Identification of the 11-*cis*-specific retinyl-ester synthase in retinal Müller cells as multifunctional *O*-acyltransferase (MFAT)

Joanna J. Kaylor[a], Jeremy D. Cook[a], Jacob Makshanoff[a], Nicholas Bischoff[a], Jennifer Yong[a], and Gabriel H. Travis[a,b,1]

[a]Jules Stein Eye Institute and [b]Department of Biological Chemistry, University of California, Los Angeles School of Medicine, Los Angeles, CA 90095

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Absorption of a photon by a rhodopsin or cone-opsin pigment isomerizes its 11-*cis*-retinaldehyde (11-*cis*-RAL) chromophore to all-*trans*-retinaldehyde (all-*trans*-RAL), which dissociates after a brief period of activation. Light sensitivity is restored to the resulting apo-opsin when it recombines with another 11-*cis*-RAL. Conversion of all-*trans*-RAL to 11-*cis*-RAL is carried out by an enzyme pathway called the visual cycle in cells of the retinal pigment epithelium. A second visual cycle is present in Müller cells of the retina. The retinol isomerase for this noncanonical pathway is dihydroceramide desaturase (DES1), which catalyzes equilibrium isomerization of retinol. Because 11-*cis*-retinol (11-*cis*-ROL) constitutes only a small fraction of total retinols in an equilibrium mixture, a subsequent step involving selective removal of 11-*cis*-ROL is required to drive synthesis of 11-*cis*-retinoids for production of visual chromophore. Selective esterification of 11-*cis*-ROL is one possibility. Crude homogenates of chicken retinas rapidly convert all-*trans*-ROL to 11-*cis*-retinyl esters (11-*cis*-REs) with minimal formation of other retinyl-ester isomers. This enzymatic activity implies the existence of an 11-*cis*-specific retinyl-ester synthase in Müller cells. Here, we evaluated multifunctional *O*-acyltransferase (MFAT) as a candidate for this 11-*cis*-RE-synthase. MFAT exhibited much higher catalytic efficiency as a synthase of 11-*cis*-REs versus other retinyl-ester isomers. Further, we show that MFAT is expressed in Müller cells. Finally, homogenates of cells coexpressing DES1 and MFAT catalyzed the conversion of all-*trans*-ROL to 11-*cis*-RP, similar to what we observed with chicken-retina homogenates. MFAT is therefore an excellent candidate for the retinyl-ester synthase that cooperates with DES1 to drive synthesis of 11-*cis*-retinoids by mass action.

Light perception begins with the absorption of a photon by an opsin pigment in the membranous outer segment (OS) of a rod or cone photoreceptor cell. The light-absorbing chromophore in most vertebrate opsins is 11-*cis*-retinaldehyde (11-*cis*-RAL). Photon capture isomerizes the 11-*cis*-RAL to all-*trans*-retinaldehyde (all-*trans*-RAL), inducing conformational changes in the protein that lead to its active meta-II state. After a brief period of signaling through the transduction cascade, meta II decays to yield apo-opsin and free all-*trans*-RAL. Light sensitivity is restored to the apo-opsin when it combines with 11-*cis*-RAL to regenerate the pigment. Conversion of all-*trans*-RAL to 11-*cis*-RAL is carried out by a multistep enzyme pathway called the visual cycle, located in cells of the retinal pigment epithelium (RPE) (1, 2). The retinoid isomerase in this pathway is Rpe65, which converts an all-*trans*-retinyl ester (all-*trans*-RE), such as all-*trans*-retinyl palmitate (all-*trans*-RP), to 11-*cis*-retinol (11-*cis*-ROL) and a free fatty acid (3–5). Retinyl esters are synthesized in RPE cells by lecithin:retinol acyl transferase (LRAT), which transfers a fatty acid from phosphatidylcholine to retinol (6, 7). LRAT converts both all-*trans*-ROL and 11-*cis*-ROL to their cognate esters with similar catalytic efficiency (8).

A second visual cycle is present in Müller cells of the retina, providing 11-*cis*-ROL to cones (9–11). Cones, but not rods, can use 11-*cis*-ROL as a chromophore precursor to regenerate bleached opsin pigments (10, 12, 13). The isomerase in the noncanonical pathway is dihydroceramide desaturase (DES1) (11). DES1 catalyzes rapid equilibrium isomerization of retinol (11). At equilibrium, 11-*cis*-ROL is much less abundant than all-*trans*-ROL, due to the 4.1 kcal/mole difference in free energy between these isomers (14). Accordingly, a secondary source of energy is required to drive the conversion of all-*trans*-ROL to 11-*cis*-ROL by DES1. Retinas from cone-dominant species contain 11-*cis*-retinyl esters (11-*cis*-REs), whereas retinyl esters are much less abundant in retinas from rod-dominant species (11, 13, 15). Homogenates from cone-dominant chicken and ground-squirrel retinas convert all-*trans*-ROL predominantly to 11-*cis*-REs in the presence of palmitoyl CoA (palm CoA) (13, 16, 17). These observations suggest that selective esterification of 11-*cis*-ROL may be the driving force for 11-*cis*-retinoid formation. In the current work, we sought to identify the protein responsible for the 11-*cis*-RE-synthase activity in Müller cells. We evaluated multifunctional *O*-acyltransferase (MFAT) as a candidate for this synthase. MFAT, also called acyl-CoA wax-alcohol acyltransferase-2 (AWAT2), catalyzes palm CoA-dependent synthesis of triglycerides, wax monoesters, and retinyl esters (18). It is present in the endoplasmic reticulum and predominantly expressed in skin (18). The retinol-isomer specificity of MFAT, and its expression in ocular tissues, has not been studied.

Results

Synthesis of 11-*cis*-ROL and 11-*cis*-RP by Chicken Retina Homogenates. We incubated homogenates of fresh chicken retinas in the presence of all-*trans*-ROL and palm CoA. After quenching, we extracted the

Significance

The noncanonical visual cycle permits sustained vision in daylight by providing chromophore precursor (11-*cis*-retinol) to cone photoreceptors at high rates. The isomerase of this pathway catalyzes equilibrium isomerization of retinol. At equilibrium, all-*trans*-retinol and 13-*cis*-retinol are strongly favored over 11-*cis*-retinol. A mechanism of mass action is hence required to drive production of 11-*cis*-retinol. Homogenates of cone-dominant retinas convert all-*trans*-retinol to 11-*cis*-retinyl esters, suggesting that secondary esterification of 11-*cis*-retinol drives production of 11-*cis*-retinoids for synthesis of chromophore. Here, we identified an enzyme, multifunctional *O*-acyltransferase (MFAT), that specifically esterifies 11-*cis*-retinol. MFAT acts cooperatively with isomerase-2 in Müller cells to drive production of 11-*cis*-retinoids. Thus, the energy of retinoid isomerization comes from hydrolysis of the thioester in an activated fatty acid by MFAT.

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[1]To whom correspondence should be addressed. E-mail: travis@jsei.ucla.edu.

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reaction mixtures and analyzed the retinoid content by normal-phase HPLC. Retinoids were identified by their elution times (Fig. 1 *A* and *B*) and absorption spectra (Fig. S1 *A* and *B*). The free energies of 13-*cis*-ROL and all-*trans*-ROL are similar, while both are lower than the free energy of 11-*cis*-ROL (14). Accordingly, synthesis of 13-*cis*-ROL is strongly favored over 11-*cis*-ROL in an equilibrium reaction. As expected, we observed ~10-fold higher synthesis of 13-*cis*-ROL versus 11-*cis*-ROL (Fig. 1*C*). Despite the preponderance of 13-*cis*-ROL, the same homogenates synthesized nearly 40-fold more 11-*cis*-RP than 13-*cis*-RP (Fig. 1*C*). These data corroborate previous observations (13, 16, 17) and suggest the existence of an 11-*cis*-specific retinyl-ester synthase in chicken retinas.

Retinol Substrate Kinetics of MFAT. To test the specificity of MFAT toward the various retinol isomers, we prepared homogenates of 293T cells transfected with a plasmid containing the human MFAT cDNA. We incubated homogenates of cells transfected with MFAT or nonrecombinant pcDNA in the presence of palm CoA and different concentrations of 11-*cis*-ROL, 9-*cis*-ROL, 13-*cis*-ROL, or all-*trans*-ROL. For each retinol isomer, we determined the initial synthesis rate (V_0) of its cognate retinyl palmitate. Michaelis–Menten analysis of these data yielded the maximum turnover rates (V_{max}) and Michaelis constants (K_M) (Fig. 2 *A*–*D*). The V_{max} of MFAT for synthesis of 11-*cis*-RP was 6.6-fold higher than for synthesis of all-*trans*-RP and 150-fold higher than for synthesis of 13-*cis*-RP. Further, the K_M of MFAT for 11-*cis*-ROL was severalfold lower than the K_M for 13-*cis*-ROL or all-*trans*-ROL (Figs. 2 *A*, *C*, and *D*). Thus, MFAT is a far more efficient synthase for 11-*cis*-RP versus 13-*cis*-RP or all-*trans*-RP. Although the maximal rates of MFAT-catalyzed 11-*cis*-RP and 9-*cis*-RP synthesis were similar, the K_M for 9-*cis*-ROL was severalfold higher than for 11-*cis*-ROL (Fig. 2 *A* and *B*). At native substrate concentrations, MFAT is probably much less efficient a synthase for 9-*cis*-REs versus 11-*cis*-REs. These kinetics may explain the nearly 10-fold higher concentration of 11-*cis*-REs versus 9-*cis*-REs found previously in dark-adapted chicken retinas (11).

MFAT Is Expressed in Müller Cells. To test for expression of MFAT in the eye, we analyzed homogenates of chicken and mouse retina and RPE by immunoblotting. MFAT was present in the retina, but not in the RPE (Fig. S2*A*). To determine whether MFAT is expressed in Müller cells, we performed quantitative real-time PCR on cDNA synthesized from primary cultured bovine and chicken Müller-cell RNA. We compared levels of the MFAT mRNA to mRNAs for the Müller-cell proteins: cellular retinaldehyde-binding protein (CRALBP) (19), glial fibrillary acidic protein (GFAP) (20), and DES1 (11). All mRNAs in bovine Müller cells were normalized to actin. MFAT mRNA levels were severalfold higher than the other Müller-cell mRNAs (Fig. S2*B*). We also compared levels of MFAT and CRALBP in RNA from primary cultured chicken Müller cells after normalizing to 18S rRNA. In these cells, MFAT and CRALBP mRNA levels were similar (Fig. S2*B*). To confirm MFAT expression in Müller cells, we performed immunohistochemistry on retina sections from wild-type (129/Sv) mice by using antisera against mouse MFAT and CRALBP. MFAT and CRALBP showed overlapping distribution in Müller cells (Fig. 3). In particular, both MFAT and CRALBP showed strong immunofluorescent labeling in the apical microvilli of Müller cells. These processes extend beyond the external limiting membrane (ELM) and into the interphotoreceptor matrix (IPM), where they interdigitate with photoreceptor inner segments (IS) (Fig. 3). These data establish that MFAT is expressed in Müller cells.

Chicken Retinas and MFAT-Expressing 293T Cells Produce 11-*cis*-REs with Similar Fatty-Acyl Profiles. We incubated homogenates of chicken retinas, and MFAT-expressing 293T cells, with 11-*cis*-ROL but no added palm CoA. Under these conditions, endogenous pools of activated fatty acids in the cell homogenates are used as acyl donors. Following incubation, we analyzed the retinoids by HPLC. Five discrete peaks of spectrally defined 11-*cis*-REs, representing different fatty-acyl esters of 11-*cis*-ROL, were present in both chromatograms (Fig. 4 *A* and *B* and Fig. S3). The profiles of these 11-*cis*-REs were remarkably similar between the two homogenates. In contrast, homogenates of 293T cells transfected with nonrecombinant pcDNA synthesized three spectrally defined 11-*cis*-RE peaks (Fig. 4*C*). Only two peaks (1 and 2) coeluted with the 11-*cis*-RE peaks synthesized by chicken-retina and MFAT-transfected 293T-cell homogenates (Fig. 4 *A* and *C*). These observations suggest that the 11-*cis*-RE-synthase in chicken retinas is MFAT.

Antibodies Against MFAT Inhibit 11-*cis*-RE Synthesis by Chicken-Retina Homogenates. To confirm that MFAT is responsible for 11-*cis*-RE synthesis in chicken retinas, we preincubated chicken-retina homogenates with IgG from two commercial polyclonal anti-human MFAT antisera (MFAT Ab-1 and -2). As positive controls, we preincubated chicken-retina homogenates with buffer alone or donkey anti-mouse IgG. Assays were performed by using all-*trans*-ROL and palm CoA as substrates. Preincubating with anti-MFAT Ab-1 reduced the synthesis of 11-*cis*-REs by 90%,

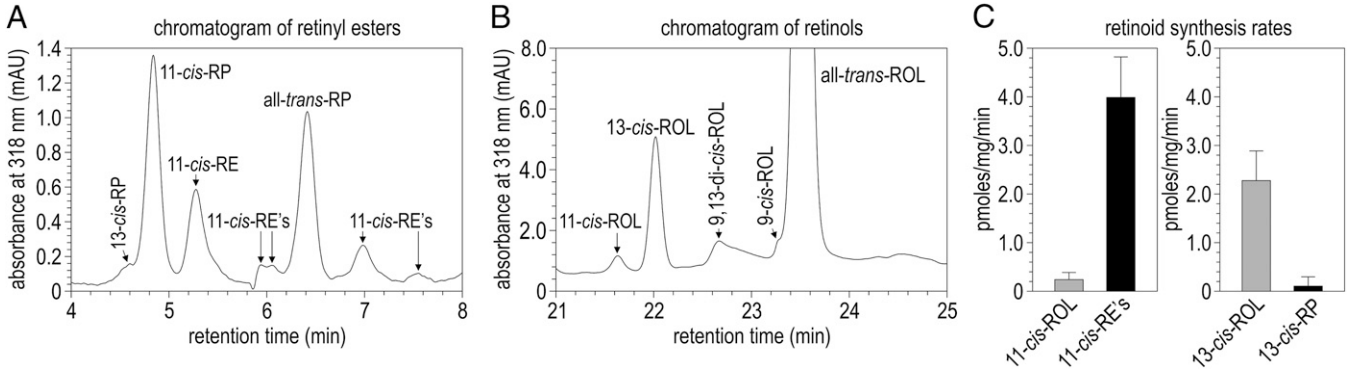

Fig. 1. Synthesis of 11-*cis*-ROLs, 11-*cis*-RPs, 13-*cis*-ROLs, and 13-*cis*-RPs from all-*trans*-ROL by chicken retina homogenates. Chicken-retina homogenates were used as the enzyme source in assays for retinol isomerization and retinyl-ester synthesis by using all-*trans*-ROL and palm CoA as substrates. (*A*) Chromatogram showing retinyl esters synthesized by chicken retina homogenates from all-*trans*-ROL. (*B*) Chromatogram showing retinols synthesized from chicken-retina homogenates from all-*trans*-ROL. (*C*) Rates of 11-*cis*-ROL and 11-*cis*-RE synthesis (*Left*), and rates of 13-*cis*-ROL and 13-*cis*-RP synthesis (*Right*) in the same reactions ($n = 3$). See Fig. S1 for peak spectra. Error bars show SEM.

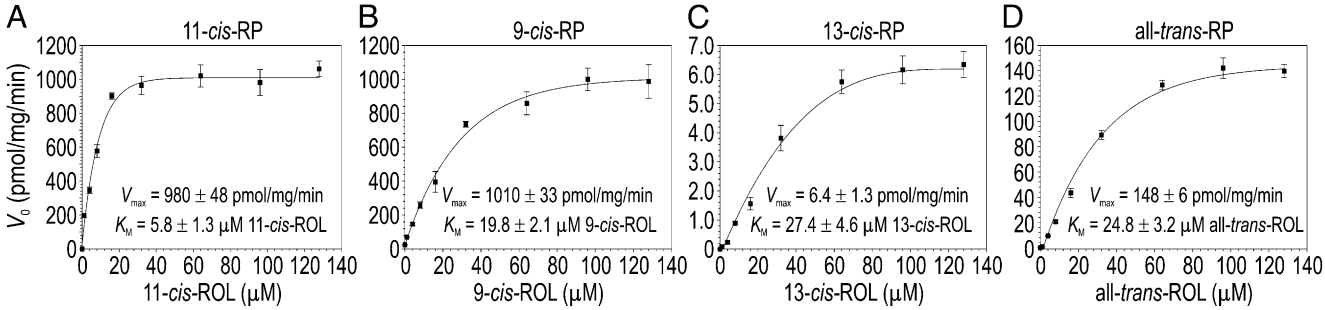

Fig. 2. Kinetic analysis of MFAT supplied with different retinol isomers. Homogenates of 293T cells expressing MFAT were assayed for palm CoA-dependent retinyl-ester synthase activities using 11-*cis*-ROL (*A*), 9-*cis*-ROL (*B*), 13-*cis*-ROL (*C*), or all-*trans*-ROL (*D*) substrate at the indicated concentrations. Nonlinear fitting of these data with the SigmaPlot Enzyme Kinetics module yielded V_{max} and K_M values for synthesis of each retinyl-ester isomer from its cognate retinol. Note the higher V_{max} and lower K_M values for 11-*cis*-ROL–dependent synthesis of 11-*cis*-RP versus 13-*cis*-RP and all-*trans*-RP. Activities are expressed as picomoles per milligram of total protein per minute (n = 3). Error bars show SEM.

whereas preincubating with anti-MFAT Ab-2 reduced the synthesis of 11-*cis*-REs by ∼70% (Fig. 5*A*). In contrast, preincubating with nonimmune IgG had no effect on the rate of 11-*cis*-RE synthesis (Fig. 5*A*). To confirm that this effect was through inhibition of MFAT, we preincubated MFAT-expressing 293T-cell homogenates with anti-MFAT Ab-1 and measured the conversion of 11-*cis*-ROL to 11-*cis*-REs. The 11-*cis*-RE-synthase activity of MFAT was 60% inhibited by anti-MFAT Ab-1, whereas nonimmune IgG had no effect on 11-*cis*-RE-synthase activity (Fig. S4). Thus, antibodies against MFAT inhibited the conversion of all-*trans*-ROL to 11-*cis*-REs by chicken retinas. These data suggest that the 11-*cis*-RE-synthase coupled to isomerase-2 in chicken retinas is MFAT.

Inhibition of 11-*cis*-RE-Synthase Activity in Chicken Retina Homogenates by 1-Hexadecanol. In contrast to most other acyl-transferases, MFAT fatty-acylates 1-hexadecanol to yield wax monoesters (18).

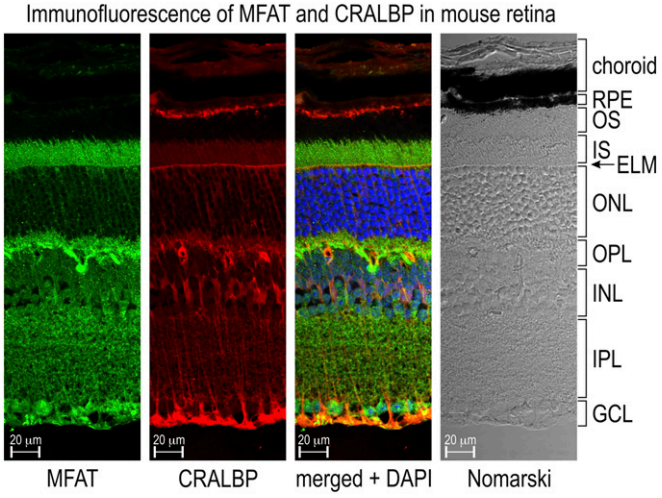

Fig. 3. MFAT expression in the retina. Immunohistochemical analysis of MFAT (green) and CRALBP (red) are shown in distal ocular sections from 6-mo-old 129/Sv mice. The merged image shows overlapping expression of DES1 and CRALBP (yellow). Nuclei were counterstained with DAPI (blue). Labels identifying retinal layers are shown to the right of the Nomarski image. ELM, external limiting membrane; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, photoreceptor inner-segments; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, photoreceptor outer-segments; RPE, retinal pigment epithelium. Müller-cell endfeet contact the vitreous within the GCL. Note expression of both MFAT and CRALBP in the apical microvilli of Müller cells, extending beyond the ELM into the IS layer. (Scale bars: 20 μm.)

At concentrations above its K_M, 1-hexadecanol should inhibit MFAT synthesis of 11-*cis*-REs by competition with retinol. The 11-*cis*-RE-synthase activity of chicken retina homogenates was reduced by 65% in the presence of 200 μM 1-hexadecanol (Fig. 5*A*). The 11-*cis*-RE-synthase activity of MFAT-expressing 293T-cell homogenates was similarly inhibited by 1-hexadecanol (Fig. S4). These observations constitute further evidence that MFAT is an important 11-*cis*-RE-synthase in chicken retinas.

MFAT Acts Cooperatively with DES1 To Synthesize 11-cis-REs. If MFAT acts cooperatively with DES1, addition of all-*trans*-ROL to homogenates of cells expressing both proteins should result in higher rates of 11-*cis*-RP versus 11-*cis*-ROL synthesis, and lower rates of 13-*cis*-RP versus 13-*cis*-ROL synthesis, similar to what we observed with chicken retina homogenates (Fig. 1*C*). We prepared a line of 293T cells that stably express MFAT (293T-M cells) and determined the rates of retinol and retinyl-ester synthesis by homogenates of 293T cells or 293T-M cells, both transiently transfected with DES1 or nonrecombinant pcDNA3.1. These assays were performed in the presence of palm CoA. Because 293T cells possess nonspecific, background acyl CoA:retinol acyl-transferase (ARAT) activity, we subtracted the retinyl esters synthesized by 293T cells from those synthesized by 293T-M cells. The ratio of 11-*cis*-RP to 11-*cis*-ROL synthesized by 293T-M homogenates was 10:1, whereas the ratio of 13-*cis*-RP to 13-*cis*-ROL was 0.06:1 (Fig. 5*B*). This trend was similar to that observed with the chicken-retina homogenates (Fig. 1*C*), suggesting that DES1 and MFAT are responsible for the conversion of all-*trans*-ROL to 11-*cis*-RP by chicken retinas (13, 16, 17).

Discussion

The goal of this project was to identify the retinyl-ester synthase responsible for the robust synthesis of 11-*cis*-REs from all-*trans*-ROL by chicken retinas (13, 16, 17). This enzyme is predicted to exhibit three properties. First, it should show higher catalytic efficiency toward 11-*cis*-ROL versus 9-*cis*-ROL, 13-*cis*-ROL, and all-*trans*-ROL substrates. Second, it should be expressed in Müller cells, which also express CRALBP (19) and DES1 (11), and are the cellular loci of the noncanonical visual cycle (9, 10). Finally, the enzyme should show cooperativity with DES1 for conversion of all-*trans*-ROL to 11-*cis*-RP. We investigated MFAT as a candidate for this enzyme as a palm CoA-dependent retinyl-ester synthase (18) of unknown specificity and ocular expression.

We established that MFAT possesses the first of these properties by demonstrating much higher catalytic efficiency for 11-*cis*-ROL–dependent synthesis of 11-*cis*-RP versus conversion of other retinol isomers to their cognate esters (Fig. 2). These observations establish that MFAT is highly specific as an 11-*cis*-RE-synthase.

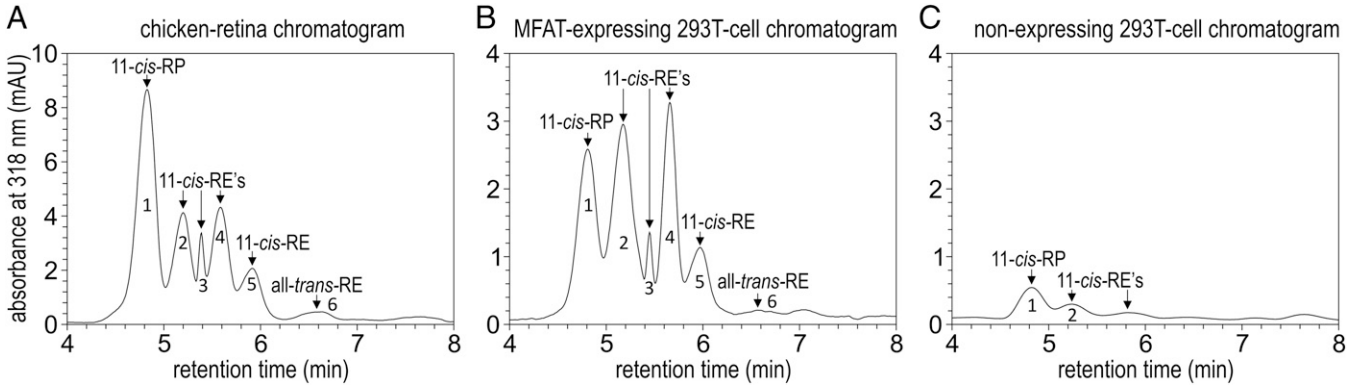

Fig. 4. Retinyl-ester synthesis from 11-*cis*-ROL by chicken retinas and 293T cells. (*A*) Chromatogram of retinyl esters were synthesized by chicken-retina homogenates using 11-*cis*-ROL as substrate and no exogenous palm CoA. The numbered peaks were identified by their elution times and spectra (Fig. S3). (*B*) Chromatogram of retinyl esters synthesized by MFAT-expressing 293T-cell homogenates. Note the similar profiles of 11-*cis*-RE fatty-acyl forms in *A* and *B*. (*C*) Chromatogram of retinyl esters synthesized by homogenates of 293T cells transfected with nonrecombinant pcDNA. Only peaks 1 and 2 coeluted with the 11-*cis*-RE peaks in *A* and *B*. This shows the background ARAT activity in 293T cells.

Both anti-human MFAT Abs-1 and MFAT Ab-2 detected bands of the appropriate size (40 kDa) on immunoblots of chicken and mouse retinas (Fig. S2*A*). We showed that MFAT is expressed in bovine and chicken Müller cells by quantitative RT-PCR (qRT-PCR) (Fig. S2*B*), and mouse Müller cells by immunocytochemistry on retina sections (Fig. 3). Despite the presence of MFAT in mouse Müller cells, 11-*cis*-REs are undetectable in mouse retinas (11). MFAT may be inactive as a retinyl-ester synthase or 11-*cis*-REs may be rapidly hydrolyzed in Müller cells of the rod-dominant mouse. Neither anti-human MFAT Ab-1 or MFAT Ab-2 yielded an immunofluorescent signal above background on chicken-retina sections, possibly due to epitope masking with the nondenatured protein. Immunocytochemistry on mouse-retina sections with MFAT and CRALBP antibodies showed colabeling of Müller endfeet, Müller-cell processes, and Müller-cell apical microvilli, which extend into the IPM. The IPM contains interphotoreceptor retinoid-binding protein (IRBP) (21) and is the site of retinoid exchange among photoreceptors, RPE, and Müller cells. Strong labeling of Müller-cell microvilli was also seen with DES1 (11). Low-level expression of MFAT in photoreceptor inner segments is also possible, although CRALBP is not expressed in photoreceptors (19). CRALBP is a water-soluble cytoplasmic protein (22), whereas MFAT is an integral-membrane protein associated with the endoplasmic reticulum (18). This observation may explain the partially overlapping distributions of MFAT and CRALBP in Müller cells (Fig. 3).

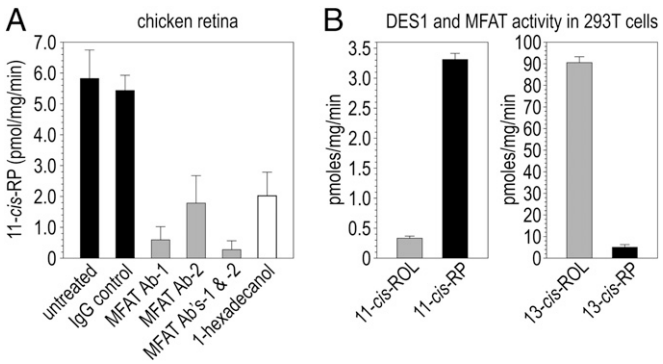

Fig. 5. Inhibition of DES1-coupled retinyl-ester synthase activities in chicken-retina and MFAT-expressing 293T-cell homogenates. (*A*) all-*trans*-ROL–dependent synthesis of 11-*cis*-RP by chicken-retina homogenates in the presence or absence of MFAT inhibitors. IgG control, homogenates were preincubated with 10 μg of donkey anti-mouse IgG; MFAT Ab-1, homogenates were preincubated with 10 μg of MFAT Ab-1; MFAT Ab-2, homogenates were preincubated with 10 μg of MFAT Ab-2; MFAT Ab's-1 & -2, homogenates were preincubated with 5 μg each of MFAT Ab-1 and MFAT Ab-2; 1-hexadecanol, homogenates were preincubated with 200 μM 1-hexadecanol; untreated, chicken-retina homogenates were preincubated with buffer only. (*B*) Synthesis of 11-*cis*-retinoids and 13-*cis*-retinoids from all-*trans*-ROL by 293T cells expressing both DES1 and MFAT. Rates of retinoid synthesis are shown in picomoles per minute per milligram of total protein after subtracting retinoids synthesized by 293T cells not expressing MFAT (n = 3). Error bars show SEM.

The addition of 11-*cis*-ROL substrate to chicken-retina or MFAT-expressing 293T-cell homogenates resulted in virtually exclusive synthesis of 11-*cis*-REs (Fig. 4 *A* and *B*). These experiments were performed with no added palm CoA, hence endogenous fatty-acyl CoAs in the homogenates served as acyl donors. Two factors determine which fatty acids are incorporated into retinyl esters: the fatty-acid preference of the ester synthase and the relative abundance of activated fatty acids in the reaction mixture. Given the phylogenetic and cell-type divergence of chicken retinas versus 293T cells, the relative concentrations of the various fatty-acyl CoAs in these two homogenates are likely different. The strikingly similar "fatty-acid fingerprints" of 11-*cis*-REs synthesized by chicken-retina and MFAT-expressing 293T-cell homogenates (Fig. 4 *A* and *B*) suggest that the 11-*cis*-RE-synthase in chicken retinas (13, 16, 17) is MFAT.

Preincubating with anti-MFAT Ab-1 dramatically inhibited the conversion of all-*trans*-ROL to 11-*cis*-RP by chicken-retina homogenates (Fig. 5*A*). Similarly, anti-MFAT Ab-1 inhibited the conversion of 11-*cis*-ROL to 11-*cis*-RP by MFAT-expressing 293T-cell homogenates (Fig. S4), confirming the inhibitory effect of this antibody on the 11-*cis*-RE-synthase activity of MFAT. The alternative acyl-receptor substrate for MFAT, 1-hexadecanol, also inhibited all-*trans*-ROL-dependent synthesis of 11-*cis*-RP by chicken retina homogenates (Fig. 5*A*) and the 11-*cis*-RE-synthase activity of MFAT-expressing 293T-cell homogenates (Fig. S4). These results suggest that MFAT accounts for much of the 11-*cis*-RE-synthase activity observed in chicken retinas (13, 16, 17).

To test for cooperativity between MFAT and DES1, we co-expressed both proteins in 293T cells and determined the rates of retinol and retinyl-ester synthesis from all-*trans*-ROL. DES1-expressing 293T cell homogenates synthesized 13-*cis*-ROL at 280-fold excess over 11-*cis*-ROL (Fig. 5*B*). This ratio is similar to the 230-fold excess of 13-*cis*-RAL over 11-*cis*-RAL in an iodine-catalyzed equilibrium reaction (14), consistent with DES1 catalyzing equilibrium isomerization of retinol (11). Despite the great preponderance of 13-*cis*-ROL versus 11-*cis*-ROL synthesized by

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these cells, the rates of 13-*cis*-RP and 11-*cis*-RP synthesis were similar. These results are in agreement with the kinetic data that showed much higher catalytic efficiency for 11-*cis*-RE versus 13-*cis*-RE synthesis by MFAT (Fig. 2 *A* and *C*). The relative ratios of 11-*cis*- and 13-*cis*-retinoids synthesized by cells expressing DES1 and MFAT (Fig. 5*C*) were similar to the ratios observed with chicken retina homogenates (Fig. 1*C*). Together, these results suggest that DES1 and MFAT act cooperatively to synthesize 11-*cis*-RP from all-*trans*-ROL. Thus, MFAT exhibits the three predicted properties of the retinyl-ester synthase responsible for synthesis of 11-*cis*-REs from all-*trans*-ROL by chicken retinas: (*i*) 11-*cis*-ROL substrate specificity; (*ii*) expression in Müller cells; and (*iii*) cooperativity with DES1.

How might synthesis of 11-*cis*-REs from all-*trans*-ROL by DES1 and MFAT facilitate regeneration of cone-opsin pigments? A model for the noncanonical visual cycle is shown in Fig. 6. In light, photobleaching of rhodopsin and cone-opsin pigments cause photoreceptors to release all-*trans*-ROL into the IPM, which is taken up by Müller cells. DES1 catalyzes rapid isomerization of all-*trans*-ROL to its *cis* isomers at ratios strongly favoring 13-*cis*-ROL over 11-*cis*-ROL. Further, because the DES1 reaction is reversible (11), newly synthesized 11-*cis*-ROL is susceptible to reisomerization. Esterification by MFAT counters this susceptibility by converting the 11-*cis*-ROL to a water-insoluble 11-*cis*-RE.

Once formed in Müller cells, how are 11-*cis*-REs mobilized for synthesis of visual chromophore by cones? CRALBP binds 11-*cis*-ROL and 11-*cis*-RAL with nanomolar affinities, but has low affinity for all-*trans* and 13-*cis*-retinoids (23, 24). CRALBP was shown to coimmunoprecipitate with DES1 (11), suggesting an interaction between these proteins. Because the K_d for 11-*cis*-ROL binding to CRALBP (23, 24) is much lower than the K_M of DES1 for 11-*cis*-ROL (11) (nanomolar versus micromolar), 11-*cis*-ROL bound to CRALBP is protected from reisomerization by DES1. Interestingly, apo-CRALBP was shown to stimulate hydrolysis of 11-*cis*-REs, whereas holo-CRALBP inhibited 11-*cis*-RE-hydrolysis (25) (Fig. 6). Thus, 11-*cis*-REs are only hydrolyzed when apo-CRALBP is available to bind the resulting 11-*cis*-ROL and prevent its reisomerization by DES1. The protein responsible for this 11-*cis*-REH activity has not been identified. Finally, CRALBP was shown to release its retinoid ligands upon association with acidic phospholipids on the inner plasma membrane (26). Together, these findings suggest a mechanism for the release by Müller cells of 11-*cis*-ROL into the IPM for delivery to cones (Fig. 6). Cones, but not rods, possess an 11-*cis*-ROL-dehydrogenase (11-*cis*-RDH) activity that oxidizes 11-*cis*-ROL to 11-*cis*-RAL. The noncanonical visual cycle therefore converts all-*trans*-ROL from photobleached rods and cones to visual chromophore for exclusive regeneration of cone opsins.

RPE cells use two enzymes to effect conversion of all-*trans*-ROL to 11-*cis*-ROL, LRAT, and Rpe65. LRAT transfers a fatty acid from the *sn*-1 fatty acid of PC to retinol (6, 7), yielding lyso-PC and an all-*trans*-RE. Rpe65 uses the all-*trans*-RE as substrate, harnessing the energy of ester hydrolysis to drive all-*trans* to 11-*cis*- isomerization (27). Resynthesis of PC from lyso-PC following deacylation by LRAT involves consumption of an activated fatty acid such as palm CoA. Thus, the energy of retinoid isomerization in the canonical pathway comes from hydrolysis of the thioester in palm CoA. In the noncanonical pathway, isomerization of retinol by DES1 involves no net energy input. Instead, 11-*cis*-ROL is secondarily esterified by MFAT, which uses an activated fatty acid such as palm CoA as the acyl donor (Fig. 6). Here again, the energy of all-*trans* to 11-*cis*-isomerization comes from hydrolysis of palm CoA. The difference is that energy input and ester synthesis are front loaded in the RPE pathway, whereas energy input occurs after isomerization in Müller cells.

Selective synthesis of 11-*cis*-REs followed by CRALBP-regulated ester hydrolysis is probably not the only mechanism of mass action driving chromophore synthesis in Müller cells. Other possibilities include selective oxidation of 11-*cis*-ROL to 11-*cis*-RAL by an 11-*cis*-ROL-dehydrogenase (11-*cis*-RDH) in cones (13) (Fig. 5) and the strongly favored recombination of 11-*cis*-RAL with apo-opsin to form a new visual pigment. The high membrane permeability of retinol (28) permits mass action to operate between nearby cells. In the latter case, the energy of isomerization comes from the photon absorbed by the visual pigment.

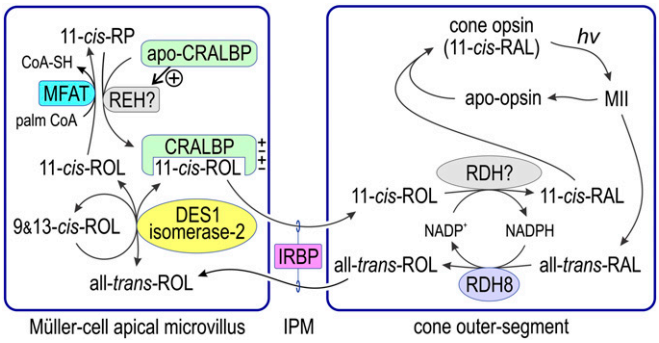

Fig. 6. Noncanonical visual cycle in Müller cells. Cone opsins use 11-*cis*-RAL as visual chromophore. Absorption of a photon by a cone opsin isomerizes the 11-*cis*-RAL to all-*trans*-RAL, as in rods. After reduction by RDH8 in the cone OS, the all-*trans*-ROL is released into the IPM and taken up by a Müller cell. Here, the all-*trans*-ROL is isomerized by DES1 to 11-*cis*-ROL, 9-*cis*-ROL, and 13-*cis*-ROL. The 11-*cis*-ROL binds to CRALBP or is fatty acylated by MFAT to yield an 11-*cis*-RE. In either event, 11-*cis*-ROL is removed from the equilibrium reaction. The other retinol isomers are reisomerized by DES1. An as-yet unidentified 11-*cis*-retinyl-ester hydrolase (REH?) hydrolyzes 11-*cis*-RP to yield 11-*cis*-ROL and palmitic acid. The 11-*cis*-REH activity was shown to be activated by apo-CRALBP and inhibited by holo-CRALBP (25). Interaction with negatively charged phospholipids on the plasma membrane causes holo-CRALBP to release its 11-*cis*-ROL ligand into the IPM, where it binds IRBP and, subsequently, is taken up by a cone outer segment (26). Binding to CRALBP provides a mechanism to protect 11-*cis*-ROL from reverse isomerization by DES1. In the cone OS, 11-*cis*-ROL is oxidized by an unknown 11-*cis*-ROL dehydrogenase (RDH?) to 11-*cis*-RAL, which combines with apo-opsin to form a new opsin pigment. Simultaneous reduction of all-*trans*-RAL and oxidation of 11-*cis*-ROL in the cone OS provides a self-renewing supply of NADPH/$NADP^+$ cofactors.

Materials and Methods

ARAT Assays on Chicken Retina and Transfected HEK 293T-Cell Homogenates. Heads from freshly killed, nonscalded adult chickens were delivered from Al Salam Polleria in the dark and on ice within 2 h after slaughter. Dissected chicken retinas were treated with white light at ~800 lx for 30 min to bleach endogenous retinoids. Assay conditions were as reported (8, 16). The assays were performed in 500-μL reactions containing 40 mM Tris-base at pH 8.0, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM DTT, with 1.0 mg/mL protein homogenates and 2% (vol/vol) BSA. After preincubating the protein samples at 37 °C for 5 min, all-*trans*-ROL and palm CoA were added (10 μM and 150 μM final concentrations, respectively) as substrates. The samples were mixed briefly and incubated for 15 min at 37 °C. Homogenates of chicken retinas and HEK 293T cells transiently expressing human MFAT were prepared and assayed as described above with modifications. After preincubating at 37 °C for 5 min, 11-*cis*-ROL was added (50 μM final concentration) and the samples were incubated with gentle agitation for 15 min at 37 °C. For the chemical and antibody inhibitor studies, the chicken retina homogenates were preincubated for 30 min at 4 °C with 200 μM 1-hexadecanol (Sigma) or 10 μg of IgG. The anti-human MFAT Abs were Ab 1 (Santa Cruz Biotechnology; SC-161526) or Ab 2 (Abcam; Ab83182). The control IgG sample was donkey anti-mouse IgG (Licor Bioscience). For the no inhibitor control, buffer alone was added to the preincubation mixtures. After incubation, the assay reactions were quenched by adding 2 mL of ice-cold methanol. Retinoid extraction is described in *SI Materials and Methods*.

MFAT Kinetic Analysis. HEK-293T cells were grown in DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS and antibiotics (100 U/mL of penicillin G and 100 μg/mL of streptomycin) at 37 °C in 5% CO_2. HEK-293T cells were transfected (PolyFect; Qiagen) with nonrecombinant pcDNA3.1

(Life Tech) or pcDNA-MFAT containing the human MFAT cDNA. After 36–42 h, the cells were suspended in media by gentle pipetting and pelleted by centrifugation at 1,000 × g for 5 min. The pellets were resuspended in PBS and repelleted. The resulting washed cell pellets were flash frozen in liquid nitrogen and stored at −80 °C until use. Assays were performed in 500-μL reactions containing 0.2 mg/mL cell homogenate, 0.5% BSA, 500 μM palm CoA, and 0, 4, 8, 16, 32, 64, 96, or 128 μM all-*trans*-ROL, 9-*cis*-ROL, 11-*cis*-ROL, or 13-*cis*-ROL substrates, as indicated. Assays were incubated for 5 min at 37 °C in 2-mL glass vials with gentle agitation. The reactions were quenched by adding ice-cold methanol, retinoids were extracted, and retinyl esters were quantitated by HPLC, as described in *SI Materials and Methods*. Data were fitted to the Michaelis–Menten equation to determine V_{max} and K_M for each isomer by using the Enzyme Kinetics Module 1.3 for SigmaPlot version 10.

MFAT Immunoblotting and Immunofluorescence in Mouse Tissues. Mouse studies were done in compliance with guidelines established by the University of California, Los Angeles, Animal Research Committee and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Protein sample preparation for immunoblot is described in *SI Materials and Methods*. Forty-microgram aliquots of mouse retina and RPE homogenate were loaded onto 12% Novex NuPAGE bis-Tris gels (Invitrogen), run with Novex NuPAGE Mops-SDS buffer (Invitrogen), and transferred to Immobilon-FL PVDF membranes (EMD-Millipore) by using the Bio-Rad semidry transfer system. Blots were subsequently probed with anti-human MFAT Ab-1 (Santa Cruz Biotechnology; SC-161526) or MFAT Ab-2 (Abcam; Ab83182) in LI-COR Blocking Buffer with 0.5% donkey serum and 0.1% Tween 20 (Sigma). Secondary antibody staining and imaging were performed with LI-COR fluorescent-tagged antibodies (LI-COR) and the LI-COR Odyssey infrared imaging system. Immunofluorescence of MFAT and CRALBP in mouse retina is described in *SI Materials and Methods*.

Bovine and Chicken Müller Cell Gene Expression Study. Müller-cell primary cultures were prepared as described in *SI Materials and Methods*. RNA from 6-wk-old primary cultured bovine or chicken Müller cells was prepared by using the Absolutely RNA Nanoprep Kit (Agilent). Synthesis of first-strand cDNA from isolated RNA was carried out by using the SuperScript III First-Strand Synthesis Supermix (Invitrogen). qRT-PCRs were carried out in the CFX96 Real Time C1000 Touch Thermal Cycler (BioRad) for 35 cycles under the conditions: 94 °C for 20 s; 56 °C for 30 s; and 72 °C for 40 s, using iTaq Universal SYBR Green Supermix (BioRad). The primers for each gene are listed in *SI Materials and Methods*.

Enzyme Assays Testing for MFAT and DES1 Cooperativity. HEK-293T cells stably expressing human MFAT (293T M cells) were generated by following procedures in the pcDNA3.1 Vector Manual using Geneticin (Invitrogen) antibiotic for selection. HEK-293T and 293T-M cells were transiently transfected with plasmid containing the chicken DES1 cDNA (11) or nonrecombinant pcDNA3.1 (100 mm plate, 8-μg plasmid, PolyFect; Qiagen). After 36 h, cells were collected, homogenized, and incubated in reaction mixtures containing 10 μM all-*trans*-ROL, 500 μM palm CoA, 500 μM NADPH, 1% BSA, and 1 mM DTT in 40 mM Tris at pH 7.2 for 15 min at 37 °C. After quenching, retinoids were extracted with hexane and analyzed by HPLC as described in *SI Materials and Methods*.

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