compared to Veh. However, bC supplementation did not alter embryonic retinol levels either in WT or in L-/-R-/- mice.

Our lab has previously reported that acute bC supplementation of vitamin A-sufficient dams at 13.5 dpc alters the mRNA expression of genes involved in both uptake and metabolism of bC in placenta in order to maintain homeostatic retinoid levels in the placental-fetal unit (Wassef et al., 2012). To determine whether such transcriptional events persist during maternal vitamin A deficiency, we performed qRT-PCR analysis on the placentas and embryos of WT and L-/-R-/- dams fed the vitamin A-deficient diet and injected with bC or Veh at 13.5 dpc. As shown in Figure 2-2A, Cmo1 mRNA expression in the placenta of Veh-treated L-/-R-/- dams was significantly greater than that of WT; however, upon bC injection, mRNA expression was reduced (p=0.05), though not to the WT level. The gene for the asymmetric bC cleavage enzyme, Cmo2, was similar in placentas of both genotypes, regardless of treatment (Fig. 2-2A). The mRNA expression of placental Rdh10 and Raldh2 were ~50% of WT Veh-treated in L-/-R-/- placentas, regardless of treatment (Fig. 2-2A). In contrast, bC treatment in WT dams reduced placental Rdh10 mRNA by ~50% compared to WT Veh-treated dams. Placental mRNA
levels of Cyp26a1 in bC-injected L-/-R/- dams showed a trend to differ from WT bC-treated (p=0.061), while Stra6 mRNA expression levels were similar in placentas of both genotypes, regardless of treatment (Fig. 2-2A).

Analysis of mRNA expression in embryos (Fig. 2-2B) revealed that Cmo1 mRNA levels were ~50% lower in L-/-R/- Veh compared to WT, and increased significantly upon bC injection without reaching the WT level. Cmo2 mRNA expression was half of the WT level in L-/-R/- embryos, regardless of treatment (Fig. 2-2B). Rdh10 mRNA levels showed a non-significant tendency (p=0.076) to decrease in bC-treated WT embryos compared to WT Veh, and were reduced in L-/-R/- Veh compared to WT but did not increase upon bC treatment (Fig. 2-2B). Raldh2 mRNA expression was significantly greater in L-/-R/- Veh embryos compared to WT, but was reduced to the WT level upon bC treatment of the L-/-R/- dams (Fig. 2-2B). Cyp26a1 mRNA was significantly reduced in L-/-R/- Veh embryos compared to WT, whereas embryonic mRNA levels of Stra6 were unaffected by genotype or treatment (Fig. 2-2B).

All together these results suggest that even severely vitamin A-deficient developing tissues activate transcriptional mechanisms to maximize the utilization of bC provided to the dams. Nevertheless, these regulatory mechanisms appear to be rather inefficient under this regimen of maternal provitamin A supplementation, as the embryonic phenotype was not improved.
Table 2-4. Phenotype distribution of L-/-R-/- embryos from dams fed a vitamin A-deficient diet, supplemented with Veh or bC from 6.5-9.5 dpc

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n [embryos, (dams)]</th>
<th>Percent Resorbed</th>
<th>Normal</th>
<th>Eye and/or Edema</th>
<th>Cleft</th>
<th>Exencephaly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh 6.5-9.5</td>
<td>22 (6)</td>
<td>51% (23/45)</td>
<td>0%</td>
<td>18%</td>
<td>82%</td>
<td>0%</td>
</tr>
<tr>
<td>bC 6.5-9.5</td>
<td>39 (8)</td>
<td>31% (19/58)</td>
<td>38%</td>
<td>59%</td>
<td>0%</td>
<td>3%</td>
</tr>
</tbody>
</table>

L-/-R-/- dams were fed a vitamin A-deficient diet from 0.5-14.5 dpc. Six dams were injected with Veh daily from 6.5-9.5 dpc (Veh 6.5-9.5); 8 dams were injected with bC daily from 6.5-9.5 dpc (bC 6.5-9.5). Percentage of resorbed embryos was calculated from the total number of implantations in the uterus. Note that 3% exencephaly represents a single embryo, as n=39.
Table 2-5. Tissue Retinol levels (14.5 dpc) of \( L/-R/- \)-dams fed a Vitamin A-deficient diet from 0.5-14.5 dpc, and injected with Veh or bC 6.5-9.5 dpc

<table>
<thead>
<tr>
<th></th>
<th>Liver (( \mu g/g ))</th>
<th>Placenta (ng/g)</th>
<th>Embryo (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( L/-R/-, ) Veh 6.5-9.5</td>
<td>0.4 ± 0.1</td>
<td>67 ± 32</td>
<td>5.6 ± 4.7</td>
</tr>
<tr>
<td>( L/-R/-, ) bC 6.5-9.5</td>
<td>0.7 ± 0.2#</td>
<td>113 ± 42</td>
<td>26.2 ±12.1</td>
</tr>
</tbody>
</table>

\( L/-R/- \)-dams were fed a vitamin A-deficient diet from 0.5-14.5 dpc, and injected with Vehicle (Veh) or \( \beta \)-carotene (bC) daily from 6.5-9.5 dpc. Data presented as mean ± SD. Statistical analysis by Student’s t test (liver, old placenta) or Mann-Whitney U test (old embryo). Sample size, \( n=3-7 \) dams; Placental and Embryonic levels include more than one embryo from each litter. \#, \( p<0.05 \) vs. \( L/-R/-, \) Veh 6.5-9.5.
Figure 2-3. Placental and embryonic mRNA expression levels of genes involved in β-carotene cleavage and retinoid homeostasis following β-carotene supplementation from 6.5-9.5 dpc.

qRT-PCR analysis was performed using mRNA from 14.5 dpc placentas (A) and embryos (B) from L-/-R/- dams treated from 6.5-9.5 dpc with Veh or bC. Tissues of L-/-R/- Veh 6.5-9.5 were set as calibrator at 1. Data presented as mean ± SD fold of L-/-R/- Veh 6.5-9.5. Sample size, n=5-6 embryos or placentas/group from different dams. Statistical analysis was performed by Student’s t test. #, p<0.05 vs. L-/-R/- Veh 6.5-9.5.
4.2. Repeated maternal supplementation with bC from 6.5-9.5 dpc

As bC supplementation at mid-gestation (13.5 dpc) was subsequent to the majority of organogenesis (Rugh, 1968), and thus unable to ameliorate the gross morphological defects of the vitamin A-deficient L-/R/- embryos, we next performed a more prolonged supplementation experiment during the critical window of mouse organ development (Rugh, 1968). L-/R/- pregnant females were fed a vitamin A-deficient diet from 0.5-14.5 dpc, and supplemented with ~40 µg/g bC or Veh daily from 6.5-9.5 dpc by IP injection. bC supplementation dramatically improved the phenotype of L-/R/- embryos, as the cleft palate was no longer observed, 38% of embryos developed as grossly phenotypically normal (Fig. 2-1E), and the percentage of resorptions was reduced in comparison to Veh-treated animals (Table 2-4). At 14.5 dpc, L-/R/- dams injected from 6.5-9.5 dpc displayed higher concentrations of bC in liver, while serum bC levels were less than those described above for dams injected at 13.5 dpc (Table 2-2). Table 2-5 shows the increased hepatic retinol concentration of L-/R/- dams injected with bC from 6.5-9.5 dpc compared to Veh-injected controls. In contrast, embryonic and placental bC (Table 2-2) and retinol (Table 2-5) were not significantly altered by prolonged bC treatment.

Since the external developmental defects were fully rescued in 38% of L-/R/- embryos from dams given bC from 6.5-9.5 dpc, we wondered whether key mediators of retinoid homeostasis had facilitated the improved phenotype. Thus, we performed qRT-PCR on malformed L-/R/- Veh embryos and normal L-/R/- bC embryos at 14.5 dpc, along with their respective placentas. Figure 2-3A shows similar placental mRNA
expression levels of \textit{Cmo1}, \textit{Stra6}, \textit{Rdh10}, \textit{Raldh2}, and \textit{Cyp26a1} at 14.5 dpc between Veh and bC-treated dams; however, \textit{Cmo2} mRNA expression was significantly reduced in the placentas of bC-injected dams. Only \textit{Cyp26a1} embryonic mRNA expression increased significantly in bC-treated dams compared to Veh, whereas all other genes analyzed were similar in embryos between the groups (Fig. 2-3B).

Taken together, these results confirm the ability of severely vitamin A-deficient tissues to utilize bC provided to the dams at early stages of development. Such a developmental window (6.5-9.5 dpc) is optimal to achieve gross improvement in embryogenesis.
5. Discussion

Both maternal dietary vitamin A intake and tissue status are critical determinants of embryonic development (Kim et al., 2008). Studies in both humans and mice have demonstrated that vitamin A deficiency is associated with cleft face/palate and defects in tissue development, including the heart and limbs (Clagett-Dame and Knutson, 2011; Wilson and Warkany, 1950). The world’s population relies on dietary intake of both preformed vitamin A and provitamin A carotenoids (including bC) to ensure adequate vitamin A levels (Grune et al., 2010). At term, correlations among maternal serum bC, placental and cord serum retinol have been observed only in mothers with subadequate serum vitamin A levels (Dimenstein et al., 1996). Thus, scarce maternal tissue retinoid and carotenoid stores jeopardize the adequate supply of retinoids to the developing embryo, especially if dietary vitamin A is limiting. The \(L-/R-/\) mouse is a useful model to examine the uptake and function of supplemented bC during pregnancy when maternal vitamin A stores are limiting (Wassef et al., 2012).

Our lab has previously shown that embryonic CMO1 can generate retinoids locally from maternal circulating bC, to support normal embryonic development (Kim et al., 2011). In the current study, we compared the ability of a single (13.5 dpc) or repeated (6.5-9.5 dpc) doses of bC to maintain embryonic retinoid homeostasis in a model of maternal dietary vitamin A deficiency (WT on vitamin A-deficient diet) and a model of severe maternal vitamin A-deficient status (\(L-/R-/\) on vitamin A-deficient diet). Given the dependence of embryonic patterning and organ development on retinoic acid (Clagett-Dame and Knutson, 2011), we were not surprised that the malformations in \(L-/R-/\) embryos persisted following a single maternal bC injection after the majority of
organogenesis was complete (e.g. at 13.5 dpc (Rugh, 1968); Table 2-1 and Fig. 2-1). Interestingly, despite the malformed appearance of the L-/R-/ embryos following the single bC injection, supplemental bC improved the retinoid status of vitamin A-deficient dams to a limited degree, even at a time when it was too late to support normal embryogenesis (retinol levels increased in maternal liver in the supplemented group; Table 2-3). While a small percentage of embryos from dams supplemented with bC at 13.5 dpc displayed a milder phenotype than those treated with Veh (eye defect and edema, versus cleft face/palate, Table 2-1), we believe this difference is physiologically insignificant, as even embryos from dams treated with Veh from 6.5-9.5 dpc showed a low percentage of eye defects and edema (Table 2-4). In contrast, when bC was supplemented during the window of organogenesis (6.5-9.5 dpc) when the retinoid requirements are higher (Satre et al., 1992), L-/R-/ embryos no longer showed cleft face/palate, but a shift towards a less severe phenotype (eye defect and edema) was observed, and nearly 40% of them appeared grossly normal (Table 2-4). In addition to this dramatic improvement of L-/R-/ external morphology, the resorption percentage of these embryos became similar to our previously published results for vitamin A-sufficient L-/R-/ dams (Kim et al., 2008), further reinforcing that a sustained bC supplementation during early development of otherwise vitamin A-deficient dams mimics a vitamin A-sufficient state.

We observed a lower recovery of normal embryos from bC-supplemented L-/R-/ dams (38%) compared to our published results of Cmo1+/Rbp/- embryos from supplemented Cmo1-/Rbp/- dams (61%, (Kim et al., 2011)) – a result that can be understood in light of the maternal expression of CMO1. In the case of Cmo1-/Rbp/-
dams, all of the injected bC could be delivered to their Cmo1+/-Rbp-/- embryos, to be cleaved locally via the enzyme produced by their single genomic copy of Cmo1. On the other hand, the L-/-R-/- dams expressed CMO1 in all of their tissues, and thus could cleave a significant portion of the supplemental bC (for example, in the liver) before it reached the embryo. Indeed, the maternal serum bC concentration at 14.5 dpc in the L-/-R-/- dams (1.43 ± 0.65 μg/dL, Table 2-2) was approximately 5-fold lower than that reported for Cmo1-/-Rbp-/- dams (7.0 ± 2.3 μg/dL, (Kim et al., 2011) injected under the same protocol. Furthermore, the bC-derived retinoids of L-/-R-/- mice cannot be transported to their embryos due to their lack of RBP.

We have previously shown that maternal dietary vitamin A intake and status alter embryonic mRNA expression of numerous retinoic acid-responsive genes responsible for maintaining retinoid homeostasis in the developing tissues, including Raldh2, Cyp26a1, and Stra6 (Kim et al., 2008). In addition, we recently demonstrated that maternally supplemented bC also regulates placental and embryonic mRNA expression of genes involved in bC cleavage and uptake to maintain retinoid homeostasis (Wassef et al., 2012). Thus, we wondered to what extent such regulatory transcriptional events would take place (and be effective) upon bC supplementation in our model of severe maternal vitamin A-deficient intake and status.

We previously showed that the placenta of L-/-R-/- mice expresses high levels of Cmo1 under a vitamin A-sufficient diet compared to that of WT (Wassef et al., 2012), and the results of the current study are in agreement with this finding, suggesting the likelihood that a constitutively elevated CMO1 activity may exist in the L-/-R-/- placenta. In this case, bC supplementation could lead to elevated retinoic acid levels (generated
upon symmetric bC cleavage), which might then suppress Cmo1 via a negative feedback loop similar to that reported in intestine (Lobo et al., 2010b), leading to reduced levels of Cmo1 in the placenta of bC-supplemented L-/R-/ (Fig. 2-2A). This down-regulation of placental Cmo1 may help preserve intact bC for delivery to the severely vitamin A-deficient L-/R-/ embryos, which in turn could cleave bC locally and produce the much needed retinoic acid. Further studies investigating this mechanism in the placenta need to be conducted.

RDH10 and RALDH2 control the two oxidative steps that result in the synthesis of retinoic acid from retinol (Niederreither et al., 1997; Sandell et al., 2007; Sandell et al., 2012). Specifically, RDH10 catalyzes the conversion of retinol into retinaldehyde and RALDH2 catalyzes the oxidation of retinaldehyde to retinoic acid. The placental mRNA expression of Rdh10 and Raldh2 in L-/R-/ were lower than that of the WT Veh-treated placenta (Fig. 2-2A). The reduced level of Raldh2 is in agreement with our previous study (Kim et al., 2008), and may be a mechanism to help spare placental retinoids (including endogenous) for delivery to the severely vitamin A-deficient embryos, where they can ultimately be converted to retinoic acid. The lack of effect of bC supplementation on Rdh10 and Raldh2 in L-/R-/ placentas may indicate that the 24-hour time point (14.5 dpc) is too early to observe feedback inhibition of retinoic acid (produced by bC cleavage) on these genes in such severely vitamin A-deficient tissues. Alternatively, retinoic acid may be shunted to the developing embryo rather than used by the placenta. bC cleaved to retinaldehyde could be transported via CRBP1/RBP/albumin to the embryo or reduced to retinol, which too can be transported to the embryo via CRBP1/RBP/albumin for local production of retinoic acid in the vitamin A-deficient
embryo (Dancis et al., 1992; Johansson et al., 2001; Quadro et al., 2004; Smith et al., 1973).

The baseline reduction of \textit{Cmo1} in \textit{L-/-R-/-} embryos (Fig. 2-2B) supports our results in vitamin A-sufficient dams lacking both LRAT and RBP (Wassef et al., 2012), although the reduction is more dramatic in the vitamin A-deficient embryos. This result yet again indicates an effect of the lack of LRAT on \textit{Cmo1} that is tissue-specific (\textit{Cmo1} is reduced in the embryo [Fig. 2-2B] and increased in the placenta [Fig. 2-2A] in the absence of LRAT) – a phenomenon that is actively under investigation in our laboratory. The increase in \textit{L-/-R-/-} embryonic \textit{Cmo1} mRNA levels upon bC supplementation is likely due to increased CMO1-mediated retinoic acid production (i.e. positive feedback). Indeed, at least in certain tissues, \textit{Cmo1} mRNA expression is positively regulated by PPAR\( \gamma \) (Boulanger et al., 2003), and \textit{Ppar}\( \gamma \) itself is up-regulated by retinoic acid (Reichert et al., 2011). However, this is the first report of a suppression of \textit{Cmo2} mRNA expression in the absence of embryonic LRAT (Fig. 2-2B). The bC-independent appearance of these mRNA reductions (bC is not present in the purified diet used in this experiments) suggests that a mechanism other than retinoic acid-mediated transcriptional regulation is responsible for the changes in mRNA levels. The lack of effect of bC supplementation on \textit{Cmo2} mRNA levels re-iterates the centrality of CMO1 as the main bC cleavage enzyme \textit{in vivo} (Amengual et al., 2010).

In a recent study by Sandell \textit{et al}. (Sandell et al., 2012), a potential retinoic acid response element (RARE) was identified in exon 5 of \textit{Rdh10}, and reduced retinoic acid was shown to increase mRNA levels of embryonic \textit{Rdh10} in the vitamin A-deficient
Raldh2-/- mouse at 9.5 dpc. Indeed, in our study, Rdh10 expression was reduced in WT embryos from bC-treated dams, suggesting that production of retinoic acid from bC cleavage may lower Rdh10 (Fig. 2-2B). Unlike our WT data or those reported by Sandell et al., Rdh10 embryonic mRNA levels did not differ between L-/-R-/- bC and Veh, although both were lower than WT Veh (Fig. 2-2B). We hypothesize that 24 hours post-injection is too early to detect negative feedback of Rdh10 in severely vitamin A-deficient tissues (L-/-R-/-).

The up-regulation of Raldh2 in vitamin A-deficient L-/-R-/- embryos (Fig. 2-2B) confirms our published results (Kim et al., 2008), while its reduction upon bC supplementation may indicate that increased retinoic acid production by CMO1 and the potential transport of retinol/retinaldehyde from the placenta reduce Raldh2 to WT levels through negative feedback (Dobbs-McAuliffe et al., 2004; Niederreither et al., 1997). Our earlier work also showed that embryonic Cyp26a1 mRNA levels were lower in vitamin A-deficient L-/-R-/- mice compared either to vitamin A-sufficient WT mice, or to L-/-R-/- mice on vitamin A-sufficient or vitamin A-excess diets (220 IU/g of diet) (Kim et al., 2008). The current study supports this finding, as L-/-R-/- Veh vitamin A-deficient embryos had lower Cyp26a1 mRNA levels than WT Veh (Fig. 2-2B), suggesting insufficient production of retinoic acid to support normal embryonic development.

When a sustained (6.5-9.5 dpc) maternal bC supplementation was performed we were only able to evaluate the transcriptional response of the developing tissues five days after the last injection of bC. This is likely the reason for the apparent difference in the transcriptional regulatory events observed in the developing tissues depending on the
window of bC supplementation. In placenta, none of the tested genes showed changes in mRNA levels, except for Cmo2 (Fig. 2-3A). Given the persistence of circulating bC and the amount in the placenta at 14.5 dpc (Table 2-2), the down-regulation of Cmo2 is interesting, and may favor the delivery of intact bC to the embryo rather than scavenging it (Amengual et al., 2010) in the placenta (Fig. 2-3A). Among all the genes tested in the embryo, only Cyp26a1, which encodes the enzyme that catabolizes retinoic acid into transcriptionally inactive forms (Niederreither et al., 2002), was significantly up-regulated when L-/R-/ dams were injected four times with bC (Fig. 2-3B). This significant increase in embryonic Cyp26a1 at 14.5 dpc may signal that sufficient retinoic acid has been generated (supported by the normal phenotype of these embryos, Table 2-4), and that any further retinoic acid should now be catabolized. The more dramatic increase in embryonic Cyp26a1 mRNA levels when bC is supplemented for four days (Fig. 2-3B), compared to a single dose (Fig. 2-2B), is reinforced by the lack of phenotype improvement following the single dose (Table 2-1). Future studies using the same experimental design and analyzing genes involved in retinoid homeostasis at 10.5 dpc for example, would provide further information on the acute changes following four days of bC supplementation during the critical stages of development. It is noteworthy that the repeated supplementation with bC also improves the maternal vitamin A status as indicated by the elevated bC levels in the livers and placentas (mildly) of L-/R-/ dams on the above-mentioned regimen.

Although our previous results indicated that Stra6 mRNA levels were reduced in vitamin A-deficient L-/R-/ placentas and embryos compared to the same genotype on
vitamin A-sufficient or vitamin A-excess diets (Kim et al., 2008), we did not observe any difference in *Stra6* mRNA levels among any groups in the present study. It may be that concentrations of preformed vitamin A, rather than provitamin A carotenoids (e.g. bC), modulate the transcription of *Stra6*. While *Stra6* is so named because it is “stimulated by retinoic acid”, our lab also has published evidence that *Stra6* mRNA expression responds to the need to efflux excessive intracellular retinol (Kim et al., 2008). Thus, although bC is indeed generating retinoic acid in bC-supplemented embryos (supported by changes in *Cyp26a1* mRNA levels and embryonic phenotype following the prolonged treatment of *L-/-R-/-* dams), cellular retinol is probably not accumulating in this system. However, in support of the previous study, WT and *L-/-R-/-* mRNA levels of *Stra6* were similar in placentas and embryos on the vitamin A-deficient diet (Fig. 2-2).

The current study highlights the importance of bC as a precursor of vitamin A during embryonic development. Even severely vitamin A-deficient developing tissues activate transcriptional mechanisms which aim to maximize the use of bC, the efficiency of which depends on the developmental window of bC supplementation. Our findings are important in view of the possibility that adequate amounts of bC given during pregnancy can attenuate the effects of vitamin A deficiency, a problem affecting millions of people worldwide.
Chapter 3

The function of β-carotene 9’,10’-oxygenase (CMO2) in mammalian embryonic development
1. Abstract

β-carotene (bC) is the main vitamin A precursor for most of the world, contributing to the supply of retinoids needed to support embryonic development. We assessed the contribution of the asymmetric β-carotene (bC) cleavage enzyme β-carotene 9’,10’-oxygenase (CMO2) to mammalian embryogenesis using knockout mice for genes including β-carotene 15,15’-oxygenase (CMO1) and retinol-binding protein (RBP). By comparing vitamin A-deprived Cmo1/-Rbp/- and Cmo1/-Rbp/-Cmo2/- mice upon bC supplementation, we showed that in the absence of CMO1, the cleavage of bC by CMO2 did not generate significant retinoids, while spontaneous oxidation of bC improved their embryonic phenotype. In Cmo2/-Rbp/- mice, vitamin A deprivation caused severe embryonic malformations which could not be fully rescued by bC despite CMO1 expression. While both the Cmo1/-Rbp/-Cmo2/- and Cmo2/-Rbp/- embryonic phenotypes were largely rescued by retinaldehyde supplementation, the greatest improvement of the Cmo2/-Rbp/- phenotype was achieved upon supplementation with β-apo-10’-carotenal, the apocarotenoid normally generated from bC by CMO2 activity. 

In vitro, β-apo-10’-carotenal interacted with the retinol-binding domain of PKCδ, a member of a mitochondrial signaling complex that enhances cellular respiration upon retinol binding. Both bC and β-apo-10’-carotenal tended to enhance cellular respiration of mouse embryonic fibroblasts (MEFs), but bC dramatically reduced cell viability at higher concentrations. β-apo-10’-carotenal did not increase oxygen consumption in PKCδ/- or PKCe/- MEFs. Overall, β-apo-10’-carotenal seemed to support embryonic
development through its ability both to generate retinoic acid and affect cellular respiration via PKC. These results indicate that β-apo-10’-carotenal is a potent nutrient that could alleviate vitamin A deficiency more effectively than bC or retinaldehyde.
2. Introduction

Normal mammalian embryonic development requires the essential nutrient, vitamin A, to support a variety of processes including cell fate specification, patterning, and differentiation (Clagett-Dame and Knutson, 2011). Vitamin A deficiency during pregnancy compromises these processes, and can lead to gross morphological defects in the eyes, limbs, heart, nervous system, and craniofacial region (Clagett-Dame and Knutson, 2011; Niederreither and Dollé, 2008). To obtain adequate vitamin A, mammals must consume it in their diet, either as preformed vitamin A (mainly retinyl esters, from animal sources), or as vitamin A precursors (pro-vitamin A carotenoids such as β-carotene [bC], from plant sources) (Harrison, 2012).

bC is the main vitamin A source for most of the human population (Grune et al., 2010; Weber and Grune, 2012), and must be converted to vitamin A in vivo by the action of carotenoid cleavage enzymes (Lobo et al., 2012a). In the intestine, this conversion is predominantly catalyzed by a cytosolic enzyme, β-carotene 15-15′-oxygenase (CMO1), which generates two molecules of retinaldehyde by symmetrically cleaving bC (Goodman and Huang, 1965; von Lintig and Vogt, 2000). Additionally, a mitochondrially-localized enzyme, β-carotene 9′-10′-oxygenase (CMO2), can generate apocarotenoids by asymmetrically cleaving bC (Amengual et al., 2010; Kiefer et al., 2001), which then can be converted to one molecule of retinaldehyde by chain shortening enzymes (Wang et al., 1996) or CMO1 (Amengual et al., 2013). In humans, the CMO1-mediated intestinal carotenoid cleavage activity is inefficient (and the Cmo1 gene is subject to polymorphisms (Leung et al., 2009; Lietz et al., 2012b)), allowing up to 45% of
dietary bC to be absorbed intact by the enterocytes (Goodman et al., 1966; Hickenbottom et al., 2002).

All retinol, regardless of its dietary origin, is esterified in enterocytes and transported (along with any intact bC) to the systemic circulation via the lymphatic system within chylomicrons (Goodman et al., 1966). The hydrolysis of triglycerides within chylomicrons by Lipoprotein Lipase (LPL) in capillary endothelial cells produces chylomicron remnants still containing retinyl esters and bC (Johnson and Russell, 1992; Ross and Zilversmit, 1977). About 75% of the chylomicron remnant retinyl ester is taken up by the liver (Goodman et al., 1965) and stored in hepatic stellate cells (Matsuura et al., 1997), while ~25% is delivered to target tissues (Goodman et al., 1965; van Bennekum et al., 1999). Hepatic retinoid stores can be released as retinol bound to its only specific carrier protein, retinol-binding protein (RBP) (Kanai et al., 1968), to deliver vitamin A to peripheral cells through STRA6, the cell-surface receptor for RBP-retinol (Kawaguchi et al., 2007; Kawaguchi et al., 2012).

In target cells, retinol can be oxidized to retinaldehyde by retinol dehydrogenases (RDHs) including RDH10 (Sandell et al., 2007; Sandell et al., 2012). bC taken up from chylomicron remnants (or re-secreted from the liver) also can be cleaved to retinaldehyde in target cells, most of which express both CMO1 and CMO2 (Lindqvist and Andersson, 2004; Lindqvist et al., 2005). Ultimately, retinaldehyde arising from provitamin A carotenoids or preformed vitamin A can be oxidized to retinoic acid irreversibly by retinaldehyde dehydrogenases (RALDHs) such as RALDH2 (Niederreither et al., 1997). Retinoic acid is the biologically active vitamin A metabolite, which regulates the transcription of hundreds of genes as a ligand for the retinoic acid receptors (RARs and
RXRs) (Niederreither and Dollé, 2008; Rhinn and Dollé, 2012). Up to a certain level, excess retinoic acid can be catabolized to form non-polar derivatives by members of the CYP26 family (Niederreither et al., 2002; Ross and Zolfaghari, 2011), and excess retinol can be stored as retinyl esters via lecithin-retinol acyltransferase (LRAT) (Batten et al., 2004; Kim et al., 2008; Kurlandsky et al., 1996; Ruiz et al., 1999) or likely also effluxed via STRA6 (Isken et al., 2008; Kawaguchi et al., 2012; Kim et al., 2011).

CMO1 has been characterized extensively by various investigators using the Cmo1-/ mouse model (Fierce et al., 2008; Hessel et al., 2007; Shmarakov et al., 2010; van Helden et al., 2010), and has been denoted as the main retinoid-generating enzyme in adult tissues of mice consuming bC (Lietz et al., 2012a). Our lab demonstrated that embryonic expression of CMO1 could allow mouse embryos to develop normally when their mothers were provided bC as the only vitamin A source (Kim et al., 2011). These results all contributed to the concept that CMO2-mediated bC cleavage is not an important source of vitamin A. However, certain feasible scenarios, such as low bC cleavage activity due to single-nucleotide polymorphisms (SNPs) in Cmo1 (Leung et al., 2009; Lietz et al., 2012b), or extremely limited dietary vitamin A and provitamin A availability, could jeopardize embryonic development if CMO1 were the only enzyme generating retinoids from bC in vivo. Additionally, recent evidence showed that “stepwise cleavage” (first by CMO2, followed by CMO1) of carotenoids having one substituted β-ionone ring (e.g. β-cryptoxanthin), could generate retinoids from provitamin A carotenoids that are otherwise poor substrates for CMO1 (Amengual et al., 2013). Given that both Cmo1 and Cmo2 are expressed in mammalian embryos, yolk sacs
and placentas throughout gestation (Kim et al., 2011), we reasoned that CMO2 may contribute to retinoid homeostasis in mammals, at least during embryogenesis when the consequences of vitamin A deficiency are so detrimental (Clagett-Dame and Knutson, 2011). Indeed, even in adult mice, Cmo2 mRNA expression was found to be up-regulated in certain Cmo1-/- tissues (Amengual et al., 2011; Shmarakov et al., 2010).

To study the retinoid-generating potential of CMO2 in vivo, we chose to study mice on the Rbp-/- background. These mice become vitamin A-deficient much more rapidly than wild-type mice when fed a vitamin A-deficient diet (Quadro et al., 1999), and thus allowed us to capture retinoid-dependent phenotypes during embryogenesis and assess the effects of bC supplementation (Quadro et al., 2005). We used the Cmo1-/-Rbp-/- mice generated in our previous study (Kim et al., 2011), as well as a novel mouse strain that we recently generated, the Cmo1-/-Rbp-/-Cmo2-/- “triple-knockout”, to determine whether cleavage of bC by CMO2 could facilitate normal embryonic development. These studies revealed that bC cleavage by CMO2 does not produce a significant/sufficient amount of retinoids to support embryonic development in the absence of CMO1. However, further studies in Cmo2-/- and Cmo2-/-Rbp-/- mice indicated that CMO2 is critically involved in other retinoid-dependent and -independent developmental processes, including retinyl ester formation and mitochondrial energy homeostasis.
3. Materials and Methods

3.1. Knockout mice, nutritional manipulation, and bC supplementation

Mice of the following genotypes were used in the present studies: wild-type (WT), \( Rbp^{-/-} \) (Quadro et al., 1999), \( Cmo1^{-/-} \) (Hessel et al., 2007), \( Cmo2^{-/-} \) (Amengual et al., 2010), \( Cmo1^{-/-}Rbp^{-/-} \) (Kim et al., 2011), \( Cmo1^{-/-}Cmo2^{-/-} \), \( Cmo2^{-/-}Rbp^{-/-} \), and \( Cmo1^{-/-}Rbp^{-/-}Cmo2^{-/-} \) (“triple-knockouts”). The latter four strains were generated in our laboratory. We first crossed \( Cmo1^{-/-}Rbp^{-/-} \) females with \( Cmo2^{-/-} \) males to produce the F1 generation (\( Cmo1^{+/-}Rbp^{+/-}Cmo2^{+/-} \)). The F1 mice and subsequent generations were inbred to obtain the genotypes listed. All mice had a mixed genetic background (C57BL/6 x Sv/129). Mice had access \textit{ad libitum} to diet and water and were maintained on a 12 hour light-dark cycle. Due to the high dietary vitamin A requirements of the \( Rbp^{-/-} \) (Quadro et al., 1999), all mice were maintained throughout life on a non-purified vitamin A-sufficient diet (18 IU/g, Prolab Isopro RMH3000 5p75, hereafter referred to as the “vitamin A-sufficient diet”) which contained a small amount of carotenoids (<1.2 \( \mu \)g/g), until the time of nutritional manipulation.

At 90 days of age, females were mated with males of their respective genotype, and were considered 0.5 days \textit{post coitum} (dpc) on the morning of vaginal plug detection. From 0.5 dpc until 14.5 dpc, dams were fed a purified vitamin A-deficient diet (Research Diets, <0.2 IU/g vitamin A, 0 \( \mu \)g/g bC), and certain dams were assigned to the bC treatment group. As the highly efficient intestinal carotenoid cleavage activity in mice prevents the absorption of intact bC (Lee et al., 1999), we bypassed the intestine by injecting bC intraperitoneally (IP) daily from 6.5-9.5 dpc (the period of most
organogenesis in mice (Rugh, 1968)). The bC solution was prepared as described previously (Wassef et al., 2012); briefly, 50 mg bC (Type I, Sigma Aldrich) was dissolved in 5 mL Vehicle (Veh) by sequential addition of ethanol, PBS and Cremophor at a ratio of 1:11:18. The concentration of the resulting solution was determined spectrophotometrically at 450 nm, and averaged ~4 mg/mL due to the poor solubility of bC. 250 μL of this solution was administered in each daily injection (6.5-9.5 dpc), resulting in an average daily dose of 1 mg bC (or ~50 μg bC/g bodyweight assuming ~22 g bodyweight), about 20 times the typical daily food intake of 25 retinol activity equivalents (RAE; 1 RAE = 1 μg retinol = 2 μg bC). This dosing regimen was shown to be non-toxic in previous studies from our lab (Kim et al., 2011; Wassef et al., 2013).

Additional pregnant Cmo2/-/Rbp-/- dams were provided with the antioxidant N-acetylcysteine (NAC) at a dose of 0.5 mg/g bodyweight in their drinking water from 0.5-14.5 dpc. Food and water intake were monitored throughout the experiment, and water was replaced every 2-3 days. These females were fed the vitamin A-deficient diet from 0.5-14.5 dpc and injected with bC IP from 6.5-9.5 dpc.

A group of Cmo2/-/Rbp-/- and triple-knockout dams was maintained on the vitamin A-deficient diet throughout gestation, but supplemented with retinaldehyde (400 μg/g diet) from 7.5-9.5 dpc, as published (Rhinn et al., 2011). Briefly, a 50 mg/mL ethanolic solution of retinaldehyde was diluted in a mixture of pulverized vitamin A-deficient diet and water (1:1, g/mL) to yield the final dose of 400 μg/g diet, assuming a daily intake of 3 g food.
A small group of Cmo2-/-Rbp-/- dams (n=3) also was fed the vitamin A-deficient diet throughout gestation, and supplemented with β-apo-10’-carotenal (~18 µg/g bodyweight, n=1; ~30 µg/g bodyweight, n=2) from 7.5-9.5 dpc. Briefly, an ethanolic solution of β-apo-10’-carotenal (~6.7 or 12 mg/mL, respectively for the different doses) was diluted in a mixture of pulverized vitamin A-deficient diet and water (1:1, g:mL) to yield the final dose of 18 or 30 µg/g bodyweight, assuming a daily intake of 4 g food. One dam from each dose was also injected with bC from 6.5-9.5 dpc as described above.

All dams were sacrificed by CO₂ inhalation at 14.5 dpc between 9:00 and 11:30 am, unless otherwise noted. At sacrifice, maternal serum and liver were collected, uteri were dissected, and embryonic phenotype was recorded. Tissues were immediately frozen on dry ice, then stored at -80 °C until analyzed.

3.2. HPLC and LC-MS

Retinoid and bC concentrations in serum, tissues, and bC preparations were analyzed by reversed-phase HPLC as described previously (Kim et al., 2008; Kim and Quadro, 2010). Briefly, 50-200 mg tissue was homogenized in 1-2 mL PBS, or 100 µL serum/diluted bC solution was measured. Internal standards (retinyl acetate for retinoid extraction, echinenone for bC extraction) were immediately added to 100-1000 µL of the sample. For retinoid extraction, samples were deproteinated by the addition of an equal volume of ethanol, then mixed with 4 mL hexanes by vortexing 1-2 minutes. After centrifugation for 3 minutes at 1400xg, the supernatants were collected and dried under N₂. For carotenoid extraction, samples were sequentially mixed by vortexing with an
equal volume of methanol (20 sec), 0.4-0.9 mL acetone (20 sec), and 1 mL petroleum ether (1-2 min); the latter step was repeated once. After centrifugation for 3 minutes at 1400xg, the supernatants were collected and dried under N₂.

The evaporated residues were resuspended in 50 μL mobile phase (acetonitrile:methanol:dichloromethane, 70%:15%:15%) and injected into a 4.6x250 mm Ultrasphere C₁₈ column (Beckman, Fullerton, CA) preceded by a C₁₈ guard column at a flow rate of 1.8 mL/min. Retinoids were detected at 325 nm, and bC was detected at 450 nm. After integration and spectral analysis of the resulting peaks, retinoid and bC concentrations were quantified using standard curves of AUC\textsubscript{compound}:AUC\textsubscript{standard} versus ng\textsubscript{compound}:ng\textsubscript{standard}. The limits of detection were <1 ng/dL and <10 ng/g for bC, and <0.1 ng/dL and <1 ng/g for retinoids.

LC-MS analysis (detection limit 1 ppm) was performed in the laboratory of William Blaner as described (Shmarakov et al., 2010).
Table 3-1. Primer sequences for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence - forward</th>
<th>Primer sequence - reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>5'-AACACAGTGCTGTCGTTG-3'</td>
<td>5'-GAAAGGTTGAAAAACGAC-3'</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5'-AATTTGGCATGTGGGAG-3'</td>
<td>5'-ACACATTTGGGTAGGAACA-3'</td>
</tr>
<tr>
<td>Cmo1</td>
<td>5'-GACCAATCAACCATTGGT-3'</td>
<td>5'-AATCAGCACCAGATTTC-3'</td>
</tr>
<tr>
<td>Cmo2</td>
<td>5'-AGGACCAGGCTGTATGGT-3'</td>
<td>5'-CGCTGGCTGAAGATAGGAC-3'</td>
</tr>
<tr>
<td>Lrat</td>
<td>5'-CTGACCAATGACAGAGGAAG-3'</td>
<td>5'-CTATCCCCAAAGACAGCCAGAC-3'</td>
</tr>
<tr>
<td>Cyp26a1</td>
<td>5'-GAACCTTACACCGCAGAT-3'</td>
<td>5'-CTCTGTATGGATTGTTTAGT-3'</td>
</tr>
<tr>
<td>Stra6</td>
<td>5'-AAGCAAGTCAGACTTCAAGG-3'</td>
<td>5'-CAGAGAGCATACCTTCTTCTC-3'</td>
</tr>
<tr>
<td>Ralh2</td>
<td>5'-TTGCAGATGCTGATGAC-3'</td>
<td>5'-TGAGGACACCTGCTAGTT-3'</td>
</tr>
<tr>
<td>Rdx1</td>
<td>5'-TATGAGGGGCACTCTCACT-3'</td>
<td>5'-GAAATCGATACATGCACAC-3'</td>
</tr>
<tr>
<td>Duox1</td>
<td>5'-TGGGAGGTACAGCGATTTG-3'</td>
<td>5'-CGCTGCAGCGAGCTTCTT-3'</td>
</tr>
<tr>
<td>p22phox</td>
<td>5'-CGTGCTACTGCTGACGT-3'</td>
<td>5'-GCACACTGCAGCGATAGAG-3'</td>
</tr>
<tr>
<td>Sod1</td>
<td>5'-GCCACATGGTTCCAGTCC-3'</td>
<td>5'-AACATGCTCTTCGCCTCCG-3'</td>
</tr>
<tr>
<td>Sod2</td>
<td>5'-AGGAGGTTAGTGGGAGCTA-3'</td>
<td>5'-AGCGGAATAAGGCTTGTGT-3'</td>
</tr>
<tr>
<td>Gpx1</td>
<td>5'-TTCCGACCCAGAAGATGG-3'</td>
<td>5'-TAAAGAGGCTGGAGCTTCTC-3'</td>
</tr>
<tr>
<td>PrxIII</td>
<td>5'-AAGAAAGATGTTGCTGGCTGGG-3'</td>
<td>5'-CGGAGGCTGAGCCTCACTC-3'</td>
</tr>
<tr>
<td>Hif1a</td>
<td>5'-TTCTGATGCTGGTCGGTCT-3'</td>
<td>5'-AAACATGCTGCCTCCATCT-3'</td>
</tr>
<tr>
<td>Gpx4-mito</td>
<td>5'-AGATGACTGGGGGGCTG-3'</td>
<td>5'-CGATGTCCTTGCTGAGAAT-3'</td>
</tr>
<tr>
<td>Gpx4-mito/cyto</td>
<td>5'-CGTGCAAGCCAGCTGTGGTG-3'</td>
<td>5'-CGATGTCCTTGCTGAGAAT-3'</td>
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<tr>
<td>Alcat1</td>
<td>5'-TCGGCCCTTTATCTTCTTGA-3'</td>
<td>5'-CAGCAATGCCAGAGCTG-3'</td>
</tr>
</tbody>
</table>
3.3. RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

RNA extraction and qRT-PCR analysis were performed as described previously (Kim et al., 2008). Briefly, 50-200 mg of tissue was homogenized in TriPure isolation reagent (2 mL/100 mg tissue), incubated 5 min at room temperature, and mixed with chloroform (0.2 mL/mL TriPure) by vigorous shaking for 15 sec. After incubation on ice for 10 minutes, samples were centrifuged 15 min at 12,000xg, and the supernatants were collected and mixed with isopropanol (0.5 mL/mL TriPure) by vortexing. Following 30 min incubation on ice and 15 min incubation at room temperature, samples were centrifuged 10 min at 12,000xg, and the RNA pellet was washed twice with cold 75% DEPC-treated ethanol. The pellet was air-dried and re-suspended in DEPC water, and this RNA extract was stored at -80 °C for later analysis. RNA concentration was determined using a Nanodrop Spectrophotometer, and RNA quality was assessed by the ratio of absorbances at 260 and 280 nm. RNA was DNAse-treated and 1 μg was reverse-transcribed to cDNA according to the manufacturer’s instructions (Roche First-strand cDNA synthesis kit).

qRT-PCR analysis was performed on duplicate/triplicate cDNA samples and controls containing no reverse transcriptase or no template, using SYBR Green Master Mix (Roche) in a Roche Light-cycler 480. Thermal cycling conditions were: 95 °C initiation for 10 minutes and 40-42 cycles of 95 °C for 30 sec, 58-60 °C annealing for 1 min, and 72 °C extension for 1 min. Primer sequences were as shown in Table 3-1. Relative gene expression was quantified using the comparative C_T method (Schmittgen and Livak, 2008), except for Gpx4 (mitochondrial and total) which were analyzed by the
standard curve method due to unequal amplification efficiencies between the gene of interest and the normalizer (β-actin). Normalized results are expressed as fold-change from the control group as indicated in figure legends.

3.4. ROS detection

Hepatic peroxide levels were assessed by the xylenol orange assay using frozen liver samples from Cmo2-/Rbp-/- dams fed the vitamin A-deficient diet from 0.5-14.5 dpc, with or without IP supplementation of bC from 6.5-9.5 dpc, and sacrificed at 14.5 dpc. Additionally, hepatic peroxides were measured in frozen liver samples from non-pregnant Cmo2-/ females maintained on the chow diet, with and without IP injection of bC for four consecutive days, and sacrificed after 24 hours. The xylenol orange assay detects the reaction of hydrogen peroxide with ferrous ions, yielding ferric ions which complex with xylenol orange to produce a purple color (Jiang et al., 1990). Hepatic lipids were extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959), and resuspended in 25 μL chloroform. This solution was incubated with the dye (xylenol orange in an acidic solution containing sorbitol and ammonium ferrous sulfate) for 30 minutes, protected from light. Absorbances were read at 560 nm, subtracted from those of controls containing no ferrous ions, and normalized to lipid content (determined gravimetrically) (Fakas et al., 2008).

Hepatic reactive oxygen species (ROS) levels were determined by the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies) in fresh liver homogenates from Cmo2-/Rbp-/- males fed the vitamin A-deficient diet for 11 days,
injected IP with bC or Veh on days 7, 8, 9 and 10. The Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit is based on the detection of resorufin, the product of the peroxidase reaction of hydrogen peroxide with 10-acetyl-3,7-dihydroxyphenoxazine (Zhou et al., 1997). Approximately 60 mg fresh liver was homogenized in a dounce with 1 mL PBS, and the homogenate was diluted 1/20 in PBS, then centrifuged 30 seconds at 13,400×g. The resulting supernatant was incubated for 30 minutes with an equivalent volume of the working solution, protected from light, according to the manufacturer’s instructions. Color development (resorufin production) was detected at 560 nm. Protein content was measured by the Bradford assay.

3.5. Cellular viability

Cells were seeded in a 96-well plate (10,000 cells/well) in complete culture medium (DMEM [Cellgro #10-017], 10% fetal bovine serum, and 1% each of Glutamax (L-glutamate) and Penicillin/Streptomycin). The next day, cells were extensively washed with PBS and incubated 24 hours with serum-free medium (DMEM, 0.05% bovine serum albumin, and 1% each of Glutamax [L-glutamate] and Penicillin/Streptomycin). Cells were then treated with bC, β-apo-10’-carotenal, or Veh (<0.2% THF/methanol, <0.2% ethanol for β-apo-10’-carotenal) overnight. The following day, cells were then incubated with 10 µL WST-1 (Roche) for 0.5-2 hours to detect cell proliferation. The absorbance was read in a microplate reader at 450 nm and 630 nm and was corrected against a cell-free sample containing medium and the reagent.
3.6. Oxygen consumption

Oxygen consumption was measured as an indicator of cellular respiration using an Oxygraph equipped with a Clark electrode. ~1 million cells were seeded per 10-cm tissue culture dish in complete medium (reduced serum advanced DMEM containing 10% FBS and 1% each of Glutamax [L-glutamate], Penicillin/Streptomycin, and non-essential amino acids). The following day, cells were washed with PBS and supplied with serum-free medium (reduced serum advanced DMEM, 0.05% bovine serum albumin, and 1% each of Glutamax [L-glutamate], Penicillin/Streptomycin, and non-essential amino acids). After overnight starvation, cells were treated with bC, β-apo-10'-carotenal, or Veh (0.5% hexane for bC, <0.2% ethanol for β-apo-10'-carotenal). Two hours later, cells were gently scraped from the dish and centrifuged for 1 minute at 1500xg. The supernatant was discarded and cells were resuspended in 1 mL of medium (glucose-free DMEM containing 1 mM pyruvate as the only energy source). Oxygen consumption was measured in a closed chamber for ~6 minutes at 37 °C, after which potassium cyanide (KCN) (3 mM) was added to inhibit cellular respiration. Oxygen consumption was calculated as the difference in slope of oxygen consumption in the absence and presence of KCN, and was normalized to protein levels as measured by the Bradford assay in sonicated cell pellets.

3.7. Western blot

Hepatic proteins were extracted from Cmo2-/Rbp-/- males fed the vitamin A-deficient diet 11 days, and injected IP with Veh or bC daily on days 7-10. RIPA complete
lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 0.5% IGEPAL, 0.87 mM Na$_3$VO$_4$, 43 mM NaF, 8.7 mM NaPyrophosphate, 1.73 mM PMSF, 3.5% Complete) was used to extract proteins, which were subsequently separated on a 15% polyacrylamide gel in a running buffer containing Tris (3 g/L), glycine (14.8 g/L), and SDS (1 g/L). Proteins were then transferred to a PVDF membrane (pore size 0.45 μm) in a transfer buffer containing Tris (5.8 g/L), glycine (2.9 g/L) and SDS (0.37 g/L). After blocking in 5% BSA (0.1% TBS-Tween), the membrane was incubated with the primary antibody (anti-SOD2 1:500 in blocking buffer, or anti-COXIV 1:2000 in blocking buffer), washed, and treated with the secondary antibody (1:5000 goat anti-rabbit HRP-conjugated IgG in blocking buffer). The membrane was then washed in 0.1% TBS-Tween and developed with ECL.

3.8. PKCδ binding assay

The zinc-finger C1b domain of PKCδ was GST-tagged and purified by affinity chromatography for use in protein fluorescence quenching assays. Tryptophan fluorescence (excitation 280 nm, emission 300-400 nm) was measured with a Horiba Fluoromax-3 (pathlength 2 mm) using 500 nM C1b in the presence of retinol or β-apo-10’-carotenal (500 nM). Other potential ligands (bC, astaxanthin, and lutein) also were tested at 500 nM. The protein buffer, vehicle (ethanol), and ligands alone were tested as controls.

3.9. Statistical analysis
SPSS statistical software (IBM SPSS Statistics, version 16) was used for statistical analysis. Normal distribution of data was determined by the Shapiro-Wilk test, and normally distributed data were analyzed by Student’s t test (for 2 groups) or analysis of variance (ANOVA, for 3 or more groups) followed by post hoc analysis (Tamhane’s analysis for groups with unequal variance, or least significant difference [LSD] for groups with equal variance). Nonparametric data were analyzed by the Mann-Whitney U test (for 2 groups) or the Kruskal-Wallis test (for 3 or more groups) followed by the Mann-Whitney U test for individual comparisons. All data are presented as mean ± standard deviation (SD) except for serum retinyl ester and hepatic bC concentrations, which are presented as the geometric mean and (range).
4. Results

4.1. Contribution of CMO2-mediated bC cleavage to retinoid generation during embryonic development in the absence of CMO1

Previous studies in our lab indicated that mouse embryos, placentas and yolk sacs express both carotenoid cleavage enzymes throughout gestation (Kim et al., 2011). The importance of CMO1 expression in the developing tissues became evident, as bC supplementation of vitamin A-deprived Cmo1-/-Rbp-/- dams during the window of organogenesis (6.5-9.5 dpc (Rugh, 1968)) prevented morphological symptoms of vitamin A deficiency syndrome in 61% of their Cmo1+/-Rbp-/- embryos (Kim et al., 2011). These data demonstrated that embryos expressing Cmo1 could generate sufficient retinoids in situ to develop normally, even in the absence of maternal Cmo1 expression. On the other hand, the contribution of CMO2 to the generation of retinoids from bC during embryonic development remained unknown, although in adult tissues its role in retinoid homeostasis appeared to be minimal (Amengual et al., 2010). Therefore, we wondered whether Cmo2 expression might contribute to retinoid homeostasis in embryos, and to address this question we took advantage of the Cmo1-/-Rbp-/- strain.
Table 3-2. Phenotype distribution of \textit{Cmo1-/-}Rbp-/- embryos from dams fed a vitamin A-deficient diet during pregnancy

<table>
<thead>
<tr>
<th>Injection</th>
<th>( n ) [embryos (dams)]</th>
<th>Percent Resorbed</th>
<th>Unsuccessful Pregnancies</th>
<th>Normal Eye and Edema</th>
<th>Cleft Exposed Brain</th>
<th>Spine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>45 (9)</td>
<td>20% (11/56)</td>
<td>31% (4/13)</td>
<td>0%</td>
<td>76%</td>
<td>22%</td>
</tr>
<tr>
<td>bC</td>
<td>61 (8)</td>
<td>22% (17/78)</td>
<td>77% (27/35)</td>
<td>8%</td>
<td>87%</td>
<td>&lt;2%</td>
</tr>
</tbody>
</table>

\textit{Cmo1-/-}Rbp-/- females were mated with \textit{Cmo1-/-Rbp-/-} males, and were fed a vitamin A-deficient diet from 0.5-14.5 dpc. A group of females was injected intraperitoneally (IP) with \( \beta \)-carotene (bC) from 6.5-9.5 dpc. Percentage of resorbed embryos was calculated from the total number of implantations in the uterus. Percentage of unsuccessful pregnancies was calculated from the total number of females in which vaginal plugs were detected and experiments were attempted.
Figure 3-1. Phenotypes of malformed embryos due to vitamin A deficiency syndrome.

Maternal vitamin A deprivation during pregnancy can lead to a range of embryonic phenotypes, depending on the mouse genotype and/or other factors. Representative images of these phenotypes are shown in order of severity (left to right). Wild-type dams generate grossly normal embryos, even after consuming a vitamin A-deficient diet from 0.5-14.5 dpc (A). However, vitamin A deprivation of knockout mouse models of various genes involved in vitamin A or carotenoid metabolism may result in embryos with defects such as malformed eyes (B), malformed eyes and peripheral edemas (the “Rbp-/-like” phenotype, C), cleft face/palate (D), or exencephaly (exposed brain, E). While a small percentage of embryos with spinal defects (F) has been observed in various genotypes, a relatively high percentage of Cmo2-/-Rbp-/- embryos from β-carotene-injected dams displayed this phenotype. All embryos are shown at 14.5 dpc.
Cmo1-/Rbp/- dams were fed a vitamin A-deficient diet (<0.22 IU vitamin A/g, 0 µg bC/g) during pregnancy, and the gross morphology of their embryos was examined. As we previously reported (Kim et al., 2011), Cmo1-/Rbp/- embryos from these dams were malformed at 14.5 dpc, displaying eye defects and peripheral edemas, as well as a certain percentage of more severe defects, including cleft face/palate and exencephaly (Table 3-2 and Fig. 3-1). To determine whether carotenoid supplementation could prevent these embryonic malformations in the absence of CMO1, Cmo1-/Rbp/- dams were supplemented with bC by daily IP injection from 6.5-9.5 dpc. At 14.5 dpc, the distribution of the embryonic phenotypes had shifted towards the milder phenotype (eye defect and peripheral edema), the cleft face/palate and exencephaly were eliminated, and a small percentage of them (8%) appeared grossly phenotypically normal (Table 3-2). These results suggest that maternal bC supplementation during the period of organogenesis can partially improve embryonic development, independent from symmetric bC cleavage by maternal or embryonic CMO1.

The above results raised the question whether the improvement of the embryonic phenotype was due to retinoic acid generated by asymmetric bC cleavage via CMO2 (Amengual et al., 2013; Wang et al., 1996). Since it has been reported that Cmo2 mRNA levels increase in certain adult tissues lacking CMO1 (Amengual et al., 2011; Shmarakov et al., 2010), we first investigated whether the partial improvement in the phenotype of Cmo1-/Rbp/- embryos from bC-supplemented dams could be attributed to increased expression of Cmo2. However, Cmo2 mRNA levels were unchanged in 14.5 dpc Cmo1-/Rbp/- embryos from bC-supplemented dams, compared with embryos from Vehicle-
Figure 3-2. *Cmo2* mRNA expression levels in *Cmo1-/-Rbp-/-* embryos from dams fed a vitamin A-deficient diet.

qRT-PCR analysis was performed using mRNA from 14.5 dpc (A) 10.5 dpc (B) embryos from *Cmo1-/-Rbp-/-* dams fed a vitamin A-deficient diet during gestation, and treated 6.5-9.5 dpc with Vehicle (Veh) or β-carotene (bC). Embryos from Veh were set as calibrator at 1. Data are presented as mean ± SD fold of Veh. Sample size, (A) n=4 embryos/group from different dams, (B) n=3-5 embryos/group (1-2 dams). Statistical analysis was performed by Student’s *t* test.
### Table 3-3. Embryonic retinoid concentrations (ng/g) of Cmo1/-/Rbp-/- (Cmo2/-) mice

<table>
<thead>
<tr>
<th>Diet/Treatment</th>
<th>Genotype</th>
<th>Cmo1/-/Rbp-/-</th>
<th>Cmo1/-/Rbp-/- Cmo2/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n [embryos (dams)]</td>
<td>Retinol</td>
<td>Retinyl Ester</td>
</tr>
<tr>
<td>Vitamin A-sufficient chow</td>
<td>35 (7)</td>
<td>37.0 ± 8.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>120 ± 38&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Purified vitamin A-deficient</td>
<td>17 (5)</td>
<td>21.4 ± 4.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57 ± 26&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Purified vitamin A-deficient + bC IP</td>
<td>13 (6)</td>
<td>30.5 ± 4.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41 ± 16&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Females of the indicated genotypes were mated with males of the same genotype, and were fed either a vitamin A-sufficient chow diet or a purified vitamin A-deficient diet from 0.5-14.5 dpc. A group of females from the latter diet was injected intraperitoneally (IP) with β-carotene (bC) from 6.5-9.5 dpc. Embryos (14.5 dpc) were analyzed by HPLC. Data are presented as mean ± standard deviation. Statistical analysis by two-way ANOVA, followed by: one-way ANOVA + LSD/Tamhane’s post hoc analysis for normally distributed data within a genotype; Student’s t test for normally distributed data between genotypes on the same treatment; Kruskal-Wallis + Mann-Whitney U test for nonparametric data within a genotype; and Mann-Whitney U test for nonparametric data between genotypes on the same treatment. Within a genotype, values with different letters differ significantly, p<0.05. *, p<0.05 vs. Cmo1/-/Rbp-/- on the same treatment.
injected dams (Veh) (Fig. 3-2A). Given that the last maternal bC dose was administered at 9.5 dpc in our supplementation scheme, we also measured Cmo2 mRNA levels in a small group of 10.5 dpc Cmo1-/-Rbp-/- embryos from supplemented dams, reasoning that transcriptional changes might be more apparent 24 hours after the last treatment. We found that even 24 hours post-IP, Cmo2 mRNA levels were equivalent in the two groups (Fig. 3-2B). All together, these data show that, in contrast to adult tissues, the absence of CMO1 in the embryo does not up-regulate the transcription of Cmo2, thus disfavoring the hypothesis that this mechanism could contribute to the partial improvement in the phenotype of Cmo1-/-Rbp-/- embryos from bC-supplemented dams. Nonetheless, the lack of transcriptional up-regulation of Cmo2 does not conflict with the hypothesis that CMO2 enzymatic activity could contribute to the generation of retinoids from bC in the developing tissues, at least in the absence of CMO1.

We therefore crossed Cmo1-/-Rbp-/-females with Cmo2-/-males to generate the “triple-knockout” mice (Cmo1-/-Rbp-/-Cmo2-/-). These mice were viable and fertile when maintained on a vitamin A-sufficient diet (18 IU/g diet). HPLC analysis revealed that the concentrations of retinol were similar in Cmo1-/-Rbp-/-Cmo2-/- and Cmo1-/-Rbp-/- embryos at 14.5 dpc, regardless of whether dams were fed a vitamin A-sufficient chow diet, or a purified vitamin A-deficient diet (<0.22 IU vitamin A/g, 0 µg bC/g) during pregnancy, and only a small but significant increase in embryonic retinyl ester levels was observed in Cmo1-/-Rbp-/-Cmo2-/- embryos compared with Cmo1-/-Rbp-/- on the vitamin A-sufficient diet (Table 3-3). However, Cmo1-/-Rbp-/-Cmo2-/- embryos from dams fed a vitamin A-deficient diet exhibited a different phenotype distribution than
Table 3-4. Phenotype distribution of Cmo1-/-Rbp-/-Cmo2-/- embryos from dams fed a vitamin A-deficient diet during pregnancy

<table>
<thead>
<tr>
<th>Treatment [embryos (dams)]</th>
<th>n</th>
<th>Percent Resorbed</th>
<th>Unsuccessful Pregnancies</th>
<th>Normal Eye and Edema</th>
<th>Cleft</th>
<th>Exposed Brain</th>
<th>Spine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>155 (22)</td>
<td>14% (26/181)</td>
<td>19% (5/27)</td>
<td>0%</td>
<td>52%</td>
<td>47%</td>
<td>0%</td>
</tr>
<tr>
<td>RAL</td>
<td>76 (10)</td>
<td>6% (5/81)</td>
<td>17% (2/12)</td>
<td>92%</td>
<td>3%</td>
<td>0%</td>
<td>4%</td>
</tr>
<tr>
<td>bC</td>
<td>144 (15)</td>
<td>8% (12/156)</td>
<td>65% (28/43)</td>
<td>2%</td>
<td>85%</td>
<td>9%</td>
<td>1%</td>
</tr>
</tbody>
</table>

*Cmo1-/-Rbp-/-Cmo2-/- females were mated with Cmo1-/-Rbp-/-Cmo2-/- males, and were fed a vitamin A-deficient diet from 0.5-14.5 dpc. A group of dams was fed with 400 μg retinaldehyde (RAL)/g diet from 7.5-9.5 dpc. Another group of females was injected intraperitoneally with β-carotene (bC) from 6.5-9.5 dpc. Percentage of resorbed embryos was calculated from the total number of implantations in the uterus. Percentage of unsuccessful pregnancies was calculated from the total number of females in which vaginal plugs were detected and experiments were attempted.*
Cmo1-/Rbp-/ embryos, as a larger percentage of triple-knockout embryos had cleft face/palate (compare Table 3-2 and Table 3-4).

Overall, our initial experiments indicated that on the Cmo1-/Rbp-/ background, CMO2 deficiency did not affect embryonic retinoid levels. However, CMO2 deficiency seemed to shift the embryonic phenotype distribution towards a more severe phenotype on a vitamin A-deficient diet (cleft face/palate). As this effect was observed in the absence of supplementation with any CMO2 substrate (although carotenoids were present in the maintenance diet at ~1.2 µg/g, carotenoids and xanthophylls were absent from the experimental purified vitamin A-deficient diet), we wondered whether the more severe phenotype of the triple-knockout mice was vitamin A-dependent. We thus sought to rescue the Cmo1-/Rbp-/Cmo2-/ embryonic phenotype via maternal retinaldehyde supplementation from 7.5-9.5 dpc (Rhinn et al., 2011). Maternal retinaldehyde treatment (400 µg/g diet) allowed 92% of Cmo1-/Rbp-/Cmo2-/ embryos to develop as grossly normal (Table 3-4). Therefore, we concluded that the triple-knockout embryonic phenotype was caused by vitamin A deficiency.

Having assessed the baseline phenotype of the triple-knockout strain and its vitamin A-dependence, we used this strain to test whether the aforementioned improvement of Cmo1-/Rbp-/ embryos from bC-supplemented dams was due to retinoids generated from CMO2-dependent bC cleavage. Following the same scheme described earlier, we examined the phenotype distribution of Cmo1-/Rbp-/Cmo2-/ embryos from dams fed a vitamin A-deficient diet throughout pregnancy, and treated with bC by daily IP injection from 6.5-9.5 dpc. bC supplementation of triple-knockout
Figure 3-3. HPLC chromatograms of carotenoids extracted from a β-carotene solution, mouse serum and liver.

Carotenoids were extracted from a β-carotene (bC) solution prepared for intraperitoneal injection (A), or from the serum (B) and liver (C) of a representative triple-knockout mouse injected with the bC solution. The extracts were separated by HPLC, and the peaks in the resulting chromatograms were analyzed according to their retention times and absorbance spectra. Aside from bC and the Internal Standard (I.S., echinenone), peaks with absorbance maxima in the range of 404-452 nm were detected (indicated by *). Note the relatively small number of peaks in the chromatogram of the bC solution compared with serum and liver.
dams led to an unexpected improvement in the embryonic phenotypes, with a marked reduction in the prevalence of cleft face/palate and a shift towards the milder phenotype of eye defect and peripheral edema, along with a smaller percentage of the embryos (2%) which appeared grossly normal (Table 3-4). (Also, a small percent of supplemented embryos showed exencephaly, which we attribute to the spontaneous appearance of this phenotype at low levels even among wild-type (WT) mice (Juriloff et al., 2001)). Overall, the phenotype of triple-knockout embryos from supplemented dams was improved to a similar extent as the Cmo1-/Rbp-/- strain (compare data in Tables 3-2 and 3-4), thus suggesting that in both strains this improvement was not due to enzymatic cleavage of bC – neither symmetric (by CMO1) nor asymmetric (by CMO2). The data also indicate that CMO2 does not significantly contribute to the generation of retinoids from bC in the developing tissues, at least when CMO1 is absent.

The surprising improvement of the triple-knockout embryonic phenotype raised the question as to how bC supplementation could affect embryonic development independent of enzymatic cleavage. Extensive literature support the notion that spontaneous oxidation of bC can occur in vitro (El-Agamey et al., 2004; Gao and Kispert, 2003; Krinsky and Yeum, 2003), which could lead to retinoid formation if bC were oxidized at its 15,15’ double bond (or even elsewhere, if the product could be converted to retinoic acid by chain shortening (Wang et al., 1996)). Thus, to investigate whether any bC oxidation products had formed in our experiments, we analyzed our bC preparations by HPLC, and compared their chromatograms with those of sera and livers from injected mice. In addition to the expected bC peak, we detected other compounds with absorbance maxima in the range of apocarotenoids (402-452 nm) in all of these samples (Fig. 3-3). A
much greater abundance and variety of such compounds was observed in the serum and liver, compared to the bC preparation (Fig. 3-3). Therefore, it appeared that spontaneous oxidation of bC in vitro, but predominantly in vivo, yielded apocarotenoids which may have been available for conversion to retinoids to improve the embryonic phenotype in the bC-treated mice. HPLC analysis revealed slight increases in retinol levels in bC-supplemented Cmo1-/Rbp-/ and triple-knockout embryos compared to untreated controls, while retinyl ester levels did not increase (Table 3-3). It is also possible that retinoic acid levels were slightly elevated in the embryos with an improved phenotype.

Although our analyses of the triple-knockout strain suggested that CMO2 does not contribute significantly to retinoid production from bC during embryonic development in the absence of CMO1, other evidence from the literature prompted us to wonder whether CMO2 deficiency might affect retinoid homeostasis and consequently embryogenesis if CMO1 were present. Specifically, Amengual and colleagues recently suggested that sequential enzymatic cleavage steps by CMO2 and CMO1 could generate retinoids from bC and other carotenoids, and showed that apocarotenoids accumulated if asymmetric carotenoid cleavage by CMO2 could not be followed by further processing by CMO1 (Amengual et al., 2013). Thus, we speculated that CMO1 deficiency in our triple-knockout strain could be “masking” the true function of CMO2 in vivo. Furthermore, the embryonic phenotype distributions of the triple-knockout strain (Table 3-4) and the Cmo1-/Rbp-/ strain (Table 3-2) revealed that the percentage of cleft/face palate (a more severe malformation, Fig. 3-1) was greater in the triple-knockouts, regardless of treatment. These observations led us to ask whether CMO2 deficiency per se might affect retinoid homeostasis in the developing tissues and consequently
embryogenesis, either in the presence or the absence of supplementation with a carotenoid substrate.
Table 3-5. Embryonic retinoid concentrations (ng/g) of various strains of mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>n [embryos (dams)]</th>
<th>Retinol</th>
<th>Retinyl Ester</th>
<th>n [embryos (dams)]</th>
<th>Retinol</th>
<th>Retinyl Ester</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitamin A-sufficient chow</td>
<td></td>
<td></td>
<td></td>
<td>Purified vitamin A-deficient</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 (6)</td>
<td>102.4 ± 17.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>230 ± 52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8 (4)</td>
<td>83.2 ± 8.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>154 ± 14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td>6 (3)</td>
<td>105.4 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>151 ± 49&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>10 (5)</td>
<td>95.2 ± 9.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>92 ± 25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cmo1/-</td>
<td></td>
<td>12 (5)</td>
<td>106.5 ± 14.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>145 ± 33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10 (5)</td>
<td>88.1 ± 10.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>90 ± 19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cmo2/-</td>
<td></td>
<td>18 (7)</td>
<td>128.4 ± 11.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>189 ± 37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10 (5)</td>
<td>84.0 ± 10.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100 ± 27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cmo1/-&amp; Cmo2/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rbp/-</td>
<td></td>
<td>8 (4)</td>
<td>53.1 ± 11.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>131 ± 36</td>
<td>6 (3)</td>
<td>31.7 ± 4.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50 ± 20</td>
</tr>
<tr>
<td>Cmo1/-&amp; Rbp/-</td>
<td></td>
<td>35 (7)</td>
<td>37.0 ± 8.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>120 ± 38</td>
<td>17 (5)</td>
<td>21.4 ± 4.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57 ± 26</td>
</tr>
<tr>
<td>Cmo2/-&amp; Rbp/-</td>
<td></td>
<td>10 (6)</td>
<td>39.8 ± 6.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>110 ± 43</td>
<td>9 (6)</td>
<td>13.5 ± 5.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41 ± 13</td>
</tr>
<tr>
<td>Cmo1/-&amp; Rbp/-&amp; Cmo2/-</td>
<td></td>
<td>7 (7)</td>
<td>43.3 ± 7.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>156 ± 41</td>
<td>16 (9)</td>
<td>19.0 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57 ± 20</td>
</tr>
</tbody>
</table>

Females of the indicated genotypes were mated with males of the same genotype, and were fed either a vitamin A-sufficient chow diet or a purified vitamin A-deficient diet from 0.5-14.5 dpc. Embryos (14.5 dpc) were analyzed by HPLC. Data are presented as mean ± standard deviation. Statistical comparisons were made separately among Rbp-expressing and Rbp-/-genotypes. Values with different letters were statistically significantly different by ANOVA followed by LSD/Tamhane’s post hoc analysis for normally distributed data, or by Kruskal-Wallis test followed by Mann-Whitney U test for nonparametric data, p<0.05.
Figure 3-4. mRNA expression levels of retinoid homeostatic genes in 14.5 dpc embryos from WT and Cmo2-/- dams fed a vitamin A-sufficient diet 0.5-14.5 dpc. qRT-PCR analysis was performed using mRNA from 14.5 dpc embryos from wild-type (WT) and Cmo2-/-- dams fed a non-purified vitamin A-sufficient diet. WT embryos were set as calibrator at 1. Data are presented as mean ± SD fold of WT. Sample size, n=5 embryos/group (from n=3-5 dams). Statistical analysis was performed by Student’s t test. *, p<0.05 vs. WT.
4.2. Contribution of CMO2 to embryonic development in the presence of CMO1

Cmo2-/ mice (Amengual et al., 2010) are viable and fertile when maintained on a vitamin A-sufficient diet, and Cmo2-/embryos develop normally even when the dams are fed a vitamin A-deficient diet during pregnancy (data not shown). Interestingly, as in the case of embryos lacking CMO1 (Kim et al., 2011), Cmo2-/ embryos at mid-gestation showed reduced levels of retinyl esters compared to WT mice, regardless of the maternal dietary regimen (Table 3-5). Accordingly, Lrat mRNA levels were reduced in Cmo2-/embryos from dams fed a vitamin A-sufficient diet (Fig. 3-4). These data suggest that CMO2 is also able to impact retinoid homeostasis of the developing tissues, independent of its ability to generate retinoids from intact bC (bC was never detected in any tissue of our animals on the maintainence diet, although the diet contained a small amount of carotenoids, <1.2 µg/g).

Since we previously showed that deficiency of CMO1 in developing tissues further exacerbated the vitamin A-deficient status of embryos lacking RBP (Kim et al., 2011), we sought to investigate the effect of the lack of CMO2 on the Rbp-/- background. Accordingly, we generated Cmo2-/Rbp-/- mice, and analyzed the phenotypes and retinoid concentrations of their embryos under different dietary regimens. Cmo2-/Rbp-/- mice were viable and fertile when fed a vitamin A-sufficient chow diet, even though their embryos also displayed reduced retinyl ester levels and significantly reduced retinol levels compared to Rbp-/- embryos (Table 3-5). When Cmo2-/Rbp-/- dams were fed a vitamin A-deficient diet during pregnancy, their embryonic retinol concentrations were significantly lower than those of Rbp-/-, Cmo1-/Rbp-/-, or even Cmo1-/Rbp-/Cmo2-/-
### Table 3-6. Phenotype distribution of Cmo2-/Rbp-/ embryos from dams fed a vitamin A-deficient diet during pregnancy

<table>
<thead>
<tr>
<th>Treatment [embryos (dams)]</th>
<th>n</th>
<th>Percent resorbed</th>
<th>Unsuccessful Pregnancies</th>
<th>Normal</th>
<th>Eye and Edema</th>
<th>Cleft</th>
<th>Exposed Brain</th>
<th>Spine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>72 (11)</td>
<td>22% (21/93)</td>
<td>27% (4/15)</td>
<td>0%</td>
<td>24%</td>
<td>76%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>RAL</td>
<td>36 (8)</td>
<td>32% (17/53)</td>
<td>38% (5/13)</td>
<td>72%</td>
<td>17%</td>
<td>3%</td>
<td>8%</td>
<td>0%</td>
</tr>
<tr>
<td>bC</td>
<td>30 (4)</td>
<td>0% (0/30)</td>
<td>87% (27/31)</td>
<td>3.3%</td>
<td>80%</td>
<td>0%</td>
<td>3.3%</td>
<td>13%</td>
</tr>
</tbody>
</table>

*Cmo2-/Rbp-/- females were mated with Cmo2-/Rbp-/- males, and were fed a vitamin A-deficient diet from 0.5-14.5 dpc. A group of dams was fed with 400 µg retinaldehyde (RAL)/g diet from 7.5-9.5 dpc. Another group of females was injected intraperitoneally with β-carotene (bC) from 6.5-9.5 dpc. Percentage of resorbed embryos was calculated from the total number of implantations in the uterus. Percentage of unsuccessful pregnancies was calculated from the total number of females in which vaginal plugs were detected and experiments were attempted.*
Figure 3-5. mRNA expression levels of retinoid homeostatic genes in 14.5 dpc embryos and livers from Rbp-/- and Cmo2-/-Rbp-/- dams fed a vitamin A-deficient diet 0.5-14.5 dpc.

qRT-PCR analysis was performed using mRNA from 14.5 dpc embryos (A) and maternal livers (B) from Rbp-/- and Cmo2-/-Rbp-/- dams fed a vitamin A-deficient diet 0.5-14.5 dpc, with or without intraperitoneal injection of β-carotene from 6.5-9.5 dpc (bC 6.5-9.5). Rbp-/- embryos were set as calibrator at 1. Data are presented as mean ± SD fold of Rbp-/-.

Statistical analysis was performed by Student’s t test for normally distributed data, or Mann-Whitney U test for nonparametric data, to compare non-injected Rbp-/- vs. Cmo2-/-Rbp-/-, and injected vs. non-injected Cmo2-/-Rbp-/-. *, p<0.05 vs. Rbp-/-; #, p<0.05 vs. Cmo2-/-Rbp-/-.
embryos from dams fed the same diet (Table 3-5). Retinyl ester levels also remained low in Cmo2-/Rbp-/ embryos compared to other RBP-deficient strains, although statistical significance was not reached (Table 3-5). Overall, these data indicate that the retinoid status of the Cmo2-/Rbp-/ was tenuous compared to other strains. Correspondingly, Cmo2-/Rbp-/ embryos from dams fed a vitamin A-deficient during gestation also showed a higher percentage of cleft face/palate than Cmo1-/Rbp-/ or Cmo1-/Rbp-/Cmo2-/ embryos (compare Tables 3-2, 3-4 and 3-6). Note that the Cmo2-/Rbp-/ embryos analyzed by HPLC were the few displaying the Rbp-/like phenotype (eye defect and peripheral edema). Overall, CMO2 deficiency affected retinoid homeostasis in the developing tissues and worsened the embryonic phenotype of a well-studied mouse model of vitamin A deficiency (Rbp-/-), independent of bC supplementation.

As our HPLC analysis revealed lower retinoid concentrations in Cmo2-/Rbp-/ embryos from vitamin A-deprived dams compared to other genotypes (Table 3-5), we also examined the mRNA levels of genes encoding enzymes that maintain cellular retinoid homeostasis in embryos (Fig. 3-5A) and maternal liver (Fig. 3-5B). Embryonic mRNA expression levels of Cmo1, Rdh10, Raldh2, and Cyp26a1 did not differ among the groups, while a small decrease in Stra6 was observed in Cmo2-/Rbp-/ embryos compared to Rbp-/ (Fig. 3-5A). Hepatic Cmo1 expression did not differ between Cmo2-/Rbp-/ and Rbp-/ dams fed a vitamin A-deficient diet, but the mRNA levels of Lrat were significantly induced and Cyp26a1 levels were reduced in Cmo2-/Rbp-/ livers compared to Rbp-/ (Fig. 3-5B). These results indicate that CMO2 deficiency affects retinoid metabolism in a tissue-specific manner, regardless of whether intact carotenoid substrates are present.
Table 3-7. Retinoid and β-carotene concentrations of mice fed the vitamin A-deficient diet 0.5-14.5 dpc and injected with β-carotene 6.5-9.5 dpc

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Genotype</th>
<th>n</th>
<th>Retinol µg/dL</th>
<th>β-carotene µg/dL</th>
<th>Retinyl Ester µg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Cmo1/-Rbp/-</td>
<td>7-8</td>
<td>1.04 ± 0.39</td>
<td>15.06 ± 2.53a</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>Cmo2/-Rbp/-</td>
<td>4</td>
<td>1.07 ± 0.24</td>
<td>1.00 ± 0.64b</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>Cmo1/-Rbp/-Cmo2/-</td>
<td>9</td>
<td>0.87 ± 0.14</td>
<td>15.79 ± 2.45a</td>
<td>nd (0-3.88)</td>
</tr>
<tr>
<td>Liver</td>
<td>Cmo1/-Rbp/-</td>
<td>7-8</td>
<td>3.5 ± 0.9a</td>
<td>2.81 (161-458)</td>
<td>248 ± 66</td>
</tr>
<tr>
<td></td>
<td>Cmo2/-Rbp/-</td>
<td>4</td>
<td>2.4 ± 0.6b</td>
<td>214 (106-748)</td>
<td>196 ± 72</td>
</tr>
<tr>
<td></td>
<td>Cmo1/-Rbp/-Cmo2/-</td>
<td>9</td>
<td>2.9 ± 0.4a</td>
<td>250 (91-511)</td>
<td>255 ± 63</td>
</tr>
<tr>
<td>Embryos</td>
<td>Cmo1/-Rbp/-</td>
<td>13 (7)</td>
<td>30.5 ± 4.8a</td>
<td>nd (0-9)</td>
<td>41 ± 16a</td>
</tr>
<tr>
<td></td>
<td>Cmo2/-Rbp/-</td>
<td>8  (4)</td>
<td>34.4 ± 6.1a</td>
<td>nd (0-5)</td>
<td>70 ± 11b</td>
</tr>
<tr>
<td></td>
<td>Cmo1/-Rbp/-Cmo2/-</td>
<td>18 (9)</td>
<td>22.2 ± 5.4b</td>
<td>28 ± 13</td>
<td>54 ± 23b</td>
</tr>
</tbody>
</table>

Females of the indicated genotypes were mated with males of the same genotype. Dams were fed a purified vitamin A-deficient diet from 0.5-14.5 dpc, and were injected intraperitoneally with β-carotene from 6.5-9.5 dpc. Embryos (14.5 dpc) were analyzed by HPLC. Data are presented as mean ± standard deviation, except for hepatic β-carotene which is expressed as geometric mean (range). The detection limit for β-carotene is <10 ng/g, and for retinyl ester is <0.1 ng/dL; data below the limit of detection are shown as non-detectable (nd). Statistical analysis by one-way ANOVA followed by LSD/Tamhane’s post hoc analysis for normally distributed data, or by Kruskal-Wallis test followed by Mann-Whitney U test for nonparametric data. For a given compound within a tissue, values with different letters differ significantly, p<0.05. Statistical analysis was not performed when metabolites were non-detectable.
We then tested the vitamin A-dependence of the Cmo2/-/Rbp/- embryonic phenotype, following the same retinaldehyde supplementation scheme described above. At 14.5 dpc, 72% of the Cmo2/-/Rbp/- embryos from retinaldehyde-supplemented dams appeared grossly normal (Table 3-6), indicating a clear dependence of the Cmo2/-/Rbp/- embryonic phenotype on the lack of vitamin A. Surprisingly, when pregnant Cmo2/-/Rbp/- females were fed a vitamin A-deficient diet from 0.5-14.5 dpc and supplemented with bC by daily IP injection from 6.5-9.5 dpc, a very high rate of unsuccessful pregnancies (87%) was observed. Of the embryos recovered at midgestation, only 3.3% (a single embryo) showed a normal gross morphology (Table 3-6). While the cleft face/palate was eliminated (80% of embryos showed only eye defects and/or peripheral edemas), 3.3% (a single embryo) displayed exencephaly and 13% of them showed a more severe phenotype that was not observed in the unsupplemented animals (a skeletal defect of the spine) (Table 3-6 and Fig. 3-1). Note that CMO1 was expressed in both maternal and embryonic tissues of the Cmo2/-/Rbp/- mice, and indeed, the bC concentration in maternal serum and embryos was very low or non-detectable in this strain (Table 3-7). Retinol and retinyl ester levels in sera, livers, and abnormal embryos from bC-supplemented Cmo2/-/Rbp/- dams did not differ dramatically from those of supplemented Cmo1/-/Rbp/- or triple-knockout dams (Table 3-7). However, it is interesting that despite the increased retinoid levels in bC-supplemented Cmo2/-/Rbp/- compared to non-treated Cmo2/-/Rbp/- on the vitamin A-deficient diet (compare Tables 3-5 and 3-7), these embryos were still malformed.

The high prevalence of cleft face/palate among Cmo2/-/Rbp/- embryos from dams fed a vitamin A-deficient diet, along with the increase in spinal defects in embryos
from bC-supplemented dams of this strain (Table 3-6), pointed to an important function of CMO2 during embryogenesis, both in the absence and in the presence of supplementation with its substrate, bC. The rescue of 72% of Cmo2-/-Rbp-/- embryos by retinaldehyde supplementation (Table 3-6) suggested that the Cmo2-/-Rbp-/- phenotype was at least partially retinoic acid-dependent. However, the failure to rescue the phenotype with bC, despite the expression of CMO1 (Table 3-6), indicated that the provitamin A carotenoid could not effectively influence retinoid homeostasis in the absence of CMO2. Indeed, upon bC supplementation, the majority of genes involved in retinoid homeostasis remained unchanged in Cmo2-/-Rbp-/- embryos (Fig. 3-5A) and maternal livers (Fig. 3-5B); only a statistically significant increase in Lrat mRNA expression bC-supplemented Cmo2-/-Rbp-/-embryos compared to untreated controls (Fig. 3-5A).

The failure to rescue the Cmo2-/-Rbp-/- embryos with bC could be explained by two potential mechanisms. It may be that not only the ability to cleave bC by CMO1, but also the proposed ability to scavenge bC (or its cleavage products) by CMO2 (Amengual et al., 2010), is required to facilitate embryonic development, at least when bC is the only available vitamin A source. Alternatively, the data may indicate that the apocarotenoids generated from bC by CMO2 may be critical for certain cellular processes during embryogenesis. Note that embryos from Cmo2-/- (n=8 dams) and Cmo1-/-Cmo2-/- (n=6 dams) fed the vitamin A-deficient diet and injected 6.5-9.5 dpc with bC developed normally (data not shown), indicating that the Cmo2-/-Rbp-/- phenotype depended not only on the lack of CMO2, but also on the severe embryonic vitamin A deficiency induced by the Rbp-/- background (Table 3-5). Overall, these data suggest that by
Figure 3-6. mRNA expression levels of redox homeostatic genes in 14.5 dpc embryos and livers from Rbp-/- and Cmo2-/-Rbp-/- dams fed a vitamin A-deficient diet 0.5-14.5 dpc.

qRT-PCR analysis was performed using mRNA from 14.5 dpc embryos (A) or maternal livers (B) from Rbp-/- and Cmo2-/-Rbp-/- dams fed a vitamin A-deficient diet 0.5-14.5 dpc, with or without intraperitoneal injection of β-carotene from 6.5-9.5 dpc (bC 6.5-9.5). Rbp-/- embryos were set as calibrator at 1. Data are presented as mean ± SD fold of Rbp-/-.

Sample size, (A) n=4-8 embryos/group (from n=3-4 dams), (B) n=3-6 livers/group. Statistical analysis was performed by Student’s t test or Mann-Whitney U test to compare non-injected Rbp-/- vs. Cmo2-/-Rbp-/-, and injected vs. non-injected Cmo2-/-Rbp-/-.* p<0.05 vs. Rbp-/-.
cleaving bC, CMO2 performs a very specific function that seems to be important for embryonic development, and cannot be overcome by the generation of retinoids via CMO1.

Considering the mitochondrial localization of the enzyme and the known effects of CMO2 deficiency on markers of oxidative stress and apoptosis in adult tissues (Amengual et al., 2010; Lobo et al., 2012b), we examined the possibility that maternal and/or embryonic CMO2 deficiency could negatively affect embryonic development by affecting oxidative stress pathways. We performed qRT-PCR analysis of gene expression levels in embryos from vitamin A-deprived Cmo2-/-Rbp-/- dams, compared to control Rbp-/- dams fed the same diet. The mRNA levels of Sod2, Gpx4, PrxIII, and p22phox were unchanged in embryos from Cmo2-/-Rbp-/- dams compared with Rbp-/-, while slight increases in the mRNA levels of Sod1, Gpx1, Duox1, and Alcat1 were observed in Cmo2-/-Rbp-/- embryos. The embryonic mRNA levels of these genes were equivalent in Cmo2-/-Rbp-/- dams fed the vitamin A-deficient diet, with and without bC supplementation from 6.5-9.5 dpc (Fig. 3-6A). Similar results were found upon qRT-PCR analysis of maternal livers, though a significant reduction of Sod2 mRNA was found in the Cmo2-/-Rbp-/- compared to Rbp-/- (Fig. 3-6B).
Figure 3-7. Hepatic markers of oxidative stress in CMO2-deficient strains.

(A) Hepatic peroxide levels were assessed by the xylenol orange assay using frozen liver samples from Cmo2/-Rbp/- dams fed the vitamin A-deficient diet from 0.5-14.5 dpc, with or without IP supplementation of bC from 6.5-9.5 dpc, and sacrificed at 14.5 dpc. Additionally, hepatic peroxides were measured in frozen liver samples from non-pregnant Cmo2/- females maintained on the chow diet, with and without IP injection of bC for four consecutive days, and sacrificed after 24 hours. Following incubation with the xylenol orange reagent, absorbances were read at 560 nm and subtracted from those of controls incubated with reagent lacking ferrous ions. Hydrogen peroxide levels were determined by a standard curve and normalized to lipid content (determined gravimetrically) (Fakas et al., 2008). Data are presented as mean ± SD. (B) Hepatic
hydrogen peroxide levels were determined by the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit in fresh liver homogenates from Cmo2-/Rbp-/- males fed the vitamin A-deficient diet for 11 days, injected IP with bC or Veh on days 7, 8, 9 and 10. Absorbances were read at 560 nm, and hydrogen peroxide levels were determined by a standard curve. Data were normalized to protein concentration as determined by the Bradford assay. Data are presented as mean ± SD. (C) Hepatic SOD2 protein levels were determined in the same Cmo2-/Rbp-/- males described in (B). The SOD2 protein level was normalized to a mitochondrial protein (COXIV) and β-actin. Data are presented as mean ± SD fold of Veh.
In addition to mRNA levels, we measured other markers of oxidative stress in Cmo2-/- (Rbp-/-) mice. Analysis of hydrogen peroxide levels in frozen liver samples using the xylenol orange assay (Fig. 3-7A), or in fresh liver samples using the Amplex Red Hydrogen Peroxide/Peroxidase assay kit (Fig. 3-7B), revealed no effect of four daily IP injections of bC in CMO2-deficient strains (Cmo2-/-Rbp/- males and pregnant females, or Cmo2-/- females). Additionally, four daily IP injections of vitamin A-deprived Cmo2-/-Rbp/- males with bC did not affect protein levels of SOD2 compared with Veh (Fig. 3-7C). Furthermore, a pilot study of N-acetylcysteine (NAC) administration to Cmo2-/-Rbp/- dams (n=2) fed the vitamin A-deficient diet from 0.5-14.5 dpc and injected with bC from 6.5-9.5 dpc, revealed no rescue of the phenotype (i.e. no grossly normal embryos, n=17), following intake of the antioxidant from 0.5-14.5 dpc in their drinking water at a dose of 0.5 mg/g bodyweight (data not shown). Together, these results disfavor the hypothesis that the Cmo2-/-Rbp/- embryonic phenotype was due to an unbalanced oxidative state.

4.3. Contribution of CMO2-mediated apocarotenoid production to embryonic development

We then asked whether the apocarotenoid-generating action of CMO2 might be required for normal embryonic development, and tested whether embryonic apocarotenoid levels were affected by CMO2 expression. We performed LC-MS analysis of 14.5 dpc embryos from Cmo1-/- and Cmo1-/-Cmo2-/- dams fed a regular chow diet, injected with bC (20-50 µg/g bodyweight) or Veh at 13.5 dpc, and sacrificed 24 hours
Figure 3-8. β-apo-10'-carotenoid acid concentrations in Cmo1/- and Cmo1/-Cmo2/- embryos from dams supplemented with β-carotene at 13.5 dpc.

LC-MS analysis was performed on 14.5 dpc embryos from Cmo1/- and Cmo1/-Cmo2/- dams fed a non-purified vitamin A-sufficient diet, injected with Vehicle (Veh) or β-carotene (bC) at 13.5 dpc. Data are presented as mean ± SD. Sample size, n=3 embryos/group from different dams. Statistical analysis was performed by two-way ANOVA with genotype and treatment as factors.
Figure 3-9. HPLC chromatogram of β-apo-10’-carotenal.

The ethanolic β-apo-10’-carotenal solution used to prepare the mouse diet was analyzed by HPLC to verify its purity and spectral characteristics. The $\lambda_{\text{max}}$ in ethanol was 448 nm, slightly different from its reported $\lambda_{\text{max}}$ in hexane of 437 nm (Eroglu et al., 2012).
Figure 3-10. Phenotypes of most-improved *Cmo2-/-Rbp-/-* embryos following treatment with retinoids, carotenoids, or apocarotenoids.

*Cmo2-/-Rbp-/-* embryos from dams fed a vitamin A-deficient diet (0.5-14.5 dpc) without additional treatments (“none”) displayed a high percentage of cleft face/palate (Fig. 3-1D), but a low number displayed the milder, *Rbp-/-*like phenotype (A). A variety of embryonic phenotypes was observed following maternal supplementation with retinaldehyde (“RAL”, 400 μg/g diet, 7.5-9.5 dpc), β-carotene (“bC”, ~40 μg/g bodyweight injected intraperitoneally 6.5-9.5 dpc), β-apo-10’-carotenal (“apo10”, ~18-30 μg/g bodyweight in the diet, 7.5-9.5 dpc), or both β-apo-10’-carotenal and β-carotene (“apo10 + bC”). Representative images are shown of the best (closest to normal) embryonic phenotype observed following maternal treatment with retinaldehyde (B), β-carotene (C), β-apo-10’-carotenal (D, ~30μg/g bodyweight), or both β-carotene and β-apo-10’-carotenal (E, ~30μg β-apo-10’-carotenal/g bodyweight; F, ~18 μg β-apo-10’-carotenal/g bodyweight). The percentage of embryos with each phenotype is as follows (out of the total number of embryos for the treatment group, n): (A) 24% [n=72], (B) 72% [n=36], (C) 3.3% [n=30], (D), 71% [n=7], (E) 100% [n=2], (F) 100% [n=5]. All embryos are shown at 14.5 dpc.
later. While we did not detect β-apo-10’-carotenol (reported by (Amengual et al., 2013)), or β-apo-10’-carotenal (the expected primary product, Fig. 1-4), we detected β-apo-10’-carotenoid acid, firstly in the Vehicle control groups, regardless of the presence of CMO2 (Fig. 3-8). These data support the notion that bC-containing animal diets also contain apocarotenoids (Shmarakov et al., 2010) and indicate that these compounds are able to cross the placental barrier. Furthermore, we found that the levels of β-apo-10’-carotenoid acid (which would be generated upon the oxidation of β-apo-10’-carotenal) tended to increase upon bC treatment of Cmo1/-/- embryos, but not Cmo1/-/-Cmo2/-/- embryos, in a small group of animals (Fig. 3-8). These data suggest that the ability of embryos to generate β-apo-10’-carotenoid acid from bC depends on CMO2. In addition, the results agree with the proposition (Amengual et al., 2013) that β-apo-10’-carotenoids can be cleaved by CMO1, as β-apo-10’-carotenoid acid accumulated in embryos lacking CMO1. Most importantly, these data suggest that the observed embryonic phenotype of the Cmo2/-/-Rbp/-/- could be due to the low levels of β-apo-10’-carotenal in combination with the limiting availability of retinoids.

To investigate this hypothesis, we attempted to rescue the embryonic phenotype of the Cmo2/-/-Rbp/-/- mice on a vitamin A-deficient diet by maternal dietary supplementation with β-apo-10’-carotenal (18 or 30 µg/g bodyweight) from 7.5-9.5 dpc (Fig. 3-9). Regardless of the dose of β-apo-10’-carotenal or whether the dams were injected with bC from 6.5-9.5 dpc, Cmo2/-/-Rbp/-/- embryos from β-apo-10’-carotenal-fed females were phenotypically normal (12 / 14 embryos from 3 dams, Fig. 3-10) or displayed a very mild eye defect. These data support the notion that not only the
symmetric bC cleavage product retinaldehyde (and its oxidized metabolite, retinoic acid), but also the asymmetric bC cleavage product β-apo-10’-carotenal, is required to facilitate normal embryonic development in the absence of dietary retinoids. Therefore, the action of CMO2 in generating β-apo-10’-carotenal appears to be important during gestational vitamin A deficiency.

As β-apo-10’-carotenal-supplemented Cmo2/-/Rbp/- dams expressed CMO1, the possibility was raised that their embryos were rescued due to the recently-demonstrated ability of CMO1 to convert of β-apo-10’-carotenol to retinaldehyde (Amengual et al., 2013), which subsequently could be oxidized to retinoic acid (Niederreither et al., 1997). While some of the supplemented β-apo-10’-carotenal may have been converted to retinoic acid, the inability of the Cmo2/-/Rbp/- embryos to develop normally when the dams were administered with bC (Table 3-6) confirmed that retinoic acid was not the only deficient compound in Cmo2/-/Rbp/- embryos, since the retinaldehyde/retinoic acid generated from bC by CMO1 was insufficient to produce a normal phenotype. Therefore, the question arose what essential function β-apo-10’-carotenal might perform in cells.

Apocarotenoids are known to transcriptionally repress retinoic acid-responsive gene expression, by competing with retinoic acid for the ligand-binding sites of retinoic acid receptors (alpha, beta and gamma) and retinoid X receptor alpha (Eroglu et al., 2010; Eroglu et al., 2012). However, the mRNA levels of various retinoic acid-regulated genes did not differ significantly between Rbp/- and Cmo2/-/Rbp/- embryos (Fig. 3-5).
Figure 3-11. Proposed mechanism whereby retinol affects the PKCδ signalosome.

The activity of the pyruvate dehydrogenase complex in the mitochondrion is regulated by the phosphorylation state of its E1 regulatory subunit. De-phosphorylation by pyruvate dehydrogenase phosphatases 1 and 2 (PDP1,2) activates the enzyme, while phosphorylation by pyruvate dehydrogenase kinase 2 (PDK2) inhibits it (reviewed in (Holness and Sugden, 2003)). This suppressive function of PDK2 is stimulated by protein kinase C epsilon (PKCε) when the latter binds retinol (Gong et al., 2012). On the other hand, PDK2 is suppressed (and hence, the PDHC is activated) when protein kinase C delta (PKCδ) is activated (Gong et al., 2012). It is suggested that PKCδ becomes activated when it binds retinol and its zinc-finger domain undergoes a conformational change due to the transfer of an electron to oxidized cytochrome c (Cyt Cox) (Acin-Perez
et al., 2010a). The activated PKCδ then is predicted to phosphorylate an unknown intermediate phosphatase (PDK2 phosphatase), which dephosphorylates (represses) PDK2, ultimately relieving the suppression of the PDHC. A portion of this figure was reprinted from (Hoyos et al., 2012), © 2012, with permission from Elsevier.
Furthermore, since apocarotenoids are repressors of retinoic acid-responsive genes, the inability to enzymatically generate such apocarotenoids (i.e. in Cmo2-/Rbp-/ mice) would have been expected to enhance transcriptional pathways that maintain retinoid homeostasis, rather than interfere with them. Therefore, we did not favor the possibility that apocarotenoid-dependent transcriptional regulation was affected in the Cmo2-/Rbp-/-, and instead considered potential mitochondrial effects of apocarotenoids. Indeed, the mitochondrial localization of CMO2 (Amengual et al., 2010), together with the lack of evidence of oxidative stress in our CMO2-deficient strains, suggested that mitochondrial functions aside from redox homeostasis could be affected in vitamin A-deprived Cmo2-/Rbp-/ mice.

Recently, retinol has emerged as an important regulator of cellular energy homeostasis as a member of a mitochondrial signaling complex, the “PKCδ signalosome” (Fig. 3-11, (Acin-Perez et al., 2010a)). Within this complex, retinol may act as a bridge to transport electrons from PKCδ to Cytochrome c, thus activating PKCδ by changing the conformation of its zinc-finger “C1b” domain. It has been suggested that activated PKCδ then phosphorylates other intermediates (yet to be determined), which ultimately activate the Pyruvate Dehydrogenase Complex to increase flux through the citric acid cycle and electron transport chain (Acin-Perez et al., 2010a). The stimulatory activity of PKCδ is modulated by the inhibitory action of another PKC isoform, PKCε (Gong et al., 2012). We wondered whether apocarotenoids, which are structurally similar to retinol and produced in mitochondria by CMO2, could also bind and activate PKCδ and/or PKCε and thus influence energy flux.
Figure 3-12. Emission spectra of the PKCδ C1b domain in the presence of several potential ligands.

Binding assays were performed in which tryptophan fluorescence of the PKCδ C1b domain (500 nM) was measured after incubation of the protein with 500 nM retinol (ROH), β-apo-10’-carotenal (apo10), astaxanthin, lutein, or β-carotene (bC). The spectra for the protein buffer alone (Buffer) and the vehicle (ethanol, “C1b + EtOH”) are also shown. With the exception of retinol, which emits at 466 nm, no emission was detected for the other ligands alone (not shown).
Wild-type (WT) MEFs were seeded at 1 million cells/dish in complete culture medium. The next day, the medium was replaced with serum-free medium. On the third day, cells were treated 2 hours with β-carotene or Vehicle (Veh, 0.5% hexane). Following treatment, cells were scraped and resuspended in glucose-free medium containing 1mM pyruvate as the only energy source. Oxygen consumption was recorded in a closed chamber for ~6 minutes using an Oxygraph equipped with a Clark electrode. 3 mM potassium cyanide (KCN) was added to inhibit cellular respiration, and oxygen consumption was determined as the difference in slope (nmol Oxygen/min) with and without KCN. The data were normalized to protein content in sonicated cell pellets. Data are presented as mean ± SD. Sample size, n=2 dishes/group. Statistical analysis was performed by one-way ANOVA. While the overall ANOVA reached statistical significance (p<0.05), significant differences were not found between treatments by LSD post hoc analysis.
Wild-type (WT), *PKCδ/-*, and *PKCe/-* MEFs were seeded at 1 million cells/dish in complete culture medium. The next day, the medium was replaced with serum-free medium. On the third day, cells were treated 2 hours with β-apo-10′-carotenal or Vehicle (Veh, <0.2% ethanol). Following treatment, cells were scraped and resuspended in glucose-free medium containing 1mM pyruvate as the only energy source. Oxygen consumption was recorded in a closed chamber for ~6 minutes using an Oxygraph equipped with a Clark electrode. 3 mM potassium cyanide (KCN) was added to inhibit cellular respiration, and oxygen consumption was determined as the difference in slope (nmol Oxygen/min) with and without KCN. The data were normalized to protein content in sonicated cell pellets. Data are presented as mean ± SD. Sample size, n=2 dishes/treatment. Statistical analysis was performed by one-way ANOVA within each genotype, followed by LSD post hoc analysis. *, p=0.05 vs. Veh. Trends for significance were observed in WT MEFs at doses of 4 μM (p=0.06) and 0.5 μM (p=0.06).
To test this hypothesis, we performed fluorescent binding assays using the retinol-binding C1b domain of PKCδ and an equimolar concentration of either retinol or β-apo-10’-carotenal (Fig. 3-12A). As expected (Hoyos et al., 2000), the tryptophan fluorescence of the C1b domain was quenched ~25% in the presence of retinol (Fig. 3-12A). Fluorescence quenching of 25% also was observed when the protein was tested in the presence of β-apo-10’-carotenal (Fig. 3-12A; preliminary measurements indicate Kd=61.8 nM, corrected for inner filtering). Eighteen percent quenching was detected with bC as the ligand, while carotenoids containing more than one substituted β-ionone ring (astaxanthin and lutein), did not significantly quench fluorescence (Fig. 3-12B). Overall, these data implicate β-apo-10’-carotenal as a potential ligand for the PKCδ signalosome, and support the hypothesis that molecules with at least one unsubstituted β-ionone ring are favored for interaction with PKCδ (Gong et al., 2012).

Given the potential that bC and β-apo-10’-carotenal interact with PKCδ, we sought to determine whether these compounds affect mitochondrial function. We tested oxygen consumption as an indicator of cellular respiration in serum-starved WT MEFs treated for 2 hours with bC or β-apo-10’-carotenal. As previously shown for retinol (Acin-Perez et al., 2010b), both bC (Fig. 3-13) and β-apo-10’-carotenal (Fig. 3-14) tended to enhance respiration at certain doses. To assess whether this enhancement was PKC-dependent, we also tested oxygen consumption in PKCδ−/−, and PKCε−/− MEFs treated with β-apo-10’-carotenal in a preliminary experiment. Although the baseline respiration was lower in these two cell lines, β-apo-10’-carotenal had no effect on oxygen consumption in PKCδ−/− or PKCε−/− MEFs (Fig. 3-14). Overall these results suggest
Figure 3-15. Cellular viability in WT MEFs treated with β-carotene or β-apo-10’-carotenal.

Wild-type (WT) MEFs were seeded in a 96-well plate at ~10,000 cells/well in complete culture medium. The next day, cells were extensively washed in PBS and the medium was replaced with serum-free medium. On the third day, cells were treated overnight with β-carotene (bC), β-apo-10’-carotenal, or Vehicle (Veh, <0.2% THF or ethanol). The next day, cells were incubated 2 hours with the cell proliferation reagent WST-1 (Roche). Absorbance was read at 450 nm and 630 nm and corrected against a sample containing WST-1 and medium but no cells. Data are presented as mean ± SD.
that β-apo-10’-carotenal modulates respiration and that the PKCδ/ε network might be the target of its action in mitochondria. Furthermore, when we assessed the viability of WT MEFs in serum-free media treated for 24 hours with different doses of bC or β-apo-10’-carotenal, we found that β-apo-10’-carotenal had a less dramatic effect on cell death compared to bC, i.e. lower toxicity (Fig. 3-15). This result highlights that bC may indeed be toxic, as previously postulated (Amengual et al., 2010), while β-apo-10’-carotenal is less harmful, and likely beneficial given the above results.

Taken together, these results demonstrate that β-apo-10’-carotenal, the product of CMO2-mediated bC cleavage, could affect a mitochondrial signaling pathway that regulates energy flux. This function could be required to support embryonic development, and so its absence could detrimentally affect embryogenesis, as in the Cmo2-/Rbp-/-.


5. Discussion

For more than a decade, there has been great interest in the two carotenoid cleavage enzymes in vertebrates and their relative contribution to animal metabolism. As both enzymes are expressed in most tissues (Lindqvist and Andersson, 2002; Lindqvist and Andersson, 2004; Lindqvist et al., 2005), it was logical to assume that their functions and substrate preferences would not entirely overlap. Over time, CMO1 emerged as the dominant retinoid-generating enzyme in both adult (Hessel et al., 2007) and embryonic tissues (Kim et al., 2011), which symmetrically cleaves (at their 15,15’ bonds) carotenoids with at least one un-substituted β-ionone ring (Lindqvist and Andersson, 2002). CMO2 was found to asymmetrically cleave a broad array of substrates at their 9’,10’ or 9,10 bonds, allowing for substituted rings in compounds including carotenes (Amengual et al., 2010; Kiefer et al., 2001), xanthophylls, and even certain retinoids (Lobo et al., 2012b). The contribution of CMO2 to retinoid homeostasis has been considered minimal, given its allowance for a variety of substrates and the lack of retinoid deficiency in Cmo2/- mice (Amengual et al., 2010). Only recently has CMO2 been identified as a potentially critical enzyme in retinoid metabolism, due to its newly uncovered function of generating apocarotenoids from substituted carotenoids such as β-cryptoxanthin, which subsequently can be converted to retinaldehyde by CMO1 (Amengual et al., 2013).

Our lab has focused on the role of the carotenoid cleavage enzymes during mammalian embryogenesis. We found that mouse embryos, placentas, and yolk sacs express both enzymes at varying levels throughout gestation (Kim et al., 2011). Local bC
cleavage by embryos expressing CMO1 rescued a large number of them from developmental defects due to maternal vitamin A deficiency (Kim et al., 2011). While these studies confirmed the importance of CMO1 for embryonic development under conditions where preformed vitamin A is limiting, the necessity of CMO2 expression in the developing tissues remained to be elucidated. The Cmo1-/-Rbp-/- mouse strain generated in this prior study became a useful model to assess the role of CMO2 in embryonic development.

5.1. CMO2 does not contribute significantly to retinoic acid formation in embryos in the absence of CMO1

Our initial experiments, wherein Cmo1-/-Rbp-/- dams were fed a vitamin A-deficient diet during pregnancy, revealed an embryonic phenotype distribution of Cmo1-/-Rbp-/- embryos similar to that previously published for Cmo1+/-Rbp-/- and Cmo1-/-Rbp-/- embryos from Cmo1-/-Rbp-/- dams fed a vitamin A-deficient diet (Kim et al., 2011). In the previous study, maternal bC supplementation from 6.5-9.5 dpc allowed ~61% of Cmo1+/-Rbp-/- embryos to develop normally. In the present study, an improvement in the embryonic phenotype was observed upon maternal bC supplementation, despite the absence of maternal or embryonic CMO1 expression (i.e. Cmo1-/-Rbp-/- embryos from dams of the same genotype). However, this improvement predominantly manifested itself as a shift towards a milder malformed phenotype; very few normal Cmo1-/-Rbp-/- embryos were observed (Table 3-2). Although Cmo2 mRNA levels were unaltered in these supplemented animals (Fig. 3-2), nevertheless the question
arose whether asymmetric bC cleavage by CMO2 could partially contribute to retinoid-dependent processes during embryonic development.

Employing the same dietary regimen and bC supplementation protocol, we explored the effects of bC treatment in a novel triple-knockout strain that we generated, the Cmo1-/-Rbp-/-Cmo2-/- mice. To our amazement, an improvement in the embryonic phenotype was observed upon maternal bC supplementation of this strain, even in the absence of maternal or embryonic CMO1 or CMO2 expression. The phenotypes and their percentage distribution were similar in Cmo1-/-Rbp-/-Cmo2-/- (Table 3-4) and Cmo1-/-Rbp-/- (Table 3-2), suggesting that these improvements were not due to enzymatic cleavage of bC, neither symmetric (by CMO1) nor asymmetric (by CMO2). On the other hand, retinaldehyde supplementation completely rescued 92% of Cmo1-/-Rbp-/-Cmo2-/- embryos, highlighting the weak contribution of supplemental bC to embryonic development in this strain.

The results of our bC supplementation of triple-knockouts raised the question of how a retinoid-dependent phenotype could be rescued by bC in the absence of either of the known carotenoid cleavage enzymes. It is generally assumed that a third mammalian carotenoid oxygenase does not exist, as CMO1 and CMO2 share amino acid sequence homology only with each other and a retinoid isomerase only expressed in the eye (RPE65) (von Lintig, 2010). Therefore, we considered the issue of spontaneous bC oxidation – a well-known fact in the literature, and the most likely reason for the phenotype improvement of the supplemented triple-knockouts. Indeed, HPLC analysis of our bC solutions revealed a variety of compounds indicative of bC degradation, which also were detected in the sera and livers of injected mice (Fig. 3-3). Given the marked
increase in such compounds in serum and tissue extracts compared to the bC preparations used for IP injection, it is likely that many of these spontaneous oxidation products were generated *in vivo*. Note that although retinoid levels did not differ markedly in embryos from bC-injected triple-knockout mice compared to controls (Table 3-3), we cannot exclude that conversion of small amounts of these apocarotenoids to retinoic acid (by chain shortening (Wang et al., 1996), given the lack of CMO1) could facilitate vitamin A-dependent developmental processes and thus partially improve the embryonic phenotype. Other possible beneficial functions of apocarotenoids during embryogenesis are discussed below. Overall, our data suggest that CMO2-mediated bC cleavage may have produced a small amount of retinoids to support embryonic development in the supplemented Cmo1-/Rbp-/ mice, but other mechanisms of bC oxidation predominated in both Cmo1-/Rbp-/ and triple-knockout mice, leading to similar degrees of phenotype improvement regardless of whether CMO2 was expressed.

5.2. CMO2 affects the metabolism of retinol and retinyl esters in embryos

Although our data failed to demonstrate a significant retinoid-generating activity of CMO2 in bC-supplemented embryos, regardless of the presence or absence of CMO1, further experiments showed that CMO2 affects embryonic development, independent of its ability to asymmetrically cleave bC. Firstly, we discovered that CMO2 appears to be involved (directly or indirectly) in embryonic retinol and retinyl ester accumulation (Table 3-5). mRNA levels of *Lrat*, which encodes the enzyme that synthesizes retinyl esters, were reduced in Cmo2-/ embryos from dams on the vitamin A-sufficient diet compared to WT (Fig. 3-4). This effect seemed to depend on CMO1 availability, as
embryonic retinoid levels were lower in embryos lacking only CMO2, compared with those lacking both CMO1 and CMO2 (compare Cmo2-/− versus Cmo1-/−Cmo2-/−, or Cmo2-/−Rbp-/− versus Cmo1-/−Rbp-/−Cmo2-/−, Table 3-5), ultimately resulting in a more severe phenotype for Cmo2-/−Rbp-/− embryos (Table 3-6). However, Lrat mRNA levels did not differ between Cmo2-/−Rbp-/− and Rbp-/− embryos from vitamin A-deprived dams, and increased upon bC-supplementation of the former (Fig. 3-5A). Of note, Lrat mRNA levels were greater in Cmo2-/−Rbp-/− maternal livers compared to Rbp-/− when both were fed a vitamin A-deficient diet (Fig. 3-5B). While the reasons for these tissue-specific (embryo versus liver), background-specific (WT versus Rbp-/−), and perhaps diet-specific (vitamin A-sufficient versus deficient) effects are unclear, the evidence that CMO2 affects LRAT warrants further investigation, including analysis of LRAT protein levels and enzymatic activity.

The present study is not the first evidence that a carotenoid cleavage enzyme can impact retinoid homeostasis apart from the supplementation of its substrate. Indeed, previous work from our lab showed that Cmo1-/− embryos had lower retinyl ester levels than WT, accompanied by lower mRNA and enzymatic activity levels of LRAT (Kim et al., 2011). In the case of the Cmo1-/−, CMO1 deficiency also reduced embryonic concentrations of other lipids (predominantly triglycerides and cholesteryl esters) and mRNA levels of the corresponding acyltransferases Dgat2, Lcat, and Acat1 (Dixon et al., 2014). We found no effect on mRNA levels of these same acyltransferases in Cmo2-/− embryos compared to WT (data not shown). Therefore, it appears that CMO1 and CMO2 affect embryonic retinoid homeostasis by different mechanisms, which are under investigation in our laboratory.
5.3. CMO2 affects the ability of retinoids and bC to support embryonic development

Retinaldehyde supplementation appeared less effective in rescuing Cmo2-/Rbp-/ embryos from vitamin A-deprived dams (Table 3-6), compared with Cmo1-/Rbp-/Cmo2-/ embryos from dams fed the same diet (Table 3-4). Although the majority (72%) of the Cmo2-/Rbp-/ embryonic phenotype could be rescued by alleviating their vitamin A deficiency (i.e. feeding retinaldehyde), a significant proportion of the retinaldehyde-supplemented Cmo2-/Rbp-/ embryos still developed abnormally. The increased appearance of exencephaly in this strain upon retinaldehyde supplementation (8%, versus <4% after bC supplementation, Table 3-6) possibly suggests embryonic vitamin A toxicity induced by the retinaldehyde treatment. Other hypotheses are discussed below to address the susceptibility of the Cmo2-/Rbp-/ embryos to more severe phenotypes.

bC supplementation interfered with the ability of inseminated Cmo2-/Rbp-/ females to conceive or carry embryos, and both improved and exacerbated the morphological defects of Cmo2-/Rbp-/ embryos from vitamin A-deprived dams (Table 3-6). These results are striking, given that both mothers and embryos expressed CMO1, and should have been able to generate retinoids to support normal development via symmetric cleavage of bC. Indeed, our previous work showed that 61% of Cmo1+/Rbp-/ embryos from bC-supplemented Cmo1-/Rbp-/ dams were grossly normal, indicating that even a single genomic copy of Cmo1 is sufficient to support a certain degree of normal embryogenesis (Kim et al., 2011). Taken together, these results suggest two possibilities. On the one hand, not only the ability to cleave bC by CMO1, but also the proposed ability to scavenge bC (Amengual et al., 2010) or its cleavage products by CMO2, could be required to facilitate embryonic development, at least when bC is the...
only available vitamin A source. Alternatively, it may be that CMO2-deficient mice are susceptible to deficiency in apocarotenoids on two levels. First, they are unable to generate new apocarotenoids from supplemental bC via CMO2. Second, they are able to deplete any stored apocarotenoids (e.g. previously ingested from the non-purified maintenance diet and stored in maternal tissues prior to conception) by cleaving them via CMO1. Effects of apocarotenoid deficiency have not been described in the literature to date, but may be supported by our data. Indeed, we showed that embryonic β-apo-10’-carotenoic acid levels tended to be elevated upon bC treatment of Cmo1/- dams but not Cmo1/-Cmo2/-, suggesting that embryonic production of β-apo-10’-carotenoids is CMO2-dependent (Fig. 3-8). Additionally, supplementation of Cmo2/-Rbp/- females with β-apo-10’-carotenal (Fig. 3-10), but not bC (Table 3-6), facilitated the development of normal embryos. Thus, it appears that in addition to (or perhaps even more than) retinoic acid, β-apo-10’-carotenal is required for embryonic development when vitamin A is limiting.

5.4. CMO2 affects mitochondrial function by forming apocarotenoids

CMO2 is well-established as an enzyme that affects mitochondrial function. The first published study on the Cmo2/- mice (Amengual et al., 2010) employed diets containing zeaxanthin or lutein but no vitamin A. The authors showed that CMO2 deficiency coupled with dietary carotenoid (xanthophyll) supplementation led to the formation of 3-dehydrocarotenoids, their accumulation in mitochondria, a reduction in hepatic mitochondrial ADP-dependent (state 3) respiration compared to untreated Cmo2-
and an up-regulation of hepatic protein levels of SOD2 (MnSOD), HIF1-α, phospho-AKT and phospho-MAPK compared to WT mice. The latter two proteins were also up-regulated in Cmo2-/ hearts following xanthophyll feeding. In HepG2 cells (a cell line that does not express CMO2) treated with the same dehydrocarotenoids detected in xanthophyll-fed Cmo2-/ mice, ROS production was induced and mitochondrial membrane potential decreased. Importantly, bC also induced ROS formation and membrane depolarization in HepG2 cells, but these phenotypes were ameliorated following transfection with a plasmid containing CMO2. These results suggested that the accumulation of (dehydro)carotenoids in mitochondria could directly affect the electron transport chain, thus triggering oxidative stress (Amengual et al., 2010).

Later work in zebrafish by the same group (Lobo et al., 2012b) showed that erythrocyte apoptosis and anemia occurred in larvae injected with antisense morpholino oligonucleotides against CMO2, or treated with a CMO2 inhibitor (4-oxo-4HPR). The targeted CMO2 knockdown also caused mRNA levels of Gpx1 and Cat to increase in isolated blood cells. Treatment of larvae with hydrogen peroxide induced Cmo2 mRNA expression in whole larvae and their blood cells, suggesting that Cmo2 is induced by oxidative stress. Indeed, antioxidant exposure (N-acetylcysteine) prevented erythrocyte anemia in 4-oxo-4HPR treated larvae. In this same paper, the authors showed that human HepG2 cells, which do not express CMO2, generated ROS and depolarized their mitochondrial membranes following bC (and canthaxanthin) treatment, whereas these did not occur in a CMO2-expressing cell line (T47D, human breast carcinoma). Ultimately, the oxidative stress in carotenoid-treated HepG2 cells led to cytochrome c release, pro-
caspase 3 and PARP1 cleavage, and chromatin condensation, all hallmarks of the apoptotic pathway (Lobo et al., 2012b).

Overall, the above studies indicated that scavenging of carotenoids (including bC) and their derivatives (dehydrocarotenoids) is an important function of CMO2 which protects mitochondria from oxidative stress. These findings led us to wonder whether CMO2 deficiency had triggered oxidative stress pathways in Cmo2-/-Rbp-/- embryos, possibly leading to the developmental defects we observed (upon bC supplementation) (Table 3-6). However, the high incidence of cleft palate among embryos from dams fed a diet devoid of carotenoids and retinoids during pregnancy (Table 3-6) also suggested that deficiency in this mitochondrial enzyme could lead to oxidative stress, even in the absence of its substrate(s) in the gestational diet. In fact, Amengual et al. found that hepatic pMAPK was induced in Cmo2-/- mice fed a chow diet compared to WT (however these authors found hepatic steatosis in carotenoid-fed Cmo2-/- but not chow-fed Cmo2-/-, and no tested proteins were up-regulated in the hearts of chow-fed Cmo2-/-) (Amengual et al., 2010). Nevertheless, in both our experiments and those of Amengual and colleagues, it is unclear to what extent the small amounts of bC and apocarotenoids in the non-purified maintenance diet could affect the phenotype of the Cmo2-/- animals prior to the provision of the purified experimental diet. In other words, it cannot be unequivocally stated that any phenotype has been observed in Cmo2-/- mice in the total absence of the substrate(s) of CMO2. This issue is further discussed in Chapter 4.

One possible cause of oxidative stress in Cmo2-/-Rbp-/- embryos could be their more severe vitamin A deficiency relative to other strains (Tables 3-5 and 3-6). Vitamin A deficiency in both rats and mice affects the hepatic activity of enzymes in oxidative
stress pathways, including Catalase, Glutathione-S-transferase, Glutathione Peroxidase (Arruda et al., 2009), DT diaphorase, and Urate Oxidase (Sohlenius-Sternbeck et al., 2000). Interestingly, whether vitamin A deficiency induces or diminishes the activity of these enzymes appears to be species-specific and may depend on the severity of the vitamin A deficiency. In rats, vitamin A deficiency also reduced the hepatic mitochondrial membrane potential and GSH/GSSG ratio, while it increased polar and nonpolar lipid and MDA levels in mitochondria, and elevated a marker of mitochondrial DNA damage (8-oxo-DG) (Barber et al., 2000). One study in vitamin A-deficient rats suggested a potential mechanism whereby vitamin A deficiency could indirectly affect oxidative stress pathways by increasing tissue accumulation of iron, a known catalyst of certain oxidation reactions (Arruda et al., 2009).

Despite the established link between vitamin A deficiency and oxidative stress, and the published evidence that CMO2 deficiency renders cells susceptible to oxidative stress (Amengual et al., 2010; Lobo et al., 2012b), under our experimental conditions we did not find striking molecular evidence of oxidative stress in Cmo2/-/(Rbp/-) in comparison to other strains or in response to bC treatment (Fig. 3-6 and 3-7). Additionally, no normal embryos were recovered in a small group of vitamin A-deprived Cmo2/-/Rbp/- dams (n=2) supplemented with the antioxidant N-acetylcysteine (NAC) throughout gestation prior to bC treatment from 6.5-9.5 dpc. Rather, our data pointed to a role of CMO2 in generating apocarotenoids that may regulate PKC signaling in the mitochondria, cellular respiration, and hence cell survival. First, we observed that in addition to retinol (Hoyos et al., 2000), β-apo-10’-carotenal, and bC to a lesser extent,
could interact with the retinoid-binding C1b domain of PKC, while other carotenoids with substituted β-ionone rings did not (Fig. 3-12). Second, β-apo-10’-carotenal tended to enhance cell respiration (Fig. 3-14), similar to retinol (Acin-Perez et al., 2010b), and while bC dose-dependently reduced cell viability, β-apo-10’-carotenal did not (Fig. 3-15). Given all these beneficial activities of β-apo-10’-carotenal, it is possible that downstream targets of PKCδ signaling (e.g. the Pyruvate Dehydrogenase Complex) could be affected in CMO2-deficient cells due to their inability to enzymatically generate β-apo-10’-carotenal.

The question then arises why Cmo2-/-Rbp-/- embryos, but not embryos from other CMO2-deficient strains (e.g. Cmo2-/- and Cmo1-/-Rbp-/-Cmo2-/-) are so sensitive to apocarotenoid deficiency. One possible answer is that both retinol and β-apo-10’-carotenal are capable of interacting with PKCδ. While retinol is the known and perhaps main activator of PKCδ, in vitamin A-deficient conditions, apocarotenoids may help to prevent the complete failure of the PKCδ signalosome that seems to regulate cellular energy homeostasis. Cmo2-/- embryos from vitamin A-deprived dams could access adequate levels of retinol for PKCδ activation due to maternal RBP, and thus would not rely on apocarotenoids to activate this signalosome; and indeed these embryos developed normally. In contrast, Cmo2-/-Rbp-/- embryos from vitamin A-deprived dams could not depend on maternal hepatic retinoid stores due to the lack of RBP to mobilize them. Indeed, the embryonic retinol levels in the Cmo2-/-Rbp-/- strain were the lowest of all of our strains (Table 3-5), in agreement with the hypothesis that a PKCδ ligand was lacking in these malformed embryos. To add insult to injury, any apocarotenoids the dams might
Exogenous treatment

*Cmo1-/-*-*Rbp-/-* + bC
(Severe)

A.

Cmo2-/--*Rbp-/-* + bC
(More severe)

C.

Cmo2-/--*Rbp-/-* + apo10
(More improved)

E.

Cmo2-/--*Rbp-/-* + RAL
(Improved)

G.

*Cmo1-/-*-*Rbp-/-*-*Cmo2-/-* + RAL
(More improved)

I.
Figure 3-16. Enzymatic pathways for the production of β-apo-10’-carotenal and retinoids in various mouse models.

Knockout mouse strains on the vitamin A-deficient diet were treated with β-carotene (bC), β-apo-10’-carotenal (apo10), or retinaldehyde (RAL). The resulting embryonic phenotype is indicated in parentheses. The left panels (A, C, E, G, I) illustrate the expected effect of the treatment (orange box) on the embryonic levels of retinoids and β-apo-10’-carotenal. A red arrow indicates a product expected to be formed via CMO1; a blue arrow indicates a product expected to be formed via CMO2; and an orange arrow indicates a compound expected to be formed/present due to the treatment itself (apart from CMO1 or CMO2 action). Gray coloring indicates that β-carotene or β-apo-10’-carotenal may have been available due to maternal hepatic stores, but were not added exogenously, or could not be formed from the exogenous treatment. The right panels (B, D, F, H, J) illustrate the expected effect of the genotype on the endogenous levels of β-apo-10’-carotenal (corresponding to the font size), which may have been available due to maternal stores of β-carotene or β-apo-10’-carotenal consumed in the pre-pregnancy chow diet.

In the Cmo1/-Rbp/- + bC (A and B), the ability to generate β-apo-10’-carotenal (from both exogenous and endogenous β-carotene) is insufficient to yield a normal phenotype, due to the inability to generate retinoic acid and retinol from β-carotene or β-apo-10’-carotenal. Under these circumstances, β-apo-10’-carotenal can be formed but cannot be converted to retinaldehyde via CMO1. In the Cmo2/-Rbp/- (C and D), even though retinoic acid and retinol can be formed by symmetric β-carotene cleavage, β-apo-
10’-carotenal cannot be formed and its endogenous levels are expected to be quite low (compared to A and B), resulting in an even worse phenotype than the Cmo1-/-Rbp-/-.

In contrast, supplementation of the Cmo2-/-Rbp-/- with β-apo-10’-carotenal (E and F) can increase the levels of both β-apo-10’-carotenal and retinoids (retinol and retinoic acid), and greatly improves the embryonic phenotype. Retinaldehyde supplementation of the Cmo2-/-Rbp-/- (G and H) can overcome its deficiency in retinol and retinoic acid, but not its deficiency in β-apo-10’-carotenal; thus, a lesser degree of improvement is achieved.

The Cmo1-/-Rbp-/-Cmo2-/- (I and J), which is expected to have higher endogenous levels of β-apo-10’-carotenal than the Cmo2-/-Rbp-/- due to the inability to metabolize it via CMO1, achieves a greater degree of rescue upon retinaldehyde supplementation.
have obtained from the chow diet (in the 90 days prior to experimental manipulation) could have been converted to retinoids by CMO1 (Amengual et al., 2013). Therefore, embryos from vitamin A-deprived Cmo2-/Rbp-/ dams had the least access to retinol and apocarotenoids of any of our strains. Indeed, even the Cmo1-/Rbp-/Cmo2-/- embryos had a better chance of receiving apocarotenoids, since at least those consumed in the pre-pregnancy chow diet could not be metabolized by CMO1.

Our data suggest that both retinoic acid (to activate retinoic acid-dependent transcription factors) and retinol/β-apo-10′-carotenal (to enhance cellular respiration) are required for normal embryonic development when preformed vitamin A is limiting in the diet (Fig. 3-16). Firstly, our evidence indicates that retinoic acid alone is insufficient to fully rescue embryonic development. Although it is unclear to what extent β-apo-10′-carotenal was metabolized to retinoic acid in the β-apo-10′-carotenal-supplemented Cmo2-/Rbp-/ dams, the failure of this strain to generate normal embryos upon bC injection (Table 3-6) supports the aforementioned hypothesis. However, even retinol could have been produced in bC-treated Cmo2-/Rbp-/ embryos if bC was cleaved to retinaldehyde by CMO1 and then reduced (i.e. by DHRS3 (Billings et al., 2013)). This may suggest that not only retinol, but also β-apo-10′-carotenal, is required for normal embryogenesis. In other words, the PKC-binding ability that β-apo-10′-carotenal seems to share with retinol may not be its only essential function. Secondly, our evidence indicates that β-apo-10′-carotenal alone (without being converted to other compounds) is insufficient to fully rescue embryonic development. Indeed, we observed only a small percentage of normal embryos in bC-supplemented Cmo1-/Rbp-/ dams despite their
expression of CMO2 (Table 3-2 and Figure 3-16A), suggesting that bC (and/or some of the β-apo-10’-carotenal produced from bC) must be converted to retinaldehyde by CMO1 (Amengual et al., 2013) and then oxidized to retinoic acid to facilitate normal development. In contrast to these restricted dietary conditions, mice consuming a non-purified vitamin A-sufficient diet would have access to apocarotenoids (to enhance cellular respiration, or perform other yet-to-be-determined functions), as well as preformed vitamin A (e.g. retinyl esters), which could be either hydrolyzed to retinol (to enhance cellular respiration) or subsequently oxidized to retinoic acid (to activate transcription factors). The Cmo2-/Rbp/- supplemented with β-apo-10’-carotenal would similarly have access both to apocarotenoids and retinoids (Fig. 3-16E).

Taken together, it appears that β-apo-10’-carotenal alone (generated by CMO2 in the absence of CMO1, Fig. 3-16A) or retinol/retinoic acid alone (generated by CMO1 in the absence of CMO2, Fig. 3-16C) cannot fully support embryonic development. However, if this is the case, it seems surprising at first that we observed a high percentage of normal embryos in retinaldehyde-fed Cmo1-/Rbp-/Cmo2/- and Cmo2-/Rbp/- dams (Table 3-4 and 3-6; Fig. 3-16G and I), which could have produced retinol or retinoic acid but not β-apo-10’-carotenal. It may be that the apocarotenoids stored in the maternal liver from the pre-pregnancy chow diet were sufficient to support any apocarotenoid-dependent developmental processes, even when apocartenoids were removed from the diet (Fig. 3-16H and J). Indeed, we observed a higher degree of rescue in retinaldehyde-supplemented triple-knockouts than Cmo2-/Rbp/- embryos (compare Table 3-4 and 3-6), which could be attributed to the theoretically lower stores of apocarotenoids in Cmo2-
/-Rbp/- maternal livers (Fig. 3-16H), and thus could support the notion that both retinaldehyde and apocarotenoids are required for normal embryogenesis.

The question remains why embryonic retinol levels were lower in Cmo2/-Rbp/- embryos compared with all other strains (Table 3-5). While the answer to this question awaits further investigation, we can only conclude that whether or not bC is supplemented, CMO2 is critical to embryonic development when dietary retinoids are limiting. For reasons that are yet unclear, this function of CMO2 depends on the expression of CMO1, as different phenotypes (including retinol levels) were observed following vitamin A deprivation and/or bC supplementation of Cmo2/-Rbp/- and Cmo1/-Rbp/-Cmo2/- mice. How the two carotenoid oxygenases can interact with one another, given their compartmental separation within the cell, will be an exciting topic of future work.
Chapter 4

General conclusion and future directions
4.1. Insights on vitamin A deficiency

Vitamin A deficiency continues to be a significant worldwide health problem that affects both developed and developing countries (Grune et al., 2010), and presents particular challenges for pregnant women (World Health Organization, 2009). Sadly, little success has been achieved in the majority of clinical trials attempting to alleviate vitamin A deficiency through supplementation of pregnant women with vitamin A or bC (Thorne-Lyman and Fawzi, 2012). At the same time, it is clear that adverse health effects can result from both unintentional and intentional exposure to high doses of certain vitamin A metabolites or their carotenoid precursors. For instance, miscarriages and fetal malformations were reported after maternal intake of isotretinoin or “Accutane” (Lammer et al., 1985)), while an increased risk of lung cancer was observed in smokers and asbestos-exposed workers following combined bC and retinyl palmitate supplementation in the CARET trial (Omenn et al., 1996)). Additionally, the precise relationship between bC intake and its provitamin A activity is constantly being redefined, as new conversion factors are described in subsets of the world population depending on their predominant food matrix, genetic polymorphisms, age, BMI, etc. (Grune et al., 2010). Overall, despite the great advances in the basic fields of vitamin A and carotenoid research in the past century, these efforts have not provided sufficient translational information to overcome the challenges of alleviating vitamin A deficiency.

Considering the persistence of vitamin A deficiency in pregnant women and their children despite supplementation attempts, the question arises what governs the ability to rescue mammals from vitamin A deficiency. The work presented here has suggested several factors influencing this ability. First, the severity of the maternal vitamin A
deficiency influences the severity of the fetal vitamin A deficiency, and hence the ability to rescue it. Our mouse model of dietary vitamin A deficiency, unaccompanied by a vitamin A deficient tissue status (WT mice fed a gestational vitamin A-deficient diet, Chapter 2), confirmed the well-established principle that retinoids stored in the maternal liver and released to the periphery bound to RBP are sufficient to support normal embryogenesis despite the lack of incoming dietary vitamin A (Quadro et al., 2005). In contrast, our model of both dietary and tissue vitamin A deficiency (Lrat-/Rbp-/- dams fed a gestational vitamin A-deficient diet) gave rise to highly malformed embryos (Fig. 2-1). In the case of the WT dams, there was no embryonic vitamin A deficiency to rescue, although the supplemental bC at 13.5 dpc appeared to be used to generate retinoic acid to a certain extent, as evidenced by the down-regulation of the retinoic acid-responsive gene Rdh10 in placentas and embryos (Fig. 2-2). For the Lrat-/Rbp-/- dams, the severity of the vitamin A-deficiency was such that neither acute (13.5 dpc) nor prolonged (6.5-9.5 dpc) bC supplementation could rescue 100% of their offspring (Tables 2-1 and 2-4), despite the transcriptional induction of several genes involved in retinoid homeostasis (Fig. 2-2).

Second, the study of the Lrat-/Rbp-/- mice indicated that the stage of gestation influences the ability of supplemental bC to improve the embryonic phenotype. As could be expected, retinoid-dependent developmental processes could not be supported by retinoids generated from bC after the critical window of organ development had elapsed (Rugh, 1968). Thus, no phenotypic improvement was observed following bC supplementation of Lrat-/Rbp-/- dams at 13.5 dpc (Table 2-1), while supplementation from 6.5-9.5 dpc allowed 38% of embryos to develop normally, and completely eliminated the cleft palate from the offspring (Table 2-4). Apparently, at least certain
forms of vitamin A are effective even later than 6.5 dpc, as retinaldehyde supplementation from 7.5-9.5 dpc rescued a large proportion of embryos from both the Cmo2/-/Rbp/- and Cmo1/-/Rbp/-/Cmo2/- strains (Tables 3-4 and 3-6), and β-apo-10'-carotenal supplementation from 7.5-9.5 dpc rescued Cmo2/-/Rbp/- embryos (Fig. 3-10). Nevertheless, it is possible that closer to 100% of embryos from these supplemented dams could have been rescued if the supplementation had been started a day earlier.

Third, our data indicate that the form of vitamin A administered impacts the success of the supplementation regimen. For instance, retinaldehyde more effectively rescued embryos from vitamin A deficiency than bC, regardless of whether the embryos expressed CMO1 (e.g. Cmo2/-/Rbp/-, Table 3-6) or not (Cmo1/-/Rbp/-/Cmo2/-, Table 3-4). Other possible interpretations of the lack of effect of bC in CMO2-deficient strains are discussed further below. Importantly, our Cmo2/-/Rbp/- data (Fig. 3-10) showed for the first time that β-apo-10'-carotenal, an asymmetric bC cleavage product, could rescue embryos from vitamin A deficiency syndrome when its precursor could not (Table 3-6).

Fourth, it appears that the organ of bC metabolism (local embryonic versus maternal tissues) affects its ability to improve the embryonic phenotype. In our previously published work on Cmo1/-/Rbp/- dams, bC supplementation promoted the normal development of 61% of their Cmo1+/-/Rbp/- embryos, despite the absence of one genomic copy of Cmo1 in the embryos (Kim et al., 2011). On the other hand, only 38% of Lrat/-/Rbp/- embryos from bC-supplemented dams were grossly normal (Table 2-4). As previously discussed (Chapter 2.5), it is likely that this difference arose from the inability of pregnant Cmo1/-/Rbp/- dams to metabolize bC in their own CMO1-deficient
livers, which thus allowed more intact bC to reach their needy embryos. Less intact bC would have been available for Lrat-/-Rbp-/- embryos to metabolize in situ after the “first pass” of bC through the maternal liver, which expressed CMO1. However, it is possible that the seeming embryonic preference for in situ bC cleavage is an artifact of the Rbp-/-genetic background, since normally retinoids generated in the maternal liver would be delivered to embryos via RBP (Quadro et al., 2005). Other models of embryonic vitamin A deficiency, such as Rdh10-/- mice (Sandell et al., 2007), could be crossed with Cmo1-/-mice to avoid the potential artifact of the Rbp-/- background, thus allowing the necessity of in situ bC cleavage to be investigated. For instance, if Rdh10-/-Cmo1+/- embryos from Rdh10-/-Cmo1/- and Rdh10-/-Cmo1+/- dams showed similar degrees of improvement following maternal bC supplementation, it could be concluded that the organ of bC cleavage is irrelevant, so long as the resulting retinoids can be transported to the fetus.

Finally, the ability to rescue mammals from vitamin A deficiency depends on the deficient metabolite of vitamin A. Historically, embryonic vitamin A deficiency syndrome has been attributed to the impairment of retinoic acid-dependent developmental processes (see Chapter 1.3.1). Therefore, it is intriguing that the Cmo2-/-Rbp-/- embryos did not develop normally, despite their ability to generate retinoic acid from bC via CMO1. In part, the same reasoning could be applied to the Cmo2-/-Rbp-/- as was applied to the Lrat-/-Rbp-/- above; namely, that maternal hepatic CMO1 activity prevented intact bC from reaching the embryos. While the concentration of bC was below the detection limit in supplemented Cmo2-/-Rbp-/- embryos (Table 3-7), these steady-state HPLC analyses cannot clarify whether this was due to maternal or embryonic CMO1 activity. Maternal hepatic retinol and retinyl ester levels tended to be lower in bC-supplemented
Cmo2/-Rbp/- dams compared with other supplemented strains (Table 3-7), raising the question of whether hepatic bC processing predominated in this strain. However, serum bC levels in supplemented dams were comparable to those of Lrat/-Rbp/- dams (i.e. far below those of Cmo1/-Rbp/- dams) (Table 3-7), reinforcing the hypothesis that maternal CMO1 was highly active in both strains.

On the other hand, as recently reviewed (Hoyos et al., 2012), the presumption may be flawed that retinoic acid (the transcriptionally active retinoid) and retinaldehyde (the visual chromophore) are the only important vitamin A derivatives, for which retinol is only an intermediate metabolite. Rather, the evidence of enhanced respiration in mitochondria or MEFs treated with retinol (which was shown not to be metabolized to other forms, (Acin-Perez et al., 2010b)) suggests that retinol itself is a metabolite with important cellular functions. Since retinol is able to enhance cellular respiration, cell survival could be jeopardized when insufficient retinol is available. Accordingly, in the present study, the severity of the embryonic phenotype appeared to correlate with the embryonic retinol level. Specifically, Cmo2/-Rbp/- embryos from vitamin A-deprived dams had a higher percentage of cleft face/palate than Cmo1/-Rbp/- or even Cmo1/-Rbp/-Cmo2/- (compare Tables 3-2, 3-4 and 3-6), corresponding to the finding that the Cmo2/-Rbp/- embryos had the lowest retinol concentrations among these strains (Table 3-6). Cell death in the midfacial region (i.e. cleft face/palate) could have resulted from insufficient cellular respiration due to reduced retinol levels. However, retinol levels of malformed bC-supplemented Cmo2/-Rbp/- embryos (Table 3-7) were comparable to those of normal Lrat/-Rbp/- embryos from dams supplemented with bC for the same duration (6.5-9.5 dpc, Table 2-5), though both were lower than WT embryonic retinol
levels (Table 3-5). This finding suggests that the un-rescued $Cmo2^{-/-}Rbp^{-/-}$ embryos were deficient in another molecule which still could not be furnished after bC supplementation (and subsequent formation of retinol or retinoic acid). We propose that this molecule is $\beta$-apo-10’-carotenal.

As discussed in Chapter 3.5.4, $Cmo2^{-/-}Rbp^{-/-}$ also would be expected to have the lowest levels of $\beta$-apo-10’-carotenol of any mouse strain we have studied. (Direct measurement of $\beta$-apo-10’-carotenol in mouse tissues by LC-MS will be an important component of future work in this area, although the existing data on the $Cmo1^{-/-}$ and $Cmo1^{-/-}Cmo2^{-/-}$ embryos (Fig. 3-8) already suggest that CMO2-deficient embryos fail to accumulate $\beta$-apo-10’-carotenoic acid in response to bC treatment.) Indeed, dietary supplementation with $\beta$-apo-10’-carotenol allowed vitamin A-deprived $Cmo2^{-/-}Rbp^{-/-}$ dams to generate normal embryos (Fig. 3-10), while IP injection with bC did not for the most part (Table 3-6). These data support the assertion that retinoic acid is not the only “deficient metabolite” of vitamin A deficiency syndrome, at least under certain conditions (in this case, the $Cmo2^{-/-}Rbp^{-/-}$ genetic background).

Our experiments showing the ability of $\beta$-apo-10’-carotenol to interact with PKC (Fig. 3-12) and affect cellular respiration (Fig. 3-14) indicate that $\beta$-apo-10’-carotenol is not only structurally, but also functionally related to retinol. Other similarities in the functions of $\beta$-apo-10’-carotenol and retinol have been discovered by others, such as the ability to bind RBP and be esterified by LRAT (Amengual et al., 2013). However, to our knowledge, our work is the first to suggest that $\beta$-apo-10’-carotenol is not only able, but required to function like retinol under certain circumstances. Such a possibility sheds
new light on the recommendation at the 2009 Hohenheim conference that some of the daily vitamin A intake should include bC (Grune et al., 2010). It remains to be clarified whether the potential necessity of β-apo-10’-carotenal extends beyond vitamin A deficiency to other physiological states, and whether β-apo-10’-carotenal is required for other cellular functions independent of those overlapping with retinol.

4.2. Insights on CMO2 function

As bC is the main vitamin A source for most of the human population (Grune et al., 2010; Weber and Grune, 2012), the challenge of alleviating vitamin A deficiency must be faced with a thorough understanding of the mechanisms by which bC yields usable forms of vitamin A. To this end, extensive studies have sought to identify and characterize the mammalian bC cleavage enzymes. Since the discovery and biochemical analysis of the ~40% homologous enzymes CMO1 and CMO2, knockout mouse models have provided helpful clues about the in vivo roles of these two known bC cleavage enzymes (reviewed in Chapter 1.4.3 and 4). Work in both our laboratory (Kim et al., 2011) and others (Amengual et al., 2013; Hessel et al., 2007) indicated that symmetric cleavage of bC by CMO1 is the main pathway by which retinoids are generated from this provitamin A carotenoid in both adult and embryonic tissues. We found that embryos expressing CMO1 could be rescued from vitamin A deficiency syndrome by taking up and cleaving intact bC from the maternal circulation (Kim et al., 2011). The more limited evidence from adult Cmo2-/ mice suggested that CMO2 functions as a scavenger localized in the mitochondrion, necessary to prevent intact carotenoids from disrupting
the electron transport chain and thus causing oxidative stress (Amengual et al., 2010; Lobo et al., 2012b). Nevertheless, considering the theoretical ability of CMO2 to generate at least one molecule of retinaldehyde after asymmetrically cleaving bC, the question arose whether its function in embryos could overlap with that of CMO1, as a backup source of retinoids.

Our studies of $\text{Cmo2}^{-/-}$ mouse strains, together with the data published by von Lintig and colleagues (Amengual et al., 2010; Amengual et al., 2013; Lobo et al., 2012b), point to at least four possible roles of CMO2: 1. As a scavenger of bC or its derivatives to protect mitochondria from oxidative stress; 2. As an asymmetric bC cleavage enzyme that generates $\beta$-apo-10'-carotenal in order to eventually form retinoids; 3. As an asymmetric bC cleavage enzyme that generates $\beta$-apo-10'-carotenal in order to activate mitochondrial signaling pathways that enhance energy flux; and 4. As an enzyme that influences retinoid homeostasis in a CMO1-dependent manner. The evidence in the third chapter of this dissertation in some cases supports, and in other cases conflicts with these hypotheses.

In support of the role of CMO2 as a mitochondrial scavenger of bC, we observed a particularly high rate of unsuccessful pregnancies in bC-injected $\text{Cmo2}^{-/-}\text{Rbp}^{-/-}$ dams, along with a percentage of supplemented $\text{Cmo2}^{-/-}\text{Rbp}^{-/-}$ embryos with a worse phenotype than those from untreated dams (the spinal defect, Table 3-6). On the other hand, when $\text{Cmo2}^{-/-}$ and $\text{Cmo1}^{-/-}\text{Cmo2}^{-/-}$ dams were fed the vitamin A-deficient diet throughout gestation and injected with bC for the same period (6.5-9.5 dpc), their embryos developed normally (data not shown), suggesting that it was not the lack of
CMO2 *per se* that caused the embryonic phenotype in response to bC. Rather, it seemed that the interaction of the vitamin A-deficient background (*Rbp-/-* strains fed a vitamin A-deficient diet) with the bC treatment interfered with embryonic development in the absence of CMO2. In a similar fashion, our cell viability assays on bC-treated WT MEFs were performed on serum-starved cells, and thus mimicked the toxic effects of bC administered to vitamin A-deficient animals. In these experiments high concentrations of bC dramatically reduced cell survival, while β-apo-10’-carotenal did not (Fig. 3-15), suggesting that bC but not its CMO2-generated cleavage product is harmful at high doses. In fact, most of the *in vivo* work published on Cmo2-/- mice involved dietary regimens in which the mice were fed for 8 weeks with a diet devoid of retinoids, containing only non-provitamin A xanthophylls (Amengual et al., 2010). Therefore, it is highly possible that the originally-described Cmo2-/- mice were vitamin A-deficient, and thus enabled the detection of the scavenging function of CMO2.

In conflict with the suggestion that CMO2 protects cells from carotenoid-induced oxidative stress, we found little direct (ROS, Fig. 3-7A and B) or indirect (mRNA and protein measurements, Fig. 3-6 and 3-7C) evidence of an unbalanced oxidative state in *Cmo2-/- (Rbp-/-)* mice, either with reference to untreated mice of the same genotype, or with reference to *Rbp-/-* controls. Antioxidant consumption (NAC) throughout gestation also did not rescue the phenotype of bC-supplemented *Cmo2-/- Rbp-/-* embryos (n=2 dams, data not shown). It may be that differences in experimental conditions between our studies and those of von Lintig and colleagues account for these apparently conflicting findings; indeed, our carotenoid supplementation never exceeded four days, whereas von
Lintig used regimens up to 8 weeks long (Amengual et al., 2010). However, von Lintig and colleagues also found that oxidative stress in the Cmo2-/- was not exclusive to carotenoid treatment, as Cmo2-/- mice fed a chow diet displayed some markers of oxidative stress (Amengual et al., 2010). At the same time, rodent chow is known to contain bC and apocarotenoids (Shmarakov et al., 2010), so the proposed scavenging role of CMO2 will need to be re-assessed in Cmo2-/- mice raised on a purified vitamin A-sufficient diet (devoid of carotenoids) throughout life, before it can be stated with certainty whether CMO2 affects oxidative stress in a carotenoid-dependent or -independent manner.

As for the second potential function of CMO2 in vivo, we found little evidence to support the hypothesis that β-apo-10'-carotenal generated by CMO2 is a significant source of retinoic acid for embryonic development, at least in the absence of CMO1. Indeed, regardless of whether CMO2 was expressed (i.e. in Cmo1-/-Rbp-/- or Cmo1-/-Rbp-/-Cmo2-/- mice), bC supplementation of vitamin A-deprived dams led to a partial improvement in the embryonic phenotype (Tables 3-2 and 3-4). This is in agreement with the recent finding that bC supplementation led to similar tissue accumulation of bC in Cmo1-/- and Cmo1-/-Cmo2-/- mice, which led the authors to conclude that CMO2 does not significantly affect “bulk bC metabolism” (Amengual et al., 2013). However, as these investigators noted, the consecutive cleavage activities of CMO2 and CMO1 on asymmetric provitamin A carotenoids like β-cryptoxanthin may be an important mechanism for retinoic acid production from carotenoids that are not direct substrates for CMO1 (Amengual et al., 2013). While we did not provide such carotenoids to our
pregnant mice, the small amounts of carotenoids in the pre-pregnancy chow diet could have been stored in the maternal liver and differentially metabolized in mice depending on their expression of CMO2. In other words, mice expressing CMO2 could have converted these stored carotenoids into β-apo-10’-carotenal, which then could have been converted to retinoic acid either by chain shortening (Wang et al., 1996) or by CMO1 activity (Amengual et al., 2013). In support of this notion, we did observe a slightly higher percentage of cleft face/palate in Cmo1-/Rbp-/Cmo2-/ embryos compared to Cmo1-/Rbp-/ embryos from dams fed a vitamin A-deficient diet (compare Tables 3-2 and 3-4), as well as a very high level of cleft face/palate in Cmo2-/Rbp-/ embryos (Table 3-6), suggesting that even at baseline, CMO2-deficient mice were more deficient in retinoic acid than mice expressing CMO2. As discussed above, this possibility could be clarified by raising mice on a purified vitamin A-sufficient diet devoid of carotenoids. Following such a maintenance regimen, the gestational vitamin A-deficient diet would be expected to yield a similarly high percentage of cleft face/palate in Cmo1-/Rbp-/Cmo2-/ embryos – if retinoic acid production is the only reason that CMO2-deficient strains are more prone to cleft face/palate.

On the other hand, our data suggest that retinoic acid production is not the only reason for the more severe phenotype of CMO2-deficient strains. Indeed, if this were the case, then the cleavage of bC by CMO1 in supplemented Cmo2-/Rbp-/ mice should have rescued the embryonic phenotype. Since this was not the case (Table 3-6), apocarotenoid deficiency seems to be the more plausible explanation for the severe phenotype of Cmo2-/Rbp-/ embryos, the failure to rescue it with bC, and most of all the
ability to rescue it with β-apo-10’-carotenal (Fig. 3-10 and 3-16). Such an apocarotenoid deficiency would validate the third suggested role of CMO2 in generating β-apo-10’-carotenal in order to activate mitochondrial signaling pathways. Aside from the in vivo evidence, in vitro evidence from WT MEFs showed that β-apo-10’-carotenal does not markedly reduce cell viability at doses below 2 μM (Fig. 3-15), and tends to enhance oxygen consumption at a dose of 0.5 μM (p=0.06, Fig. 3-14). The effect of β-apo-10’-carotenal on oxygen consumption may well be due to its ability to interact with PKC (Fig. 3-12A). Indeed, given the structural similarity between β-apo-10’-carotenal and retinol (Fig. 1-4), it is feasible that, like retinol (Fig. 3-11), β-apo-10’-carotenal could activate the PKCδ signalosome (Acin-Perez et al., 2010a;Acin-Perez et al., 2010b) and thus stimulate flux through the Pyruvate Dehydrogenase Complex, ultimately increasing glucose metabolism and cellular respiration. Our results in MEFs lacking PKCδ or its opponent PKCe (Gong et al., 2012) suggest that the effect on cellular respiration by β-apo-10’-carotenal is mediated by this signalosome (Fig. 3-14). In future studies, it will be important to further examine the specific interactions of β-apo-10’-carotenal with these two PKC isoforms.

It should also be noted that the potential role of CMO2 in generating apocarotenoids that activate PKC would be carotenoid-specific. While carotenoids and apocarotenoids with at least one unsubstituted β-ionone ring were able to interact with PKC, doubly-substituted carotenoids were not (Fig. 3-12). Thus, supplementation of Cmo2/- mice with doubly-substituted xanthophylls (as in the studies of von Lintig and colleagues (Amengual et al., 2010)) would not be expected to highlight this function of
CMO2. This discrepancy may further explain why these former studies implicated CMO2 as a scavenging enzyme rather than a producer of bioactive compounds.

Finally, our studies of CMO2-deficient mouse strains uncovered another function of CMO2 not previously described in the literature – a CMO1-dependent influence on embryonic retinoid homeostasis, in the absence of carotenoid supplementation. Like Cmo1-/ mice (Dixon et al., 2014; Kim et al., 2011), Cmo2-/ embryos from dams fed a vitamin A-sufficient chow diet had lower retinyl ester levels (Table 3-5) and Lrat mRNA expression levels (Fig. 3-4) than WT embryos. Cmo2-/Rbp-/- embryos from dams fed the vitamin A-deficient diet had the lowest retinol and retinyl ester levels of any of the Rbp-/ strains (Table 3-5). It appears that this puzzling effect is CMO1-dependent, as strains lacking both CMO1 and CMO2 generally had greater retinol and/or retinyl ester levels than strains lacking only CMO2 (Table 3-5). Similarly, triple-knockout embryos were rescued by retinaldehyde supplementation to a greater extent than Cmo2-/Rbp-/- embryos (compare Tables 3-4 and 3-6), suggesting that the absence of CMO2 was more detrimental to embryonic retinoid homeostasis in the presence of CMO1. In this case, the theoretically lower levels of β-apo-10’-carotenal in Cmo2-/Rbp-/- embryos could explain the lesser phenotypic improvement upon retinaldehyde supplementation (Fig. 3-16H).

While the above results were obtained in mice not injected with bC, as previously discussed, some of these results may be artifacts of the maintenance chow diet, which contained small amounts of bC and apocarotenoids (see embryos fed this diet in Fig. 3-8). Therefore, any future efforts to clarify this effect of CMO2 on retinoid homeostasis should be performed on animals raised on a purified vitamin A-sufficient diet, in order to
verify its carotenoid independence. However, since apocarotenoids have been reported to be repressors of retinoic acid-dependent transcription rather than direct activators of transcription factors (Eroglu et al., 2010; Eroglu et al., 2012; Ziouzenkova et al., 2007a), we do not favor the possibility that the effects of CMO2 on LRAT are transcriptionally-mediated (i.e. the lack of apocarotenoids in CMO2-deficient mice would if anything remove a transcriptional repressor, and so would not be expected to reduce Lrat mRNA levels). Co-immunoprecipitation studies of CMO2 may be the best approach to determine whether this enzyme interacts with other mitochondrial proteins that could somehow influence cellular levels of retinol and retinyl esters. Whatever the interaction may be, it is expected to provide a signal between the mitochondrion and the cytosol in a way that also involves CMO1.

### 4.3. Future directions

The studies presented here have provoked exciting questions to be addressed in future investigations. In vivo, the priority will be to characterize retinoid homeostasis in CMO2-deficient mouse strains on a baseline of complete carotenoid deficiency (i.e. purified vitamin A-sufficient maintenance diet), to confirm whether this enzyme is more than a simple carotenoid oxygenase. Since CMO2 is expressed not only in embryos but also in most adult tissues (Lindqvist et al., 2005), other tissues should be analyzed to determine whether such functions are particular to development or common to multiple tissues. The health effects of β-apo-10'-carotenal also require further investigation in vivo. For instance, WT embryonic phenotypes should be assessed in response to various
doses of β-apo-10’-carotenal to determine whether this compound is toxic to embryos. It will also be informative to raise WT mice for several generations on a purified vitamin A-sufficient diet, to determine whether apocarotenoids (such as those in chow diets) are required in addition to retinoids for normal embryonic development (i.e. for functions that do not overlap with retinol), as suggested in Figure 3-16. Knowing that apocarotenoids can cross the maternal-fetal barrier (Fig 3-8), the mechanisms of their delivery should also be investigated.

In vitro, it will be important to elucidate the molecular mechanisms whereby β-apo-10’-carotenal alters cellular respiration, using not only PKC-deficient MEFs, but also MEFs from all our CMO2-deficient mouse strains. Since different mitochondrial isoforms of PKC (δ and ε) regulate the Pyruvate Dehydrogenase Complex in opposite directions (Gong et al., 2012), it will be key to understand whether β-apo-10’-carotenal preferentially activates one or the other. Aside from oxygen consumption, other markers of Pyruvate Dehydrogenase Complex activation (such as subunit phosphorylation and ATP production) should be measured. Competition assays with β-apo-10’-carotenal and other carotenoids or retinoids will provide further insights into the affinity of the PKC signalosome for this apocarotenoid.

Additionally, since carotenoid cleavage enzymes seem to interact with one another despite their different sub-cellular localizations (within the mitochondrion and in the cytosol), it will be interesting to investigate how carotenoids and/or their cleavage products travel between these two compartments. Do carotenoid binding proteins (Sakudoh et al., 2007) exist in mammalian mitochondrial membranes? Does an
intermediary protein facilitate contact between CMO1 and CMO2? The answers to these questions will improve our knowledge of the intracellular trafficking of retinoids and carotenoids.

The overall aim of these future studies will be to increase our understanding of what comprises vitamin A deficiency, and how it can be prevented by CMO2 activities, both dependent on and independent of carotenoid intake. The dogma that retinoic acid is “the” biologically active form of vitamin A controlling embryonic development, seems to giving way to a new proposition – that the alcohols and aldehydes generated by both symmetric and asymmetric cleavage of carotenoids have essential mitochondrial functions, without which cellular energy homeostasis suffers. Given the variety of carotenoid cleavage enzymatic activities in the human population (Leung et al., 2009), these findings suggest that not only bC, but also apocarotenoids, should be recommended components of the human diet to ensure optimum health, especially during pregnancy.
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Oxygenase (Bcox) is Essential for Pattern Formation and Differentiation during Zebrafish Embryogenesis. *Development* **130**, 2173-2186.


