Published in final edited form as:

FEBS Lett. 2020 February ; 594(3): 540–552. doi:10.1002/1873-3468.13633.

Impacts of deletion and ichthyosis prematurity syndrome - associated mutations in fatty acid transport protein 4 on the function of RPE65

Songhua Li[1], **John F. Green**[1,#], **Minghao Jin**[1,2,*]

[1]Neuroscience Center of Excellence Louisiana State University School of Medicine, New Orleans, LA 70112, USA

[2]Department of Ophthalmology, Louisiana State University School of Medicine, New Orleans, LA 70112, USA

Abstract

The retinal pigment epithelium-specific 65 kDa (RPE65) isomerase plays a pivotal role in photoreceptor survival and function. RPE65-catalyzed synthesis of 11-*cis*-retinol from all-*trans*-retinyl esters in the visual cycle is negatively regulated, through a heretofore unknown mechanism, by the fatty acid transport protein FATP4, mutations in which are associated with ichthyosis prematurity syndrome (IPS). Here, we analyzed the interaction between deletion mutants of FATP4 and RPE65 as well as the impacts of IPS-associated FATP4 mutations on RPE65 expression, 11-*cis*-retinol synthesis, and all-*trans*-retinyl ester synthesis. Our results suggest that the interaction between FATP4 and RPE65 contributes to inhibition of RPE65 function and that IPS-associated nonsense and missense mutations in FATP4 have different effects on the visual cycle.

Keywords

retinoid visual cycle; retinal pigment epithelium; photoreceptor; acyl-CoA synthetase; ichthyosis prematurity syndrome; Leber congenital amaurosis; lecithin:retinol acyltransferase

INTRODUCTION

Continuous regeneration of the 11-*cis*-retinal chromophore of rhodopsin and cone opsin visual pigments is essential for sustaining the visual function and survival of the rod and cone photoreceptors in the retina. RPE65, an endoplasmic reticulum (ER)-associated protein (1), is a key retinoid isomerase in the retinal pigment epithelium (RPE)-dependent visual cycle that produces 11-*cis*-retinal (2). It catalyzes the synthesis of 11-*cis*-retinol from all-

*Corresponding author: Minghao Jin, Neuroscience Center, LSU School of Medicine, 2020 Gravier St., New Orleans, LA 70112, USA, Phone: +1 504-568-2141, Fax: +1 504-599-0891, mjin@lsuhsc.edu.

#Present address: Department of Ophthalmology, University of Mississippi Medical Center, Jackson, MS 39216, USA

Author Contributions: SL and JFG performed experiments; SL and MJ designed experiments, analyzed data and wrote the manuscript; MJ conceived and supervised the study.

Conflict of Interests: None

trans-retinyl fatty acid esters, such as all-*trans*-retinyl palmitate, synthesized by lecithin:retinol acyltransferase (LRAT) and other enzymes in the RPE (3–8). RPE65 is also the isomerase enzyme responsible for the production of *meso*-zeaxanthin, one of the three macular pigments that function as potent antioxidants and light screening pigments to protect macular from blue light damage and reactive oxygen species (9,10). In agreement with the pivotal roles, mutations in the *RPE65* gene cause vision loss and photoreceptor degeneration in affected patients, canines and mice (11,12). In humans, over 100 different mutations in the *RPE65* gene have been linked to retinal degenerative diseases, such as Leber congenital amaurosis and retinitis pigmentosa (The Human Gene Mutation Database: http://www.hgmd.cf.ac.uk/ac/index.php).

Although RPE65 has such important functions, its higher expression levels and activities are associated with increase in both retinal susceptibility to light-induced degeneration (13,14) and the accumulation rates of the visual cycle-derived cytotoxic bisretinoids, the major autofluorescent components of lipofuscin implicated in Stargardt disease and geographic atrophy form of age-related macular degeneration (15–19). Conversely, elimination or reduction of RPE65 function through genetic or chemical approaches protected retinal photoreceptors from light-induced degeneration in rodent models (20–25). These facts suggest that defining the mechanisms that regulate RPE65 expression, stability and function may help the development of an effective precautionary measure to protect retinal photoreceptors from photodamages.

Through an unbiased screening of bovine RPE cDNA libraries, we have previously identified fatty acid transport protein 4 (FATP4), the most abundant FATP in the RPE (26), as a negative regulator of 11-*cis*-retinol synthesis catalyzed by RPE65 retinoid isomerase (26). FATP4 is a member of FATP family that facilitates transport of long-chain fatty acids across the cell membrane (27,28). Among the six members of the mammalian FATP family, FATP4 is the only FATP whose ER localization domain has been identified (29). FATP4 also has fatty acyl-CoA synthetase activity with specificity toward saturated and monounsaturated long-chain and very long-chain fatty acids (30,31). A spontaneous mutation in the mouse *Fatp4* gene resulted in neonatal death of the mutant mice (named wrinkle-free, *wrfr*) due to severe defects in the epidermal barrier formation and breathing difficulties secondary to their tight skin phenotype (32). Transgenic expression of FATP4 in the keratinocyte can prevent the neonatal death of the *wrfr* mutant mice (33). This rescued transgenic mouse line, which lacks FATP4 in the RPE, exhibits a significant increase in the retinoid isomerase activity, the visual cycle rate as well as susceptibility of rod and cone photoreceptors to light-induced degeneration (26). Although *in vitro* assays suggest that FATP4 may function as a mixed-type inhibitor of RPE65 (26), the mechanisms by which FATP4 inhibits 11-*cis*-retinol synthesis are still not clear.

Recently, several point mutations (A92T, C168X, S247P, and Q300R) in the human *FATP4* gene have been identified in patients with ichthyosis prematurity syndrome (IPS) (34,35), a recessive disorder characterized by premature birth, neonatal asphyxia, and thick caseous desquamating epidermis with severe ichthyotic scaling (36). Except for the C168X nonsense mutation, the missense mutations are mapped on the putative ER-localization domain (A92T) or the highly conserved ATP-dependent AMP-binding (S247P and Q300R) domain

of FATP4, respectively (34,35). Fibroblasts from a patient with the C168X nonsense mutation showed a significant reduction in the acyl-CoA synthesis and lipid-incorporation activities toward erucic acid, a monounsaturated omega-9 very long-chain fatty acid (22:1ω9) (34).

In the present study, we investigated the impacts of IPS-associated point mutations and a series of deletion mutations in FATP4 on RPE65 expression, 11-*cis*-retinol synthesis, all-*trans*-retinyl ester synthesis, and interaction of deletion mutants with RPE65. The results suggest that direct interaction between RPE65 and FATP4 is an important mechanism by which FATP4 inhibits the synthesis of 11-*cis*-retinol and that the IPS-associated nonsense and missense mutations have significantly different effects on the functions of the visual cycle enzymes.

MATERIALS AND METHODS

Plasmids and mutagenesis:

The pRK-based mammalian expression vectors for RPE65 (37) and FATP4 (26) were used to express these two wild-type proteins. Point and deletion mutations were introduced into the FATP4 expression plasmid using PCR with either the QuikChange II XL Site-directed Mutagenesis Kit (Agilent Inc.) or *PfuUltra* High-Fidelity DNA polymerase (Agilent Inc.). A C-terminally fused Flag epitope was also introduced into the wild-type and mutant FATP4 constructs using the PCR-based method. Primers used in the PCR are shown in Table 1. Following mutagenesis, sequences of the constructs were confirmed by DNA sequence analysis in both directions. Plasmid DNA for transfection was purified using the PureLink HiPure Plasmid DNA Purification Kit (Thermo Fisher Scientific Inc.).

Cell culture and transfection:

The 293T-C and 293T-LC cells stably expressing cellular retinaldehyde-binding protein (C) or lecithin:retinol acyltransferase and cellular retinaldehyde-binding protein (LC) (3) were maintained in the Dulbecco's modified Eagle's medium (Thermo Fisher Scientific Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin G and 100 μg/ml streptomycin) at 37°C under 5% CO_2. Transfection of the cells with plasmid DNA was performed using the PolyJet transfection reagent (SignaGen Laboratories) (38).

Retinoid isomerase and LRAT assays in living cells:

For time course analysis of 11-*cis*-retinol synthesis, 293T-LC cells transfected with pRK-RPE65 and pRK were incubated with 7 μM all-*trans*-retinol for 1~18 hours. To analyze the effects of wild-type and mutant FATP4 on the synthesis of 11-*cis*-retinol and all-*trans*-retinyl esters, 293T-LC cells co-transfected with pRK-RPE65 and pRK-FATP4 construct or pRK mock vector were incubated with 7 μM all-*trans*-retinol in the media for 4 hours (for retinoid isomerase assay) or 30~90 minutes (for LRAT assay). After removing the medium, the cells were washed in phosphate buffered saline (PBS), pelleted by centrifugation, and lysed in 0.5 ml of lysis buffer (0.1% SDS in 10 mM HEPES buffer, pH 7.2). Ten to twenty microgram proteins of the cell homogenates were subjected to immunoblot analysis. Activities of

RPE65 and LRAT were determined by monitoring the synthesis of 11-*cis*-retinol or all-*trans*-retinyl esters, respectively. To detect 11-*cis*-retinol, retinoids in the cells were saponified with potassium hydroxide as described previously (3).

In vitro retinoid isomerase assay:

Homogenates (containing 0.5–1 mg proteins) of 293T-C cells co-transfected with pRK-RPE65 and mutant or wild-type FATP4 construct were incubated with 10 μM all-*trans*-retinyl palmitate in 20 mM HEPES buffer (pH 7.2) containing 100 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 20 μM leupeptin, 6 mM sodium cholate and 6% bovine serum albumin (BSA) for 2 hours. After quenching the reaction by adding 2 volumes of methanol, retinoids were extracted with hexane and analyzed by high-performance liquid chromatography (HPLC).

HPLC analysis of retinoids:

Retinoids were analyzed by normal-phase HPLC as previously described (39). In brief, retinoids in hexane extractions were evaporated, re-dissolved in 100 μl of hexane, and separated on a silica column (Zorbax-Sil 5 μm, 250 × 4.6 mm, Agilent Technologies) by gradient (0.2–10% dioxane in hexane at 2.0 ml/min flow rate) or non-gradient (10% dioxane in hexane at 1.0 ml/min flow rate) elution of mobile phase in an Agilent 1100 HPLC system.

Immunoblot analysis:

Protein concentrations in the cell homogenates were determined with the micro BCA protein assay kit (Thermo Fisher Scientific Inc.). Ten to twenty micrograms of proteins in Laemmli buffer were denatured at 70°C for 10 min, separated in a 10% or 12% polyacrylamide gel by SDS-PAGE, and then transferred onto an Immobilon-P membrane (MilliporeSigma Co.). The membrane was incubated with blocking buffer, a primary antibody against RPE65 (40) or Flag (Cell Signaling Technology, Cat# 2368), and a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc.). Before and after incubating with the antibodies, the membrane was washed in 0.1% Tween-20/PBS. Immunoblots were visualized with the ECL Prime Western blotting detection reagent and ImageQuant LAS 4000 (41). Signal intensity of each band was quantified using ImageQuant TL software.

Immunoprecipitation:

Homogenates of 293T-LC cells expressing RPE65 and mutant FATP4-Flag fusion proteins were incubated with anti-Flag M2 affinity gel (MilliporeSigma Co., Cat# A2220) in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 and protease inhibitors for 4 hours, then precipitated and washed as described previously (42). The precipitated proteins were analyzed by immunoblot analysis using antibodies against RPE65 or Flag epitope.

Immunocytochemistry:

Cells grown on a glass coverslip were transfected with plasmid DNA, fixed with 4% paraformaldehyde and incubated with 0.2% Triton X-100 in PBS for 15 minutes. After blocking with 10% FBS and 2% goat serum in PBS for 1 hour, the cells were incubated with

an antibody against RPE65 (MilliporeSigma Co., Cat# mAB5428) or Flag epitope and then with Alexa Fluor 488 or 555 conjugated antibody as described previously (43). Before and after incubating with antibodies, the cells were washed with 0.1% Tween-20/PBS three times. Images were captured with a Zeiss LSM710 Meta confocal microscope with a 40X oil-immersion objective (44).

Statistical analysis:

Data were expressed as means ± standard deviation (SD) from at least three experiments. Statistical significance was determined with a paired, two-tailed Students *t*-test. *P* values less than 0.05 were considered to be statistically significant. All analyses were done using SigmaPlot version 11 software (Systat Software, Inc.).

RESULTS

FATP4 mutant containing the ER-association, AMP-binding and FATP domains strongly bound with RPE65

To know what regions of FATP4 are important for interacting with and/or inhibiting RPE65, we generated a series of deletion mutants of FATP4 with a C-terminally fused Flag epitope tag (Fig. 1A,B). We co-transfected the 293T-LC cells with RPE65 and the FATP4 deletion mutants or pRK mock vector. Immunoblot analysis of the cell lysates confirmed that RPE65 is strongly expressed in all transfected cells (Fig. 1C, upper panel). We then performed immunoprecipitation with an anti-Flag antibody. As shown in Fig 1C (middle and bottom panels), N1-FATP4 mutant, which contains the N-terminal transmembrane and ER-localization (ERx) domains, bound with RPE65. This binding seems weak, because the binding was detectable only when the immunoblot membrane was exposed for longer than 4 min on the ImageQuant LAS 4000. Under the same experimental conditions, we failed to detect binding of C1-FATP4 mutant with RPE65. Immunocytochemistry revealed that the N1-FATP4 mutant, but not the C1-FATP4 mutant, colocalized with RPE65 (Fig. 2). C2-FATP4 mutant that contains the C1 mutant region and the conserved fatty acid transport protein (FATP)/very long-chain fatty acyl-CoA synthetase (VLACS) domain showed a weak binding with RPE65. In contrast, C3-FATP4 mutant containing the C2 mutant region plus the ATP-dependent AMP-binding domain exhibited a strong binding with RPE65. C4-FATP4 mutant that contains the C3 mutant region and the ERx domain exhibited more strong binding affinity for RPE65 (Fig. 1C).

FATP4 mutants with high binding affinity for RPE65 inhibited 11-*cis*-retinol synthesis, but not all-*trans*-retinyl ester synthesis

To determine appropriate time of substrate incubation *in cellulo* isomerase assay, we analyzed time course of 11-*cis*-retinol in the RPE65-transfected 293T-LC cells incubated with 7 μM all-*trans*-retinol for different times. As shown in Fig. 3A, the synthesis of 11-*cis*-retinol was increased as the incubation time increased. However, it showed a linear increase in the first 6-hour incubation (Fig. 3A), and it was easy to identify the peak of 11-*cis*-retinol when the substrate was incubated for 4 hours (Fig. 3D,E). We therefore determined to use 4-hour as the substrate incubation time for the following isomerase assays in living cells. To know whether the interaction between FATP4 and RPE65 contributes the FATP4-mediated

inhibition of 11-*cis*-retinol synthesis, we performed retinoid isomerase assay in living cells. We incubated 293T-LC cells co-transfected with RPE65 and a FATP4 mutant construct with 7 μM all-*trans*-retinol for 4 hours. We then analyzed expression levels of RPE65 and the synthesis of 11-*cis*-retinol in the cells. Expression levels of RPE65 was reduced by approximately 40% or 25% in the cells co-transfected with N1-FATP4 or C2-FATP4, respectively, as compared to pRK mock vector-cotransfected cells. Expression levels of RPE65 in the other FATP4 mutant-cotransfected cells were similar to that in the pRK control cells (Fig. 3B,C). Figure 3D and F show representative HPLC chromatograms of retinoids indicating the synthesis activity of 11-*cis*-retinol in the cells co-transfected with RPE65 and pRK or C4-FATP4 mutant. We confirmed our identification of 11-*cis*-retinol by UV-spectral analysis (Fig. 3E). HPLC data showed that the C1-FATP4 had no significant effect on 11-*cis*-retinol synthesis whereas the N1 and C2 FATP4 mutants reduced the 11-*cis*-retinol synthesis by approximately 55% or 40%, respectively, as compared to pRK control. However, after normalizing by the expression levels of RPE65 in Fig. 3C, N1-FATP4 and C2-FATP4 inhibited the synthesis of 11-*cis*-retinol by approximately 13% or 15%, respectively (Fig. 3G). In contrast, C3-FATP4 and C4-FATP4 reduced 11-*cis*-retinol synthesis by ~30% or ~50%, respectively (Fig. 3G). Inhibitory effect of C4-FATP4 on the 11-*cis*-retinol synthesis was similar to that of wild-type FATP4 (Fig. 4C).

RPE65 uses all-*trans*-retinyl esters, such as all-*trans*-retinyl palmitate, as its substrate (3,45,46). Since all-*trans*-retinyl palmitate is a highly hydrophobic molecule, we used all-*trans*-retinol as the substrate in the isomerase assay using living 293T-LC cells. In this assay, RPE65 used intracellular all-*trans*-retinyl ester synthesized from the all-*trans*-retinol by LRAT as its substrate. Therefore, the reduced synthesis of 11-*cis*-retinol described above (Fig. 3G) might be due to inhibition of the LRAT function by the C3-FATP4 and C4-FATP4 mutants. We tested this possibility by measuring synthesis of all-*trans*-retinyl esters in the cells. As shown in Fig. 3H, only N1-FATP4 slightly inhibited the synthesis of all-*trans*-retinyl esters in the cells incubated with all-*trans*-retinol for 60 min. All other FATP4 mutants did not inhibit the synthesis of all-*trans*-retinyl esters under the same experimental conditions (Fig. 3H). These results suggest that 1) N1-FATP4 inhibited both 11-*cis*-retinol synthesis and all-*trans*-retinyl ester synthesis; 2) C1-FATP4 had no inhibitory activity on the functions of RPE65 and LRAT; and 3) C3-FATP4 and C4-FATP4 inhibited 11-*cis*-retinol synthesis (RPE65 function) without reducing all-*trans*-retinyl ester synthesis (LRAT function).

To confirm the effects of the C1 and C3 mutants on the synthesis of 11-*cis*-retinol, we performed *in vitro* isomerase assay using all-*trans*-retinyl palmitate as the substrate. We used sodium cholate to increase the solubility of all-*trans*-retinyl palmitate in the cell homogenates of the assay (3). Similar to the results described above, the C1-FATP4 did not reduce 11-*cis*-retinol synthesis whereas the C3-FATP4 and wild-type FATP4 inhibited the synthesis of 11-*cis*-retinol by approximately 30% or 60%, respectively (Fig. 3I). Immunoblot analysis showed that expression levels of RPE65 in the cells transfected with C1-FATP4 were similar to those in the cells cotransfected with C3-FATP4 or wild-type FATP4 (Fig. 3J). These results indicate that C3-FATP4, but not C1-FATP4, has a relatively strong inhibitory effect on the RPE65-catalyzed synthesis of 11-*cis*-retinol.

Different impacts of IPS-associated nonsense and missense mutations in FATP4 on the synthesis of 11-*cis*-retinol and all-*trans*-retinyl esters

Recently several point mutations, including a non-sense and three missense mutations (Fig. 1A), are identified in the *FATP4* gene of patients with ichthyosis prematurity syndrome (IPS). We evaluated the effects of these mutations (C168X, A92T, S247P and Q300R) of FATP4 on the levels of RPE65 expression as well as synthesis of 11-*cis*-retinol and all-*trans*-retinyl esters in transfected 293T-LC cells. Similar to the N1-FATP4, the C168X FATP4 markedly reduced expression level of RPE65 in its cotransfected cells (Fig. 4A,B). Synthesis of 11-*cis*-retinol was also reduced by 30% in the C168X FATP4-transfected cells, as compared to the pRK-transfected control cells. After normalizing by the expression level of RPE65 (Fig. 4A,B), C168X FATP4 inhibited 11-*cis*-retinol synthesis by approximately 15% (Fig. 4C). Fig. 4D is the representative chromatograms of retinoids showing the retinoid isomerase activities in the 293T-LC cells transfected with pRK vector alone (isomerase negative control), pRK plus RPE65 (isomerase positive control), RPE65 plus wild-type FATP4, or RPE65 plus S247P FATP4 mutant. Immunoblot analysis and HPLC data indicated that the three IPS-associated FATP4 mutants with an A92T, S247P or Q300R substitution did not reduce RPE65 expression and all-*trans*-retinyl ester synthesis, but they inhibited 11-*cis*-retinol synthesis in both *in cellulo* and *in vitro* isomerase assays (Fig. 4A–F).

DISCUSSION

The RPE65-catalyzed all-*trans* to 11-*cis* isomerization is the most critical enzymatic reaction in the regenerating 11-*cis*-retinal chromophore from the all-*trans*-retinal produced by the photoisomerization. This rate-limiting step in the visual cycle (47) has been an important therapeutic target to protect the retinal photoreceptors from photodamage (21,22,25) and to reduce formation of the visual cycle-derived cytotoxic bisretinoids (48,49). In this study, we showed a potential mechanism of FATP4 function in inhibiting RPE65 function as well as different impacts of IPS-associated nonsense and missense mutations in FATP4 on the synthesis of 11-*cis*-retinol and all-*trans*-retinyl esters.

FATP4 is a transmembrane protein of the FATP family proteins (FATP1–6) encoded by *SLC27A* family genes (*SLC27A1–6*) (50,51). FATP4 is closely related to FATP1 in the phylogenetic analysis while FATP2 is more related to FATP3 (51). In addition to FATP4, FATP1 and FATP2 have also been shown to inhibit the synthesis of 11-*cis*-retinol catalyzed by RPE65 (52,53). All of these FATP members possess a putative transmembrane, an ATP-dependent AMP-binding (ATP/AMP) and a FATP/VLACS domains in different regions of the proteins (27–29). These proteins do not contain a particular known functional domain in their C-terminal region. Our immunoprecipitation assay showed that the C-terminal 89 amino acid segment of FATP4 (C1-FATP4) did not interact with RPE65 (Fig. 1). In agreement with this result, immunocytochemistry revealed that the C1-FATP4 did not colocalize with RPE65 (Fig. 2). In addition, neither expression level of RPE65 nor synthesis of 11-*cis*-retinol and all-*trans*-retinyl esters were reduced by C1-FATP4 in the cotransfected cells (Fig. 3). These results suggest that the C-terminal region is not involved in the FATP4-mediated inhibition of the RPE65 function.

In contrast, we found that the N1-FATP4 containing the N-terminal 176 amino acids significantly reduced RPE65 expression level as well as the synthesis of 11-*cis*-retinol and all-*trans*-retinyl esters (Figs. 1–3). Consistent with these results, the IPS-associated FATP4 with the C168X nonsense mutation also caused a significant decrease in RPE65 expression and synthesis of 11-*cis*-retinol and all-*trans*-retinyl esters in the cells (Figs. 1,4). One of the possibilities for the reduction in the synthesis of 11-*cis*-retinol is the decreased expression level of RPE65 in the cells. Previous studies by us and others showed that the synthesis activities of 11-*cis*-retinol depend on expression levels of RPE65 (3,4,37). An alternative possibility is that the reduced synthesis of all-*trans*-retinyl esters, the substrate of RPE65, resulted in the decrease in the synthesis of 11-*cis*-retinol in the cells expressing N1-FATP4 or C168X FATP4. In the cells expressing N1-FATP4 or C168X FATP4, all-*trans*-retinyl ester synthesis was reduced by approximately 20%, as compared to the pRK-transfected control cells (Figs. 3,4). A previous study showed that the synthesis of all-*trans*-retinyl esters was reduced by 45–50% in the $Lrat^{+/-}$ mouse eyes, as compared to the wild-type eyes, whereas the synthesis activity of 11-*cis*-retinol in the $Lrat^{+/-}$ mouse ocular tissues were similar to that in wild-type mice (54). These results suggest that the reduced synthesis of 11-*cis*-retinol in the cells expressing N1-FATP4 or C168X FATP4 is unlikely due to the reduced synthesis of all-*trans*-retinyl esters in the cells.

Then, why was RPE65 reduced in the cells expressing N1-FATP4 or C168X FATP4. FATP4 is the only FATP family member whose ER-localization domain has been identified (29). In the mouse RPE and epidermic granular keratinocytes, FATP4 colocalized with the ER luminal protein markers (26,55). Reportedly, the first 102 amino acid residues including the putative transmembrane and the ER-targeting (amino acids 47–102) domains facilitate the localization of FATP4 to the ER (29). Both N1-FATP4 and C168X FATP4 contain the transmembrane and ER-targeting domains. Immunocytochemistry showed that wild-type FATP4 and N1-FATP4, but not C1-FATP4, colocalized with RPE65 (Fig. 2), which is an ER membrane/microsome-associated protein (1). Unlike wild-type FATP4 and C1-FATP4, N1-FATP4 formed aggregate-like structures in the cells. This observation suggests that N1-FATP4 mutant might be misfolded and caused ER stress. Many mutations in membrane and secretion proteins render the mutated proteins prone to form aggregates in the ER of the cells (38,56), which in turn reduce the ER function in the protein translation. The reduced synthesis of 11-*cis*-retinol and all-*trans*-retinyl esters might be secondary to the cytotoxic effects of misfolded N1-FATP4 and C168X FATP4 in their expressing cells. Although we did not check whether C168X also form the aggregates-like structure, the similar reduced expression of RPE65 and the similar N-terminal region suggest that C168X FATP4 and N1-FATP4 have the same effects on the cells. Nevertheless, it will be important to analyze whether C168X FATP4 inhibits the visual cycle by reducing RPE65 expression in the RPE of IPS patients with the mutant FATP4.

One of the purposes of this study was to determine whether FATP4 has a particular segment(s) that is important for inhibiting or interacting with RPE65. We found that C3-FATP4 and C4-FATP4 relatively strongly bound with RPE65 in immunoprecipitation assay whereas N1-FATP4 and C2-FATP4 bound with RPE65 in a relatively weaker affinity. The C2-FATP4, C3-FATP4, and C4-FATP4 reduced 11-*cis*-retinol synthesis by approximately 15%, 30% or 50%, respectively, without reducing RPE65 expression and all-*trans*-retinyl

ester synthesis (Fig. 3), while C1-FATP4 neither bound with nor inhibited RPE65. These results indicate that the binding affinities of the deletion mutants with RPE65 are positively correlated with their inhibitory effects on the synthesis of 11-*cis*-retinol (Figs. 1,3), This finding suggests that interaction between RPE65 and FATP4 is involved in the mechanisms whereby FATP4 inhibits the synthesis of 11-*cis*-retinol. It will be important to identify the mechanisms that regulate the FATP4-RPE65 interaction and expression of FATP4 in the RPE.

Studies using protein chimeras have identified a 71–73 amino acid segment between the ATP/AMP and FATP domains as an important domain for the fatty acid transport activities of FATP1 and FATP4 (57). This domain is overlapping with the C-terminal part of the peripheral membrane-association domain (amino acids 258–475) of FATP1 (50). These two domains are completely included in C3-FATP4 while C2-FATP4 contains only a small part of these domains. These two domains might contribute to the relatively strong interaction of C3-FATP4 with RPE65. The ER-association domain of FATP4 might further increase the binding affinity of C4-FATP4 with RPE65, whose mouth of the substrate entry tunnel is surrounded by hydrophobic residues that are likely responsible for anchoring RPE65 to the ER membranes (58). In the skin epidermis, FATP4 interacts with Ichthyin/NIPAL4 whose mutations have been linked to congenital ichthyosis (59). Ichthyin/NIPAL4, a transmembrane protein having homologies to G protein-coupled receptors and magnesium transporter proteins (60,61), may function as a receptor for trioxilins A3 and B3 (60). It will be interesting to test whether the interaction between FATP4 and Ichthyin/NIPAL4 is involved in the mechanisms of the FATP4-mediated inhibition of 11-*cis*-retinol synthesis in the RPE.

Mutations in the *SLC27A4/FATP4* gene are associated with ichthyosis prematurity syndrome (IPS) (34,35). Except for the C168X nonsense mutation, which has been shown to reduce the very long-chain fatty acid-CoA synthetase activity of FATP4 (34), the other three missense mutations (A92T, S247P and Q300R) have not yet been studied for their effects on the fatty acid metabolisms. The similar skin phenotypes and clinical course of IPS patients with the mutations (34,35) suggest that the three missense mutations also reduced the function of FATP4 in the very long-chain fatty acid metabolisms in the epidermis. However, we found that the nonsense mutation and the missense mutations displayed significantly different impacts on RPE65 expression and FATP4-medicated inhibition of 11-*cis*-retinol synthesis. The three missense mutations neither significantly reduce RPE65 expression nor eliminate the inhibitory function of FATP4 in the 11-*cis*-retinol synthesis catalyzed by RPE65 (Fig. 4). The inhibitory activity of FATP4 on the 11-*cis*-retinol synthesis was reduced only 10–15% by the missense mutations. This result is consistent with the previous studies: 1) lignoceroyl (C24:0)-CoA inhibited 11-*cis*-retinol synthesis by approximately 20% at its concentration that was 3-fold higher than the substrate concentration of all-*trans*-retinyl palmitate in the *in vitro* RPE65 assay (26), 2) long-chain fatty acyl-CoA synthetases (ACSLs) inhibited 11-*cis*-retinol synthesis in *cellulo* RPE65 assay (53), and 3) triacsin C, a potent ACSL inhibitor that does not reduce the fatty acyl-CoA synthetase activity of FATP4 (62), inhibited the function of RPE65 in the synthesis of 11-*cis*-retinol (53). Triacsin C has been shown to inhibit ACSL-catalyzed synthesis of palmitoyl-CoA by binding with palmitic acid (62) while both FATP1 and FATP4 are insensitive to inhibition by triacsin C although

both FATP1 and FATP4 have a long-chain fatty acyl-CoA synthetase activity (30,63). These studies suggest that proteins involved in the saturated long-chain and very long-chain fatty acid metabolisms regulate the visual cycle enzymes (RPE65 and LRAT) in a very complicated mechanism. Elucidating the mechanism may provide valuable information for to the development of a safe and effective approach that can reduce bisretinoid formation and retinal light damage.

Acknowledgements:

This work was supported by NIH grants EY021208 (M.J.), EY028572 (M.J.), RR016816 (NG Bazan), GM103340 (NG Bazan), Research to Prevent Blindness and Louisiana Lions Eye Foundation grants (LSU Department of Ophthalmology) and a Research Enhancement Fund from LSU School of Medicine (M.J.).

Abbreviations:

FATP	fatty acid transport protein
IPS	ichthyosis prematurity syndrome
ACS	acyl-CoA synthetase
RPE	retinal pigment epithelium
HPLC	high-performance liquid chromatography
SD	standard deviation
ER	endoplasmic reticulum
LRAT	lecithin:retinol acyltransferase

REFERENCES

1. Hamel CP, Tsilou E, Pfeffer BA, Hooks JJ, Detrick B, and Redmond TM (1993) Molecular cloning and expression of RPE65, a novel retinal pigment epithelium-specific microsomal protein that is post-transcriptionally regulated in vitro. J. Biol. Chem 268, 15751–15757 [PubMed: 8340400]
2. Redmond TM, Yu S, Lee E, Bok D, Hamasaki D, Chen N, Goletz P, Ma JX, Crouch RK, and Pfeifer K (1998) Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. Nat. Genet 20, 344–351 [PubMed: 9843205]
3. Jin M, Li S, Moghrabi WN, Sun H, and Travis GH (2005) Rpe65 is the retinoid isomerase in bovine retinal pigment epithelium. Cell 122, 449–459 [PubMed: 16096063]
4. Moiseyev G, Chen Y, Takahashi Y, Wu BX, and Ma JX (2005) RPE65 is the isomerohydrolase in the retinoid visual cycle. Proc. Natl. Acad. Sci. U. S. A 102, 12413–12418 [PubMed: 16116091]
5. Redmond TM, Poliakov E, Yu S, Tsai JY, Lu Z, and Gentleman S (2005) Mutation of key residues of RPE65 abolishes its enzymatic role as isomerohydrolase in the visual cycle. Proc. Natl. Acad. Sci. U. S. A 102, 13658–13663 [PubMed: 16150724]
6. Ruiz A, Winston A, Lim YH, Gilbert BA, Rando RR, and Bok D (1999) Molecular and biochemical characterization of lecithin retinol acyltransferase. J. Biol. Chem 274, 3834–3841 [PubMed: 9920938]
7. Kaschula CH, Jin MH, Desmond-Smith NS, and Travis GH (2006) Acyl CoA:retinol acyltransferase (ARAT) activity is present in bovine retinal pigment epithelium. Exp. Eye Res 82, 111–121 [PubMed: 16054134]

8. Kaylor JJ, Radu RA, Bischoff N, Makshanoff J, Hu J, Lloyd M, Eddington S, Bianconi T, Bok D, and Travis GH (2015) Diacylglycerol o-acyltransferase type-1 synthesizes retinyl esters in the retina and retinal pigment epithelium. PLoS One 10, e0125921 [PubMed: 25974161]

9. Bernstein PS, Li B, Vachali PP, Gorusupudi A, Shyam R, Henriksen BS, and Nolan JM (2016) Lutein, zeaxanthin, and meso-zeaxanthin: The basic and clinical science underlying carotenoid-based nutritional interventions against ocular disease. Prog. Retin. Eye Res 50, 34–66 [PubMed: 26541886]

10. Shyam R, Gorusupudi A, Nelson K, Horvath MP, and Bernstein PS (2017) RPE65 has an additional function as the lutein to meso-zeaxanthin isomerase in the vertebrate eye. Proc. Natl. Acad. Sci. U. S. A 114, 10882–10887 [PubMed: 28874556]

11. Gu SM, Thompson DA, Srikumari CR, Lorenz B, Finckh U, Nicoletti A, Murthy KR, Rathmann M, Kumaramanickavel G, Denton MJ, and Gal A (1997) Mutations in RPE65 cause autosomal recessive childhood-onset severe retinal dystrophy. Nat. Genet 17, 194–197 [PubMed: 9326941]

12. Aguirre GD, Baldwin V, Pearce-Kelling S, Narfstrom K, Ray K, and Acland GM (1998) Congenital stationary night blindness in the dog: common mutation in the RPE65 gene indicates founder effect. Mol. Vis 4, 23 [PubMed: 9808841]

13. Wenzel A, Reme CE, Williams TP, Hafezi F, and Grimm C (2001) The Rpe65 Leu450Met variation increases retinal resistance against light-induced degeneration by slowing rhodopsin regeneration. J. Neurosci 21, 53–58 [PubMed: 11150319]

14. Li S, Sato K, Gordon WC, Sendtner M, Bazan NG, and Jin M (2018) Ciliary neurotrophic factor (CNTF) protects retinal cone and rod photoreceptors by suppressing excessive formation of the visual pigments. J. Biol. Chem 293, 15256–15268 [PubMed: 30115683]

15. Parish CA, Hashimoto M, Nakanishi K, Dillon J, and Sparrow J (1998) Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. Proc. Natl. Acad. Sci. U. S. A 95, 14609–14613 [PubMed: 9843937]

16. Weng J, Mata NL, Azarian SM, Tzekov RT, Birch DG, and Travis GH (1999) Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knockout mice. Cell 98, 13–23 [PubMed: 10412977]

17. Kim SR, Fishkin N, Kong J, Nakanishi K, Allikmets R, and Sparrow JR (2004) Rpe65 Leu450Met variant is associated with reduced levels of the retinal pigment epithelium lipofuscin fluorophores A2E and iso-A2E. Proc. Natl. Acad. Sci. U. S. A 101, 11668–11672 [PubMed: 15277666]

18. Schmitz-Valckenberg S, Bindewald-Wittich A, Dolar-Szczasny J, Dreyhaupt J, Wolf S, Scholl HP, Holz FG, and Fundus Autofluorescence in Age-Related Macular Degeneration Study, G. (2006) Correlation between the area of increased autofluorescence surrounding geographic atrophy and disease progression in patients with AMD. Invest. Ophthalmol. Vis. Sci 47, 2648–2654 [PubMed: 16723482]

19. Wu Y, Yanase E, Feng X, Siegel MM, and Sparrow JR (2010) Structural characterization of bisretinoid A2E photocleavage products and implications for age-related macular degeneration. Proc. Natl. Acad. Sci. U. S. A 107, 7275–7280 [PubMed: 20368460]

20. Grimm C, Wenzel A, Hafezi F, Yu S, Redmond TM, and Reme CE (2000) Protection of Rpe65-deficient mice identifies rhodopsin as a mediator of light-induced retinal degeneration. Nat. Genet 25, 63–66 [PubMed: 10802658]

21. Sieving PA, Chaudhry P, Kondo M, Provenzano M, Wu D, Carlson TJ, Bush RA, and Thompson DA (2001) Inhibition of the visual cycle in vivo by 13-cis retinoic acid protects from light damage and provides a mechanism for night blindness in isotretinoin therapy. Proc. Natl. Acad. Sci. U. S. A 98, 1835–1840 [PubMed: 11172037]

22. Mandal MN, Moiseyev GP, Elliott MH, Kasus-Jacobi A, Li X, Chen H, Zheng L, Nikolaeva O, Floyd RA, Ma JX, and Anderson RE (2011) Alpha-phenyl-N-tert-butylnitrone (PBN) prevents light-induced degeneration of the retina by inhibiting RPE65 protein isomerohydrolase activity. J. Biol. Chem 286, 32491–32501 [PubMed: 21785167]

23. Wu L, Ueda K, Nagasaki T, and Sparrow JR (2014) Light damage in Abca4 and Rpe65rd12 mice. Invest. Ophthalmol. Vis. Sci 55, 1910–1918 [PubMed: 24576873]

24. Zhang J, Dong Z, Mundla SR, Hu XE, Seibel W, Papoian R, Palczewski K, and Golczak M (2015) Expansion of first-in-class drug candidates that sequester toxic all-trans-retinal and prevent light-induced retinal degeneration. Mol. Pharmacol 87, 477–491 [PubMed: 25538117]
25. Shin Y, Moiseyev G, Petrukhin K, Cioffi CL, Muthuraman P, Takahashi Y, and Ma JX (2018) A novel RPE65 inhibitor CU239 suppresses visual cycle and prevents retinal degeneration. Biochim Biophys Acta Mol Basis Dis 1864, 2420–2429 [PubMed: 29684583]
26. Li S, Lee J, Zhou Y, Gordon WC, Hill JM, Bazan NG, Miner JH, and Jin M (2013) Fatty acid transport protein 4 (FATP4) prevents light-induced degeneration of cone and rod photoreceptors by inhibiting RPE65 isomerase. J. Neurosci 33, 3178–3189 [PubMed: 23407971]
27. Hirsch D, Stahl A, and Lodish HF (1998) A family of fatty acid transporters conserved from mycobacterium to man. Proc. Natl. Acad. Sci. U. S. A 95, 8625–8629 [PubMed: 9671728]
28. Stahl A, Hirsch DJ, Gimeno RE, Punreddy S, Ge P, Watson N, Patel S, Kotler M, Raimondi A, Tartaglia LA, and Lodish HF (1999) Identification of the major intestinal fatty acid transport protein. Mol. Cell 4, 299–308 [PubMed: 10518211]
29. Milger K, Herrmann T, Becker C, Gotthardt D, Zickwolf J, Ehehalt R, Watkins PA, Stremmel W, and Fullekrug J (2006) Cellular uptake of fatty acids driven by the ER-localized acyl-CoA synthetase FATP4. J. Cell Sci 119, 4678–4688 [PubMed: 17062637]
30. Hall AM, Wiczer BM, Herrmann T, Stremmel W, and Bernlohr DA (2005) Enzymatic properties of purified murine fatty acid transport protein 4 and analysis of acyl-CoA synthetase activities in tissues from FATP4 null mice. J. Biol. Chem 280, 11948–11954 [PubMed: 15653672]
31. Jia Z, Moulson CL, Pei Z, Miner JH, and Watkins PA (2007) Fatty acid transport protein 4 is the principal very long chain fatty acyl-CoA synthetase in skin fibroblasts. J. Biol. Chem 282, 20573–20583 [PubMed: 17522045]
32. Moulson CL, Martin DR, Lugus JJ, Schaffer JE, Lind AC, and Miner JH (2003) Cloning of wrinkle-free, a previously uncharacterized mouse mutation, reveals crucial roles for fatty acid transport protein 4 in skin and hair development. Proc. Natl. Acad. Sci. U. S. A 100, 5274–5279 [PubMed: 12697906]
33. Moulson CL, Lin MH, White JM, Newberry EP, Davidson NO, and Miner JH (2007) Keratinocyte-specific expression of fatty acid transport protein 4 rescues the wrinkle-free phenotype in Slc27a4/Fatp4 mutant mice. J. Biol. Chem 282, 15912–15920 [PubMed: 17401141]
34. Klar J, Schweiger M, Zimmerman R, Zechner R, Li H, Torma H, Vahlquist A, Bouadjar B, Dahl N, and Fischer J (2009) Mutations in the fatty acid transport protein 4 gene cause the ichthyosis prematurity syndrome. Am J Hum Genet 85, 248–253 [PubMed: 19631310]
35. Sobol M, Dahl N, and Klar J (2011) FATP4 missense and nonsense mutations cause similar features in Ichthyosis Prematurity Syndrome. BMC Res Notes 4, 90 [PubMed: 21450060]
36. Bygum A, Westermark P, and Brandrup F (2008) Ichthyosis prematurity syndrome: a well-defined congenital ichthyosis subtype. J. Am. Acad. Dermatol 59, S71–74 [PubMed: 19119129]
37. Jin M, Yuan Q, Li S, and Travis GH (2007) Role of LRAT on the retinoid isomerase activity and membrane association of Rpe65. J. Biol. Chem 282, 20915–20924 [PubMed: 17504753]
38. Li S, Yang Z, Hu J, Gordon WC, Bazan NG, Haas AL, Bok D, and Jin M (2013) Secretory defect and cytotoxicity: the potential disease mechanisms for the retinitis pigmentosa (RP)-associated interphotoreceptor retinoid-binding protein (IRBP). J. Biol. Chem 288, 11395–11406 [PubMed: 23486466]
39. Jin M, Li S, Nusinowitz S, Lloyd M, Hu J, Radu RA, Bok D, and Travis GH (2009) The role of interphotoreceptor retinoid-binding protein on the translocation of visual retinoids and function of cone photoreceptors. J. Neurosci 29, 1486–1495 [PubMed: 19193895]
40. Mata NL, Moghrabi WN, Lee JS, Bui TV, Radu RA, Horwitz J, and Travis GH (2004) Rpe65 is a retinyl ester binding protein that presents insoluble substrate to the isomerase in retinal pigment epithelial cells. J. Biol. Chem 279, 635–643 [PubMed: 14532273]
41. Li S, Samardzija M, Yang Z, Grimm C, and Jin M (2016) Pharmacological Amelioration of Cone Survival and Vision in a Mouse Model for Leber Congenital Amaurosis. J. Neurosci 36, 5808–5819 [PubMed: 27225770]

42. Li S, Izumi T, Hu J, Jin HH, Siddiqui AA, Jacobson SG, Bok D, and Jin M (2014) Rescue of Enzymatic Function for Disease-associated RPE65 Proteins Containing Various Missense Mutations in Non-active Sites. J. Biol. Chem 289, 18943–18956 [PubMed: 24849605]
43. Sato K, Li S, Gordon WC, He J, Liou GI, Hill JM, Travis GH, Bazan NG, and Jin M (2013) Receptor interacting protein kinase-mediated necrosis contributes to cone and rod photoreceptor degeneration in the retina lacking interphotoreceptor retinoid-binding protein. J. Neurosci 33, 17458–17468 [PubMed: 24174679]
44. Li S, Hu J, Jin RJ, Aiyar A, Jacobson SG, Bok D, and Jin M (2015) Temperature-sensitive retinoid isomerase activity of RPE65 mutants associated with Leber Congenital Amaurosis. J Biochem 158, 115–125 [PubMed: 25752820]
45. Gollapalli DR, and Rando RR (2003) All-trans-retinyl esters are the substrates for isomerization in the vertebrate visual cycle. Biochemistry (Mosc) 42, 5809–5818
46. Moiseyev G, Crouch RK, Goletz P, Oatis J Jr., Redmond TM, and Ma JX (2003) Retinyl esters are the substrate for isomerohydrolase. Biochemistry (Mosc) 42, 2229–2238
47. Gollapalli DR, and Rando RR (2004) The specific binding of retinoic acid to RPE65 and approaches to the treatment of macular degeneration. Proc. Natl. Acad. Sci. U. S. A 101, 10030–10035 [PubMed: 15218101]
48. Radu RA, Mata NL, Nusinowitz S, Liu X, Sieving PA, and Travis GH (2003) Treatment with isotretinoin inhibits lipofuscin accumulation in a mouse model of recessive Stargardt's macular degeneration. Proc. Natl. Acad. Sci. U. S. A 100, 4742–4747 [PubMed: 12671074]
49. Kubota R, Boman NL, David R, Mallikaarjun S, Patil S, and Birch D (2012) Safety and effect on rod function of ACU-4429, a novel small-molecule visual cycle modulator. Retina 32, 183–188 [PubMed: 21519291]
50. Anderson CM, and Stahl A (2013) SLC27 fatty acid transport proteins. Mol. Aspects Med 34, 516–528 [PubMed: 23506886]
51. Watkins PA, Maiguel D, Jia Z, and Pevsner J (2007) Evidence for 26 distinct acyl-coenzyme A synthetase genes in the human genome. J. Lipid Res 48, 2736–2750 [PubMed: 17762044]
52. Guignard TJ, Jin M, Pequignot MO, Li S, Chassigneux Y, Chekroud K, Guillou L, Richard E, Hamel CP, and Brabet P (2010) FATP1 inhibits 11-cis retinol formation via interaction with the visual cycle retinoid isomerase RPE65 and lecithin:retinol acyltransferase. J. Biol. Chem 285, 18759–18768 [PubMed: 20356843]
53. Eroglu A, Gentleman S, Poliakov E, and Redmond TM (2016) Inhibition of RPE65 Retinol Isomerase Activity by Inhibitors of Lipid Metabolism. J. Biol. Chem 291, 4966–4973 [PubMed: 26719343]
54. Batten ML, Imanishi Y, Maeda T, Tu DC, Moise AR, Bronson D, Possin D, Van Gelder RN, Baehr W, and Palczewski K (2004) Lecithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver. J. Biol. Chem 279, 10422–10432 [PubMed: 14684738]
55. Lin MH, and Miner JH (2015) Fatty acid transport protein 1 can compensate for fatty acid transport protein 4 in the developing mouse epidermis. J. Invest. Dermatol 135, 462–470 [PubMed: 25184958]
56. Liu Y, and Vollrath D (2004) Reversal of mutant myocilin non-secretion and cell killing: implications for glaucoma. Hum. Mol. Genet 13, 1193–1204 [PubMed: 15069026]
57. DiRusso CC, Darwis D, Obermeyer T, and Black PN (2008) Functional domains of the fatty acid transport proteins: studies using protein chimeras. Biochim. Biophys. Acta 1781, 135–143 [PubMed: 18258213]
58. Kiser PD, Golczak M, Lodowski DT, Chance MR, and Palczewski K (2009) Crystal structure of native RPE65, the retinoid isomerase of the visual cycle. Proc. Natl. Acad. Sci. U. S. A 106, 17325–17330 [PubMed: 19805034]
59. Li H, Vahlquist A, and Torma H (2013) Interactions between FATP4 and ichthyin in epidermal lipid processing may provide clues to the pathogenesis of autosomal recessive congenital ichthyosis. J. Dermatol. Sci 69, 195–201 [PubMed: 23290633]

60. Lefevre C, Bouadjar B, Karaduman A, Jobard F, Saker S, Ozguc M, Lathrop M, Prud'homme JF, and Fischer J (2004) Mutations in ichthyin a new gene on chromosome 5q33 in a new form of autosomal recessive congenital ichthyosis. Hum. Mol. Genet 13, 2473–2482 [PubMed: 15317751]

61. Quamme GA (2010) Molecular identification of ancient and modern mammalian magnesium transporters. American journal of physiology. Cell physiology 298, C407–429 [PubMed: 19940067]

62. Vessey DA, Kelley M, and Warren RS (2004) Characterization of triacsin C inhibition of short-, medium-, and long-chain fatty acid: CoA ligases of human liver. J. Biochem. Mol. Toxicol 18, 100–106 [PubMed: 15122652]

63. Hall AM, Smith AJ, and Bernlohr DA (2003) Characterization of the Acyl-CoA synthetase activity of purified murine fatty acid transport protein 1. J. Biol. Chem 278, 43008–43013 [PubMed: 12937175]

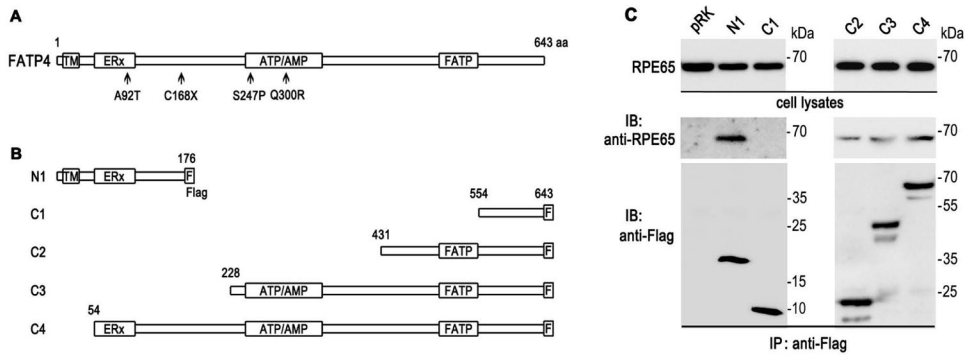

Figure 1.

(*A*) Schematic overview of FATP4 functional domains and positions of ichthyosis prematurity syndrome (IPS)-associated mutations. TM, transmembrane; ERx, ER-localization; ATP/AMP, ATP-dependent AMP-binding; FATP, fatty acid transport protein domain also known as very long-chain fatty acyl-CoA synthetase (VLACS) domain. **(*B*)** Schematic show of a series of deletion mutants of FATP4. F in the C-terminal box denotes Flag epitope tag. **(*C*)** Immunoblot analysis and immunoprecipitation of RPE65. Expression of RPE65 in the 293T-LC cells cotransfected with pRK-RPE65 and a Flag-tagged FATP4 deletion mutant or pRK mock vector was determined by immunoblot analysis using an anti-RPE65 antibody (upper panel). Homogenates of the cells were immunoprecipitated with anti-Flag affinity gel. The precipitates were then probed with antibodies against RPE65 (middle panel) or Flag epitope (bottom panel). Molecular weights (kDa) of markers are shown at right sides of immunoblots.

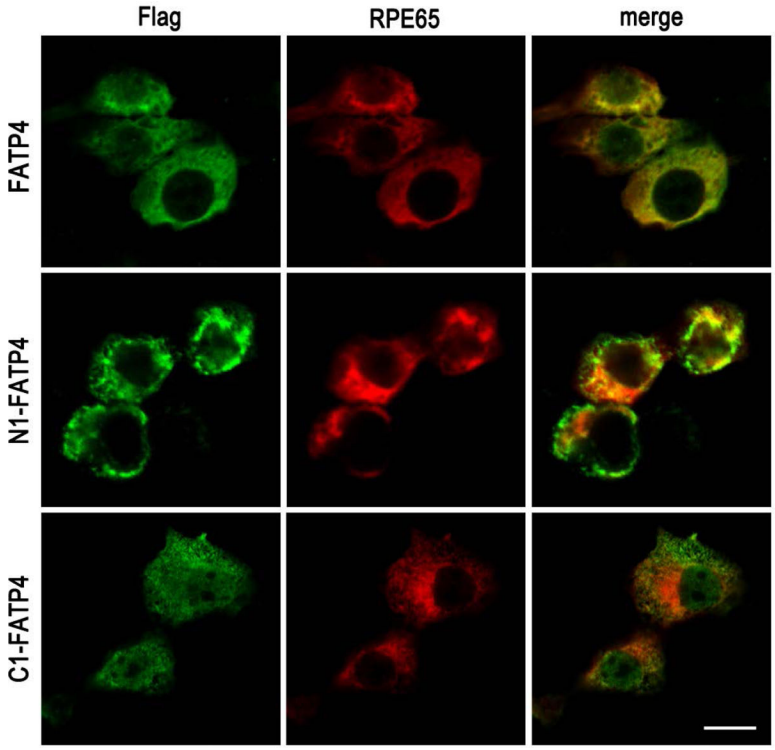

Figure 2.

Subcellular localization of wild-type and deletion mutant FATP4 in the cells expressing RPE65. 293T-LC cells cotransfected with pRK-RPE65 and a FATP4-Flag fusion construct were double-stained with antibodies against Flag epitope (green) or RPE65 (red). Note that wild-type FATP4 and N1-FATP4, but not C1-FATP4, colocalized with RPE65. Also, note that most of N1-FATP4 and RPE65 are accumulated in aggregate-like structures in the cells. Scale bar denotes 10 μm.

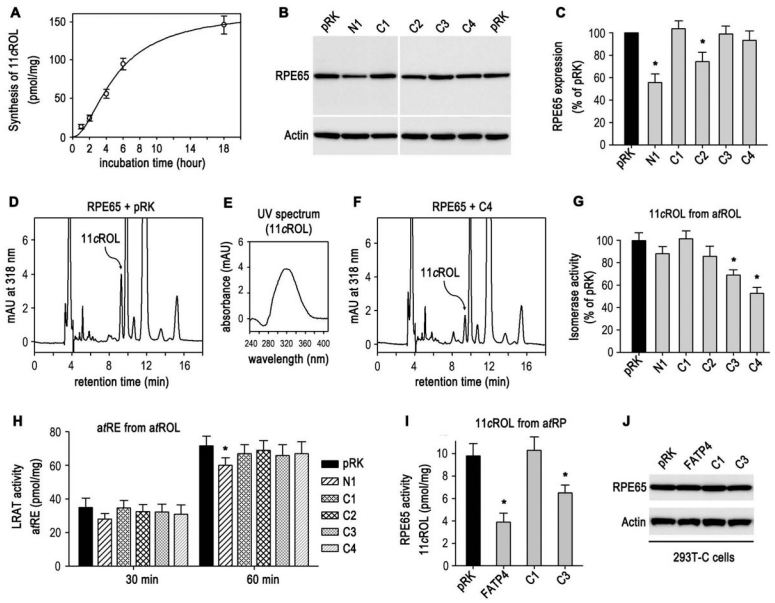

Figure 3.

Effects of deletion mutants of FATP4 on RPE65 expression as well as synthesis of 11-*cis*-retinol (11*c*ROL) and all-*trans*-retinyl esters (a*t*RE). **(*A*)** Time course of 11*c*ROL synthesis from 7 μM all-*trans*-retinol (a*t*ROL) substrate in the media of 293T-LC cells transfected with RPE65 and pRK. **(*B*)** Immunoblot analysis of RPE65 in 293T-LC cells that cotransfected with RPE65 and pRK mock vector or the indicated FATP4 deletion mutant. Beta actin was detected as a loading control. **(*C*)** Relative expression levels of RPE65 in the deletion mutant FATP4-cotransfected 293T-LC cells were normalized by actin levels and expressed as percent of RPE65 level in the pRK-cotransfected cells. **(*D*)** A representative HPLC chromatogram of retinoids in the 293T-LC cells that were transfected with RPE65 and pRK vector, and were incubated with 7 μM a*t*ROL substrate for 4 hours. The peak corresponding to 11*c*ROL is indicated with an arrow. **(*E*)** UV spectrum acquired from the 11*c*ROL peak in (*D*). **(*F*)** A representative chromatogram of retinoids in the 293T-LC cells that were transfected with RPE65 and C4-FATP4 mutant, and were incubated with all-*trans*-retinol substrate. Note the decrease in 11*c*ROL peak pointed by the arrow. **(*G*)** Relative activities of 11*c*ROL synthesis normalized by the expression levels of RPE65 in (*C*). Transfected 293T-LC cells in (*B*) were incubated with 7 μM a*t*ROL for 4 hours and retinoids in the cells were analyzed by HPLC. **(*H*)** LRAT assay showing synthesis of a*t*RE from the a*t*ROL substrate incubated for 30 or 60 min with the 293T-LC cells transfected with pRK (control) or the indicated FATP4 plasmids. **(*I*)** *In vitro* isomerase assay showing synthesis of 11*c*ROL from the substrate of all-*trans*-retinyl palmitate (a*t*RP) incubated with homogenates of 293T-C cells transfected with the indicated constructs. All asterisks indicate significant differences between pRK control and mutant (or WT) FATP4 ($p \leq 0.05$); error bars denote SD ($n \geq 3$). **(*J*)** Immunoblot analysis showing expression levels of RPE65 in the cells in (*I*).

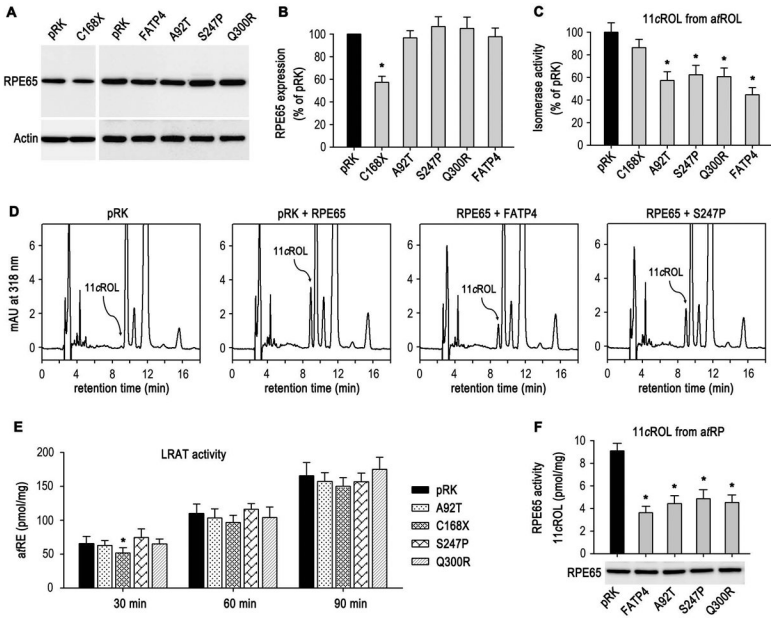

Figure 4.

Impacts of IPS-associate mutations in FATP4 on RPE65 expression, 11-*cis*-retinol (11*c*ROL) synthesis and all-*trans*-retinyl ester (a*t*RE) synthesis. **(*A*)** Immunoblot analysis of RPE65 in 293T-LC cells cotransfected with RPE65 and pRK or FATP4 having one of the indicated IPS mutations. Beta actin was detected as a loading control. **(*B*)** Relative expression levels of RPE65 normalized by actin levels in the cells cotransfected with RPE65 and one of the indicated plasmids. **(*C*)** Relative activities of 11*c*ROL synthesis normalized by the expression levels of RPE65 in (*B*). **(*D*)** Representative chromatograms of retinoids in the 293T-LC cells that were transfected with pRK vector alone (isomerase negative control), pRK plus RPE65, RPE65 plus wild-type FATP4, or RPE65 plus S247P FATP4 mutant, and incubated with 7 μM all-*trans*-retinol (a*t*ROL) substrate. The peaks corresponding to 11*c*ROL are indicated with arrows. The small 11*c*ROL-like peak in the pRK-transfected cells was not able to identify as 11*c*ROL by UV-spectral analysis. **(*E*)** Relative LRAT activities showing synthesis of a*t*RE in the 293T-LC cells that were transfected with the indicated plasmids and incubated with a*t*ROL for 30–90 min. **(*F*)** *In vitro* isomerase assay showing synthesis of 11*c*ROL from the substrate of all-*trans*-retinyl palmitate (a*t*RP) incubated with homogenates of the 293T-C cells transfected with the indicated constructs. Asterisks denote significant differences between pRK control and mutant (or WT) FATP4 ($p \leq 0.05$); error bars indicate SD ($n \geq 3$).

Table 1:

Primer pairs used for making FATP4 mutants

Primers	Sequence (5'−3')
C1-F	AGGGGATCCAGCATGGCCGCTGTGGCC
C1Flag-R[¥]	TCCCTCGAGTCACTTGTCGTCGTCATCCTTGTAGTCTCCCAGCTTCTCCTGGCCCGC
C2-F	GGGGGATCCATGGAGCTGATCCGGGGC
C3-F	AAGGGATCCATGGAAGATGCCCCCAAGC
C4-F	GGGGGATCCATGGAGACTGTCAGGCGTGATATCTTCGG
N1-F	AAGGGATCCATGCTGCTTGGGGCGTCTC
N1Flag-R	TCCCTCGAGTCACTTGTCGTCGTCATCCTTGTAGTCCTGGGAGGAGGTCAGGCAG
A92T-F	CCACCCGGACAAGACAACCCTGATCTTCGAGGGC
A92T-R	GCCCTCGAAGATCAGGGTTGTCTTGTCCGGGTGG
C168X-F	CGCATTGCTCCACTGACTGACCTCCTCCCAGG
C168X-R	CCTGGGAGGAGGTCAGTCAGTGGAGCAATGCG
S247P-F	GATAAGCTCTTCTACATCTACACGCCAGGCACCACCGGG
S247P-R	CCCGGTGGTGCCTGGCGTGTAGATGTAGAAGAGCTTATC
Q300R-F	ATCGTGGGGATAGGGCGGTGCCTGATCCACGGC
Q300R-R	GCCGTGGATCAGGCACCGCCCTATCCCCACGAT

[¥]The primer C1Flag-R was used as the reverse primer to generate the C2-FATP4, C3-FATP4, and C4-FATP4 that contain a C-terminal Flag epitope.