Chemical Chaperone TUDCA Preserves Cone Photoreceptors in a Mouse Model of Leber Congenital Amaurosis

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Purpose. Mutations in either retinoid isomerase (RPE65) or lecithin-retinol acyltransferase (LRAT) lead to Leber congenital amaurosis (LCA). By using the *Lrat*$^{-/-}$ mouse model, previous studies have shown that the rapid cone degeneration in LCA was caused by endoplasmic reticulum (ER) stress induced by S-opsin aggregation. The purpose of this study is to examine the efficacy of an ER chemical chaperone, tauroursodeoxycholic acid (TUDCA), in preserving cones in the *Lrat*$^{-/-}$ model.

Methods. *Lrat*$^{-/-}$ mice were systemically administered with TUDCA and vehicle (0.15 M $NaHCO_3$) every 3 days from P9 to P28. Cone cell survival was determined by counting cone cells on flat-mounted retinas. The expression and subcellular localization of cone-specific proteins were analyzed by western blotting and immunohistochemistry, respectively.

Results. TUDCA treatment reduced ER stress and apoptosis in *Lrat*$^{-/-}$ retina. It significantly slowed down cone degeneration in *Lrat*$^{-/-}$ mice, resulting in a ~3-fold increase in cone density in the ventral and central retina as compared with the vehicle-treated mice at P28. Furthermore, TUDCA promoted the degradation of cone membrane–associated proteins by enhancing the ER-associated protein degradation pathway.

Conclusions. Systemic injection of TUDCA is effective in reducing ER stress, preventing apoptosis, and preserving cones in *Lrat*$^{-/-}$ mice. TUDCA has the potential to lead to the development of a new class of therapeutic drugs for treating LCA. (*Invest Ophthalmol Vis Sci.* 2012;53:3349–3356) DOI: 10.1167/iovs.12-9851

Retinoid isomerase (RPE65) and lecithin-retinol acyltransferase (LRAT) are key enzymes in the retinal pigment epithelium (RPE) required for 11-*cis* retinal recycling. LRAT catalyzes the esterification of all-*trans* retinol to all-*trans* retinyl esters,[1] which in turn are the substrates for the retinoid isomerase, RPE65, to produce 11-*cis* retinol.[2–4] Mutations in RPE65 or LRAT cause Leber congenital amaurosis (LCA), the most severe retinal dystrophy causing visual impairment in early childhood.[5–8] RPE65-LCA (LCA2) is more common than LRAT-LCA.[5] Two mouse models, *Rpe65*$^{-/-}$ and *Lrat*$^{-/-}$, capture many salient pathologic features of human LCA.[9–11] Both rod and cone functions are severely compromised due to the lack of 11-*cis* retinal.[9,10] Apo-rhodopsin is transported normally to the rod outer segments (ROS) and the rod photoreceptors degenerate slowly (>10 months). In contrast, the cone opsins (S-opsin and M-opsin) fail to traffic from the cone inner segment to the cone outer segment (COS) and the cone photoreceptors in the central/ventral regions degenerate rapidly (<4 weeks).[11–14] Concomitantly, cone membrane–associated proteins—e.g., cone transducin α-subunit ($G\alpha_{t2}$), cone phosphodiesterase 6α′ (PDE6α′), and G-protein–coupled receptor kinase 1 (GRK1)—are not transported to the outer segment properly and are degraded. Early loss of foveal cones also occurs in RPE65-deficient patients.[15,16] Rod degeneration is caused by the constitutive activity of the rhodopsin apoprotein,[17] while rapid cone degeneration is caused by endoplasmic reticulum (ER) stress induced by S-opsin aggregation.[18]

In this work, we examine the efficacy of an ER chemical chaperone, tauroursodeoxycholic acid (TUDCA), in preventing the rapid cone degeneration in the *Lrat*$^{-/-}$ model. TUDCA is a hydrophilic bile acid, which has been shown to be effective in alleviating ER stress and preventing apoptosis in many disease models, including type 2 diabetes,[19] hereditary hemochromatosis[20] acute kidney injury,[21] acute pancreatitis,[22] retinal degeneration,[23–28] Alzheimer's disease,[29–34] and Parkinson's disease.[35] This study indicates that systemic injection of TUDCA is effective in reducing ER stress, preventing apoptosis, and preserving cones in *Lrat*$^{-/-}$ mice.

Methods

Animals and TUDCA Injection

Lrat$^{-/-}$ mice were generated and described previously.[10] WT (*C57BL/6J*) mice were purchased from Jackson Laboratory. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah and were performed in accordance with the ARVO Statement for the Use of Animal in Ophthalmic and Vision Research. Mice were reared under cyclic light (12-hour light/12-hour dark).

Lrat$^{-/-}$ and WT mice were treated with TUDCA (TCI America) or vehicle (0.15 M $NaHCO_3$, pH 7.0) following a published procedure.[25] Several studies showed that subcutaneous injection of TUDCA (500 mg/kg b.w.) every 3 days preserved the structure and function of both cone and rod in mouse models of retinitis pigmentosa.[24–26,28] Thus, the same dose and injection schedule was adopted in this study. Mice were

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weighed and injected subcutaneously at the nape of the neck starting at P9. The starting date of P9 was selected because others have shown that early intervention by TUDCA is crucial to avoid treatment variability.[36]

Cone Cell–Density Analysis in Flatmounted Retinas

Retinal flatmounts were prepared as described previously,[12,14] except that cone arrestin was used to label cone cells. Briefly, eyes were fixed in 4% formaldehyde solution in PBS for 10 minutes. After removing the cornea, iris, lens, and RPE-choroid, retinas were fixed for 2 hours. After three washes with PBS, the retinas were incubated with a blocking buffer (0.6% triton X-100, 1% BSA in PBS) for 1 hour. The retinas were incubated with a cone arrestin antibody[14] overnight at 4°C. After washing three more times with PBS, retinas were incubated with Cy3-conjugated goat anti-rabbit secondary antibody. Retinas were again washed three times in PBS, mounted on a slide, and cover-slipped. Samples were viewed with a confocal microscope (Olympus FV1000; Olympus America Inc., Center Valley, PA). Cones were counted from three retinal areas: central (within 500 μm of the optic nerve), dorsal, and ventral. Data were expressed as the mean number of cones/mm^2 ± SEM.

Immunohistochemistry

The immunohistochemical procedure was performed as previously described.[14,18,37] Cryostat sections (10–15 μm) were incubated with various primary antibodies, and were visualized with Alexa 488- or Cy3-conjugated secondary antibodies. Nuclear dye DAPI was included in the secondary antibodies. The following primary antibodies were used, anti-M-opsin (AB5405) from Millipore, anti-S-opsin (MBO) (generous gift of J. Chen, USC);[38] anti-CHOP (L63F7); anti-active-caspase-3 (#9669) from Cell Signaling Technology; anti-GRK1 (SC-13,078); anti-$G\alpha_{t2}$ (SC-390) from Santa Cruz Biotechnology; anti-cone-arrestin (prepared according to reference 39); and monoclonal anti-rhodopsin antibody1D4 (generous gift of R. Molday, UBC, Vancouver, Canada).

Western Blot

Retinas from both eyes of the mouse were sonicated in 150 μL of radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) plus a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein concentration was measured by Bio-Rad protein assay kit; 15 μg of protein from each sample was loaded for SDS-PAGE, except that samples were diluted 100 times for detecting rhodopsin. The primary antibodies were detected with goat anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase. The primary antibodies were the same as used in immunohistochemistry. Additional immunoblot antibodies were monoclonal anti-$G\alpha_{t1}$/$G\alpha_{t2}$ (TF15, Cytosignal) (the amount of $G\alpha_{t2}$ from cones is negligible in comparison with $G\alpha_{t1}$ from rods on western), anti-PDE6α' (generous gift of T. Li, NEI), rabbit monoclonal anti-VCP (EPR3307[2]; Epitomics, Inc., Burlingame, CA), and anti-β-actin (AC-15; Sigma-Aldrich, St. Louis, MO).

Statistics

Data were presented as mean ± SEM, and the differences were analyzed with unpaired two-sample Student's *t*-test. P values < 0.05 were considered statistically significant.

Results

TUDCA Significantly Slows Down Cone Degeneration in *Lrat*$^{-/-}$ Mice

Cone photoreceptors degenerate rapidly in *Lrat*$^{-/-}$ mice.[11–14] To determine whether TUDCA can prevent or slow down this rapid cone degeneration, *Lrat*$^{-/-}$ mice were injected subcutaneously once every 3 days starting at P9. Treated mice were euthanized at P28 and cone cell densities were determined at different retinal regions from fluorescence images of cone-arrestin–stained retinal flatmounts. There was significant cone degeneration in the ventral and central regions of retina in vehicle injected *Lrat*$^{-/-}$ mice (Figs. 1A, 1B), as described previously.[11–14] Frozen sections probed with an anti-cone arrestin antibody revealed cone fragments in the inner segment and pedicle area in a few remaining severely degenerate cones (Fig. 2, red arrows). TUDCA treatment resulted in a significant increase (~3-fold, $P < 0.001$) in cone density in the ventral and central retina compared with the vehicle (Fig. 1). Cone cell morphology was greatly improved, as evidenced by the presence of intact cone structures (Fig. 2). TUDCA treatment also increased cone numbers (25%) in the dorsal retina, although the difference was not statistically significant ($P = 0.057$). Although TUDCA provided significant protection of central and ventral cones of *Lrat*$^{-/-}$ mice, the cone densities are still lower than those of WT (54.5% and

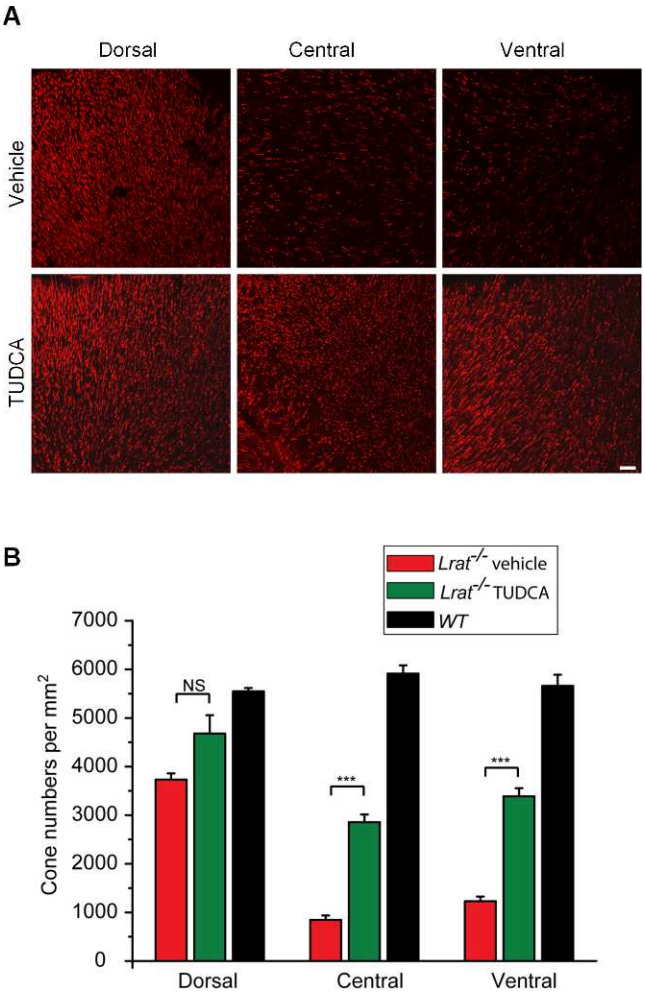

Figure 1. Quantification on the protective effect of TUDCA on *Lrat*$^{-/-}$ cones. (**A**) Cones of TUDCA or vehicle injected *Lrat*$^{-/-}$ mice (P28) were visualized by labeling cone arrestin in flatmounted retinas. Scale bar = 50 μm. (**B**) Cone photoreceptors were counted in the ventral, central, and dorsal sections in TUDCA injected *Lrat*$^{-/-}$ ($n = 8$), vehicle-injected *Lrat*$^{-/-}$ ($n = 6$), and untreated WT ($n = 5$). Data were expressed as the average numbers of cones per mm^2 (mean ± SEM). Untreated WT mice were included as controls to evaluate the efficacy of TUDCA. ***$P < 0.001$, NS, not significant.

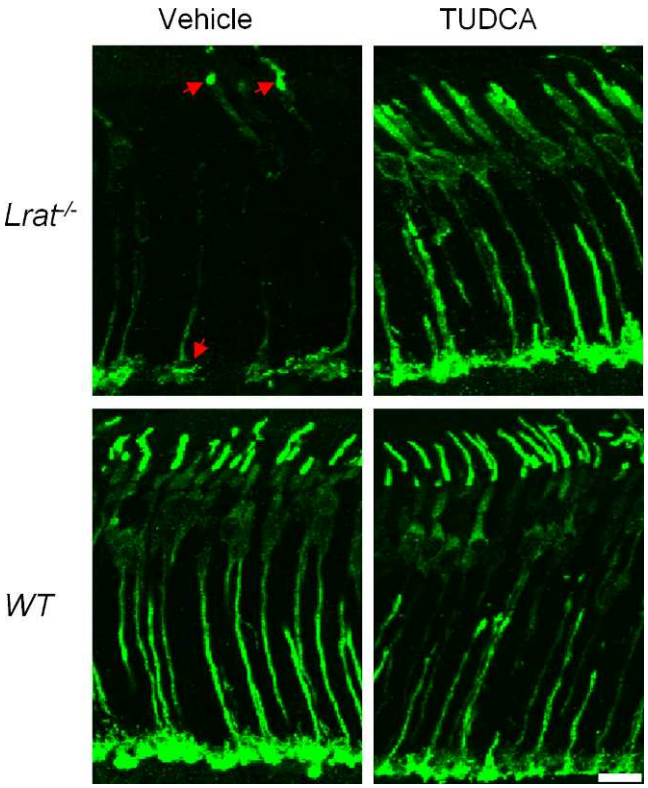

Figure 2. Comparison of cone cell morphology between TUDCA and vehicle-treated P28 mice (*Lrat*$^{-/-}$ and WT). Cones of TUDCA or vehicle-injected *Lrat*$^{-/-}$ or WT mice were visualized by labeling cone arrestin in retinal sections. *Red arrows* indicate a few remaining severely degenerate cones of *Lrat*$^{-/-}$ mice with "patched" labeling in the outer segments and synaptic pedicels. Scale bar = 10 μm.

62.7% lower for central cones and ventral cones, respectively). In contrast to its protective effect on cones, TUDCA treatment had no effect on rod photoreceptors of P28 *Lrat*$^{-/-}$ and WT mice in terms of both morphology and cell numbers (indicated by the thickness of the outer nuclear layer (ONL, Fig. 3).

TUDCA Reduces ER Stress and Apoptosis in *Lrat*$^{-/-}$ Retina

Due to S-opsin aggregation, central and ventral cones are under much more intense ER stress than dorsal cones in the *Lrat*$^{-/-}$ mice.[18] Therefore, this study examined whether TUDCA treatment had an effect on CHOP (C/EBP homology protein, a B-ZIP transcription factor) activation. CHOP is a well-characterized UPR (unfolded protein response) target gene and an ER stress marker associated with apoptosis.[40] Indeed, a substantial reduction of CHOP was observed in the ventral retina of TUDCA-treated *Lrat*$^{-/-}$ mice compared with that in vehicle-treated littermates at P18 (Fig. 4A). Furthermore, the TUDCA injection virtually eliminated apoptotic signals in the ONL of *Lrat*$^{-/-}$ mice judged by caspase-3 activation (Fig. 4B).

TUDCA Does Not Correct Mistrafficking of Membrane-Associated Proteins in *Lrat*$^{-/-}$ Cones

Previous work showed that several membrane-associated proteins (e.g., M/S opsins, GRK1, $G\alpha_{t2}$) involved in cone phototransduction failed to traffic to the outer segment of *Lrat*$^{-/-}$ or *Rpe65*$^{-/-}$ cones.[14] The mistrafficked proteins were degraded through a posttranslational mechanism,[14] except that

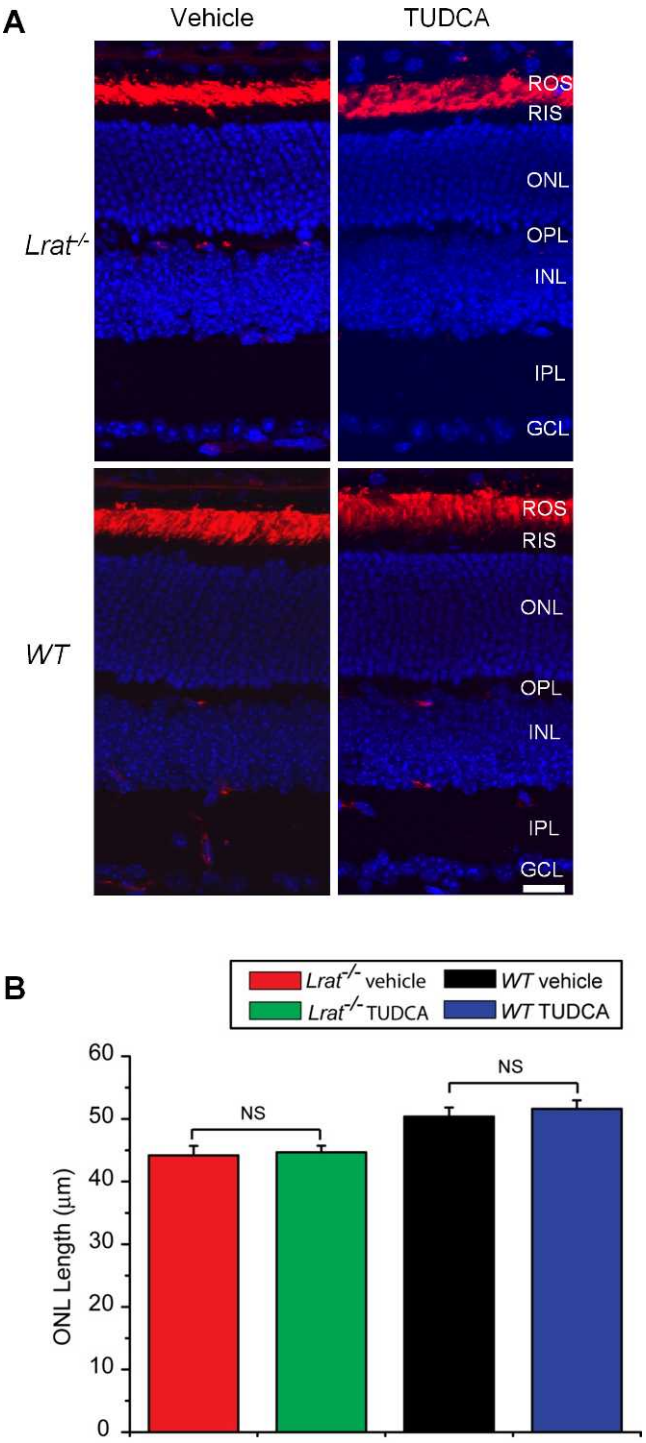

Figure 3. Effect of TUDCA treatment on rod photoreceptors. (**A**) P28 *Lrat*$^{-/-}$ and WT retinal sections were labeled with rhodopsin antibody 1D4 (in *red*). Nuclei were stained with DAPI (*blue*). Scale bar = 20 μm. (**B**) ONL length was measured from TUDCA-injected *Lrat*$^{-/-}$ ($n = 8$), vehicle-injected *Lrat*$^{-/-}$ ($n = 6$), TUDCA-injected WT ($n = 6$), and vehicle-injected WT ($n = 6$). Data were presented as mean ± SEM. NS, not significant.

S-opsin aggregated and resisted cellular degradation.[18] This study examined by immunohistochemistry whether TUDCA could rescue the trafficking of these membrane proteins. In vehicle-injected P18 *Lrat*$^{-/-}$ mice, transmembrane proteins such as M- and S-opsins were mislocalized in the inner segment, cell body, axon, and synaptic pedicle of cones, as

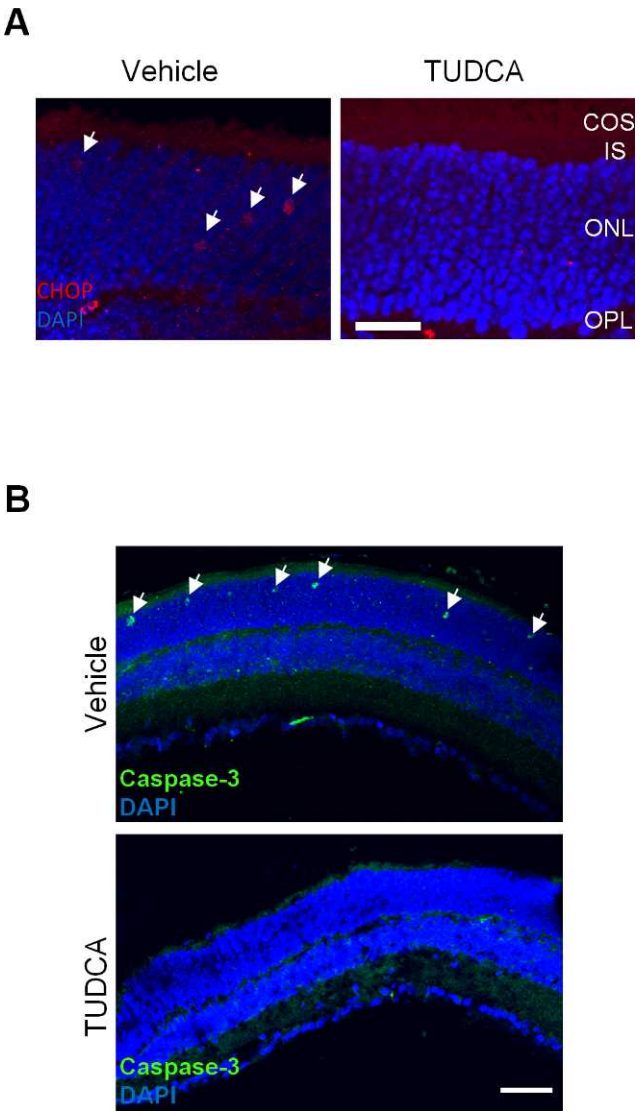

Figure 4. Effect of TUDCA treatment on CHOP and caspase-3 activation in *Lrat*$^{-/-}$ retina. (**A**) The ventral retinal sections of P18 *Lrat*$^{-/-}$ mice were labeled with an anti-CHOP antibody (*red*) and DAPI (*blue*). *White arrows* indicate CHOP signals in the ONL of the ventral retina of vehicle-injected *Lrat*$^{-/-}$ mice. Red signals in the OPL were due to the labeling of retinal vessels by the Cy3-conjugated goat anti-mouse secondary antibody. Scale bar = 20 μm. (**B**) Retinal sections of P18 *Lrat*$^{-/-}$ mice (vehicle or TUDCA injected) were labeled with an antibody against the active caspase-3 (*green*) and DAPI (*blue*). *Arrows* indicate caspase-3 signals in the ONL of the vehicle-injected mice. Scale bar = 50 μm.

shown previously[11,14] (Fig. 5). For peripheral membrane-associated proteins, GRK1 was not detected in *Lrat*$^{-/-}$ cone outer segment (COS) while the signal of $G\alpha_{t2}$ was much reduced similar to the previous work.[14] TUDCA treatment did not correct the mistrafficking of either cone transmembrane proteins (S-opsin and M-opsin) or peripheral membrane–associated proteins ($G\alpha_{t2}$ and GRK1).

TUDCA Promotes the Degradation of Membrane-Associated Proteins in *Lrat*$^{-/-}$ Cones

It has been shown previously that S-opsin aggregation causes rapid central/ventral cone degeneration in *Lrat*$^{-/-}$ mice.[18] This study then examined by immunoblot the possibility that TUDCA exerted its protective effect on *Lrat*$^{-/-}$ cones by reducing S-opsin aggregation. At P18, an early stage of cone degeneration, M-opsin was markedly reduced, whereas S-opsin accumulated in *Lrat*$^{-/-}$ cones as previously described[18] (vehicle-injected *Lrat*$^{-/-}$ versus vehicle-injected *WT,* Fig. 6A). TUDCA treatment made no difference in the protein levels of M- and S-opsins in either *Lrat*$^{-/-}$ or *WT* mice, suggesting TUDCA did not reduce S-opsin aggregation. As shown previously by study authors[18] and others,[41] S-opsin of *Lrat*$^{-/-}$ cones (both vehicle and TUDCA treated) exhibited slower mobility in SDS-PAGE compared with WT S-opsin (Fig. 6A), which was attributed to incomplete N-glycan processing.[41] Misfolded S-opsin aggregates in the ER, which may prevent the normal N-glycan trimming in the Golgi, resulted in higher molecular-weight products. However, the nature and significance of this "mobility shift" deserves further study. At P28, an advanced stage of cone degeneration, TUDCA treated *Lrat*$^{-/-}$ retina contained far more S-opsin and cone arrestin than vehicle controls (Fig. 6B), consistent with a significant increase of cone numbers in the central and ventral retina (Fig. 1). Surprisingly, an increase of other COS proteins (e.g., M-opsin, $G\alpha_{t2}$, and PDE6α′) was not observed, despite the large increase of cone numbers in TUDCA-treated *Lrat*$^{-/-}$ retina. In fact, the level of $G\alpha_{t2}$ decreased in TUDCA-treated *Lrat*$^{-/-}$ retina. This result suggests that TUDCA promotes the degradation of COS proteins (except S-opsin) in *Lrat*$^{-/-}$ cones (see more in Discussion). In contrast to *Lrat*$^{-/-}$ cones, TUDCA had no effect on protein stability of membrane-associated proteins in *Lrat*$^{-/-}$ rods (i.e., $G\alpha_{t1}$ and rhodopsin) (Fig. 6B). TUDCA may promote the degradation of COS proteins by enhancing the ER-associated degradation (ERAD) pathway. To examine this possibility, this study compared the expression level of valosin-containing protein (VCP/P97)—an important component of the ERAD machinery that provides the main driving force for extraction of poly-ubiquitinated ERAD substrates through the ER membrane[42,43]—in retinal extracts between TUDCA and vehicle-treated *Lrat*$^{-/-}$ mice. Western blotting analysis showed that TUDCA increased the level of VCP (Fig. 6B), indicating enhanced ERAD.

Discussion

In the *Lrat*$^{-/-}$ LCA model, cone membrane-associated proteins (M-opsin, $G\alpha_{t2}$, PDE6α′, and GRK1) fail to traffic properly to the COS and are significantly reduced through a posttranslational mechanism.[14] One exception is S-opsin that resists proteasome degradation and aggregates, resulting in ER stress and rapid cone degeneration in the ventral and central retina.[18] In this work, an ER chaperone (TUDCA) was used to preserve *Lrat*$^{-/-}$ cones. Study authors found that TUDCA treatment significantly slowed down ventral and central cone degeneration (Fig. 1). Previous work suggested that cone opsins require 11-*cis* retinal to fold correctly and to traffic to COS.[14] Other COS proteins may require cone opsins as guides to be co-transported and targeted correctly. In the absence of 11-*cis* retinal, both M-opsin and peripheral membrane–associated proteins (e.g., prenylated PDE6α′ and GRK1, as well as acylated $G\alpha_{t2}$), which are processed in the ER, are likely degraded by ERAD. ERAD is an important quality control system in the ER of eukaryotic cells to ensure that only properly folded and assembled proteins (or complexes) are allowed to exit for delivery to their intended sites of function.[44,45] Inefficient clearance of misfolded and misassembled proteins from the ER leads to ER stress, which has been implicated in the pathogenesis of several major diseases (e.g., diabetes, inflammation, and neurodegenerative disorders

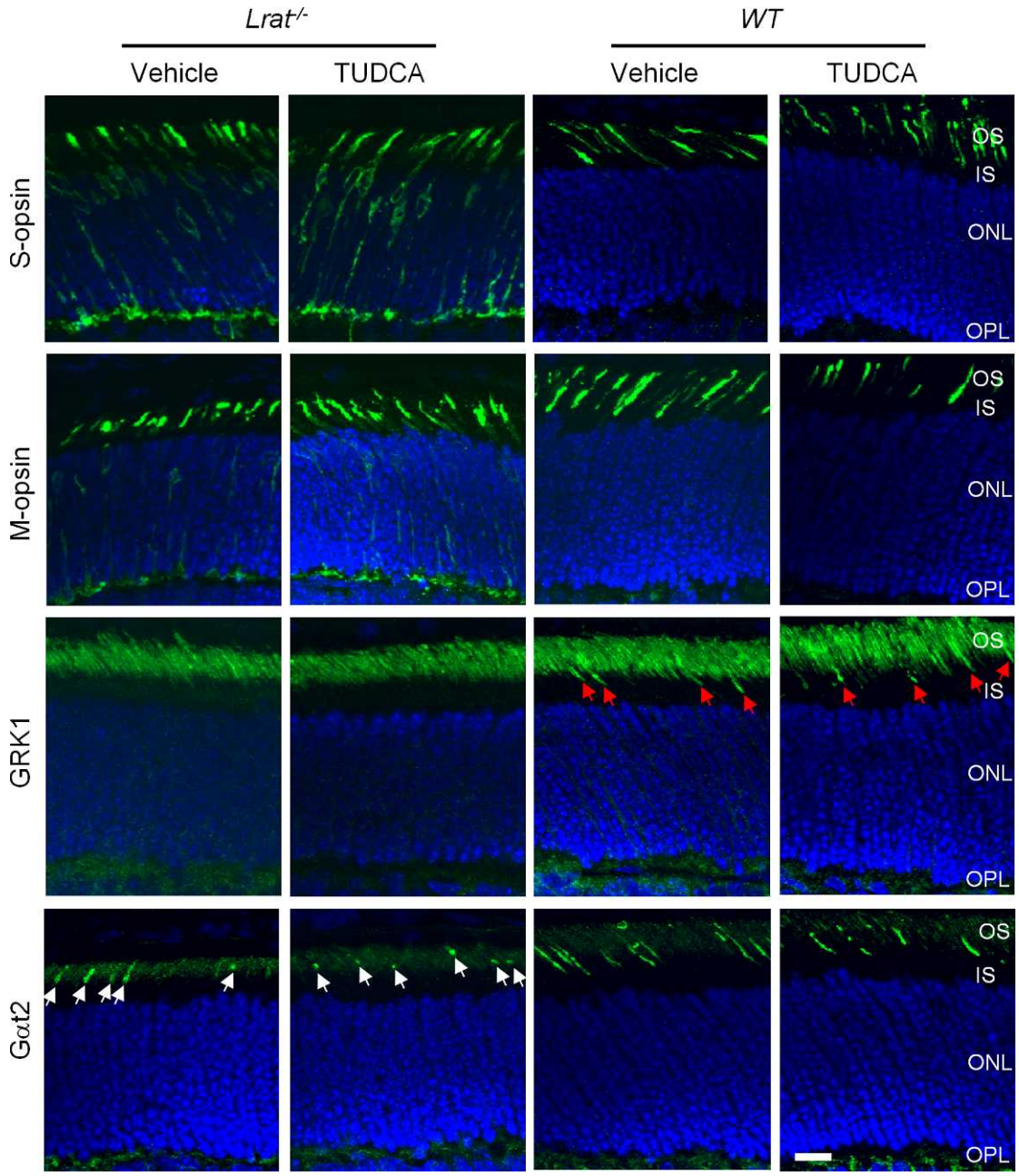

Figure 5. Immunolocalization of membrane-associated proteins (S-opsin, M-opsin, GRK1, and $G\alpha_{t2}$) in the retinas of TUDCA or vehicle-injected *Lrat*$^{-/-}$ and WT mice. P18 *Lrat*$^{-/-}$ and WT retinas were stained with antibodies against S-opsin, M-opsin, GRK1, and $G\alpha_{t2}$ (*green*). Nuclei were stained with DAPI (*blue*). *Red arrows* indicate GRK1 signals in the WT (both vehicle and TUDCA treated) COS while *white arrows* indicate reduced $G\alpha_{t2}$ signals in the *Lrat*$^{-/-}$ COS. Scale bar = 10 μm.

including Alzheimer's disease and Parkinson's disease[40]). TUDCA enhanced the degradation of COS proteins via ERAD (Fig. 6B) and reduced CHOP activation (Fig. 4A), suggesting that TUDCA protects cones by enhancing the ER's capability in dealing with stress. TUDCA may also protect cones via its potent anti-apoptotic property.[36,46] TUDCA has no effect on cytoplasmic proteins (e.g., arrestin) or ROS proteins (e.g., $G\alpha_{t1}$ and rhodopsin), probably because the folding and trafficking of these proteins were not affected by the absence of 11-*cis* retinal, and thus less affected by ERAD. Furthermore, TUDCA neither reduces S-opsin aggregation nor corrects the mistrafficking of membrane-associated proteins in cones, suggesting it does not stabilize the conformations of these proteins (or prevent their aggregation) as other chemical chaperones do.[47,48]

Currently, there is no FDA-approved treatment to prevent vision loss in LCA2 patients. There are two strategies under active study in the field: gene therapy delivering AAV2-RPE65 by subretinal injection and drug-based therapy by retinoid analogs. Gene therapy offers great promise with successful phase I and II clinical trials.[49–54] However, gene therapy has a limited application window, as a virus with a replacement gene must be injected before significant retinal degeneration occurs.[52,53] This is especially true in rescuing cones. A recent study concluded that there is no benefit and some risk in treating the cone-rich fovea.[54] Moreover, subretinal delivery of

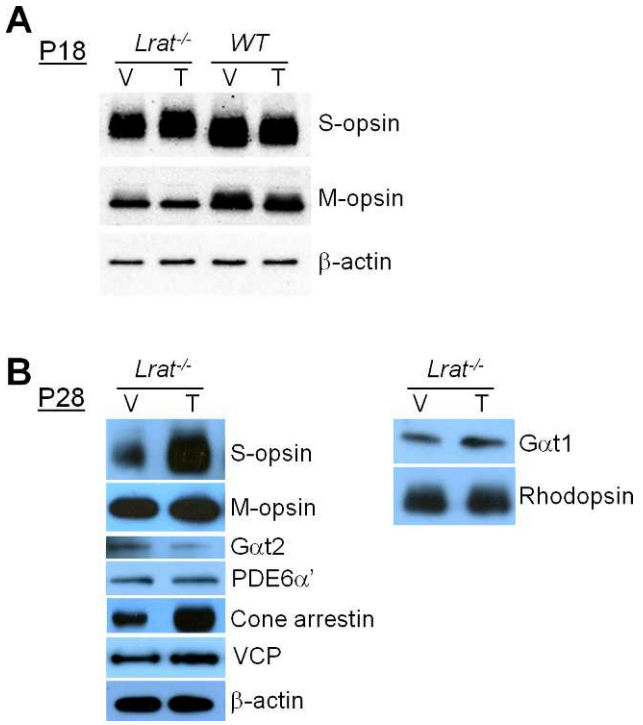

Figure 6. Western blotting analysis of various proteins in the retinas. (**A**) P18 *Lrat*$^{-/-}$ and WT mice. (**B**) P28 *Lrat*$^{-/-}$ mice. Mice were injected with vehicle or TUDCA. The results shown are representative of several blots. β-actin was included as the internal loading controls.

AAV-RPE65 is unlikely to cover the entire retina, leaving treated areas vulnerable to secondary degeneration caused by negative bystander effects from neighboring untreated cells.[55] Retinoid therapy can rescue both rod and cone function in animal models.[11,13,16,56,57] It also produced encouraging results in a phase Ib open label trial.[58] However, it is much more difficult to preserve cones than rods. In fact, a recent study showed that retinoid treatment is ineffective in preventing cone degeneration of *Rpe65*$^{-/-}$ mice raised in normal light/dark cycles and is only effective under constant darkness.[59] Therefore, development of novel drugs that prevent or delay the rapid cone degeneration in LCA2 will have considerable impact on the therapeutic strategy. Reagents that can protect cones of untreated regions from degeneration will minimize bystander effects and preserve untreated photoreceptors eligible for future treatment. The results of this study suggest that TUDCA may be a good candidate in treating LCA2 with several advantages: (1) TUDCA is not light sensitive and is therefore effective under normal light-dark cycle; (2) The hydrophilic TUDCA can be delivered to the eye by oral intake; and (3) TUDCA has already been approved for treating various liver and gallbladder diseases with outstanding safety records.[60,61] Therefore, TUDCA-based ER chaperones may be used as substitutes to gene therapy for protecting cones of LCA2 patients at very young ages when gene therapy might be too traumatic to the developing eye (i.e., before 4 years). TUDCA may also be used as a supplement to gene therapy at older ages to maximize the preservation of cones. Furthermore, since retinoid therapy is effective in preserving rods while TUDCA may be effective in preserving cones, the combination of the two may be used to protect both rods and cones.

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