Supplemental Information for:

Retinal Bioavailability and Functional Effects of a Synthetic Very-Long-Chain Polyunsaturated Fatty Acid in Mice

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Supplemental Methods

Chemical synthesis of 32:6 n-3: (see **Figure 1a** for additional details). To a 0.2 M solution of DHA in dry THF at 0 °C was added $LiAlH_4$ (4.1 equivalents relative to DHA). The resulting solution was stirred at 0 °C until the reaction was judged complete by thin layer chromatography (ca. 2 hr). The reaction was quenched with saturated aqueous sodium sulfate (Na_2SO_4) (0.004 equivalents relative to DHA). The resulting mixture was warmed to room temperature (rt) and stirred for an additional 1 hr. The solution was filtered through celite, and the solvent was removed under reduced pressure to give DHA alcohol in 97% yield that was used in the subsequent oxidation reaction without additional purification.

To a 0.5 M solution of DMSO (8.1 equivalents relative to DHA alcohol) in CH_2Cl_2 at -78 °C was slowly added oxalyl chloride (8.7 equivalents relative to DHA alcohol). After the resulting mixture had stirred for 0.5 hr, a 0.5 M solution of DHA alcohol in CH_2Cl_2 was then added slowly. The resulting mixture was stirred for an additional 2 hr. To this was slowly added a 6.4 M solution of triethyl amine (17 equivalents relative to DHA alcohol) in CH_2Cl_2 over a 1 hr time period. Once the addition was completed, the solution was stirred at -78 °C for an additional 1 hr and then warmed to rt. The reaction was quenched by adding water (equivalent volume to CH_2Cl_2). The layers were separated, and the aqueous phase was extracted three times with CH_2Cl_2. The combined organic layers were washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure. The product was purified by flash column chromatography using silica gel that had been neutralized using 2% triethylamine. The eluent for the purification consisted of a mixture of ethyl acetate and hexanes giving 22:6 n-3 aldehyde in 84% yield.

To a 0.2 M solution of 10-bromodecanol in CH_2Cl_2 at 0 °C was added dihydropyran (1.6 equivalents relative to 10-bromodecanol) and *p*-toluene sulfonic acid monohydrate (0.1 equivalents relative to 10-bromodecanol). The solution was warmed to RT and stirred overnight, after which the reaction was quenched with water (equivalent volume to CH_2Cl_2). After separation,

the aqueous phase was extracted three times with CH_2Cl_2. The organic layers were combined and dried (Na_2SO_4). The solvent was removed under reduced pressure, and the resulting residue was purified via flash column chromatography using a mixture of ethyl acetate and hexanes to give 2-((10-bromodecyl)oxy)tetrahydro-*2H*-pyran in 89% yield.

Magnesium turnings were subjected to 1 M HCl for 5-10 min, after which the turnings were washed three times with water, three times with MeOH, three times with acetone, and dried in an oven at 120 °C overnight. These turnings (2 equivalents relative to the bromide from above) were added to a round-bottom flask under N_2. To this was added a 0.5 M solution of the bromide from above in THF along with a catalytic amount of dibromoethane. The reaction was allowed to stir at rt for 2 hr. Formation of the Grignard was monitored by ^{1}H NMR for the disappearance of the CH_2 signal at 3.40 ppm. The reaction mixture was then cooled to 0 °C, and DHA aldehyde (0.83 equivalents relative to bromide) was added slowly. The reaction mixture was warmed to rt and stirred for an additional 2 hr, after which the reaction was quenched with the addition of saturated aqueous ammonium chloride (equivalent volume to THF). The phases were separated, and the aqueous layer was extracted three times with ether. The extracts were combined, dried (Na_2SO_4), and concentrated under reduced pressure. The resulting residue was purified via flash column chromatography using a mixture of ethyl acetate and hexanes to give 32:6 n-3 2° alcohol in 73% yield.

To a 0.08 M solution of 32:6 n-3 2° alcohol in CH_2Cl_2 at 0 °C was sequentially added triethyl amine (10 equivalents relative to the 2° alcohol) followed by the slow addition of methyl sulfonyl chloride (3 equivalents relative to the 2° alcohol). The reaction mixture was warmed to rt immediately after the addition was complete. The mixture was stirred for an additional 2 hr before the reaction was quenched with water (equivalent volume to CH_2Cl_2). The phases were separated, and the aqueous phase was extracted three times with CH_2Cl_2. The combined organic extracts were washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure. Purification via

flash column chromatography using a mixture of ethyl acetate and hexanes gave 32:6 n-3 2° mesylate in 90% yield.

To a 0.03 M solution of the mesylate from above in THF at 0 °C was added lithium aluminum hydride (1.3 equivalents relative to the mesylate). The resulting mixture was warmed to rt and stirred at that temperature for 8 hr, at which point the reaction mixture was cooled to 0 °C. A saturated aqueous solution of Na_2SO_4 (0.004 equivalents relative to the mesylate) was added to the reaction mixture to quench the reaction. After the mixture had stirred for an additional 1 hr, the slurry was filtered through celite, and the solution was concentrated. The resulting thick pale yellow oil consisting of 32:6 n-3 THP acetal was used in the subsequent reaction without additional purification.

To a 0.05 M solution of 32:6 n-3 THP acetal in a 1:1 (v/v) mixture of MeOH and THF at rt was added p-toluene sulfonic acid monohydrate (0.1 equivalents relative to the THP acetal). After the reaction mixture had stirred overnight, it was concentrated. The resulting residue was immediately purified by flash column chromatography using a mixture of ethyl acetate and hexanes to give 32:6 n-3 1° alcohol in 75% yield for the two steps.

To a 0.5 M solution of DMSO (8.1 equivalents relative 32:6 n-3 1° alcohol from above) in CH_2Cl_2 at -78 °C was slowly added oxalyl chloride (8.7 equivalents relative to the 1° alcohol). After the resulting mixture had stirred for 0.5 hr, a 0.5 M solution of the 1° alcohol in CH_2Cl_2 was then added slowly. The resulting mixture was stirred for an additional 2 hr. To this was slowly added a 6.4 M solution of triethyl amine (17 equivalents relative to 1° alcohol) in CH_2Cl_2 over a 1 hr time period. Once the addition was complete, the solution was stirred at -78 °C for an additional 1 hr and then warmed to rt. The reaction was quenched by adding water (equivalent volume to CH_2Cl_2). The layers were separated, and the aqueous phase was extracted three times with CH_2Cl_2. The combined organic layers were washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure. The product was purified by flash column chromatography using silica gel that

had been neutralized using 2% triethylamine. The eluent for the purification consisted of a mixture of ethyl acetate and hexanes, giving 32:6 n-3 aldehyde in 91% yield after concentration.

To a 0.1 M solution of the aldehyde from above in dimethylformamide at rt was added oxone (1.1 equivalents relative to the aldehyde). The resulting mixture was stirred for 3 hr and then filtered through a short plug of silica gel. Concentration and purification using flash column chromatography with a mixture of ethyl acetate and hexanes as the eluent gave VLC-PUFA 32:6 n-3 in 49% yield as a thick pale yellow oil.

Western blot: Harvested mouse retinas were washed three times with PBS and homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 0.2 mM (phenylmethylsulfonyl fluoride) to prepare total protein extracts. Then, the protein samples were loaded and separated on 4–15% gradient SDS–PAGE and transferred to 0.45 μm nitrocellulose membranes. After blocking with 5% nonfat milk for 1 hour, the membrane was washed with Tris buffered saline three times and incubated with the primary antibody, anti-Elovl4 (1:1500 dilution of 55023-1-AP, Proteintech Group, Inc., Rosemont, IL, USA), overnight at 4 ºC. Following two changes of buffer, the membrane was incubated with the secondary antibody, Goat anti-Rabbit IgG (H+L) (1:2000 dilution of 20029, Alpha Diagnostic Intl, Inc., San Antonio, Texas), for 2 hr at room temperature. To visualize protein bands, the membrane was developed using ECL Plus Western blot detection reagents (Amersham Biosciences, Pittsburgh, PA).

LC-MS/MS: Briefly, we extracted the lipids in dicholoromethane: methanol (2:1 v/v), water (500 μL) was added to separate the phases, and the sample was centrifuged at 5000 *g* for 10 min. The lower phase was collected and washed with water to remove the residues and dried under nitrogen. The dried samples were resuspended in 500 μL IPA:ACN:H_2O (10:10:1 *v/v/v*) and analyzed on an LC-MS/MS system. Lipid extracts are separated on a Waters Acquity BEH HILIC 1.7 μm, 2.1 x 100 mm column maintained at 40°C connected to an Agilent HiP 1290 Sampler, an Agilent 1290 Infinity pump, and an Agilent 6490 triple quadrupole (QqQ) mass spectrometer

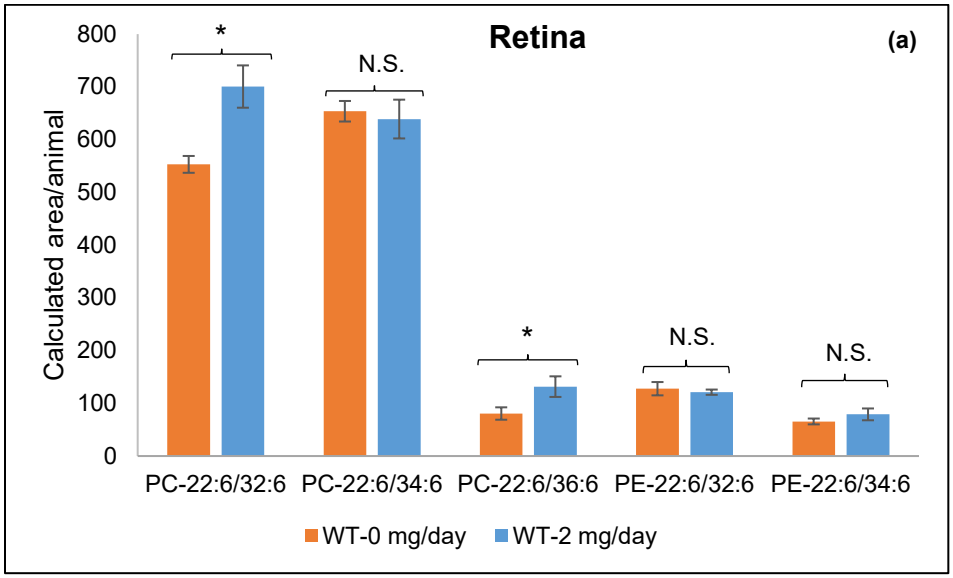
(1). Lipids were detected using dynamic multiple reaction monitoring (dMRM) in negative ion mode. Source gas temperature was set to 210°C, with a N_2 gas flow of 11 L/min and a nebulizer pressure of 35 psi. Sheath gas temperature is 400°C, sheath N_2 gas flow was 12 L/min, capillary voltage was 4000 V, nozzle voltage was 500 V, high pressure RF was 90 V, and low pressure RF was 60 V. Mobile phase A consisted of H_2O in 7 mM ammonium acetate, and mobile phase B consisted of ACN:H_2O (96:4 *v/v*) in 7 mM ammonium acetate. The chromatography gradient started at 100% mobile phase B and decreased to 84% B over 10 min. Post-time was 9 min, and the flowrate was 0.4 mL/min throughout. Needle wash was isopropyl alcohol containing 1.0% cyclohexane. Collision energies and cell accelerator voltages were optimized using lipid standards. Lipids without available standards were identified based on HR-LC/MS, quasi-molecular ion, and characteristic product ions. Retention times were either taken from HR-LC/MS data or inferred from available lipid standards. Samples were injected in a randomized order with a 2 µL injection volume. Phosphatidylcholines were monitored by the precursor to product ion transitions of $[M+HCO2]^-$ → T1_$RCOO^-$ and T2_$RCOO^-$. Phosphatidylethanolamines were monitored by the precursor to product ion transitions of $[M-H]^-$ → T1_$RCOO^-$ and T2_$RCOO^-$. Results from LC-MS experiments were collected using an Agilent Mass Hunter Workstation and analyzed using the software package Agilent Mass Hunter Quant B.07.00.

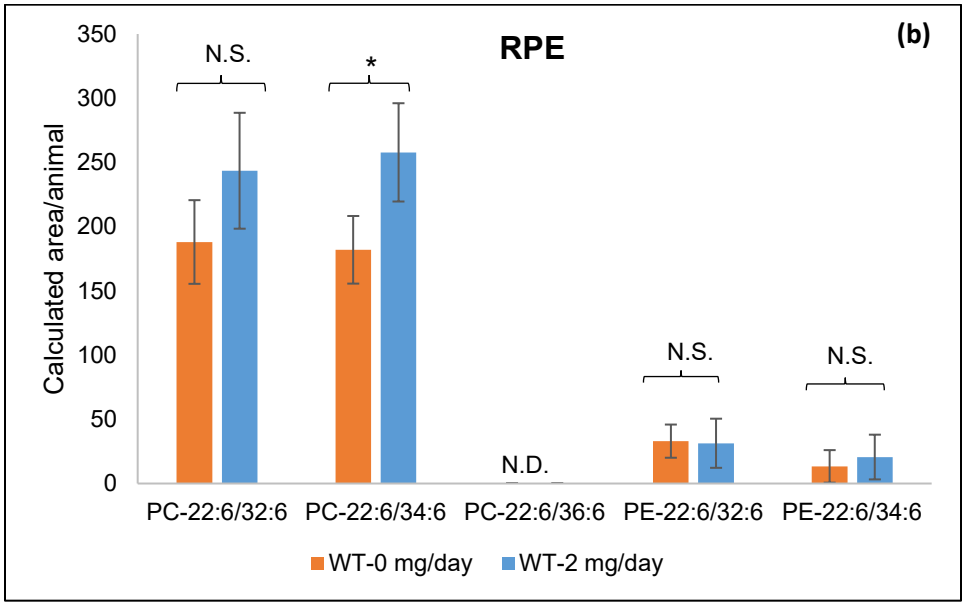
Optical coherence tomography (OCT)/fundus imaging: Mice were anesthetized with ketamine/xylazine (90/10 mg/kg BW). OCT images were captured using a Phoenix Micron IV image-guided OCT system (Pleasanton, CA, USA) (2, 3). In each eye, three OCT images were averaged to generate mean retinal thickness and outer nuclear layer (ONL) thickness. InSight software was used for sectioning individual retinal layers and average ONL thicknesses with n=3/group.

Histology: Eyecups were prepared for cryosectioning as previously described (4). Briefly, eyecups were placed in 15% sucrose for three hours, 30% sucrose overnight at 4ºC, and

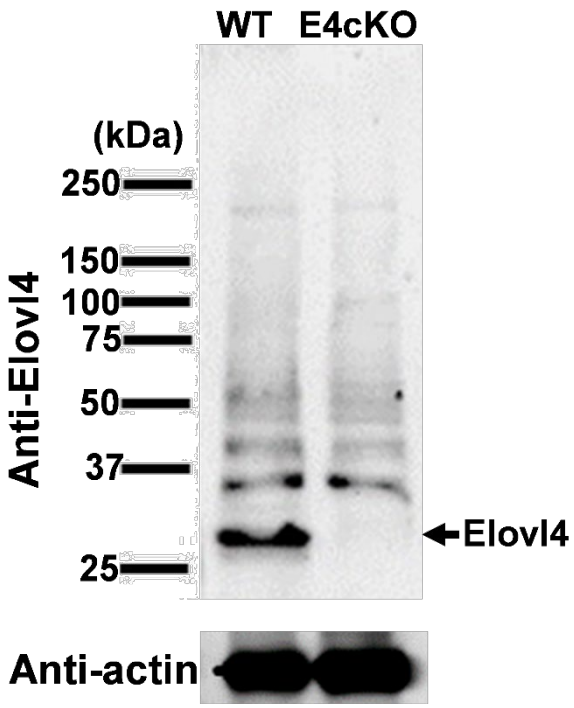
embedded in 2:1 *v/v* Optimal Cutting Temperature Compound (OCT, Sakura Finetek USA, Inc., Torrance, CA, USA):30% sucrose over dry ice. Frozen embedded eyecups were sectioned with a cryostat (Thermo Fisher Scientific, Cheshire, England) at 12 µm. Sections were placed onto charged microscope slides and stored at -20ºC. Sections were washed once with 1X PBS and blocked for one hour with 0.1% Triton X-100 blocking solution at room temperature. The permeabilized sections were mounted in DAPI Fluoromount-G (SouthernBiotech, Birmingham AL, USA). Cryosections of retina were imaged at 20X on a BZ-X810 fluorescent microscope (Keyence, Itaska, IL, USA). Images were imported into FIJI software version 1.8 (NIH, Bethesda, MD, USA), and outer nuclear layer (ONL) thickness and total retinal thickness measurements were performed as previously described (5).

Supplemental Figures

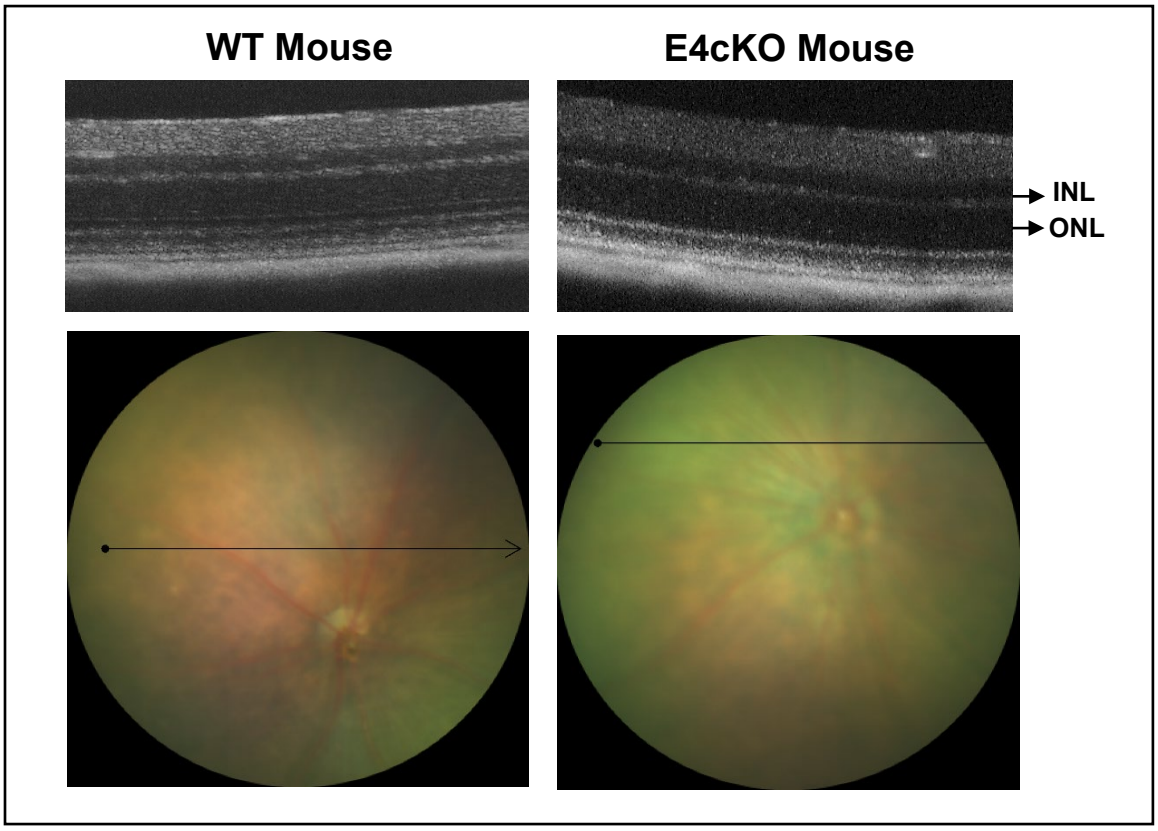




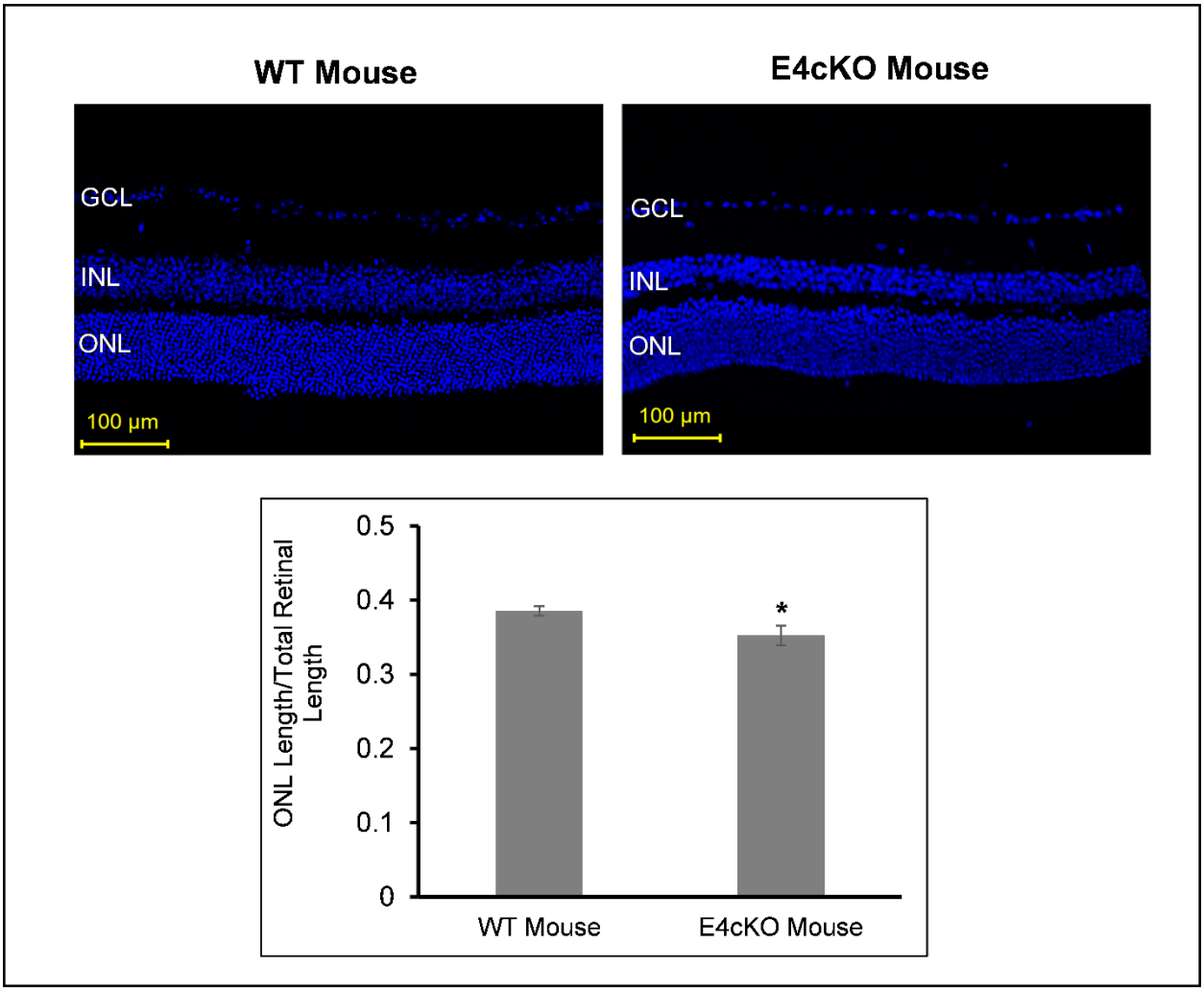
Supplemental Figure S1: Phospholipid analysis of wild type (WT) mice after 15 days of gavage feeding of VLC-PUFA 32:6 n-3 (2 mg/day). **(a)** Retina. **(b)** RPE. Data presented as mean ± SEM (n=8 mice/group) (p values: *p<0.05; N.S.: not significant; N.D.: not detectable).



Supplemental Figure S2: Western blotting results to confirm the absence of Elovl4 protein expression in an *Elovl4* rod-cone conditional KO (E4cKO) mouse compared to a wild type (WT) mouse at 8 months of age.



Supplemental Figure S3: Optical coherence tomography (OCT) and color fundus images of 8-month-old wild type (WT) and *Elovl4* rod-cone conditional KO (E4cKO) mice. INL- inner nuclear layer; ONL-outer nuclear layer.



Supplemental Figure S4: Histological analysis of an *Elovl4* rod-cone conditional KO (E4cKO) retina at 8 months of age in comparison to an age-matched wild type (WT) retina. Representative images of DAPI-stained cryosections (top) and quantification of ONL/total retinal thickness per 100 µm in mid-peripheral retina (bottom). Data are expressed as mean ± SEM for n=20 measurements (*p<0.05). GCL- ganglion cell layer; INL- inner nuclear layer; ONL-outer nuclear layer.

Supplemental References

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