Carotenoid Metabolism at the Intestinal Barrier

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Abstract

Carotenoids exert a rich variety of physiological functions in mammals and are beneficial for human health. These lipids are acquired from the diet and metabolized to apocarotenoids, including retinoids (vitamin A and its metabolites). The small intestine is a major site for their absorption and bioconversion. From here, carotenoids and their metabolites are distributed within the body in triacylglycerol-rich lipoproteins to support retinoid signaling in peripheral tissues and photoreceptor function in the eyes. In recent years, much progress has been made in identifying carotenoid metabolizing enzymes, transporters, and binding proteins. A diet-responsive regulatory network controls the activity of these components and adapts carotenoid absorption and bioconversion to the bodily requirements of these lipids. Genetic variability in the genes encoding these components alter carotenoid homeostasis and induce various pathologies in research animals. We here summarize the advanced state of knowledge about intestinal carotenoid metabolism and its impact on carotenoid and retinoid homeostasis of other organ systems, including the eyes, liver, and immune system. The implication of the findings for science-based intake recommendations for these essential dietary lipids is discussed.

Keywords

Carotenoid; Apocarotenoid; Retinoid; Intestine; Metabolism; Abbreviations

Introduction

Carotenoids are a class of terpenoid pigments built from eight isoprene units in plants, fungi, and bacteria [1]. Scientists took great interest in isolating these pigments from nature in the 19th century and referred to them by common names long before their structures were determined (Figure 1). The eponymous β-carotene was the first carotenoid to be isolated from nature by Wackenroder, and its chemical structure was the first of any vitamin or provitamin that was determined in 1930 by Karrer [2], who was awarded a Nobel Prize for his work.

Carotenoids possess an extended polyene chromophore with up to eleven double bonds that can carry terminal hexyl-rings. More than 1000 carotenoids occur in nature [3]. Their enormous chemical diversity results from chemical modulations of the core structure,

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including the shifting of conjugated double bonds and the addition of functional groups to the terminal rings (Figure 1). The short chain apocarotenoids (< C40) also are members of the carotenoid substance class that derive from oxidative cleavage at specific double bonds of the parent carotenoid molecules.

The role of carotenoids in human health is currently a subject of intense investigation. Low status of total plasma carotenoids and individual carotenoids is associated with vitamin A deficiency and a number of degenerative diseases including cardiovascular disease, cognitive impairments, and age-related macular degeneration [4–8]. A number of potential mechanisms through which carotenoids can benefit human health have been proposed. The most commonly cited is their capability of acting as antioxidants, e.g., as free radical scavengers, in lipophilic environments such as membranes and lipoproteins [9]. The antioxidant action of carotenoids may decrease lipid peroxidation and eventually reduce oxidative stress and inflammation responses in cells and tissues [10].

The blue light filtering properties of carotenoids compose another mechanism of protection of cellular components from the environment. Macular pigments have been chemically identified as the carotenoids lutein, zeaxanthin, and meso-zeaxanthin [11]. These carotenoids are enriched in the fovea in primate retinas and confer its yellow colour. Hence, the fovea is traditionally known as the *macula lutea*, or 'yellow spot'. The macular pigments can protect the retina against light damage [12, 13] and reduce the adverse impact of light scattering and chromatic aberration, thereby optimizing contrast sensitivity of the retina [14]. The light filtering properties of carotenoids also can provide modest protection against ultraviolet -induced erythema in the skin [15].

Importantly, carotenoids make a crucial contribution to vision and gene transcription. Seminal research in the molecular basis of these actions led to the discovery of visual G protein-coupled receptors and nuclear hormone receptors [16, 17]. Among the carotenoid family are provitamin A carotenoids (β-carotene, α-carotene, and β-cryptoxanthin) that undergo obligate metabolic conversion to retinaldhyde. Retinaldhyde is the chromophore of visual pigments that mediate phototransduction in the retina [18]. Retinaldehyde can be further metabolized to retinol (vitamin A), retinyl esters, and retinoic acid by endogenous enzymes [19]. Retinol and retinyl esters are the transport and storage form of the vitamin, respectively [20]. Retinoic acid binds to nuclear receptors [21], which are ligand activated transcription factors that control the expression of genes which are involved in processes such as cell differentiation, embryonic development, immunity, and metabolism [22–24].

Mammals cannot synthesize carotenoids and must absorb these pigments from the diet. The major site of their absorption and metabolism is the small intestine. A number of studies observed that vitamin A status, individual genetics, and disease states affect this process and contribute to the observed variability of plasma responses to dietary carotenoids in clinical and epidemiological studies [25–27]. In recent years, molecular players of carotenoid metabolism have been identified and their interactions have been studied in animal models. The principles governing this metabolism and the involved transcription factors, metabolizing enzymes, binding proteins, and transporters enzymes are reviewed here. The

implications of these studies for recommendations for carotenoid intake in health and disease are discussed.

2. Carotenoid absorption in the intestine

2.1 The formation of mixed micelles

Fifty carotenoids are typically ingested with the diet. Of these, 20 are commonly found in human tissues [28]. These carotenoids are divided into carotenes (pure hydrocarbons) and xanthophyll (oxygenated carotene metabolites). The six main carotenoids circulating in the human blood are β-carotene, α-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene (Figure 1).

Mammals exclusively acquire carotenoids from the diet. The absorption and bioconversion of dietary carotenoids primarily takes place in the intestine, where carotenoids form mixed micelles with amphiphilic and hydrophobic compounds including bile salts, cholesterol, fatty acids, monoacylglycerides and phospholipids. The hydrophobicity of individual carotenoids is the key physiochemical factor that determines their bioaccessibility (octanol-water partition coefficients log PC > 8) in naturally produced mixed micelles as well as their postprandial blood response [29]. Polar side groups of ionone rings play a minor role, though it is often stated in the literature that xanthophylls are more readily bioavailable than carotenes [30]. In many fruits and vegetables, xanthophylls exist as esters of fatty acids [31]. Before carotenoid esters are absorbed, the fatty acid moiety is cleaved by pancreatic carboxyl ester lipase to form free xanthophylls in the gastrointestinal tract [32]. Xanthophyll ester hydrolysis appears to be an efficient process because the absorption of lutein and β-cryptoxanthin esters is comparable to that of the free carotenoid forms [33]. Furthermore, the uptake of zeaxanthin esters may be even greater than that of the parent carotenoid [34].

Additionally, the geometric form of carotenoids, which easily undergo isomerization from the prevalent all-*trans*-form to *cis*-isomers following exposure to heat and/or light, affects their bioavailability. The relative absorption of geometric isomers varies between carotenoids. For example, absorption is reduced for β-carotene *cis*-geometric isomers, whereas uptake is increased for lycopene *cis*-geometric isomers compared to their respective all-(E)-form [35, 36].

The absorption efficiency of carotenoids is also influenced by food matrix, formulation, and food processing. The context, or matrix, in which carotenoids is provided, is another important factor which determines the bioavailability of these lipids [25, 27]. Carotenoids prepared in oil (e.g. red palm oil), or commercially available water-soluble beadlets, have superior bioavailability compared with those in raw fruits or vegetables [25, 27]. Fat intake, like a modest amount of salad dressing, increases carotenoid uptake by facilitating the formation of mixed micelles [37]. The differences in absorption are likely explained by the occurrence of carotenoids in different cellular compartments (chromoplast versus chloroplast) and aggregate forms in plant tissues. Crystalline or protein-bound carotenoids are less well solubilized in mixed micelles than carotenoids that exist in oil droplets and membranes. Mild heating and food processing elevate the bioavailability of carotenoids by disrupting plant cell walls, binding proteins, and organelles, thus liberating carotenoids for

uptake. Fiber, olestra, plant sterol, and stanol esters are dietary compounds that decrease the absorption of carotenoids. Antioxidants, such as vitamins C and E, may increase the stability of the carotenoids in the gastrointestinal tract and thereby facilitate their absorption [38].

2.2   Molecular components facilitating cellular uptake of carotenoids

Upon being made bioaccessible in mixed micelles, carotenoids are absorbed by enterocytes across a membrane bilayer for further metabolic processing within the brush border cells. Studies in polarized CaCo-2 cells, a model for the brush border membrane, revealed that this process is saturable and selective [39], indicating the involvement of proteins. The proteins facilitating carotenoid uptake from mixed micelles were identified as class B scavenger receptors [40–45]. Studies in CaCo-2 cells revealed that scavenger receptor class B type 1 (SR-B1) facilitate carotenoid absorption from synthetic and mixed micelles [46, 47]. The involvement of SR-B1 in the absorption of these micronutrients was later confirmed in knockout mice which display significantly reduced intestinal absorption of carotenes and xanthophylls [40, 43]. Studies in mice also show that SR-B1 facilitates the intestinal absorption of fat soluble vitamins E and K [41, 42]. Later it was demonstrated that cluster determinant 36 (CD36) also contributes to the absorption of carotenoids from mixed micelles [48, 49].

CD36 and SR-B1 are highly expressed in the proximal parts of the mouse intestine and at lower levels in distal parts [50]. CD36 and SR-B1 are glycosylated transmembrane proteins with a large extracellular domain. Structural prediction, based on the crystal structure of the CD36 family member lysosomal membrane protein 2 (LIMP II), [51] indicate the presence of a large cavity traversing the entire length of the protein that serves as a tunnel for lipid transfer from extracellular to cellular compartments [52, 53].

Outside of the intestine, the functions of CD36 and SRB1 are diverse, and the proteins have broad substrate specificity due to their ability to recognize similar molecular patterns rather than specific epitopes. CD36 ligands include carotenoids, long chain fatty, native or modified lipoproteins, thrombospondin-1, collagen, apoptotic cells, amyloid B, and malaria-infected erythrocytes [54]. CD36 is expressed in muscle, adipose tissue, intestine and the capillary endothelium, where it facilitates long chain fatty acid uptake into target cells in capillary beds of tissues [55, 56], [57]. CD36-deficient mice display among other defects, significantly increased levels of circulating fatty acids [58]. SR-B1 binds high-density lipoproteins (HDLs) [59] and facilitates selective cellular uptake of cholesterol in mammalian steroidogenic tissues [60]. SR-B1 is expressed in the liver, intestine, macrophages, adrenal gland, and ovary [58, 61]. SR-B1-deficient mice develop hypercholesterolemia and multiple pathologies, including male sterility [60].

It was proposed that SR-B1 is also involved in intestinal cholesterol absorption [62]. However, these findings could not be confirmed in SR-B1-deficient mice which exhibit relatively normal intestinal cholesterol absorption and trans-intestinal cholesterol efflux [63]. Furthermore, the cholesterol absorption inhibitor ezetimibe effectively inhibits cholesterol absorption in SR-BI-deficient mice [64]. Similarly, CD36 knockout mice display no gross alterations in intestinal fatty acid absorption [65]. The minor role of CD36 in this process might be explained by the relatively high concentration (micromolar) of fatty acids in the

intestinal lumen. This allows for protein-independent uptake of dietary fatty acids by brush border cells, whereas absorption of fatty acids in capillary beds (nanomolar concentration) requires facilitators such as CD36 (reviewed in [58]).

The role of class 2 scavenger receptors in the absorption of dietary carotenoids is evolutionarily well conserved. Initial evidence for this function of class 2 scavenger receptors was provided in Drosophila. The neither inactivation nor after potential D (ninaD) protein was identified in a screen of *Drosophila* mutants that lack functional visual pigments [66]. Nonsense mutations in *ninaD* render flies deficient in carotenoids, retinoids [44, 66], and tocopherols [45]. Biochemical studies revealed that the *ninaD* gene encodes a transmembrane protein which facilitates uptake of zeaxanthin and β-carotene from synthetic Tween 40 micelles [45]. In *Drosophila* larvae, NinaD is expressed in the midgut and its expression is essential for the uptake of carotenoids for retinoid production during compound eye development [67]. In silkworms, a NinaD homologous scavenger receptor has been identified to be critical for acquiring dietary carotenoids for silk coloration [68, 69]. Silkworm mutants display white cocoons because of a lack of yellow carotenoids. In canary birds, SR-B1 is needed for carotenoid coloration of feathers and skin [70]. The white recessive canary bird possesses a splice site mutation which renders SR-B1 inactive [70]. The mutant bird shows white feather coloration and very low levels of carotenoids in blood and tissues. These birds also suffer from severe vitamin A deficiency and depend on supplementation with preformed vitamin A via the diet [71].

3. Carotenoid metabolism in the intestine

Following absorption, carotenoids with pro-vitamin A activity are converted to retinoids and other possible apocarotenoid metabolites (Figure 2). The primary conversion product retinaldehyde is further converted to retinol and retinyl esters. Retinyl esters are incorporated into chylomicrons together with non-cleaved carotenoids and other dietary lipids and then released into the lymph before entering the general circulation [72, 73]. Retinaldehyde, the primary cleavage product, can further be converted to the corresponding acids, alcohols, and esters by endogenous enzymes. The different carotenoid and retinoid processing enzyme classes will now be introduced (Figure 3).

3.1 Carotenoid Cleavage Dioxygenases

In 1930, Moore described the production of vitamin A from β-carotene in the intestine of research animals [74]. In 1965, the respective enzymatic activity was independently characterized by Olson and Goodman [75, 76]. In these initial reports, β-carotene was observed to be cleaved symmetrically at the C15,C15′ double bond to yield two molecules of retinaldehyde. Later on, enzyme activity for the eccentric cleavage of β-carotene was described in cell-free homogenates of the mammalian intestine [77, 78].

The cloning of two genes, encoding carotenoid cleavage dioxygenases (CCDs) - with distinct region selectivity for the cleavage of carotenoids provided the molecular basis of this metabolism [79, 80]. CCDs introduce molecular oxygen at specific double bonds of the carotenoids polyene backbone, resulting in the formation of two apocarotenoid molecules [81–83]. The existence of two CCDs with symmetric and eccentric cleavage modes was

subsequently confirmed in many species, including human, macaque, ferret, chicken, bovine, rat, and zebrafish [79, 84–92]. Structural analyses showed that CCDs share a common fold of a seven-bladed propeller covered by a half dome. The active center of the enzymes is accessible through a long tunnel lined with hydrophobic amino acid residues. These non-heme iron oxygenases, with characteristic catalytic centers, catalyze oxidative cleavage and geometric isomerization of carbon double bonds [93–96]. Biochemical analyses with the recombinant BCO1 and BCO2 defined their region specificity of double bond cleavage and substrate specificity [88, 89, 91, 92, 97–99]. BCO1 cleaves across the C15,C15′ double bond adjacent to a canonical β-ionone ring site of carotenoids and β-apocarotenoids [88, 91, 100, 101]. Thus, provitamin A carotenoids and apocarotenoids (> C20) with at least one β-ionone ring are substrates for this enzyme. In fact, β-apo-10'-carotenal and BCO1 can rescue normal embryonic development in mice maintained on a diet free of preformed vitamin A [102]. Additionally, recombinant BCO1 converts the open chain lycopene into acyclic retinoids [86, 88], a reaction that may maintain retinoid signaling under condition of severe vitamin A deficiency [103]

Though originally characterized as β-carotene metabolizing enzyme [79], BCO2 displays broad substrate specificity for carotenoids (Figure 4). The enzyme catalyzes conversion of acyclic lycopene, zeaxanthin, lutein, and cantaxanthin [89, 90, 92, 97–99, 104]. Although BCO2 cleaves across the C9′,C10′ positions adjacent of chemical diverse ionone ring sites [97], it shows higher turnover rates with substrates with hydroxylated ionone ring sites [97]. Mouse and human BCO2 also converts apocarotenoids into dicarbonyl compounds ([97, 99], unpublished). In contrast, chicken BCO2 does not catalyze the latter reaction [89]. On the one hand, the different substrate specificities of mammalian and chicken BCO2s might be attributed to species-specific differences. Birds accumulate 3-hydroxy-dehydro-β-10-apocarotenol (galloxanthin) in oil droplets of their retinas whereas mammals do not accumulate long-chain apocarotenoids when supplemented with xanthophyll [98, 99, 105]. On the other hand, there are potentially methodological limitations with enzymatic assays and product analysis. Dialdehyde cleavage products of apocarotenoids are chemically labile and difficult to detect by standard chromatographic methods when produced in small amounts.

BCO1 and *BCO2* are expressed in the intestine and several additional tissues [79, 91, 106, 107]. BCO1 is a monomeric, soluble, and cytosolic enzyme. The cytosolic localization indicates that BCO1 interacts with other cellular components, such as membranes, lipid droplets, and proteins, in order to extract its lipophilic substrates [91, 100, 108]. BCO2 is a mitochondrial enzyme that associates with the inner membrane [89, 99, 104]. This localization of BCO2 was discovered by sub-fractionating of isolated hepatic mitochondria of mice and by electro-microscopy and immunogold staining of human mitochondria [109]. To achieve mitochondrial localization, BCO2 is expressed with a signal peptide sequence in many vertebrate species, including human. The signal peptide is removed during mitochondrial import to yield a mature protein [109]. Rodent BCO2s contain a shorter signal sequence though they also are enriched at the inner membrane of mitochondria [109]. BCO2 substrates such as zeaxanthin and lutein accumulate at the inner mitochondrial membrane in BCO2-deficient hepatic mitochondria of mice [109]. Accumulation of xanthophylls at the inner mitochondrial membrane has also been reported in birds [110], but little is known

about how carotenoids are shuttled between different organelles and membranes within in the cell.

The generation of mouse lines, carrying loss-of-function mutations in *BCO1* and *BCO2* genes, set the stage for an in-depth analysis of the physiological roles of these enzymes. Phenotypically, these mutants are characterized by a yellow fat phenotype [99, 101, 111]. Studies in CCD knockout mice revealed that BCO1 is the major β-carotene metabolizing enzyme and the key enzyme for vitamin A production [101, 109, 111, 112]. In its absence, only trace amounts of β-apo-10'-carotenoids were produced from supplemented β-carotene by BCO2 [101, 113]. Consistent with BCO2's enzymatic properties and subcellular localization, xanthophylls accumulated in hepatic mitochondria of BCO2-deficient mice [99, 109]. In *Bco1* and *Bco2* single knockout mice, carotenoid levels were highest in liver and adipose tissue [92, 99, 101, 111]. While β-carotene accumulated unmodified in mouse tissues [114], xanthophyll accumulated in oxidized form [99, 114]. 4',5'-Didehydro-retro-β-carotene-3,3'-dione (rhodoxanthin) and (6RS,6RS)-ɛ,ɛ-Carotene-3,3'-di-one were respectively identified as zeaxanthin oxidation products in white adipose tissues [43] and in blood and liver [99, 114] (Figure 5). Oxidation of xanthophyll has also been reported to occur in wild type mice [115, 116]. Lower levels of oxidized carotenoids were measured in lung and heart of CCD deficient mice [99, 101, 111]. β-Carotene and xanthophyll also accumulate in the eyes of knockout mice with higher levels in the retinal pigment epithelium than in the neuronal retina [43, 117]. However, levels of carotenoids in the eyes were low when compared to other tissues and blood [92]. This observation clearly reflects a lack of a mechanism for ocular accumulation of carotenoids in rodents though the mouse retina expresses high levels of glutathione-S-transferase, GSTP [117]. GSTP1 has been implicated in ocular accumulation of zeaxanthin in the human macula [118]. BCO2-deficient mice also accumulate lycopene in different tissues, whereas levels of supplemented lycopene were indistinguishable between wild type control and BCO1-deficient mice [119].

The roles of BCO1 and BCO2 in carotenoid metabolism are well conserved throughout evolution. Similar to mice, humans carrying mutations in the *BCO1* gene display elevated β-carotene and reduced vitamin A levels [108]. Mutations in the *BCO2* gene have not yet been described in humans but are prevalent in other vertebrates. Mutations in the *BCO2* gene associate with a yellow skin phenotype in chicken [120] and reptiles [121]. Loss-of-function mutations in BCO2 gene in cows, rabbits, and sheep cause a yellow fat phenotype [122–125]. It was speculated that the genetic variability in the *BCO2* gene in domestic farm animals provides a mechanism to survive in environments with carotenoid shortage [126]. Given the importance of carotenoids in vision, development, reproduction, and immunity, it is possible that *BCO2* mutations in domestic animals helped to adapt them to dietary conditions that are vastly different from those of their wild-living ancestors. Notably, Paul Bernstein's laboratory proposed that a four amino acid long insertion renders human BCO2 inactive and favors the accumulation of the eye protective macula pigment [117]. However, it is controversial whether the human *BCO2* gene encodes an enzymatically inactive BCO2 mutant variant. Macaque BCO2 shows overall sequence identity with human BCO2, and the recombinant protein displays robust enzymatic activity [92]. Additionally, it was recently demonstrated that recombinant human BCO2 is enzymatically active when expressed in *E. coli* cells [127]. Moreover, a recent study provided an alternative explanation for carotenoid

accumulation in central parts of the human retina. This study shows that *BCO2* mRNA is highly expressed in the peripheral retina and at low level in the central (macula) retina of the human eye. This pattern of expression may explain the selective accumulation of carotenoids in the macula regions of the human retina [128].

The need of tissues to express an enzymatically active BCO2 is indicated by several studies. BCO2-deficiency has been associated with oxidative stress in adult tissues [129–132]. Additionally, studies in zebrafish and mouse show that BCO2 prevents carotenoid toxicity in vertebrate embryos [102, 104]. Accordingly, BCO2-deficient mice exhibit oxidative stress in tissues and reduced mitochondrial respiration rates [99, 114, 129]. *In vitro* studies in human hepatoma cells indicate that carotenoid accumulation increases ROS production in mitochondria [99, 104]. Oxidative stress is associated with several disease states, including cardiovascular and neurodegenerative disease, type 2 diabetes, cardiovascular disease, and cancer. Interestingly, human *BCO2* is expressed as an oxidative stress-inducible gene [92]. This regulation may prevent excessive accumulation of carotenoids in human tissues and explain the observation that carotenoid levels decrease in chronic disease states.

3.2 Metabolism of long chain apocarotenoids

Trace amounts of apocarotenoids were detected in mammalian tissues using modern chromatography techniques and sensitive detection methods, such as mass spectrometry [133–139]. Emerging evidence implicates these compounds as biologically active modulators of physiological processes [103, 140–142]. For instance, pharmacological doses of apo-10'-lycopenoic acid can protect mouse liver from damage [143]. Furthermore, β-13-Apo-carotenone and certain lycopenoids act as retinoic acid receptor (RAR) antagonist and can antagonize vitamin A action [135, 141]. However, it is still not clear whether these apocarotenoids derive from the diet or are synthesized from dietary carotenoids by endogenous enzymatic pathways. A few studies provide evidence that β-10'-apo-carotenol is synthesized from carotenoids in a BCO2-dependent manner from β-carotene [113] and β-cryptoxanthin [97, 101]. Interestingly, it was shown that β-10'-apo-carotenol was transported and metabolized by proteins that were originally characterized in the context of retinoid metabolism [97, 101]. Though β-10'-apo-carotenol accumulated to some extent in mouse tissues upon carotenoid supplementation, no gross transcriptional changes were observed in the liver, fat, and lung under the applied conditions [144]. More recently, a study provided compelling evidence that BCO2 is involved in the production of a bona fide signal molecule that controls lipid transfer in the placenta [145]. While the production and occurrence of β-10'-apo-carotenol is now well-documented in mouse tissues, zeaxanthin, lutein, and lycopene-derived apocartenoid metabolites have yet not been detected [98, 99, 119]. Intervention studies in humans also failed to detect these compounds upon carotenoid supplementation [146, 147]. These observations indicate that apocarotenoid metabolites of carotenoids only exist transiently and are rapidly metabolized. As outlined above, both BCO1 and BCO2 can convert long chain apocarotenoids to smaller chemical entities [88, 97] by pathways that await chemical characterization. Thus, further research is warranted to clarify the metabolism and physiological roles of these molecules in mammalian biology.

3.3   Metabolism of Retinoids

The metabolism of vitamin A and its metabolites has been recently reviewed in several excellent articles [18, 20, 148, 149]. The formal first step in this metabolic pathway is the generation of retinaldehyde from carotenoid precursors by CCDs. Retinaldehyde can be either oxidized to all-*trans*-retinoic acid (RA) or reduced to all-*trans*-retinol. All-*trans*-retinol is converted to retinyl esters. The alcohol and ester form of vitamin A are the predominant retinoids in most tissues, including the intestine. In contrast, the acidic form of the vitamin exists only in very low (nanomolar) concentrations in most tissues [150]. The tissue levels of retinoic acid are tightly controlled, and even small amounts of this hormone-like compound are sufficient to elicit profound cellular responses through the activation of retinoic acid receptors (RARs) [151, 152]. RARs are transcription factors of the nuclear hormone receptor gene family, which in conjunction with retinoid X receptors (RXRs), control transcription by binding to conserved DNA motifs (retinoic acid response elements) in promoter regions of about 500 target genes in the human genome [153]. The amount of retinoic acid in tissues is tightly controlled throughout the mammalian life cycle by cytochrome P450-dependent hydroxylases [154–157].

The conversion of retinol to retinal is catalyzed by cytosolic alcohol dehydrogenases (ADHs) and microsomal retinol dehydrogenases (RDH) [21, 158]. The latter belong to the short chain dehydrogenase/reductase protein family (SDR). Adenine dinucleotide cofactors NAD(H) and NADP(H) are the redox carriers of these reactions. The enzymes bind their cofactors by a conserved sequence motif, the Rossmann-fold, which consists of six to seven parallel β-strands flanked by three to four α-helices [18, 159]. ADHs and RDHs use different catalytic mechanisms with either a zinc atom or a tyrosine in the active center, respectively. In enzymatic assays, ADHs and RDHs catalyze the bidirectional inter-conversion of retinol to retinal, dependent upon the oxidative state of their redox carriers. Under physiological conditions, the ratio of NAD/NADH is around 700 in the cytoplasm, and the ratio of NADP/NADPH is 0.005. Thus, enzymes using NAD as redox carriers catalyze the oxidation while enzymes using NADP catalyze reduction of retinoids.

Though ADHs and RDHs can use retinol and retinal as substrates, most of these enzymes can metabolize other alcohols including sterols [21]. Much of what is known about the physiological roles of different ADHs and RDHs stems from loss-of-function studies in experimental animals, such as knockout mice [21, 158, 160]. The important role of RDHs (RDH5, 8, 10, 11, 12, 13, and 14) in the visual cycle has just been reviewed [18]. For extra-ocular retinoid metabolism, RDH10 plays a critical role for the conversion of retinol to retinal in retinoic acid production [161]. Rdh10 mutations are embryonically lethal [161]. The expression of RDH10 in adult tissues, including the eyes, indicates that the RDH10 contributes to retinoid homeostasis throughout the mammalian life cycle [162]. Evidence from knockout mice suggests that RDH1 contributes to retinoid homeostasis in adult tissues as well [158, 163, 164]. Though not lethal, mutations in the corresponding gene are associated with increased hepatic retinyl ester stores and altered body fat mass [164]. Additionally, a critical retinaldehyde reductase (DHRS3) has been identified. DHRS3 reduces vitamin A aldehyde to retinol, and the levels of this protein are transcriptionally regulated by retinoic acid signaling [165]. Loss-of-function studies in mice indicate that

DHRS3 is required to control embryonic retinoic acid levels by limiting the availability of retinal for oxidation to retinoic acid [165]. Because DHRS3 is expressed in adult tissue, the enzyme might be critical for tissue control of retinoic acid levels post-developmentally [162, 166]. Notably, DHRS3 expression is highly increased in ISX null mice with increased rates of intestinal retinaldehyde production and all-trans retinoid acid (RA) levels [167]. Recently, studies suggested RDH11 functions as an retinaldehyde reductase *in vivo* and is essential for the maintenance of all-*trans*–retinol steady-state levels in mouse liver and testis [168].

Esterification of retinol also seemingly contributes to the control of retinoid homeostasis in the gut [72]. Early studies demonstrated two independent enzymatic activities for the formation of retinyl esters, namely a lecithin-dependent acyl transfer facilitated by lecithin retinol acyltransferase (LRAT). LRAT is expressed in most tissues except adipocytes, and the acyl-CoA-dependent activity is present in a variety of mammalian tissues such as small intestine, liver, adipocytes, skin, testis, and retina. LRAT has been molecularly cloned from several vertebrate species, and the generation of LRAT-deficient mice confirmed its pivotal role in retinoid metabolism and vitamin A homeostasis [169–171]. LRAT is a 25 kDa protein that localizes to the endoplasmic reticulum and is an integral membrane protein with a single membrane-spanning helix localized at the C-terminus. On the basis of its amino acid sequence and predicted tertiary structure, LRAT is classified as a member of the ancestral NlpC/P60 thiol peptidase protein superfamily [172, 173]. Besides LRAT, the human genome encodes seven genes belonging to this protein family. Their general structural motif is reminiscent of papain-like proteases, and consists of a four-strand antiparallel β-sheet and three α-helices. The conserved catalytic residues Cys161, His60, and His72 define the active site. LRAT adopts an analogous catalytic strategy as thiol peptidases, whereby the deprotonated Cys161 serves as a nucleophile to attack the carbonyl carbon of an ester bond at the SN1 position of phosphatidylcholine, eventually leading to an acyl transfer and trans-esterification of retinol. LRAT-deficient mice lack liver and lung retinyl ester stores and are highly susceptible to vitamin A deficiency [72, 174]. Mice deficient for LRAT display highly elevated hepatic levels of CYP26A1, a major retinoic acid catabolizing enzyme, when maintained on vitamin A sufficient diet It is likely that in the absence of LRAT and retinol esterification, CYP26A1 is activated to metabolize retinoic acid in a compensatory mechanism [174].

In contrast to LRAT, which utilizes lecithin as an acyl-donor, acyl-CoA:retinol acyltransferase (ARAT) takes advantage of a pre-activated acyl-moiety coupled to coenzyme A. ARAT has never been purified or cloned, however, the existence of such vitamin A ester forming enzymes is supported by several lines of evidence. Studies of LRAT-deficient mice revealed that the intestinal absorption decreased to 10% of that in wild type animals after gavage of a physiological dose of the vitamin [72, 73]. Though the critical function of LRAT for intestinal vitamin A metabolism is evident, the consequences of mutations in the *LRAT* gene for provitamin A metabolism have yet not been investigated.

4. Quality control of vitamin A production in the intestine

Dissection of the carotenoid metabolic pathway using knockout mouse models clearly showed that BCO1 is required for β-carotene metabolism [101, 111], and that BCO2 is

required for the metabolism of non-provitamin A carotenoids [99, 119]. Provitamin A carotenoids must contain at least one unsubstituted β-ionone ring and include the symmetric β-carotene as well as asymmetric β,ε-carotene (α-carotene) [175] and 3R-β,β-Caroten-3-ol (β-cryptoxanthin) [176]. In the test tube, recombinant BCO1 splits asymmetric provitamin A precursors into canonical and non-canonical retinaldehyde moieties [88, 91, 97]. Recombinant BCO2, also converts these compounds into various apocarotenoid products [89, 90, 92, 97, 101]. Hence, the enzymatic conversion of a single asymmetric provitamin A carotenoid could result in many apocarotenoid products which can be further metabolized to alcohols, acids, and geometric isomers. Surprisingly, the main apocarotenoids in mammals are retinoids, indicating that there must be a mechanism for their selective production.

Using β-cryptoxanthin as the model carotenoid, evidence has been provided that the enzymatic properties of BCO1 and BCO2 provide a quality control mechanism for vitamin A production [97, 101]. In this pathway, BCO2 removes the non-canonical ionone rings, followed by cleavage of the resulting long chain apocarotenoid into retinaldehyde by BCO1 (Figure 6A). This mechanism produces one molecule of retinoid from one molecule of carotenoid without non-canonical retinoid byproducts. In the mouse intestine, all metabolites of this pathway were identified [97]. Notably, a two-step mechanism for the conversion of the asymmetric provitamins 5,6-epoxy-β-carotene was already proposed in 1966 [177]. These authors proposed a removal of the non-canonical ionone ring site by eccentric cleavage which is then followed by a chain shortening of the β-apo-10'-carotenal by a β-oxidation-like mechanism.

The two-step enzymatic processing of β-cryptoxanthin is made possible by the different ring site selectivity of the two CCDs. The enzymes' respective centric and eccentric cleavage mode complements this process [79] (Figure 6). Biochemical studies showed that recombinant BCO2 favors non-canonical ionone ring sites over canonical β-ionone ring sites, and that this mode of cleavage primarily leads to the production of β-10'-apocarotenal from asymmetric provitamins [97]. In the test tube, recombinant human BCO1 centrically cleaves β-cryptoxanthin into retinal and 3-hydroxy-retinal [88, 91]. However, 3-hydroxy-retinal was not produced from supplemented β-cryptoxanthin in the mouse intestine [97]. Previously, Lindqvist and Andersson [91] elegantly demonstrated that the catalytic efficiency of human BCO1 for β-crypoxanthin is very poor. This enzymatic property is consistent with the lack of β-cryptoxanthin cleavage in mice [97].

Determination of the structure of CCDs provide an explanation for the substrate selectivity of BCO1 and BCO2. Structural prediction indicates that the enzymes share the common CCD fold that consists of a rigid seven bladed propeller with a long, non-polar substrate tunnel running through the middle [178]. The mouth of this tunnel is surrounded by large hydrophobic patches that aid membrane interaction of the enzyme. In the active site in the tunnel, there is a Fe^{2+} cofactor, necessarily coordinated by four His residues, which activates oxygen for an otherwise spin-forbidden reaction. Once in the tunnel, the substrate is cleaved at this site [93]. In homology models for BCO1 and BCO2, the retinoid isomerase RPE65 was used as the template for structural prediction [179]. RPE65, a retinoid isomerase in the visual cycle [180–182], is the third family member of the mammalian CCD enzyme family. When comparing the predicted structures of BCO1 and BCO2, researchers reasoned that the

diameter of the mouth of the BCO2 tunnel should be wider than that of BCO1 because BCO2 can accommodate bulky hydroxylated ionone ring sites in the substrate tunnel. Structural comparison identified two candidate amino acid residues at the mouth of the substrate tunnel. In BCO1, these amino acids narrow the entrance (Trp270 and a Leu168) and were well conserved in BCO1 across different species. Exchange of these amino acids with the corresponding amino acids of BCO2 (Trp270Phe and Leu168Gly) by site-directed mutagenesis produced a mutant BCO1 enzyme that is able to cleave zeaxanthin with two hydroxylated ionone rings [97]. Thus, a small change in an overall conserved fold provides the structural basis of the ring site specificity of BCO1 and BCO2. In the future, it remains to be clarified whether the described pathway for β-cryptoxanthin metabolism can be applied to the conversion of other asymmetric carotenoids with only one β-ionone ring site.

Distinct enzymatic properties of BCO1 and BCO2 also contribute to the metabolism of β-carotene diastereomers such as 9-*cis*-β-carotene (Figure 6). Symmetric cleavage of this dietary compound would result in one molecule of all-*trans*-retinal and one molecule of 9-*cis*-retinal. The early literature considered 9-*cis*-retinal as a precursor for 9-*cis*-retinoic acid that can bind RXRs and control transcriptional activities [183, 184]. Furthermore, 9-cis-retinal can bind to opsin to form isorhodopsin in the eyes [185] and can be used as a chromophore surrogate in treatment of blinding diseases [186]. Additionally, 9-*cis*-13,14-dihydroretinoic acid has been implicated as a natural endogenous ligand of this nuclear receptor [187]. However, there is presently no consensus as to whether these retinoid diasteromers are produced in meaningful amounts in mammals [24].

Studies in gerbils showed that 9-*cis*-β-carotene can be used as a dietary source of vitamin A but with only about 38% efficiency as compared to all-*trans*-β-carotene [188]. The lower efficiency of 9-*cis*-β-carotene in providing vitamin A might be partially explained by lower intestinal absorption of the 9-*cis*-geometric isomer when compared to the all-*trans*-β-carotene isomer [35, 189]. Notably, no increase of 9-*cis*-retinoid levels was observed in the mouse liver upon 9-*cis*-β-carotene supplementation [190]. Studies in humans brought up the proposal that the 9-*cis*-double bond of β-carotene is isomerized to an all-*trans*-double bond during absorption, and that this mechanism explains the lack of 9-*cis*-retinoid production [35]. After supplementation with [^{13}C]-labeled 9-*cis*- β-carotene, the resulting [^{13}C]-retinoids existed mainly in the all-*trans*-configuration [191]. Upon BCO1 cloning, biochemical studies with the recombinant murine enzyme showed that 9-*cis*-β-carotene is converted into the all-*trans*-retinal, 9-*cis*-retinal, and 13-*cis*-retinal stereoisomer in a molar ratio of 9:3:1 [190] (Figure 6). Notably, Jim Olson's group previously obtained a similar result with cell free extracts of rat intestine [192], indicating that BCO1 possesses intrinsic isomerase activity. This finding contrasts with a reports suggesting that 9-*cis*- β-carotene is converted to 9-*cis*-retinal and all-*trans*-retinal in one to one molar ratio [193, 194]. It also contrasts the more recent proposal that 9-*cis*-β-carotene is not enzymatically converted by recombinant human BCO1 [88]. Isomerase activity has been also described in other CCD family members, including the insect vitamin A forming enzyme NinaB [195, 196] and the mammalian retinoid isomerase RPE65 [180–182]. Biochemical analysis with insect NinaB provided a reaction mechanism for a combined isomerization and oxidative cleavage reaction of double bonds of carotenoid substrates [83]. A similar mode of action of BCO1

would explain why 9-*cis*-β-carotene is mainly converted to the all-*trans*-retinoid diastereoisomer.

Taken together, with the large variety of carotenoids available in food, mammals require a reliable and robust mechanism for vitamin A production. Recent biochemical studies indicate that ring site selectivity and intrinsic isomerase activity of involved CCD enzymes play an important role in this process. These enzymatic properties of CCDs are essential for avoiding production of abberant apocarotenoid metabolites which can interfere with vitamin A-dependent physiological processes.

5. Regulation of carotenoid absorption and vitamin production

Several studies indicate that the vitamin A status of the host affects carotenoid absorption [197, 198] and metabolism [199] in the intestine. Early biochemical studies revealed that activity of the vitamin A forming BCO1 is higher in the intestine of vitamin A deficient rats [200]. Van Vliet et al. [201] showed that high doses of vitamin A or its precursor β-carotene decreased intestinal BCO1 enzyme activity, indicating that BCO1 activity is under negative feedback regulation of vitamin A. After the cloning of BCO1 gene, Bachmann and colleagues provided evidence that the negative feedback regulation occurs at the transcriptional level by showing that retinoic acid decreased the intestinal expression of *Bco1* mRNA in the gut but not in the liver of chickens [202].

The protein that controls the intestinal *Bco1* mRNA expression was discovered by a screening for gut specific transcription factors [203]. The intestine specific homeodomain transcription factor ISX is a 242-amino protein highly expressed in epithelial cells of the brush border membranes. ISX expression initiates early in development just prior to the transition from a gut endoderm to a columnar epithelium [203]. *Isx*$^{-/-}$ mice are grossly normal and healthy when raised on a standard vitamin A-rich mouse chow [203]. Analysis of their intestinal transcriptome suggested that ISX regulates intestinal expression of SR-B1 [203]. Seino and colleagues then showed that similar to *SR-B1*, intestinal expression of *Bco1* is significantly increased in *Isx*$^{-/-}$ mice, suggesting that ISX is involved in the regulation of vitamin A production and absorption [204] (Figure 7). This conclusion was supported by the finding that intestinal expression of *Isx* is low in vitamin A deficiency and high in vitamin A sufficiency [204]. A mechanistic explanation for the vitamin A responsiveness of ISX expression was provided by the identification of an RAR binding element in the promoter of the human *ISX* gene [205]. It was further shown that RA can induce intestinal *Isx* expression, and that this induction is accompanied by a down-regulation of *SR-B1* and *Bco1* expression [205]. The increased expression of *Bco1* and *SR-B1* in the absence of ISX is driven by other transcription factors that control the activity of these genes [206, 207].

For ISX to control *Bco1* and *Scarb1* expression, it must interact with the promoter region of their genes. Using gel retardation assays, a 21-bp stretch about 1.3 kb upstream of the start ATG of the mouse *Bco1* gene was identified as the ISX binding region [208]. ChIP experiments in human CaCo-2 cells indicated that human ISX also interacted with this binding motif. Furthermore, studies in CaCo-2 cells showed that the identified promoter

element decreased expression of a luciferase reporter gene in the presence of ISX. A similar binding motif was later identified in the murine *SR-B1* promoter [43]. Thus, ISX acts as a transcriptional repressor via binding a conserved DNA motif. Interestingly, the putative ISX binding site in the human *BCO1* promoter region is afflicted by a common genetic polymorphism that associates with serum and tissue carotenoid levels [208–210].

The physiological basis of the ISX-dependent regulation of carotenoid absorption and vitamin A production was demonstrated in mice. ISX-deficient mice produce significantly higher amounts of vitamin A from dietary β-carotene than wild type mice [208]. Amounts of stored vitamin A in these animals well exceed that of mice fed with preformed vitamin A. The absolute increase of retinyl esters was most pronounced in the liver, where ~80% of postprandial retinyl esters are stored [20]. Peripheral tissues such as the lung and white adipose tissue also showed a significant increase in retinyl ester accumulation [208]. The vitamin A-dependent regulation of *SR-B1* expression by ISX also explains the reported interactions between xanthophyll and β-carotene absorption. For instance, it has been observed that dietary vitamin A reduces xanthophyll absorption in chickens [211]. Studies in mice clearly demonstrated that BCO1 and β-carotene can affect intestinal SR-B1 expression and xanthophyll absorption [43].

In the intestine of vitamin A sufficient mice, ISX is expressed at higher levels in distal rather than in proximal parts [204]. The ISX target genes, encoding BCO1 and SR-B1, reveal a reverse pattern of expression [204]. Thus, in the vitamin A deficient state, the expression of BCO1 and SR-B1 spreads to more distal parts of the intestine. This pattern of expression significantly enlarges the surface of the intestine available for carotenoid and fat soluble vitamin absorption by SR-B1. Similarly, SR-B1 and BCO1 are highly expressed throughout the intestine in ISX-deficient mice. The consequences for intestinal vitamin A production on a diet rich in provitamin A are depicted in Figure 8. In wild type mice, retinol and retinyl esters content is low. In contrast, the levels of these compounds are significantly increased in all parts of the intestine with highest levels in the jejunal parts. Thus, the ISX dependent regulation does not only increase the expression levels of SR-B1 and BCO1 but also the surface area by which carotenoids and fat soluble vitamins can be acquired from the food matrix.

There is an ongoing debate about the 'right' supplementation strategy for vitamin A, especially in early postnatal life [212]. Studies in animal models demonstrate that imbalances in vitamin A supply have significant effects on the offspring. For example, elevated maternal vitamin A affects lymphocyte differentiation in the developing gastrointestinal tract, and too much dietary vitamin A promotes aberrant immune responses and inflammation in the mouse intestine [167, 213, 214]. A recent study reported on the long-term epigenetic effects of preformed vitamin A on early neonatal life [215]. The analyses in rat pups revealed that even modest supplementation of preformed vitamin A during the suckling phase can impact methylation marks in developing white adipose. Physiologically, the amount of preformed vitamin A in breast milk is relatively constant and not affected by dietary fluctuations. The vitamin A-induced genomic imprinting of adipocytes of the pups is in keeping with the finding that neonatal vitamin A supplementation favors body fat gain later in life [216]. Notably, dietary β-carotene does not

exert such effects on adipocytes in the same experimental setup [217], most likely because its absorption and bioconversion is tightly controlled by bodily demands via ISX (28, 29).

Taken together, the diet-responsive regulation of vitamin A production provides an elegant mechanism to cope with seasonal fluctuations in food supplies [218] and unbalanced diets [219]. Central to this regulation is the negative feedback mechanism regulated by the transcription factor ISX which controls the expression of the genes encoding the vitamin A forming enzyme BCO1 and the SR-B1 protein in epithelial cells of the intestine [43, 205, 208]. This regulation secures effective carotenoid absorption and vitamin A production when the dietary supply of these nutrients is limited and prevents excessive production of vitamin A when the supply with β-carotene is abundant [43, 208]. This regulation also affects the absorption of xanthophyll and other fat soluble vitamins [43, 208]. In the future, the specific roles of ISX in fat soluble vitamin A and lipid homeostasis needs further elucidation [24].

6.　Cross talk between carotenoid metabolism and immunity

The intestine constitutes an effective barrier against toxins, antigens, and enteric flora. Hence, the intestinal mucosa harbors one of the largest populations of lymphocytes in the body. Several studies showed that sufficient intake of dietary vitamin A is critical for maintaining immune homeostasis at the intestinal barrier. The vitamin A metabolite retinoic acid induces the expression of gut-homing receptors on T and B cells, promotes conversion of regulatory T cells, and provokes T cell-independent IgA switches in naïve B cells [220–222].

Accordingly, vitamin A deficiency compromises intestinal immunity and makes mice more susceptible to infection with bacterial pathogens [223, 224]. Vitamin A deficient mice also display a dramatic increase of interleukin-13 (IL-13)-producing type 2 innate lymphoid cells and display resistance to nematode infection [224]. However, too much dietary vitamin A may also compromise immunity. Increased intestinal retinoic acid in conjunction with IL-15 can act as an adjuvant to enhance secretion of IL-12 and IL-23 [213]. This scenario can promote inflammation [225, 226] and loss of tolerance to dietary antigens [213]. Thus, an increasing amount of studies indicate that imbalances in dietary vitamin A affect immune cell differentiation and function.

A recent study shows that ISX acts as a sensor for dietary vitamin A and has a profound impact on intestinal immune homeostasis [167] (Figure 8). ISX-deficient mice display an increase in the size and number of secondary lymphoid organs in the gut [167]. This increase can already be maternally imprinted in the offspring when ISX-deficient dams are maintained on β-carotene-rich food during pregnancy [167]. The immune phenotype of ISX-deficient mice can be likely attributed to alterations in intestinal carotenoid absorption and bioconversion. ISX-deficient mice show elevated intestinal retinoic acid levels when fed a β-carotene-rich diet. This retinoic acid synthesis can take place in dendritic cells of lymphoid organs [227] as well as in epithelial and stromal cells of the mucosal lymphoid tissues [228–230] where BCO1 is expressed [106]. In wild type mice, excessive retinoic acid production is prevented by the retinoic acid -dependent induction of ISX that regulates the absorption and bioconversion of β-carotene.

Interestingly, ISX-deficient mice develop pancreatic insulitis that is associated with decreased insulin production and systemic deregulation of glucose metabolism [167]. In this context, it is worth mentioning that failure to establish tolerance against commensal flora and food antigens has been implicated in the pathology of type 1 diabetes and other autoimmune diseases [231]. retinoic acid is an important signal for the differentiation of regulatory T-cells in the intestine [23, 232, 233], and the invasion of activated gut-derived T helper cells has been reported in pancreatic islet lesions of diabetic mice [234]. Thus, uncontrolled retinoic acid production in ISX-deficient mice most likely impairs T cell immunity and caused the diabetic phenotype of this mouse mutant.

Taken together, ISX plays an important role in the control of intestinal vitamin A homeostasis as required immunity and tolerance at the intestinal barrier. In vitamin A deficiency, the ISX-dependent regulation of vitamin A production guarantees that even minute amounts of dietary carotenoids are used for vitamin A production to maintain immunity. In periods of excess supplies with carotenoids, ISX prevents excessive vitamin A production that eventually can trigger inflammatory responses in the intestine. In future studies, the ISX-deficient mouse will be a versatile model to study the molecular details of these processes. A better understanding of the factors which control mucosal immune development and tolerance will aid nutritional intervention strategies to improve health in neonatal and adult life.

7. Transport and body distribution of carotenoids and retinoids

7.1 Formation of Chylomicrons

For transport and distribution in the body, intact carotenoids and retinyl esters are packaged into chylomicrons along with other dietary lipids [235] (Figure 10). Pre-chylomicron assembly starts at the endoplasmic reticulum and requires surface proteins apolipoprotein B48 (apoB-48) and apolipoprotein A-IV. For initiation of this assembly, amphipathic surface lipids (phospholipids and cholesterol) and neutral core lipids (triacylglycerol and cholesterol esters) must be present. Microsomal triglyceride transport protein (MTP) catalyzes the lipidation of apoB-48 in the inner leaflet of the ER [236]. The addition of triacylglycerol, cholesterol esters, and phospholipids to apoB-48 generates secretion-competent primordial chylomicrons. The assembled pre-chylomicrons translocate to the Golgi apparatus. In this compartment, pre-chylomicrons acquire apolipoprotein A1, and apoB-48 undergoes significant glycosylation. Mature chylomicrons are then secreted into the lymph and reach the circulation at the level of the subclavian vein. In the circulation, chylomiocrons interact with lipoprotein lipase of peripheral tissues [237]. The resulting chylomicron remnant is taken up by the liver for processing of its remaining lipid cargo. Retinyl esters are cleaved into fatty acid and retinol by a yet not molecularly identified retinyl ester hydrolase(s) and transported to stellate (Ito) cells. Here, they are re-esterified by LRAT and stored in lipid droplets [20, 72].

7. 2 Transport of stored vitamin A

Retinol is released from hepatocytes, bound to RBP4, into the circulation [20, 238] (Figure 10). This complex is named holo-RBP4 and is the major retinoid in the fasting circulation.

Holo-RBP4 forms a complex with the 55 kDa transthyretin (TTR) homotetramer at a 2:1 molar ratio [239]. The cellular uptake of retinol from holo-RBP4 is mediated by a receptor localized at the cytoplasm membrane of target tissues [240–243]. This receptor is encoded by the *Stra6* gene [244, 245]. Structural analysis revealed that STRA6 is assembled as an intricate dimer with 18 transmembrane helices (nine per protomer) and two long horizontal intramembrane helices interacting at the dimer core [246]. The receptor complex displays a lipophilic cleft to which holo-RBP4 binds with high affinity [244, 246]. Studies in cell lines indicate that STRA6 facilitates the bidirectional flux of retinol between RBP4 and cells [247, 248]. Cellular accumulation of retinol is driven by esterification by LRAT [247–250]. STRA6 allows for a higher flux of vitamin A into cells than is possible when this process is reliant on passive diffusion [251]. The evolutionary adaptation to store the fat-soluble vitamin and distribute it in a controlled manner provides a key pro-survival advantage to endure periods with little to no dietary supply of the essential nutrient [248, 249, 251–255]. In healthy subjects, blood holo-RBP4 levels are homeostatic and only decline under conditions of severe vitamin A deficiency (VAD) when body retinoid stores become exhausted [148, 256].

The pathological consequences of mutations in the *STRA6*, *RBP4*, or *LRAT* genes highlight the importance of vitamin A transport for ocular health. To date, 24 missense and nonsense mutations in the *STRA6* gene have been identified to cause Matthew-Wood syndrome (MWS) [257–259]. MWS is characterized by severe bilateral microphthalmia, often in combination with pulmonary dysplasia, cardiac defects, and diaphragmatic hernia, among other anomalies and malformations [257]. The symptoms of MWS are consistent with the pivotal role of vitamin A in mammalian embryonic development [260] but are highly variable even within the same family ranging from isolated microphthalmia to fatal syndromes [261]. Similarly, mutations in the *RBP4* gene can cause congenital eye malformations, including microphthalmia [262–264]. Again, even within the same family, large variations in the phenotypic manifestation of the RBP4 mutation exist [262]. It has been suggested that there is a maternal mode of inheritance of the phenotype, [262] and that the availability of dietary vitamin A impacts the severity of birth defects in affected patients [252, 262]. Mutations in the LRAT gene are associated with blindness in humans [265]. Currently, there are 11 identified mutations in the *LRAT* gene that cause Leber congenital amaurosis or retinitis pigmentosa in humans. Although effects of these mutations on LRAT's activity have not been studied systematically, the severity likely depends on the degree of inactivation of the enzyme [149].

Mouse models for all major players of vitamin A transport have been established and used for an in-depth analysis of this important process: early in life, *Stra6*$^{-/-}$ mice largely display decreased ocular vitamin A levels and visual impairment including reduced ERG responses and shortened outer segments [252, 255]. Blood and other tissues such as the lungs, fat, and liver display normal vitamin A levels when mice are bred and raised on vitamin A-rich diets [252, 254]. A comparable phenotype has been reported for RBP4-deficient mice [238, 252]. These findings clearly indicate that retinyl ester in chylomicrons, when dietary supply with the vitamin is abundant, can compensate at least in part for the RBP4/STRA6 system. Genetic disruption of the *Lrat* gene renders mice blind because of the inability to acquire vitamin A and produce retinyl ester for visual chromophore production [169]. LRAT-

deficient mice also lack major retinoid stores of the body and are highly susceptible to dietary vitamin A deficiency [72, 174].

7. 3   Transport of carotenoids to the periphery

For hepatic secretion, carotenoids are packaged into very-low-density lipoproteins (VLDL) and secreted into the blood. Carotenoids are not equally distributed among lipoproteins in the fasting circulation. The hydrophobic carotenes are found predominantly in low-density lipoproteins (LDL), while the more hydrophilic xanthophylls tend to be found in higher amounts in high-density lipoproteins (HDL) (Figure 11).

Genetic studies in Drosophila revealed that the *Santa maria* gene is required for uptake of carotenoids from circulating insect lipoproteins for visual chromophore production [67]. The *Santa maria* gene encodes a class two scavenger receptor that is required to render carotenoids available from lipoproteins to support photoreceptor development and vision. Studies in human retinal pigment epithelia cell lines indicate that SR-B1 facilitates uptake of carotenoids from HDL [266, 267]. In the circulation, zeaxanthin and lutein exist in higher concentration in HDL than in low density and very low density lipoproteins [114, 267]. The retinal pigment epithelium cells acquire fat soluble vitamins and other nutrients to deliver them to the adjacent neuronal retina to support photoreceptor function. This mechanism may play an important role in the uptake of macula pigments that are concentrated in the fovea of the macula region in the retina. β-Carotene in circulating LDL is absorbed by LDL receptor mediated endocytosis in to the retinal pigment epithelium [267] (Figure 11). Human RPE cells express relatively high levels of BCO1, the vitamin A forming enzyme, which converts β-carotene to retinaldehyde, and local vitamin A synthesis may contribute to ocular retinoid homeostasis [268].

Carotenoid content and composition is variable between tissues. The high concentration of carotenoids in the fovea region of the retina of the human eyes supports the idea of active uptake and retention mechanism for these lipids. An isoform of glutathione-S-transferase, GSTP1, and a steroidogenic acute regulatory domain protein, StARD3, were suggested as zeaxanthin and lutein binding proteins in the human retina [269, 270]. However, given the very high levels of xanthophylls in the macula, it is unlikely that these pigments predominantly exist in protein-bound form unless GSTP1 and StARD3 would be present in comparable amounts.

8.   Implication for carotenoid intake levels and human health benefits

In 2000, the Food and Nutrition Board reviewed the evidence for role(s) of carotenoids as antioxidants and determined that specific functions beyond that related to provitamin A activity had not yet been sufficiently identified [271]. No dietary recommendations for individual non-provitamin A carotenoid intake exist, as it was thought that their absence from the diet causes no specific deficiency symptoms. Recent research challenges this statement and indicates urgent need for action. Studies in primates showed that deficiency of lutein and zeaxanthin can impair vision and make the retina susceptible to light damage [6, 13]. The outcome of the Age-Related Eye Disease Study 2 (AREDS2) study provided compelling evidence for beneficial effects of low dose zeaxanthin and lutein (2 and 10 mg)

supplementation for eye health [272, 273]. While the importance of these carotenoids for eye health has been now acknowledged [11], intake of 10 mg/d have been advocated by several researchers [11, 274].

For provitamin A carotenoids, the Food and Nutrition Board of the National Research Council initially established conversion values of 2:1 for purified all-*trans*-β-carotene from supplements and 6:1 for dietary all-*trans*-β-carotene from food. Other dietary provitamin A carotenoids (mainly α-carotene and β-cryptoxanthin) were considered to have half the vitamin A activity of dietary all-*trans*-β-carotene. In 2001, the Food and Nutrition Board revised the original conversion values based on human trials revealing that the bioavailability of all-*trans*-β-carotene from foods was half of what was originally believed [275]. To decrease confusion in making the changes, a new term was coined: "retinol activity equivalent" (RAE = 1 μg all-*trans*-retinol). The RAE ratios of conversion for purified all-*trans*-β-carotene in oil, dietary all-*trans*-β-carotene, and other dietary provitamin A carotenoids were set at 2:1, 12:1, and 24:1, respectively.

In 2009, a consensus conference on β-carotene in Hohenheim, Germany, concluded that the mean dietary intake of β-carotene in Europe is in the range of 1.5–1.8 mg/d, and that provitamin A intake is <3 mg/d in most European countries. Since provitamin A carotenoids are the major source of vitamin A, it was recommended to increase the β-carotene consumption to 7 mg/d [276]. The role of carotenoids as vitamin A source will become even more important with the growing trend for vegetarian and vegan life styles.

A problem with recommendations for dietary carotenoid intake in health and disease is the variable plasma responses upon supplementation. As reviewed here, many host-related factors affect the bioavailability and bioconversion of carotenoids. It is now generally acknowledged that intestinal absorption of carotenoids is a saturable and protein-mediated process. Moreover, the conversion rate of carotenoids to retinoids is affected by vitamin A status and genetic makeup of the host [26]. The regulation of the bioconversion of carotenoids to vitamin A helps humans to adapt to the fluctuating dietary supplies of these lipids [19]. A study in Zambian children impressively reports the consequences of this regulation under conditions of excessive dietary vitamin A supplies [277]. During mango harvest, a period of high provitamin A supply, these children experienced hypercarotenodermia. This indicate that provitamin A carotenoid bioconversion to vitamin A by BCO1 is inhibited by negative feedback regulation. The hypercarotenodermia is elicited by the uptake of carotenoids by other receptors such as CD36, which are not regulated by ISX. The equivalency ratios for provitamin A carotenoids do not consider vitamin A status and one can assume that the ratio differs significantly between vitamin A sufficient and deficient populations.

The need to control intestinal carotenoid metabolism is also indicated by the critical role of vitamin A in immunity [23]. There is increasing body of evidence that excessive dietary levels of preformed vitamin A can promote inflammation and loss of tolerance at the intestinal barrier [213, 278]. A failure to establish tolerance against commensal flora and food antigens has been implicated in autoimmune disease such as celiac disease, Crohn's disease, and type 1 diabetes [231]. These diseases are on the rise in many industrialized

countries. Studies in mice revealed that mammalian genomes specifically devote a transcription factor to control intestinal vitamin A production from dietary provitamin A [167]. Accordingly, genetic variation in the *ISX* gene has been associated with inflammatory responses and proliferation of cells [279, 280]. Moreover, *ISX* gene has been associated with inflammatory bowel disease in genome wide studies [281]. At the state of the present knowledge it can be assumed that the supplementation with preformed vitamin A has a different impact on gastrointestinal immunity than the supplementation with provitamin A which is absorbed and converted to retinoids in a controlled fashion. More research is needed to better understand this crosstalk between diet and immunity in the intestine.

Genome wide association studies and candidate gene studies identified single base pair polymorphisms (SNPs) that affect carotenoid blood and tissue levels [26]. Significant SNPs were found in the genes encoding BCO1, SR-B1, and the transcription factor ISX [198, 209, 282, 283]. Interestingly, SNPs in BCO1 are associated with macula pigment density [284]. This finding is surprising at the first glance since xanthophyll is not a substrate for the vitamin A forming enzymes. The vitamin A-dependent regulation of *SR-B1* expression in the intestine explains the interaction between xanthophyll accumulation and β-carotene metabolism. Interestingly, it was previously observed that dietary vitamin A reduces xanthophyll absorption in chickens [211]. Genetic makeup influences the conversion rate of provitamin A carotenoids and may constitute the basis for the low and high responder phenotypes described in the general European male population [285]. Additionally, several SNPs in proteins that affect lipid and lipoprotein metabolism have been associated with carotenoid blood levels [26]. The latter association indicates significant interactions between carotenoid and lipid metabolism. Such interactions have been recently demonstrated in mouse models [114]. Most of the genetic data in humans stem from the analyses of β-carotene, lycopene, and lutein fasting blood levels, but it is assumed that these genetic variations also modulate the bioavailability of the other carotenoids found in human blood and tissue [26].

Additionally, carotenoid absorption and bioconversion can be affected by several chronic disease state of the gut. Atrophic gastritis, a common condition of aging, results in insufficient gastric acid secretion and decreased carotenoid uptake, by disturbing the formation and absorption of mixed micelles [30]. Parasitic infections, prevalent in vitamin A deficient populations, can also have a dramatic negative effect on carotenoid absorption [286]. Pancreatic insufficiency in cystic fibrosis also impacts the absorption of carotenoids and their apocarotenoid metabolites [287]. Additionally, Crohn's disease and celiac disease have been reported to affect carotenoid levels in blood and tissues of affected individuals [288]. Thus, specific dietary recommendation for carotenoid intake are required in disease states of the gastrointestinal tract.

In conclusion, an increasing body of evidence indicates critical roles of carotenoids in mammalian biology as antioxidants, blue light filters, and vitamin A precursor. Therefore, many nutritionists and clinicians advocate for recommendations for carotenoid intake. The establishment of such recommendations is based on the relationships between carotenoid intake, blood, and tissue concentrations. This relation is not linear and is influenced by many host and environmental factors. As reviewed here, studies in experimental animals provided

a molecular framework for how diet and genetics affect the bioavailability and bioconversion of carotenoids. This framework provides a controlled production of vitamin A and prevents excessive accumulation of carotenoids and retinoids. An important aspect in the coming years will be to refine our knowledge about this framework and to determine its specifics in human physiology. Such knowledge will eventually lead to science-based recommendations for the intake of these essential lipids.

Acknowledgments

Abbreviations

ADH	alcohol dehydrogenase
BCO1	β-Carotene-15,15'-Dioxygenase
BCO2	β-Carotene-9,10'-Dioxygenase
CCD	Carotenoid Cleavage Dioxygenases
CD36	Cluster of Differentiation 36
Cyp26a1	Cytochrome P450, Family 26 A1
Intestine	Specific Homeobox: Transcription factor
NinaD	neither inactivation nor afterpotential mutant D
LRAT	lecithin: retinol acyl transferase
RPE65	Retinal Pigment Epithelium-Specific Protein 65kDa
RBP4	Retinol Binding Protein 4
SR-B1	Scavenger Receptor Class B, Member 1
STRA6	Stimulated by Retinoic Acid 6
RALDH1	Retinal Dehydrogenase 1 Family
RAR	retinoic acid receptor
RDH	retinol dehydrogenase
RXR	retinoid X receptor
SDR	short chain dehydrogenase/reductase

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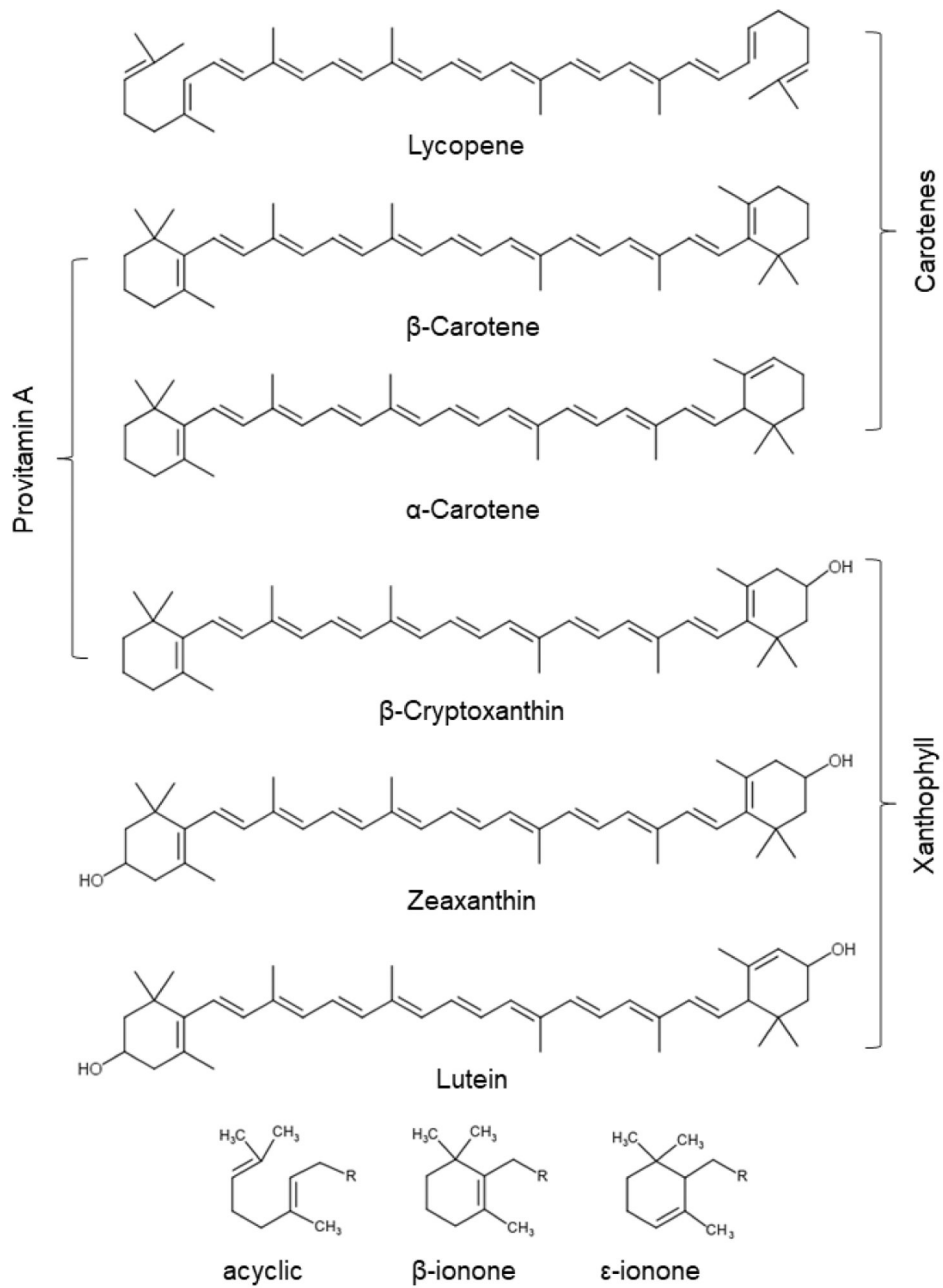
Figure 1.
Chemical structures and names of the six major carotenoids in human blood. Their classification into carotenes, xanthophyll, and provitamin A carotenoids is indicated. The chemical characteristics of different end-groups is provided at the bottom of the panel.
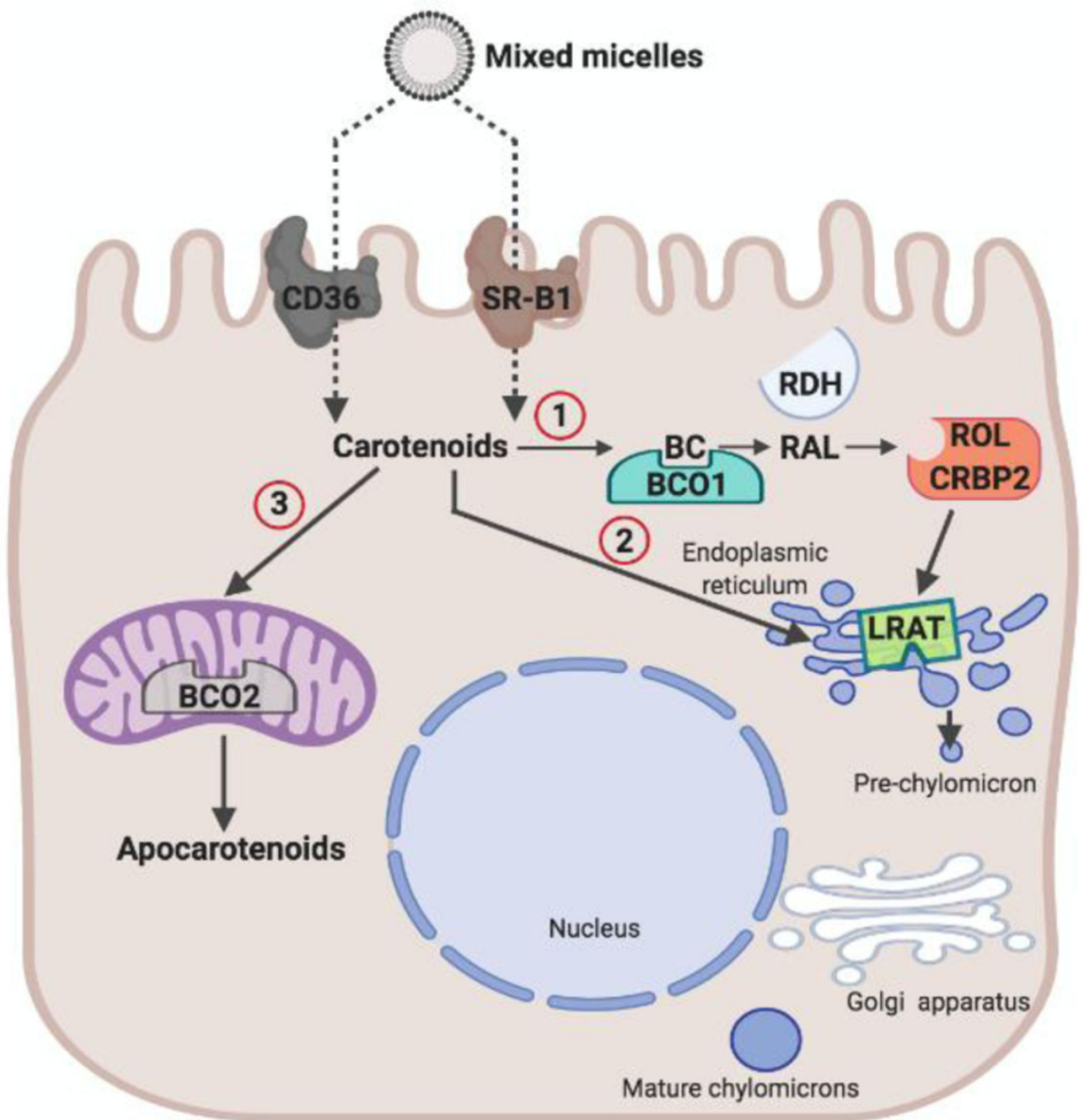
Figure 2.
Schematic of carotenoid absorption in an enterocyte. Carotenoid absorption from mixed micelles is facilitated by scavenger receptor class B type 1 (SR-B1) and cluster of differentiation 36 (CD36). Absorbed carotenoids can undergo three metabolic fates. (1) Carotenoids such as β-carotene (BC) can be converted to retinal (RAL). The conversion of BC is catalyzed by β-carotene-15,15'-dioxygenase (BCO1). RAL is reduced to retinol (ROL) by retinal dehydrogenases (RDH) in a process that involves cellular retinol binding protein (CRBP2). ROL is converted to retinyl esters (RE) by lecithin: retinol acyl transferase (LRAT) and packed in chylomicrons for body distribution. (2) Unprocessed carotenoids can be packaged into chylomicrons for body distribution. (3) Carotenoids can be converted by

mitochondrial BCO2 (β-carotene-9',10'-dioxygenase) into apocarotenoids different than retinoids.
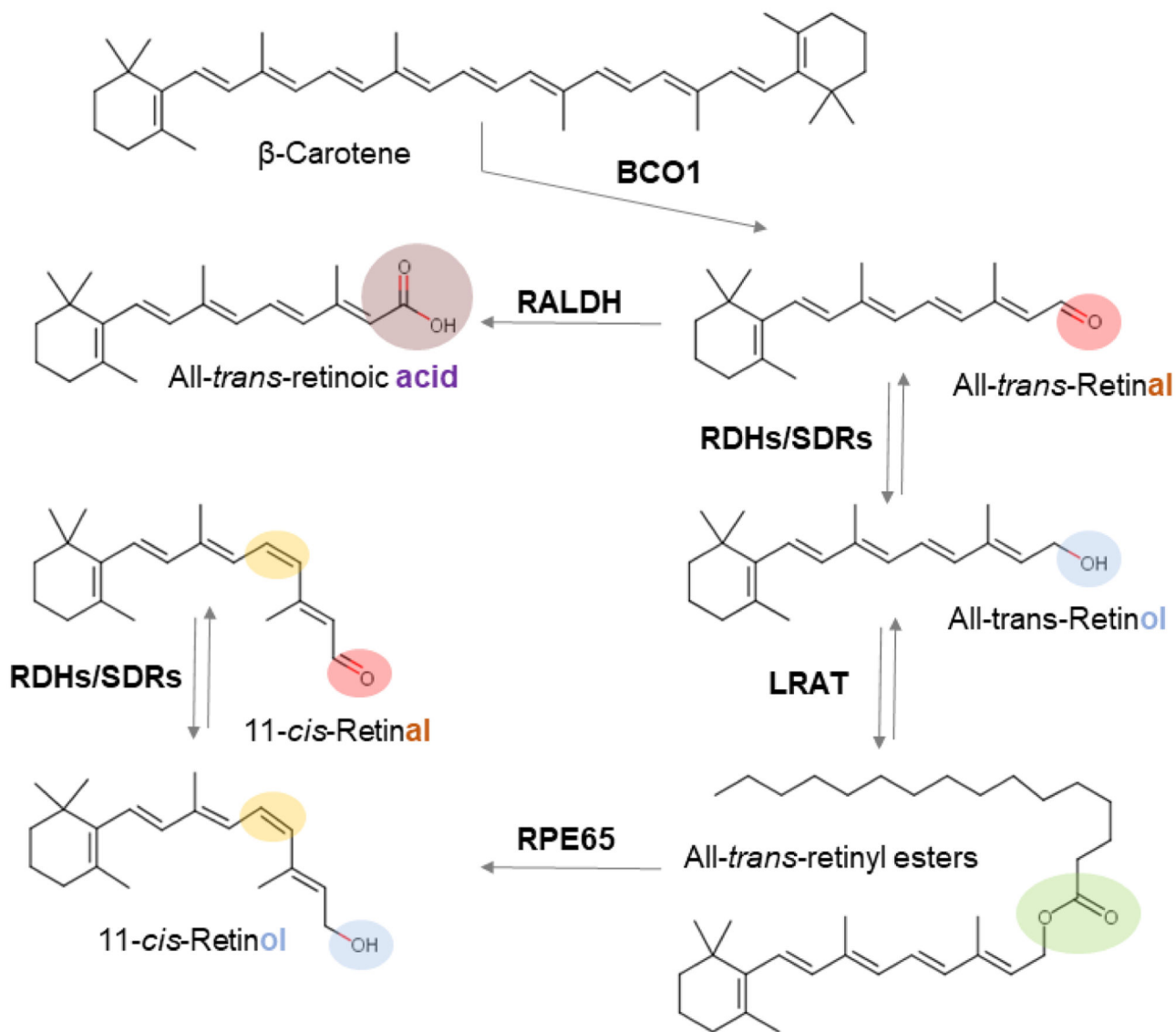
Figure 3.
Overview of vitamin A metabolism. The different functional groups of retinoids are indicated by a color code (red, aldehyde; blue, alcohol; green, ester; purple, acid). The 11-*cis*-retinoid diastereoisomer is indicated by yellow color. Major retinoid metabolizing enzyme classes (see main text) catalyzing the chemical transformations of retinoids are highlighted.
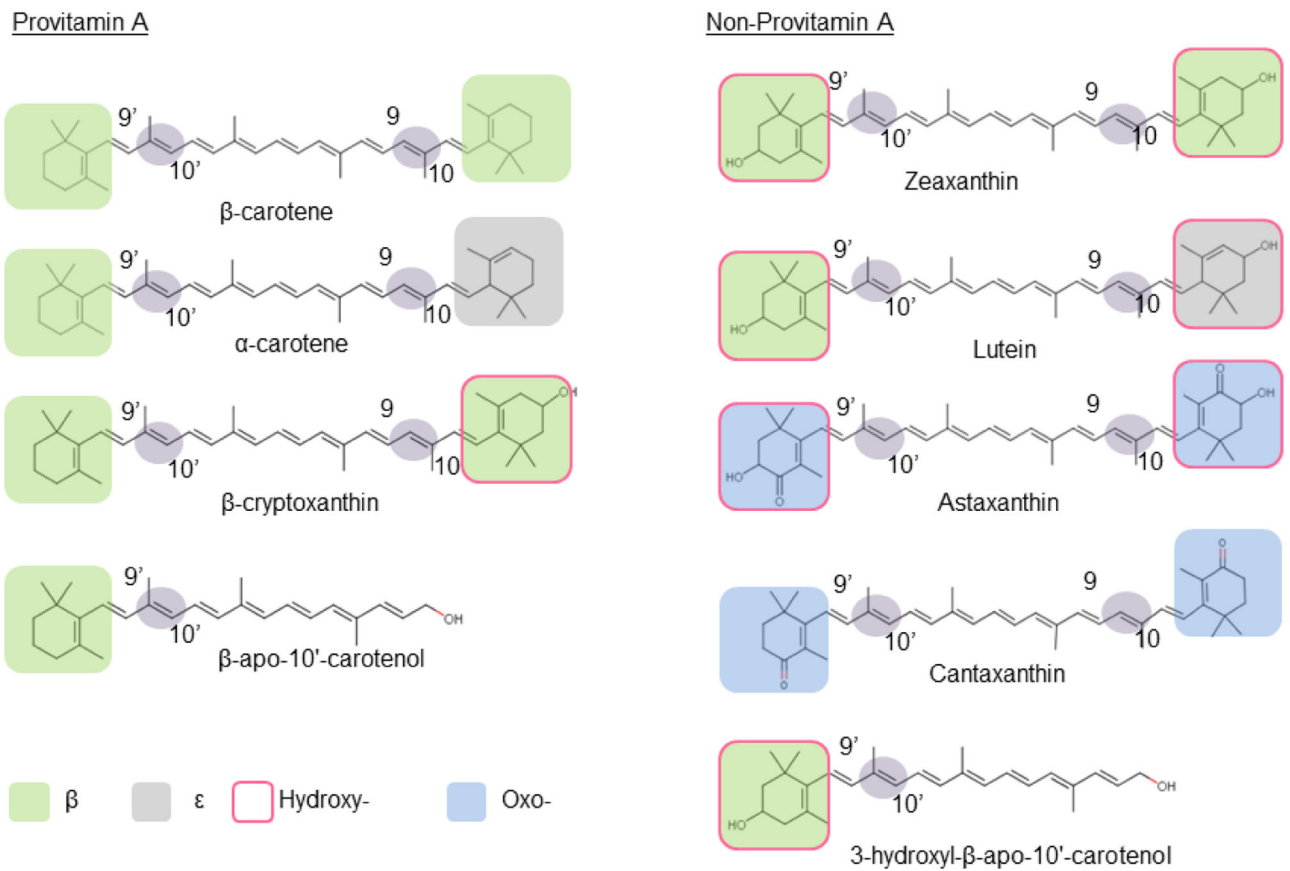
Figure 4.
Recombinant BCO2 converts a large panoply of different carotenoids with various ionone ring structures by oxidative cleavage at position 9,10 and 9',10' of the carbon skeleton. Confirmed provitamin A and non-provitamin A substrates of recombinant mouse BCO2 are displayed in the panel.
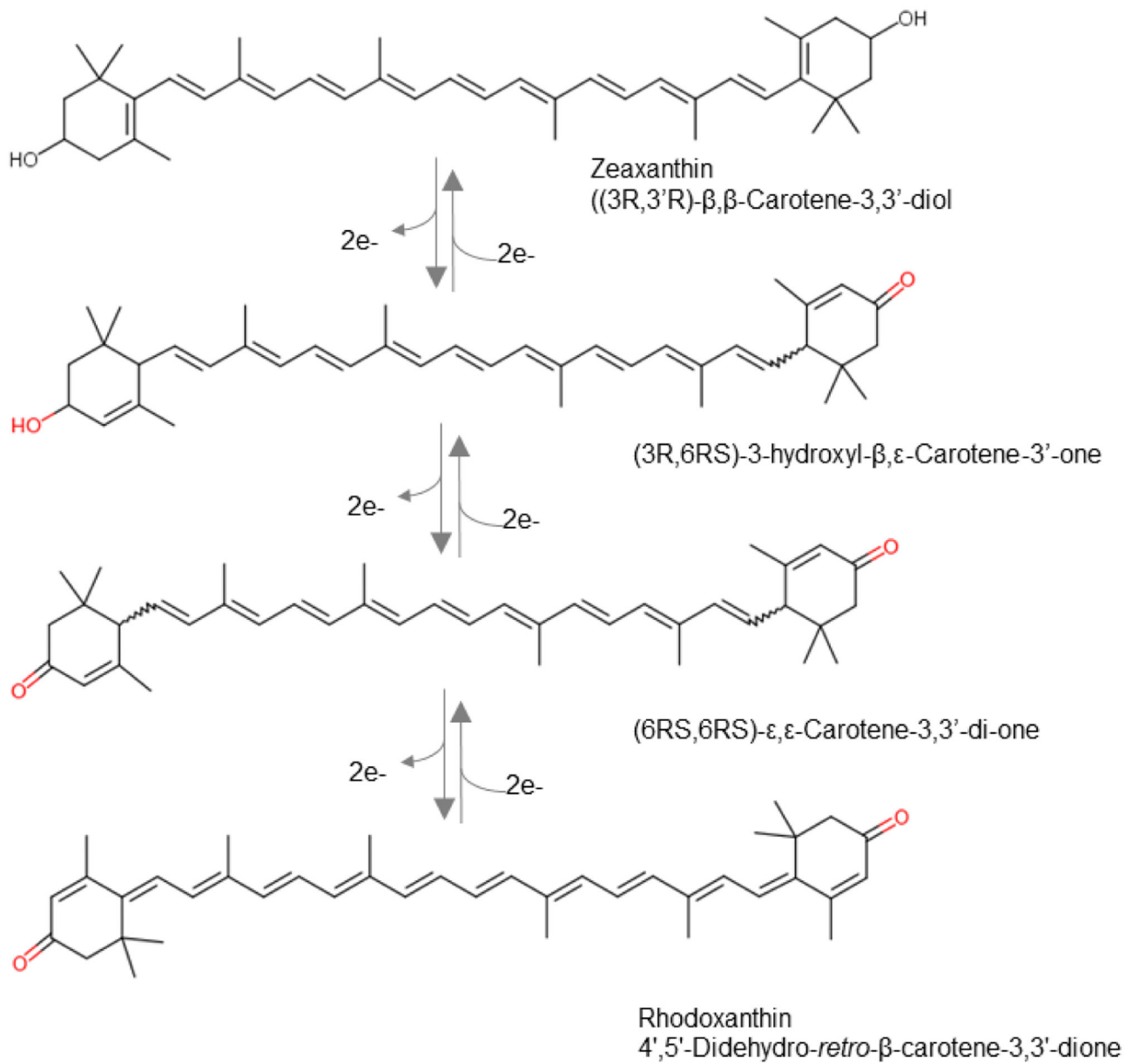
Figure 5.
Structures of zeaxanthin and its oxidation products in tissues of mice.
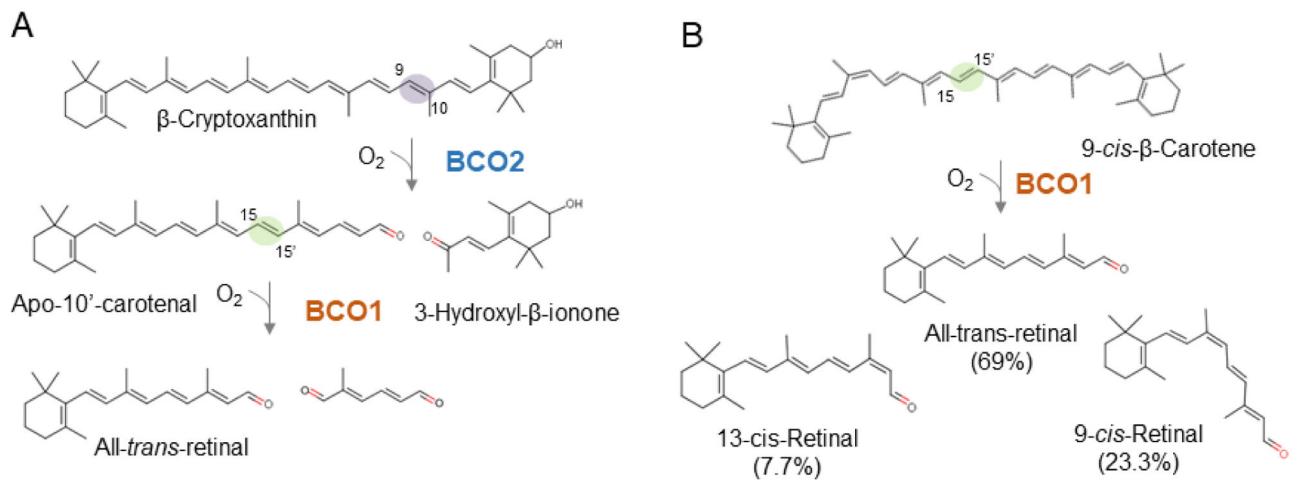
Figure 6.
Conversion of asymmetric provitamin A carotenoids into all-*trans*-retinal. (A) β-cryptoxanthin is converted by a two-step mechanism with a β-10'-apocarotenal intermediate in a pathway that involves BCO1 and BCO2 enzymes. (B) 9-*cis*-β-carotene is converted by BCO1 into all-trans-, 13-*cis*, and 9-*cis*-retinal. Notably, the all-*trans*-diastereoisomer exist in significantly higher amount than the 9-*cis*-retinal diastereomer. Trace amounts of 13-cis-retinal are also present. The distribution of the different retinal-diastereomers indicates that BCO1 possesses an intrinsic isomerase activity.
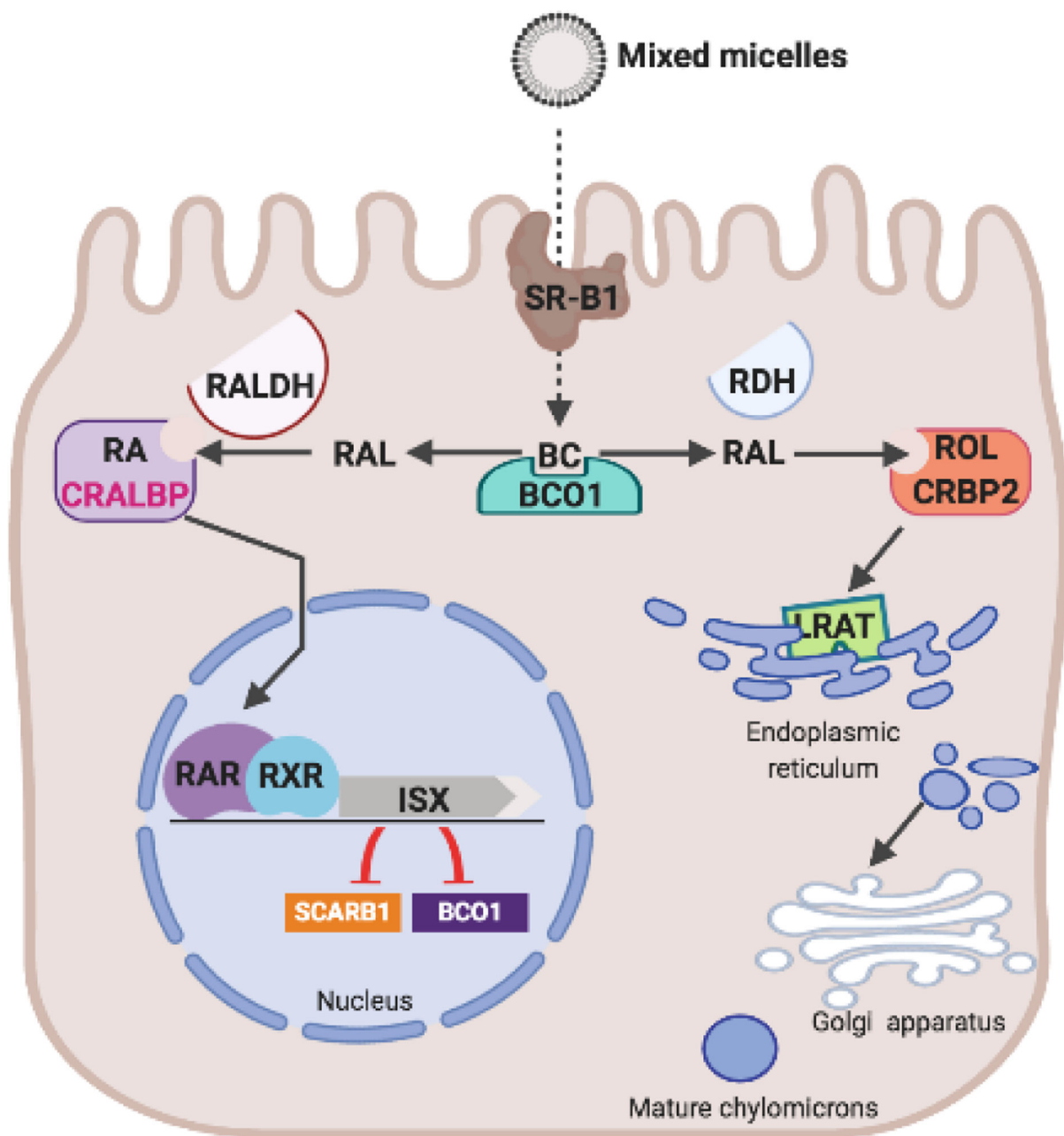
Figure 7.
A diet-responsive regulatory network controls intestinal vitamin A production and carotenoid absorption. β-Carotene (BC) absorption is facilitated by scavenger receptor class B type 1 (SR-B1). The conversion of BC by β-carotene-15,15'-dioxygenase (BCO1) is an important branching point in retinoid metabolism. The primary product retinaldehyde (RAL) is reduced to retinol (ROL) by retinal dehydrogenases (RDH) in a process that involves cellular retinol binding protein (CRBP2). ROL is the converted to retinyl esters (RE) by lecithin: retinol acyl transferase (LRAT) and packed in chylomicrons for body distribution. Some RAL is also converted to retinoic acid (RA) by retinal dehydrogenase (RALDH). RA binds to retinoic acid receptors (RARs) that in conjunction with retinoid X receptors (RXR)

regulate the expression of intestine specific homeobox (ISX) transcription factor. ISX is a transcriptional repressor of the genes encoding SCRAB1 and BCO1.
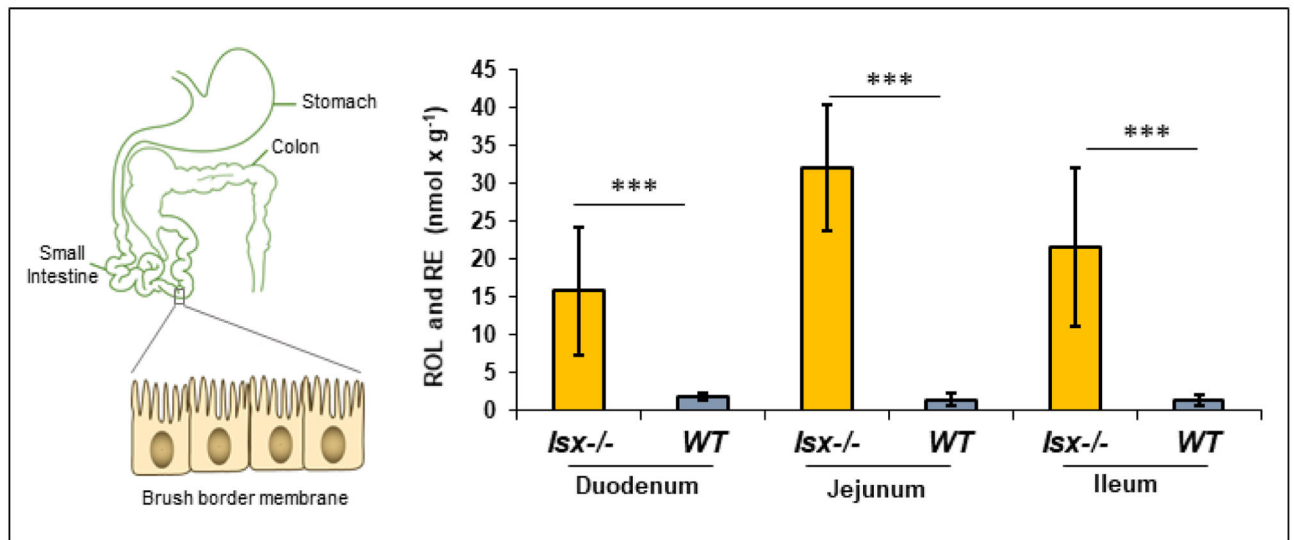
Figure 8.
ISX controls vitamin A homeostasis in the intestine. Retinol (ROL) and retinyl ester (RE) levels in different parts of the intestine of *Isx*$^{-/-}$ and WT mice fed with a β-carotene-rich (25 mg/kg) diet. The values represent five individual animals per genotype. The p values (<0.001) in student T test are indicated by three asterisks.
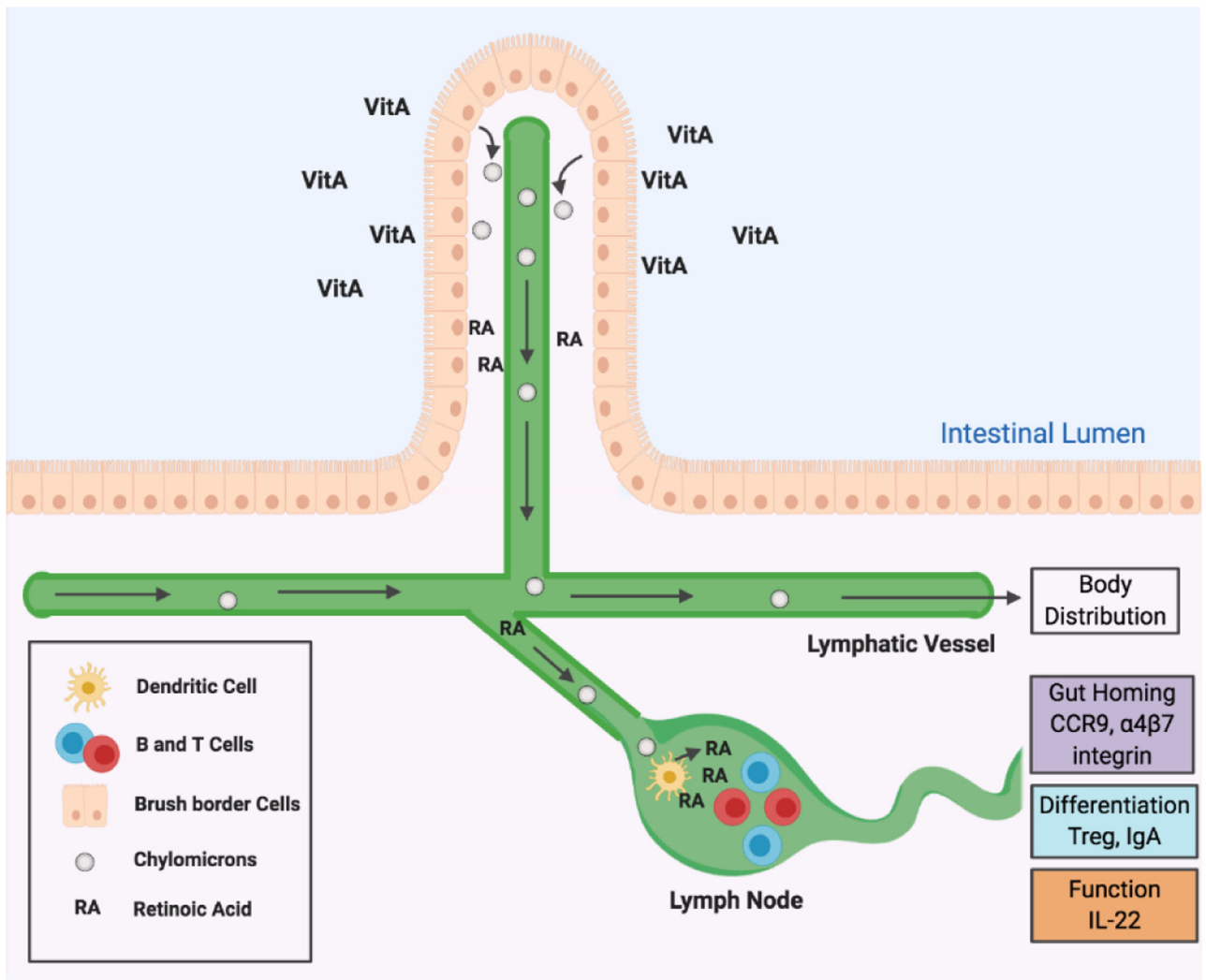
Figure 9.
Intestinal vitamin A metabolism and immunity. Brush border cells acquire dietary vitamin A precursors from the intestinal lumen and convert them to retinyl esters. Retinyl esters are packaged into chylomicrons for body distribution. Brush border cells and dendritic cells in the lymphatic system can convert dietary vitamin A also into retinoic acid (RA). RA can promote gut homing, differentiation, and functions of B and T lymphocytes.
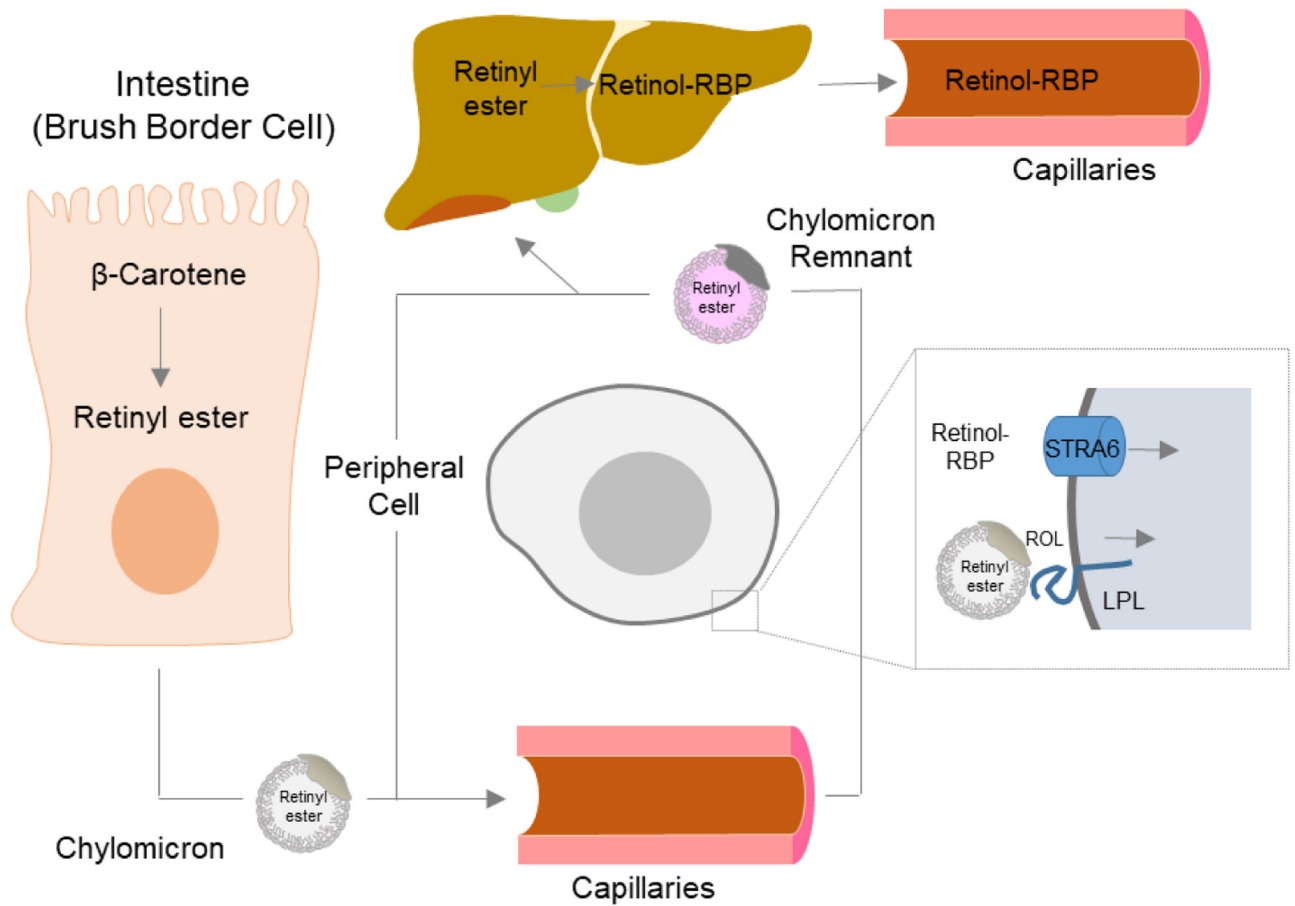
Figure 10.
Transport of vitamin A throughout the body. Carotenoids such as β-carotene are absorbed in brush border cells in the intestine, where they are converted into retinyl esters (RE), packaged into chylomicrons, and released into the circulation. The RE in chylomicrons can either be delivered to peripheral cells and taken up in a liproprotein lipase-mediated process or be transported for receptor-mediated endocytosis and storage to the liver. In the liver, stored RE are converted back to retinol which binds to RBP. The retinol-RBP complex is released into the circulation. Vitamin A is taken up from holo-RBP in a STRA6-mediated transport process.
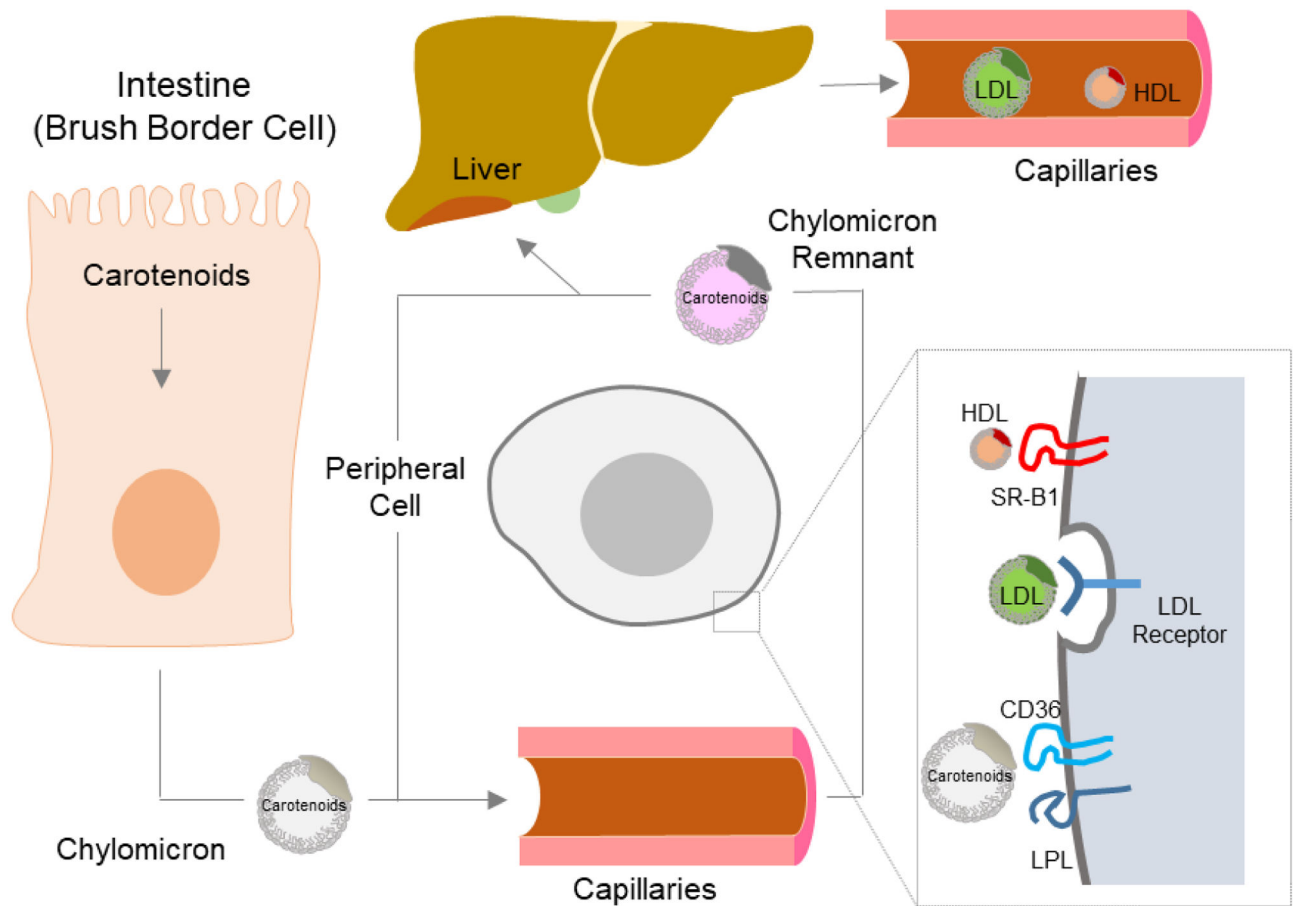
Figure 11.
Transport of carotenoids throughout the body. Carotenoids are absorbed in brush border cells in the intestine, where they are packaged into chylomicrons, and released into the circulation. The carotenoids in chylomicrons can be delivered to peripheral cells and taken up in a lipoprotein lipase-mediated process. This uptake may also involve the scavenger receptor CD36. Carotenoids in chylomicron remnants are transported to the liver. In the liver, carotenoids are packaged into Very Low Density Lipoproteins (VLDL). VLDL might be converted to Low Density Lipoproteins (LDL) in the circulation. Carotenoids from LDL are taken up by LDL receptor mediated endocytosis into peripheral cells. Additionally, carotenoids in HDL are taken up by SR-B1 facilitated interactions between HDL and peripheral cells.