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Expression and Localization of CERKL in the Mammalian Retina, its Response to Light-Stress, and Relationship with *NeuroD1* Gene

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Abstract

Mutations in the *Ceramide kinase like (CERKL)* gene are associated with retinitis pigmentosa (RP26) and cone-rod dystrophy. CERKL is homologous to *Ceramide kinase (CERK)*, and its function is still unknown. The purpose of this study was to test the expression and distribution of this gene and its protein in rat and in mouse tissues, in light-stressed rat retinas and in the retinas of *NeuroD1* knock-out mice to understand the role of CERKL in the retina. Expression of *Cerkl* and *Cerk* mRNA was determined by quantitative RT-PCR. Expression of the protein was determined by Western blotting with anti-CERKL antibody. Localization of the protein was determined by using immunofluorescence microscopy. With qRT-PCR, we revealed that the relative mRNA expression of *Cerkl* was the highest in the retina among all the rat tissue tested; it was >10-fold higher than in the brain. On the other hand, *Cerk* has ubiquitous expression and its relative abundance is >2 fold of *Cerkl* in the retina. *Cerkl* was expressed minimally in the developing mouse eyes and reached a peak at retinal maturity at 2 months. Western blots of retinal tissues revealed two major CERKL protein bands: 59 kDa (C1) and 37 kDa (C2). However, only C2 CERKL was found in the rat retinal rod outer segment (ROS) at level of that was not changed in light vs. dark adaptation. In the light-stressed retina, expression of *Cerkl* mRNA increased significantly, which was reflected in only on C2 CERKL protein. The CERKL protein localized prominently to the ganglion cells, inner nuclear layers (INL), retinal pigment epithelial (RPE) cells, and photoreceptor inner segments in the retinal sections. Nuclear localization of CERKL was not affected in RPE, INL and the ganglion cell layers in the light-stressed retina; however, the perinuclear and outer segment locations appear to be altered. In the *NeuroD1* knock-out mouse retina, the expression of *Cerkl* mRNA and protein decreased and that decrease also pertains to C2 CERKL. In conclusion, the retina had the highest level of *Cerkl* mRNA and protein expression, which reached its maximum in the adult retina; CERKL localized to ROS and RPE cells and the

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light adaptation did not change the level of CERKL in ROS; light-stress induced *Cerkl* expression in the retina; and its expression decreased in *NeuroD1* knock-out retina. Thus, CERKL may be important for the stress responses and protection of photoreceptor cells.

Keywords

ceramide kinase like (CERKL); ceramide kinase (CERK); NeuroD1; retina; photoreceptor; light-induced retinal degeneration; gene expression

INTRODUCTION

The *Ceramide kinase like (CERKL)* gene is one of the newest members of the retinitis pigmentosa (RP) family. Mutations of *CERKL* are associated with recessive, nonsyndromic retinitis pigmentosa (RP26) with significant macular involvement during the early stages of the disease (Ali et al., 2008; Auslender et al., 2007; Bayes et al., 1998; Tuson et al., 2004). Although Bayes et al., (1998) described cases of what they called recessive RP with appreciated heterogeneity in the phenotype, they also reported that younger patients (age 23 and 24 years) had macular alteration and significant central scotoma, which may indicate an early macular phenotype (Bayes et al., 1998). In 2004, Tuson et al. identified this gene and its mutation within members of the same family (Tuson et al., 2004). All affected individuals were homozygous for a nonsense mutation (R257X; CGA→TGA) in exon 5. The gene was named '*Ceramide kinase like*' based on its homology with *Ceramide kinase (CERK)* (Tuson et al., 2004). From the discovery of new mutations and from further characterization of the phenotype of the previously identified mutations, *CERKL* mutations are now considered as the cause of cone-rod dystrophy (CRD), which progresses to an RP-like phenotype in advanced stages (Aleman et al., 2009; Avila-Fernandez et al., 2008; Littink et al., 2010; Tang et al., 2009).

CERKL was initially considered as a retinal ceramide kinase. However, no kinase activity so far has been identified for this protein. CERKL expression is highly complex; more than 20 transcripts, which may generate various protein products, have been found in human and mouse tissues (Garanto et al., 2011). Attempts have been made to generate *Cerkl* knock-out mice; however, the transcriptional complexity of the gene makes it challenging to develop knock-out mice completely ablated for CERKL function (Garanto et al., 2012). CERKL has been shown to be expressed in various cell types in the retina, and a cone-dominant expression in mouse photoreceptors supports the notion that cone cell death precedes rods in humans with the *CERKL* mutation (Vekslin and Ben-Yosef, 2010). CERKL is also expressed significantly in ganglion cells and patients with *CERKL* mutations is known to develop significant pathology in the inner retina (Aleman et al., 2009). Given this transcriptional complexity, the *CERKL* mutation pathology is also complex.

In this study, we analyzed the expression and tissue distribution of *Cerkl* in rat tissues, confirmed its expression in mouse tissues and generated novel data on its expression in embryonic and developing mouse eyes to gain a better understanding of the role of this gene in the retina during embryogenesis and development. Because CERKL has previously been speculated as a retinal CERK (ceramide kinase), we performed a side-by-side comparative analysis of the expression of *Cerkl* in every tissue and at developing stages. In a recent study, Nevet et al. showed an interaction between CERKL and neuronal calcium sensor (NCS) proteins, including guanylate cyclase activating protein 1 (GCAP1), GCAP2, and recoverin in the photoreceptor cells (Nebet et al., 2012). We compared expression of these genes with *Cerkl* and *Cerk* expression in developing eye tissues. From previous *in vitro* studies, CERKL was attributed to have a protective effect against oxidative stress (Tuson et al.,

2009). We used the light-stressed rat retina model in which photoreceptor cell death occurs by oxidative stress and measured the expression of the *Cerkl* gene and its protein and determined the localization of CERKL protein to understand whether CERKL is involved in retinal protection against stress.

We further analyzed expression of CERKL in the *NeuroD1* knock-out mouse retina. BETA2/NeuroD1 is a neuronal transcription factor; it is highly expressed in the developing retina, its genomic location is immediately adjacent to the 5' of *CERKL*, and it is conserved in all known mammalian species (Cho et al., 2007). Interestingly, *NeuroD1* knock-out mice develop a characteristic retinal degeneration phenotype; both rod and cone cells begin to degenerate during the very early postnatal days (Pennesi et al., 2003). We speculated that these linked genes may also be functionally related.

MATERIALS AND METHODS

Animal and Tissue Collection

All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the University of Oklahoma Health Sciences Center (OUHSC) Guidelines for Animals in Research. All protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the OUHSC, the Dean A. McGee Eye Institute (DMEI) and the University of Texas M. D. Anderson Cancer Center. All animals were maintained on a 12-h dim cyclic light (5–10 lux at the cage level) /12-h dark cycle, and tissues were collected at the end of a dark cycle at the appropriate age. We collected the fore brain, hind brain, retina, eyecup containing RPE-choroid-sclera (eyecups = RPE+choroids+sclera; no retina), heart, lung, liver, spleen, kidney, skin, testis, and ovary from adult Sprague Dawley (SD) rats. Mouse eye balls (6 to 8) were collected from embryonic day E15 and E18, and postnatal days P1, P3, P7, P15, P30, P60, P90, P210 to study the expression of the genes of interest in the whole eye. To study the distribution of this gene expression in mouse eye tissue, the distalmost 3-mm optic nerve (ON) cut from the scleral surface of the eyeballs, eyecups, retina, lens, iris-ciliary body (I-CB) and cornea were collected after dissection. Retinas from *NeuroD1* knock-out mouse were collected at P30 for mRNA and protein expression studies.

For the immunofluorescence microscopy, adult albino (BALBc) mice and *NeuroD1* knock-out mice (at P60) eyeballs were fixed in 4% paraformaldehyde and embedded in OCT. Cryosections (10 μ m) were prepared for immunolocalization studies of the CERKL protein.

Light damage of Albino Rats

Eight- to ten week-old SD rats were exposed to damaging light (white cool light) for 6 h (9 AM to 3 PM) at an intensity of 2,700 lux following previously published protocols (Mandal et al., 2011; Mandal et al., 2009). After light damage, the retinas and eyecups were harvested at select time points (0 h, 3 h, 6 h, 12 h, and 24 h after the completion of light-damage), snap frozen in liquid N₂, and prepared for RNA and protein analysis. For studying the localization of the CERKL protein in the light-exposed retina, dark-adapted non-light-damaged (NLD) and light-damaged (LD) eyes at 0 h and 24 h after LD were harvested, fixed in 4% paraformaldehyde, embedded in OCT and sectioned.

RNA Isolation and cDNA Synthesis for Quantitative RT-PCR (qRT-PCR)

RNA was isolated from all of the collected tissues using TRIzol (Invitrogen) reagent following the manufacturer's protocol. First-strand cDNA synthesis was carried out (SuperScript II First-Strand Synthesis System; Invitrogen) for qRT-PCR. Primers for qRT-PCR were designed in such a way that they spanned at least one intron, which eliminated the

possibility of amplification from residual genomic DNA contamination. Degenerative forward and reverse primers for qRT-PCR were designed to amplify both mouse and rat *Cerkl* sequences. As *Cerkl* is known to have several transcripts (Garanto et al., 2011; Tuson et al., 2009), the primers were selected from exon 11 (forward) and exon 12 (reverse) of the known protein encoding the *Cerkl* transcript (ensemble.org: Cerkl-001 ENSMUST00000143974; NCBI CCDS database: Mouse CCDS set: CCDS38157). These two exons are common in almost all of the known transcripts of *Cerkl* (Garanto et al., 2011), so that the amplification can represent the total *Cerkl* expression. Primer pairs were forward: 5'-AACAATGGAAGCATGGCTCT-3'; reverse, 5'-CTCCTGTGGGCTGTATCCAT-3' for *Cerkl* and forward: 5'-GTCCTTCCTCCAGCACAG-3'; reverse, 5'-GCACTTCCGGATAAGGATGA-3' for *Cerk*. The sequence of primers used for other retinal genes will be obtained from the corresponding author upon request. Quantitative PCR (using iQ SYBR Green Supermix; Bio-Rad, Hercules, CA) and melt-curve analysis (using iCycler; Bio-Rad) were performed. Mean values (\pm SD) were calculated and are presented as fold over the most consistent housekeeping genes, *RPL19*, following previously published analysis methods (Mandal and Ayyagari, 2006; Mandal et al., 2009; Mandal et al., 2006a; Mandal et al., 2006b).

Immunofluorescence Microscopy

The localization of the CERKL protein in mouse and rat cryosections was determined by using immunofluorescence microscopy with a rabbit polyclonal anti-CERKL antibody (Abcam Inc. Cambridge, MA) as described previously (Mandal and Ayyagari, 2006; Mandal et al., 2006a; Mandal et al., 2006b; Sherry et al., 2005). Frozen sections were thawed, rehydrated, and treated with 1% NaBH₄ to reduce autofluorescence. Nonspecific labeling was blocked by using a 'blocker' containing 10% normal horse serum, 5% bovine serum albumin, 1% fish gelatin, and 0.5% Triton X-100 in PBS. Excess blocker was removed, and primary antibody was applied overnight at 4°C. Sections were rinsed and incubated in anti-rabbit secondary antibody with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature to visualize labeling. Sections were rinsed and cover-slipped with ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA, USA). Omission of primary antiserum eliminated immunolabeling and served as control.

Confocal microscopy was performed by using an Olympus FluoView FV500 microscope (Olympus Microsystems, Center Valley, PA, USA). To ensure quantitative image quality, laser power, pinhole settings, photomultiplier tube settings, and intensity thresholds were kept constant for each retinal section imaged. Images from NeuroD1 retina captured using epifluorescence microscope.

Preparation of Rat Retinal ROS

Rats were dark adapted for 12 h and their retinas were harvested under red light to prepare dark-adapted (D) rod outer segment (ROS); another group of rats were light adapted for 1 h after 12 h dark adaptation, and their retinas were harvested and used to prepare light-adapted (L) ROS. Four retinas from 2 rats formed one sample and we prepared ROS from 6 independent light-adapted and 6 independent dark-adapted samples. Retinal homogenates were used to isolate the ROS fractions by using methods described by Papermaster and Dreyer (Papermaster and Dreyer, 1974) with slight modification, as published earlier (Brush et al., 2010; Martin et al., 2005). The band containing purified ROS was collected, as well as the pellet from each sample. The collected fragments were diluted with homogenization buffer, and pelleted by centrifugation at 27,000g for 30 min at 4°C. The pellets were then used for protein extraction.

Protein Isolation and Western Blotting

Total protein was extracted from mice and rat retinal tissues, rat ROS and the pellet fraction using the lysis buffer T-Per (Pierce, Rockford, IL) with a protease inhibitor cocktail (Roche, Indianapolis). This was followed by centrifuging at 10,000g for 15 min at 4°C to collect the supernatants. After protein concentrations were determined by using BCA reagent (Pierce), equal aliquots (25–30 µg) of protein samples were applied to 10% sodium dodecyl sulfate polyacrylamide gels and electrophoretically separated. Resolved proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad) and blocked with 5% BSA for 1 h at room temperature. The membranes were incubated with anti-CERKL (1:1000) and anti-β-Actin (1:2000) antibodies for 16 h at 4°C, after which they were incubated with the appropriate secondary antibodies for 1 h at room temperature. Proteins were visualized by using chemiluminescence signals generated after the addition of Supersignal Chemiluminescence Substrate (Pierce, Rockford, IL). The intensities of the protein bands were determined by using Image J 1.32j software.

Statistical Analyses

Statistical analyses were performed by using Graphpad Prism 5.0 software. The quantitative data are expressed as mean ± SD for each group. An unpaired Student's *t* test and one-way ANOVA were performed for the means of two unmatched groups.

RESULTS

Expression Distribution of *Cerkl* in Murine Ocular Tissue

Mutations in the *CERKL* gene cause nonsyndromic RP or CRD in humans (Aleman et al., 2009; Ali et al., 2008; Auslender et al., 2007; Bayes et al., 1998). To understand the expression distribution of *Cerkl* in different tissues, we tested mRNA expression in various rat tissues. The distribution of *Cerkl* in human and mouse tissues has been reported and since its discovery *CERKL* has been speculated as retinal CERK (Bornancin et al., 2005; Garanto et al., 2011; Vekslin and Ben-Yosef, 2010). We tested expression and performed a side-by-side comparison of *Cerkl* with *Cerk* in rat tissues including the retina. The other tissues that were included were the forebrain, hindbrain, liver, lung, heart, skin, testis, ovary, spleen, kidney, and eyecup. *Cerkl* expression was abundant in the retina (Figure 1A). Other tissues that expressed *Cerkl* included the hind-brain, lung, kidney and eyecup (Figure 1A); however, the level of expression in those tissues was at least 10 fold lower than the expression in the retina (Figure 1A). We found that *Cerk* was also highly expressed in the retina and brain and that the relative abundance of *Cerk* was ~2 fold of *Cerkl* in the retina. All other tissues had some levels of expression of *Cerk* (Figure 1A). Therefore, the tissue distribution data indicated *Cerkl* is predominantly a retina-expressing gene. *Cerk*, on the other hand, has ubiquitous expression and its level of expression in the retina is higher than *Cerkl*, suggesting that *Cerkl* may not be an alternative of *Cerk* in the retina, as previously indicated (Vekslin and Ben-Yosef, 2010).

Using mouse and human tissues, *Cerkl* mRNA expression was found in the retina (Garanto et al., 2011; Vekslin and Ben-Yosef, 2010). We asked whether *Cerkl* was expressed in other eye tissues besides the retina, especially in the eyecup that includes RPE-choroids and sclera, which may indicate expression in the RPE cells. We detected *Cerkl* expression in mouse eyecup, I-CB and ON (Figure 1B). The highest level of expression was detected in the retina, which was similar to its expression in rat tissues (Figure 1A). However, *Cerk* had ubiquitous and higher levels of expression in all tested ocular tissues (Figure 1B).

Cerkl expression has been noted in mouse embryonic eyes (E14) and newborn eyes (P0), and a higher level of expression is found in 5-month-old eyes (Vekslin and Ben-Yosef,

2010). We studied the expression of *Cerkl* with other genes at various timepoints starting at E15 to P210. We included in this study the *Cerk* and two other genes, *Gcap1* and *Recoverin*, protein products of which have recently been shown to interact with CERKL in the photoreceptor cells (Nevet et al., 2012). We detected only a minimal level of expression of *Cerkl* in prenatal and postnatal developing eyes (Figure 1C). We found that a peak in the expression *Cerkl* at P7, the stage at which the photoreceptor cells start developing their outer segments (Figure 1C). However, its expression reached its maximum at the maturity of the retina (P60) and was maintained for 7 months (in mice) (Figure 1C) and 14–18 months (in rats, data not shown), the highest time-points examined. *Cerk* expression also had a similar pattern; however, embryonic tissues had significantly higher levels of *Cerk* expression than *Cerkl* (Figure 1C). As expected, the photoreceptor-specific *Gcap1* and *Recoverin* expression jump started at P7 and reached their maximum with the maturity of the eye (P60) and then maintained their levels in the mature retina (Figure 1C). The development and differentiation of mouse photoreceptor layer takes place between P7 and P30 (Smith et al., 2002). Increase in *Cerkl* expression at P7 may indicate its presence in the photoreceptor cells at a stage when photoreceptor outer segment starts developing and a role for CERKL in photoreceptor outer segment development or function or both. However, maintenance of an increased level of *Cerkl* in the adult retina may also indicate its presence in the photoreceptor outer segment, which is renewed and replaced continuously. This is true for other photoreceptor proteins such as Rhodopsin (Mandal et al., 2004), GCAP1 and Recoverin (Figure 1C). CERKL's interaction with GCAPs and Recoverin indicates its presence in the outer segment (Nevet et al., 2012).

Effect of Damaging Light on *Cerkl* mRNA Expression

In vitro studies suggested that CERKL is a stress response protein (Tuson et al., 2009). To examine its expression under stress, we used our light-damaged retinal model in which photoreceptor cell death occurs by apoptosis mainly due to oxidative stress. Apoptosis starts after 8–12 h and by 24 h almost all of the photoreceptor cells in the central retina enter into apoptosis (Gordon et al., 2002; Hafezi et al., 1997; Mandal et al., 2011; Mandal et al., 2009). We observed that *Cerkl* mRNA expression increased by 2 fold by 3 h and 4 fold by 6 h after light-damage (Figure 2A); and the expression remained at normal levels or higher until 24 h. However, the expression of *Cerk* was not affected until 6 h after light-damage and was significantly reduced at 12 h and 24 h after light-damage (Figure 2A), the time when the cells actively enter into apoptosis.

Expression of both *Cerkl* and *Cerk* in the eyecup tissue was reduced significantly immediately after (LD+0h) and 24 h after light damage (Figure 2B). We then tested some known photoreceptor genes in the rat retinas immediately after light-damage (LD+0). We found expression of the outer segment protein genes, namely, *rhodopsin (Rho)*, *phosphodiesterase 6A*, *cGMP-specific*, *rod*, *alpha (Pde6a)*, *phosphodiesterase 6B*, *cGMP*, *rod receptor*, *beta (Pde6b)*, *ATP-binding cassette*, and *sub-family A (ABC1)*, *member 4 (Abca4)* were significantly reduced (Figure 2C). The reduction was much higher in samples harvested at 24 h after light damage (data not shown).

Effect of Damaging Light on CERKL Localization in the Retina

Similar to the data previously shown for the mouse retina (Garanto et al., 2011; Vekslin and Ben-Yosef, 2010), we also detected CERKL localization in various regions and cell types (arrow-heads) of the retinas from albino mouse (Figure 3B) and rat (Figure 3C). Figure 3A is a serial mouse retinal section in which the primary antibody was omitted and served as negative control. Very prominent CERKL expression was found on ganglion cells, which appeared to be mostly nuclear (Figure 3B and 3C). The inner nuclear layer (INL) also had very prominent expression of CERKL (Figure 3B and 3C). CERKL was found to localize in

the inner and outer segments of the photoreceptor cells, and lightly on the intranuclear spaces of the ONL (Figure 3B and 3C). In addition to the previous observations we found distinct CERKL labeling on the RPE cells, especially on the RPE nucleus in both mouse and rat retina (Figure 3B and 3C).

CERKL can localize in the nucleus and CERKL protein contains a nuclear localization signal and nuclear export signals (Inagaki et al., 2006; Tuson et al., 2009), suggesting that CERKL might be a signal transporter or carrier protein that communicates between the nucleus and cytoplasm. To determine whether CERKL localization in the retina changes upon stress (light-damage), we studied CERKL localization in detail in the light-damaged rat retina at 0 h and 24 h after LD; the dark-adapted, non-light-damaged (NLD) retina served as control (Figure 4). In Figure 4A we present low magnification images of the entire width of retina in which Figure 4Ai, 4Aiii, and 4Av present the no primary antibody control for NLD (Figure 4Aii), LD+0 (Figure 4Aiv), and LD+24 (Figure 4Avi) samples. In Figure 4B, we present the magnified portions of the retina, regions 1–4 (boxed, Figure 4Aii) from all three samples. The RPE cells are almost disappeared or changed in morphology in the central retina at 24 h after light damage and therefore not included in the comparison in Figure 4B region 1.

Immediately after light-damage, CERKL labeling appeared greater in the outer segments (Figure 4Aiv). At 24 h CERKL labeling pattern changed in the disappearing RPE cells, localization moved from the nuclear to the base of apical membrane (Figure 4Avi). The CERKL labeling in the ONL in LD+0 retina changed from continuous perinuclear to diffused and punctuated (Figure 4Biv vs. 4Biii). With photoreceptor cells entering into apoptosis, the CERKL labeling in the ONL appeared to be intensified in the perinuclear region of some of the nuclei and disappearing from the dying photoreceptor nuclei (Figure 4Avi, 4Bv). CERKL protein localizes to the nucleus in the RPE cells, inner retinal neurons and in the ganglion cells (Figure 4Aii, 4Bi, 4Bvi, and 4Bix). No change in the localization was observed in the RPE cells immediately after light-damage (Figure 4Bii, compare with Figure 4Bi); in the ganglion cells immediately and 24 h after light damage (Figure 4Bx, 4Bxi; compare with Figure 4Bix). However, labeling intensity appeared to be increased in the nucleus of the inner retinal neurons after light damage (Figure 4Bvii, 4Bviii; compare with 4Bvi).

Effect of Light-stress and Light-adaptation on CERKL Protein Expression

Using the anti-CERKL antibody, we detected two major protein bands of 59 kDa and 37 kDa in rat retinal tissues and designated those as C1 and C2, respectively (Figure 5A-NLD, and Supplement Figure 1). When we examined the expression of the CERKL protein in the light-damaged retina we found no change in the levels of the C1 protein; however, the levels of C2 (37 kDa) protein increased significantly (3–5 fold) after light damage (Figure 5A, 5B), which was consistent with the increase of the *Cerkl* mRNA (Figure 2A).

CERKL protein has been shown to be present in photoreceptor outer segments (Garanto et al., 2011; Vekslin and Ben-Yosef, 2010). Its presence and interaction with outer segment proteins has recently been demonstrated using bovine photoreceptor outer segments (Nevet et al., 2012). We asked whether the level of CERKL changes with light and dark adaptation in rat retinal ROS. Proteins from purified ROS from light- and dark-adapted rats were analyzed by Western blotting. We detected only the C2 band (~ 37 kDa) in the ROS samples, and no difference was found in the level of this protein between the dark-adapted and light-adapted samples (Figure 5C). In this analysis, we included a few pellet samples from the same ROS prep and detected both C1 (though lighter) and C2 bands (Figure 5C). Also we did not detect any difference in the intensity of the CERKL pellet bands between D and L samples (Figure 5C).

CERKL Expression in *NeuroD1* Knock-out Mice

In the human chromosome 2q31.3, *CERKL* is linked to the *NEUROD1* gene, which is located immediate upstream of *CERKL* in *cis* configuration. This region is conserved in most of the mammalian genome including the mouse and rat genomes. BETA2/NeuroD1 is a helix-loop-helix transcription factor that is expressed widely in the developing nervous system including the retina (Cho et al., 2007; Cho and Tsai, 2006). Interestingly, *NeuroD1* knock-out mice develop a retinal degeneration phenotype, in which both rod and cone photoreceptor cells degenerate with age (Pennesi et al., 2003). Although, *NeuroD1* is expressed in most of the retinal neurons, it is the photoreceptors at the ONL that are affected by the loss of the protein (Pennesi et al., 2003). We hypothesized that these two linked genes (*Cerkl* and *NeuroD1*) are also related functionally, and that NeuroD1 may affect the transcription of *Cerkl* since NeuroD1 is a transcription factor. Recently, Garanto et al. (2011) showed that one of the transcription initiation sites for *Cerkl* belongs to the *NeuroD1* sequence (Garanto et al., 2011). It would therefore be interesting to see *Cerkl* expression in the *NeuroD1* retina. In a preliminary experiment, we observed that *Cerkl* mRNA expression was significantly reduced (38%) in the retina of one-month old *NeuroD1* knock-out mice (Figure 6A). When we tested other rods and cone cell markers in the same sample, we detected variable expression: the short wave-length cone opsin increased and cone arrestin decreased significantly (6A). In the protein analysis, we found only a reduction in the 37 kDa protein band (C2) and not in the 59 kDa band (C1) (Figure 6B, arrow). This decrease in protein levels was also reflected by a decrease in the intensity of the CERKL labeling in 2-month-old *NeuroD1* knock-out retinal sections (Figure 6C).

DISCUSSION

CERKL mutations are involved in both macular and peripheral pathologies. Full-field ERG findings indicate degeneration of both rod and cone photoreceptors and early maculopathy. All of the cases reported thus far from different groups of patients with different mutations by independent investigators point towards a common pathology whereby younger patients (20–30 year old) present with early macular alterations, which ultimately progress towards an RP-like phenotype with peripheral retinal degeneration (Aleman et al., 2009; Ali et al., 2008; Auslender et al., 2007; Avila-Fernandez et al., 2008; Bayes et al., 1998; Littink et al., 2010). Also all of these different mutations identified cause similar phenotypic consequences, which may otherwise rule out the possibility of differential penetrance of different *CERKL* mutations.

Although *CERKL* has homology with *CERK*, the function of this protein is not known. A knock-out mouse was developed by the Bornancin group (*Cerkl*^{-/-} mice), but its role as a kinase has yet to be established (Graf et al., 2008). The retinas of *Cerkl*^{-/-} mice were not characterized well by using histological and electrophysiological parameters and therefore it did not result in the generation of much information on *CERKL* mutation-mediated pathologies. Recently, Garanto et al. (2012) reported another mouse model where they substantially knocked-down the *Cerkl* expression in the retina but those mouse failed to develop any photoreceptor phenotype (Garanto et al., 2012). The *CERKL* gene in humans and mice has unusually higher varieties of transcripts that result from alternative exon splicing, alternative promoter uses, and alternative translation initiation sites (Garanto et al., 2011). Therefore, developing a clean knock-out mouse is challenging (Garanto et al., 2012). While we performing our studies on *Cerkl* expression and distribution in rat and mouse tissues, two papers were published showing the distribution of *Cerkl* gene expression in mouse tissues and the localization of the protein in the retina (Garanto et al., 2011; Vekslin and Ben-Yosef, 2010). In our studies of rat tissues, we found that the retina had the highest levels of expression of the *Cerkl* gene and proteins (Figures 1 and S1). This may explain why so far known *CERKL* mutations in humans cause mostly nonsyndromic retinal

pathology; *CERKL* has more of a retinal-specific expression. Garanto et al. (2011) suggested that higher levels of *Cerk1* were expressed in the mouse liver; however, we could not detect any measurable levels of *Cerk1* in the rat liver and no expression was found in the human liver as well (Bornancin et al., 2005; Garanto et al., 2011). Since its discovery, *CERKL* has been predicted to be a retinal CERK. However, no ceramide kinase activity has been demonstrated for *CERKL* *in vitro* and *in vivo*. Vekslin and Ben-Yosef (2010) reported that the mouse retina had significantly higher *Cerk1* expression than *Cerk* expression (Vekslin and Ben-Yosef, 2010). Our quantitative RT-PCR data indicates that *Cerk* expression was >2 fold higher than *Cerk1* expression in both mouse and rat retinas (Figure 1), that *Cerk* transcripts were present in all of the tissues that we tested, and that the expression of *Cerk* was always higher than the *Cerk1* in the tissues that also expressed *Cerk1* (Figure 1). This is similar to the data on expression in human tissues in which both *CERKL* and *CERK* expression was measured quantitatively (Bornancin et al., 2005). A recent study, however, provided a possible functional role of *CERKL* away from CERK in the retina. Nevet et al. (2012) showed the interaction of *CERKL* with neuronal calcium sensor proteins, including GCAP1, GCAP2, and recoverin in photoreceptor cells (Nebet et al., 2012). These proteins are very important for photoreceptor functioning and therefore may provide clues to how *CERKL* mutation leads to photoreceptor degeneration pathology. Thus *CERKL* could have an independent role in the retina and may not be an alternative for CERK in the retina.

For our developmental studies, we used whole mouse eyes. In the whole eye, the major contributing tissue was the retina (Figure 1B). Expression of *Cerk1* was significantly higher in the adult eyes than in the developing eyes and the higher levels of expression were maintained until an older age. This may indicate that the role of *CERKL* in the retina is more for maintenance of the retina for its day-to-day activity than its involvement in retinal development. Like previous studies (Garanto et al., 2011; Tuson et al., 2004; Vekslin and Ben-Yosef, 2010), our study found that *CERKL* localization in various layers of the retina including photoreceptors (Figure 3). We also found *CERKL* localization in the RPE cells, specifically to the RPE nucleus. *CERKL* localization in various sub-cellular organelles including the nucleus has been reported (Inagaki et al., 2006; Tuson et al., 2009). We performed a detailed study of *CERKL* localization in the light-stressed retina (Figure 4). We detected changes in the localization of *CERKL* in and around the photoreceptor nuclei and increased labeling to the photoreceptor outer segments under light stress. However, nuclear localization of *CERKL* in the RPE, inner nuclear layer and the ganglion cells were not altered (Figure 4). This is not surprising though, because, photoreceptor cells are the primary target for light damage and the damaging effect of light starts with rhodopsin activation (Humphries et al., 1997; Noell and Albrecht, 1971; Noell et al., 1971; Noell et al., 1966). Alteration in *CERKL* expression and localization in photoreceptor cells under light stress may indicate a role of *CERKL* in the stress response of these cells, or it may be a consequence of the induction of apoptosis in photoreceptor cells.

We detected two major protein products, C1 at 59 kDa and C2 at 37 kDa, respectively, from the *Cerk1* gene in the retinas of mice and rats. The expression levels of both the mature proteins were very high in the retina (Figures 5A, S1), which correlated with the mRNA expression profile of the gene, suggesting that both might have resulted from the *Cerk1* gene transcription. However, we found only C2 protein in the ROS preparation (Figure 5C) and we found both C1 and C2 in pellet (Figure 5C), but the intensity of the C1 band is much weaker in the pellet than that of the C1 band obtained from the soluble protein from the whole retina as in Figure 5A (NLD). This may indicate C2 is a membrane-associated protein and thus can separate with ROS and can associate with other membranes and get pelleted during the ROS preparation. On the other hand, C1 is the soluble protein. The membrane-associating PH domain has been identified and characterized in *CERKL* (Rovina et al., 2009). Under light stress, we observed that *Cerk1* mRNA expression was induced in the rat

retina (Figure 2A) and found that this induction was specific to the C2 protein (37 kDa) (Figure 5A, 5B). C2 is present in the photoreceptor outer segments (Figure 5C); an increase of this protein during light-stress makes reasonable sense because photoreceptor outer segments receive the primary insult of light stress. Nevet et al. (2012) showed that CERKL plays a role in calcium signaling pathway in photoreceptor outer segments (Nebet et al., 2012). Our study supports the presence of CERKL in the outer segments and its role in the functioning of the photoreceptor cells. CERKL increased under light stress may also indicate its protective role in the stressed retina. CERKL was previously reported to have a protective effect from oxidative stress in cell cultures (Tuson et al., 2009).

CERKL can localize in the nucleus and some CERKL protein contains a nuclear localization signal and nuclear export signals (Inagaki et al., 2006; Tuson et al., 2009), suggesting that CERKL might be a signal transporter or carrier protein that communicates between the nucleus and cytoplasm. However, we did not detect any alteration in the nuclear localization of the CERKL protein in various nuclear layers under light stress (Figure 4). Similarly, we did not detect any changes in the level of the C1 protein (Figure 5B) and when we subjected the insoluble fraction (containing nucleus) of the proteins from the same samples used in the experiment represented in Figure 5A, we detected the presence of only C1 protein in those fractions (data not shown), indicating the nuclear fraction does not contain C2. This provides an indication that the soluble C1 CERKL may contain nuclear export and localization signals and may have an independent role that may or may not be related to the C2 CERKL.

We noticed a significant down-regulation of *Cerk* in the light-stressed retina and both *Cerk* and *Cerkl* in light-stressed eyecups (containing RPE) (Figure 5). We tested some known retinal genes and found a reduction in the expression of those related to rhodopsin functioning and the expression of all those genes related to photoreceptor maintenance goes significantly down after induction of apoptosis. The increase in *Cerkl* expression in light-stressed retina may therefore indicate an additional role other than maintaining the normal retinal function, which could be a role in combating cellular oxidative stress as proposed earlier (Tuson et al., 2009). However, the decrease in *Cerk* expression may be related to general down-regulation of other genes involved in normal functioning. Further, we can't rule out the possibility that increased *Cerkl* expression in the light-stressed retina with concomitant reduction in *Cerk* expression may indicate a compensatory role of CERKL for some CERK-related functioning during retinal stress.

The *BETA2/NeuroD1* gene is tightly linked to *CERKL* and is conserved in mammalian genomes. BETA2 is a transcription factor located immediately before *CERKL* on the 5' end. We studied the expression of CERKL in the *NeuroD1* knock-out retina with a speculation that these linked genes might be functionally related and that BETA2 may regulate the transcription of *CERKL*. In the one-month-old *NeuroD1* retina, we found significant reductions in the *Cerkl* mRNA (Figure 6A), a reduction in cone arrestin to a similar extent, and a decrease in the level of C2 CERKL protein (Figure 6B); however, the photoreceptor cells (ONL thickness and photoreceptor nuclear layers) appeared to not be reduced significantly at the age of 2 months (Figure 6D). The statistically significant reduction in thickness of the ONL of *NeuroD1* knock-out mice was not observed by two months of age, but the thickness of ONL become significantly different after 2-month of age (Cho JH, unpublished data). The photoreceptor cells, especially cone photoreceptors in *NeuroD1* knock-out mice degenerate starting as early as P10; the development and numbers of other neurons appear normal and by 3 months of age more than 50% photoreceptor cells die in the *NeuroD1* knock-out mice (Pennesi et al., 2003). When this studies were underway, Garanto et al. showed that one of the major mouse *Cerkl* transcripts has a transcription initiation sequence from *NeuroD1* gene and that is a highly expressed isoform in the mouse retina (Garanto et al., 2011). It is possible that knocking out *NeuroD1* by inserting a *LacZ*-neo

cassette in the *NeuroD1* exon II (Naya et al., 1997) disrupted the promoter and transcription initiation site of the *Cerkl* transcripts that make the CERKL C2 protein. However, further investigation is needed to prove that the C2 CERKL is the product of the mRNA transcript that has initiation in the *NeuroD1* sequence. In one-month-old retina we also observed significant reduction in cone arrestin but not in cone opsin (Figure 6A). Cone dominant expression of CERKL and a significant presence of CERKL in the cone outer segments have been demonstrated by independent studies (Garanto et al., 2011; Vekslin and Ben-Yosef, 2010). A reduction in C2 CERKL in the *NeuroD1* knock-out retina might therefore reflect the loss or dysfunction of cone photoreceptor cells. Further investigation is needed, however, to rule out the possibility of NeuroD1 acting as a *trans* acting transcription factor for *Cerkl* in cone or rod photoreceptors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

CERKL	ceramide kinase like
CERK	ceramide kinase
SD	Sprague Dawley
qRT-PCR	quantitative RT-PCR
ROS	rod outer segment

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- We confirmed that retina has the highest levels of expression of *Cerkl* mRNA among all the rat tissues we have tested. *Cerkl* mRNA expression is highest in the mature retina and a higher level is maintained in the ageing retina.
- CERKL protein localizes to the RPE cells, photoreceptor cells, inner retinal neurons, and ganglion cells. Nuclear localization of CERKL was detected in the RPE, ganglion and the inner retinal neurons. In photoreceptor cells, CERKL protein localizes to perinuclear regions, inner segments and rod outer segments (ROS).
- In light-stressed retina, *Cerkl* mRNA increases and the increase is specific to a CERKL protein small isoform. This is the isoform found in the ROS.
- In NeuroD1 knock-out mouse retina, the expression of *Cerkl* mRNA and protein decreases.

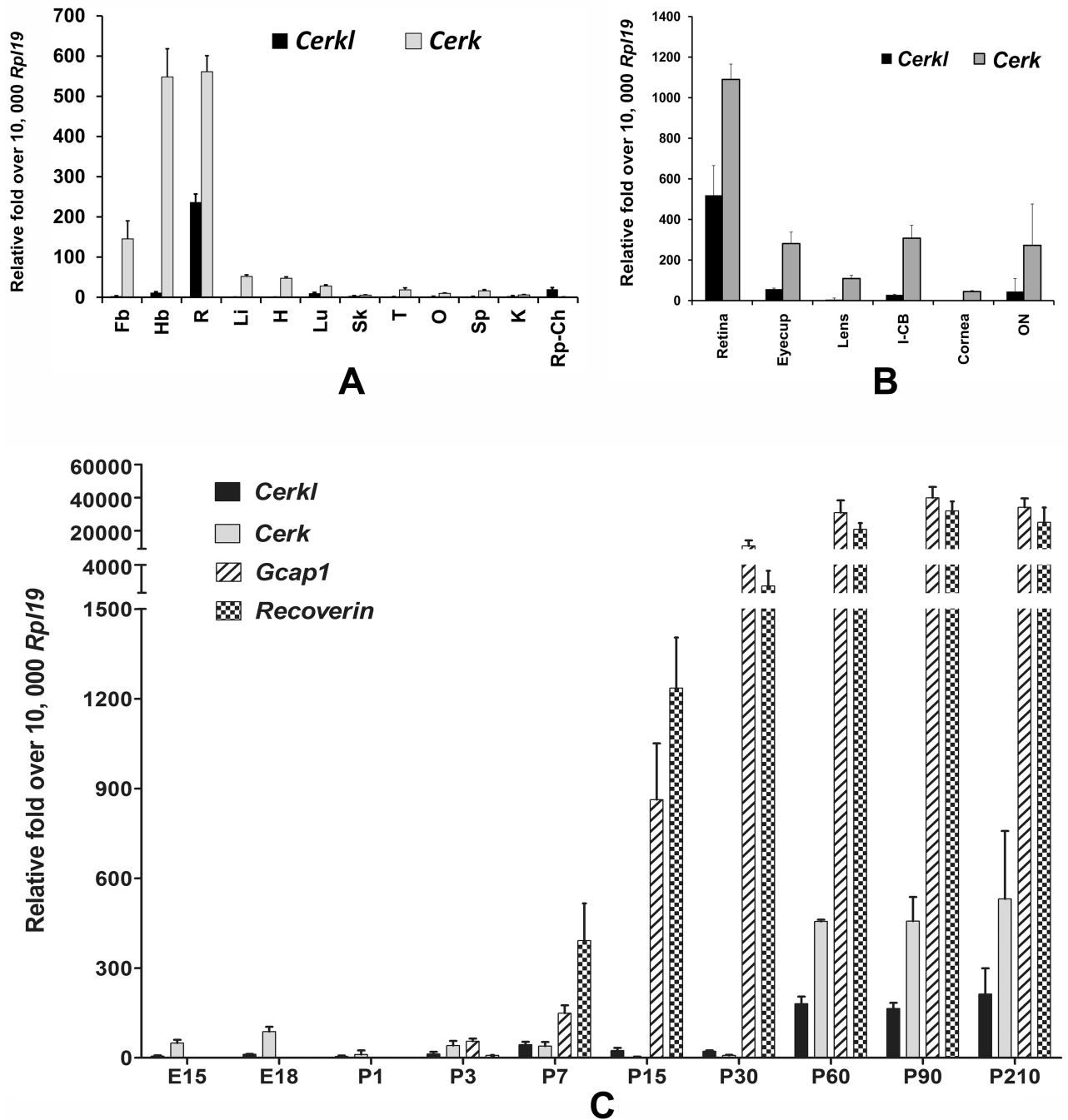


FIGURE 1.

A. Expression and distribution of *ceramide kinase like (Cerkl)* and *ceramidekinase (Cerk)* genes in rat tissues. Quantitative RT-PCR was performed with the primers that selectively amplified the major reported transcript of the *Cerkl* gene; *Cerk* expression was tested for comparison. Three independent reactions were performed per sample per primer pair, and the mean values of 3–4 samples (mean + SD, n = 3–4) were then compared with the expression of housekeeping gene *Rp19*. Fb, forebrain; Hb, hindbrain; R, retina; Li, liver; H, heart; Lu, lung; Sk, skin; T, testis; O, ovary; Sp, spleen; K, kidney; and Rp-Ch, RPE-choroids. **B. Distribution of *Cerkl* in mouse eye tissues.** *Cerk* expression was tested

for comparison. Eyes from adult mice were dissected in nuclease-free PBS, and a clean tissue pool of the optic nerve (ON; 3-mm distal-most optic nerve cut from the surface of the sclera), the eyecup containing RPE-choroid-sclera, retina, iris-ciliary body (I-CB), lens, and cornea were collected for RNA isolation and cDNA synthesis. Three independent reactions were performed per sample per primer pair, and mean values (+ SD, n = 4) are presented over the values of mouse *Rpl19* gene that is amplified in parallel replica reactions for each sample. **C. Expression of *Cerkl* in developing mouse eyes and eyes with age.** Expression of *Cerk*, *Gcap1* and Recoverin was tested for comparison. RNA was isolated from mouse whole eyes from embryonic days 15 and 18 (E15 and E18) and postnatal days 1 to 210 (P1 to P210) and was used to measure the quantitative expression of *Cerkl* and *Cerk* genes. Quantitative RT-PCR was performed and data were analyzed as described in Figure 1A (mean+ SD, n = 4).

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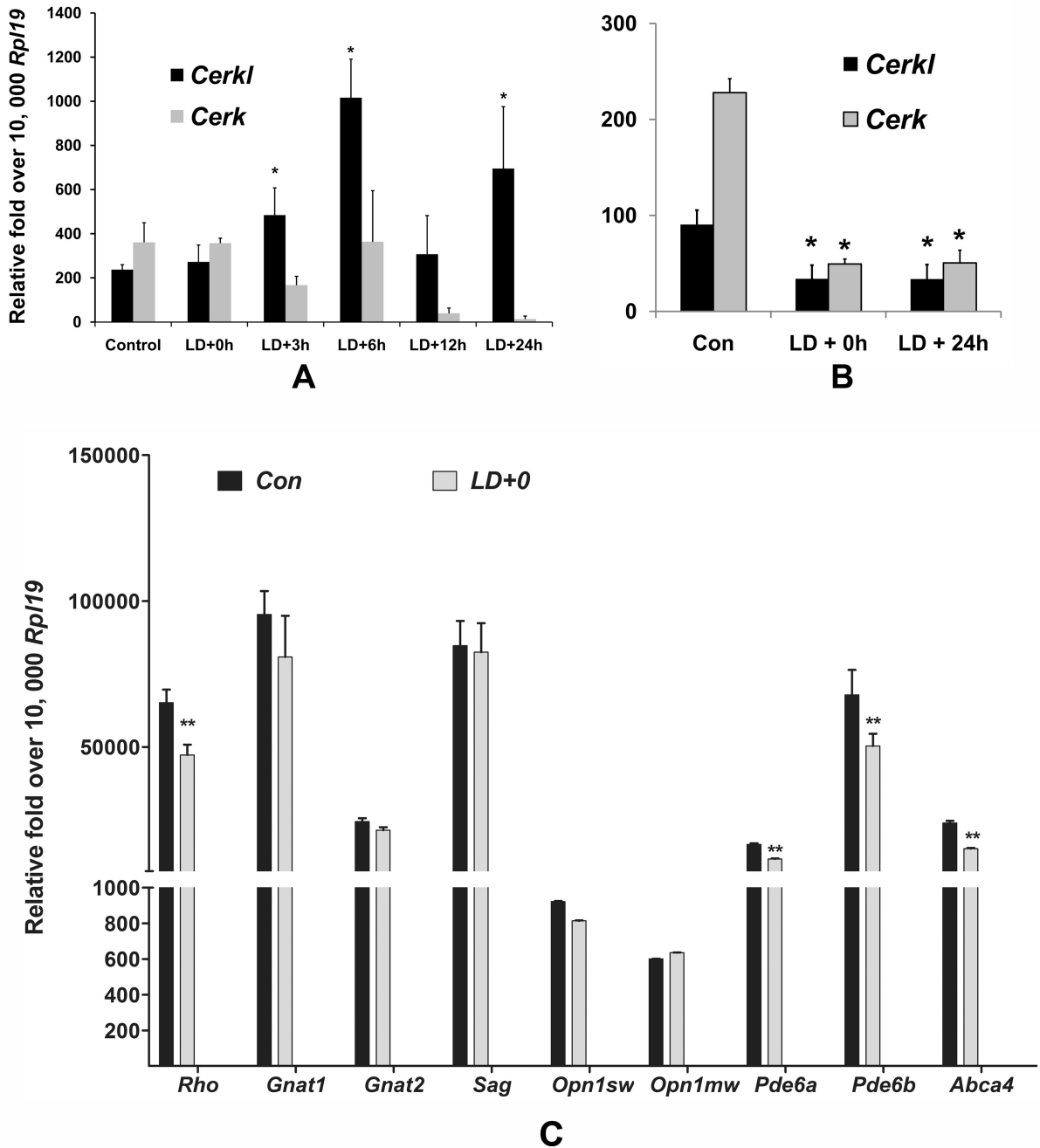


FIGURE 2.

A. Light-stress modulates *Cerkl* expression in the rat retina. SD rats were exposed to damaging light at an intensity of 2,700 lux for 6 h. The retinas and eyecups were harvested at different time points after light damage starting at 0 h (LD+0h), 3 h (LD+3h), 6 h (LD+6h), 12 h (LD+12h), and 24 h (LD+24h) and prepared for RNA extraction and subsequent qRT-PCR. Expression data were calculated by dCt methods after normalizing with three housekeeping genes (*Gapdh*, *Hgprt* and *Rpl19*) and presented against the most consistent housekeeping gene, *Rpl19* (* = $p < 0.01$; $n=4-6$; Student's t test). **B. Expression *Cerkl* and *CerK* in the eyecups after light damage.** Expression in the eyecups was tested at two time

points after light damage, Ld+0 and LD+24 (* = $p < 0.01$; $n=4-6$; Student's t test). **C. Expression of some photoreceptor markers after light damage.** Expression of Rho, guanine nucleotide binding protein, alpha transducing 1 (Gnat1), Gnat2, Rod arrestin (Sag), cone opsin short wave-length (Opn1 sw), cone opsin medium wave-length (Opn1 mw), Pde6a, Pde6b, and Abca4 was tested in the retinas harvested immediately after light damage (** = $p < 0.005$; $n=6$; one-way ANOVA). The retinas from non-light exposed rats served as controls (Con)..

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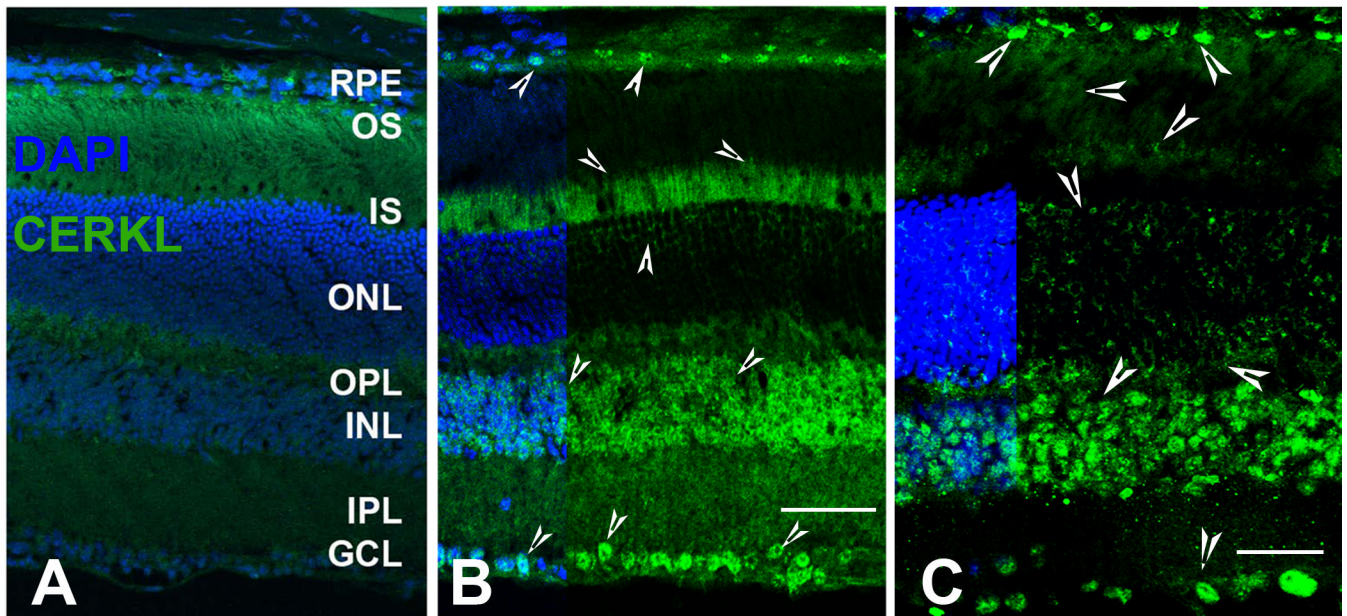


FIGURE 3. Localization of the CERKL protein in the albino mouse (BALBc) and rat retinas
 CERKL protein localization was detected by rabbit polyclonal anti-CERKL antibodies (Abcam Inc.). **A.** Retinal cryosections incubated with normal rabbit serum, served as negative control. **B.** BALBc retinal sections incubated with anti-CERKL antibodies. **C.** SD rat retinal sections incubated with anti-CERKL antibodies. CERKL localization is shown by arrow-heads. CERKL labeling was observed in various layers of the retina including ganglion cells, inner nuclear layers, photoreceptor cells, and in the RPE cells. RPE, retinal pigment epithelium; OS, photoreceptor outer segments; IS, photoreceptor inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

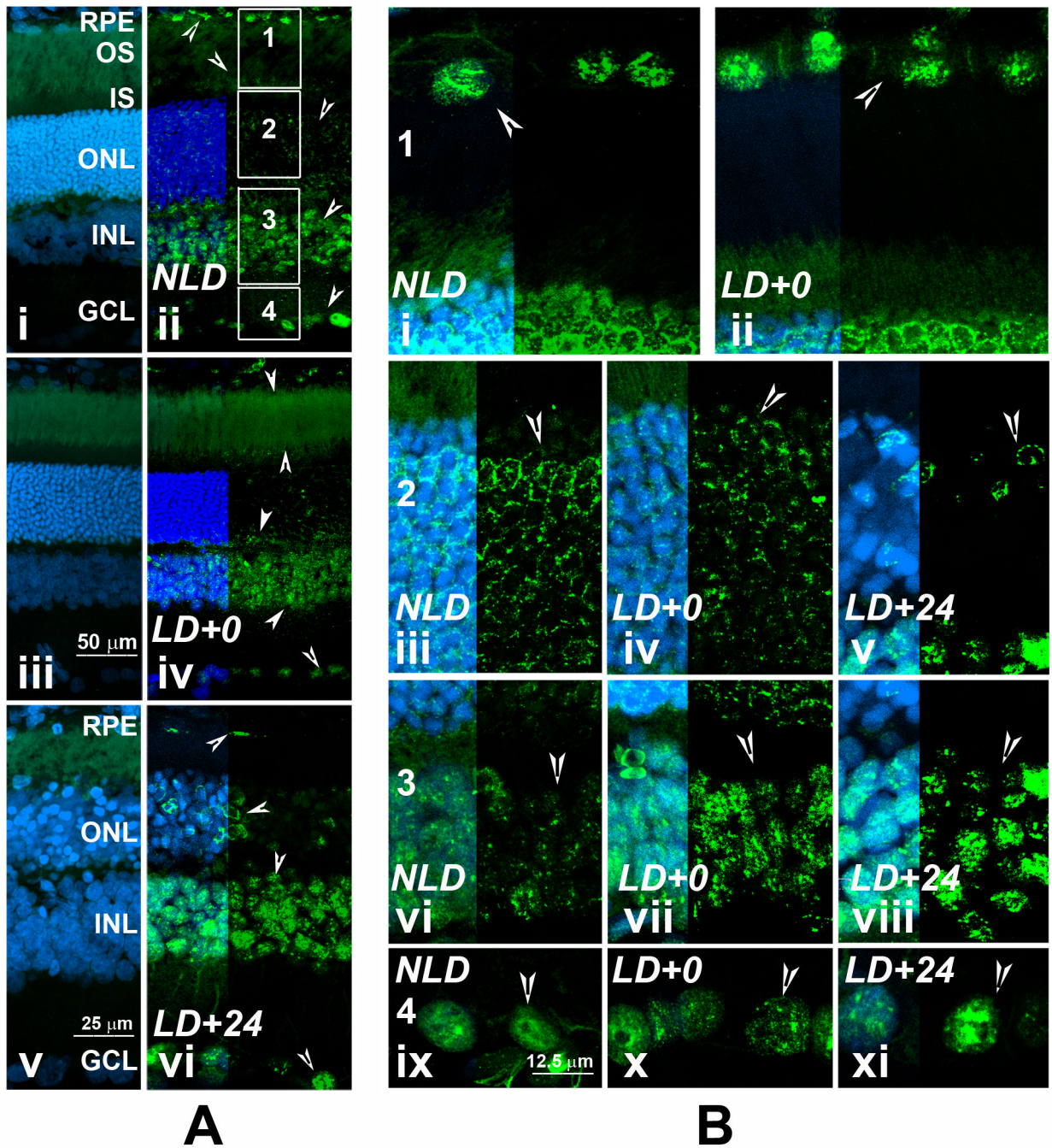


FIGURE 4. Localization of CERKL protein in rat retina after light stress

A. Retinal cryosections incubated with normal rabbit serum, served as the negative control (4Ai, 4Aiii, 4Av). SD rat retinal sections incubated with anti-CERKL antibodies: 4Aii, no-light-damaged retina (NLD); 4Aiv, LD+0 h retina; 4Avii, LD+24 h retina. **B.** Four regions of the retinal sections shown in 4Aii were captured at higher magnification to better visualize the localization of CERKL after light-stress. CERKL expression is shown by arrow-heads. Labeling intensity of CERKL increases in the outer segments at LD+0 (Compare 4Aiv with 4Aii). CERKL labeling pattern changed from the nuclear to the base of apical membrane in the disappearing RPE cells (4Avi). The CERKL labeling in the ONL in

LD+0 retina changed from continuous perinuclear to diffused and punctuated (4Biv, compare with 4Biii) and in LD+24 the labeling intensified in the perinuclear region of some of the nuclei and disappearing from the dying photoreceptor nuclei (4Bv, compare with 4Biii). No change in the nuclear localization was observed in the RPE cells immediately after light-damage (4Bii, compare with 4Bi); in the ganglion cells immediately and 24 h after light damage (4Bx, 4Bxi; compare with 4Bix). The CERKL labeling intensity appeared to be increased in the nucleus of the inner retinal neurons after light damage (4Bvii, 4Bviii; compare with 4Bvi). Abbreviations of the retinal layers are as in figure 3.

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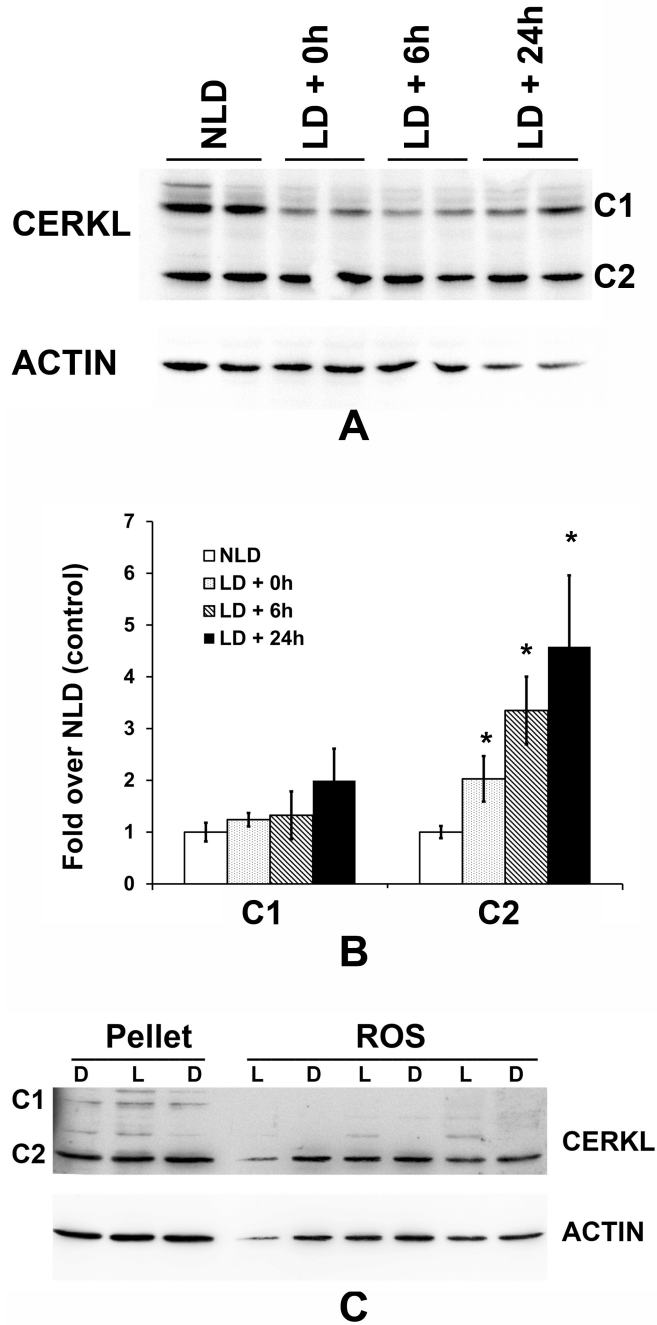


FIGURE 5. Light-stress increased expression of the CERKL protein in the retina
A. Anti- CERKL antibody recognized two principal CERKL proteins in the retina: approximately 59 kDa (C1) band and 37 kDa (C2) band. **B.** Densitometric analysis of the C1 and C2 polypeptides obtained from the light-stressed rat retinas, which are presented as fold over nolight-damaged control (NLD). (LD+0h, LD+6h, LD+24h represent samples harvested at 0 h, 6 h, and 24 h after light damage, respectively). (* = $p < 0.01$; $n=4$; Student's *t* test). **C.** **CERKL expression in retinal rod outer segments (ROS).** ROS was prepared from dark- (D) and light-(L) adapted retinas and subjected to Western blotting with anti-CERKL antibody. Pellet from the ROS prep were also analyzed.

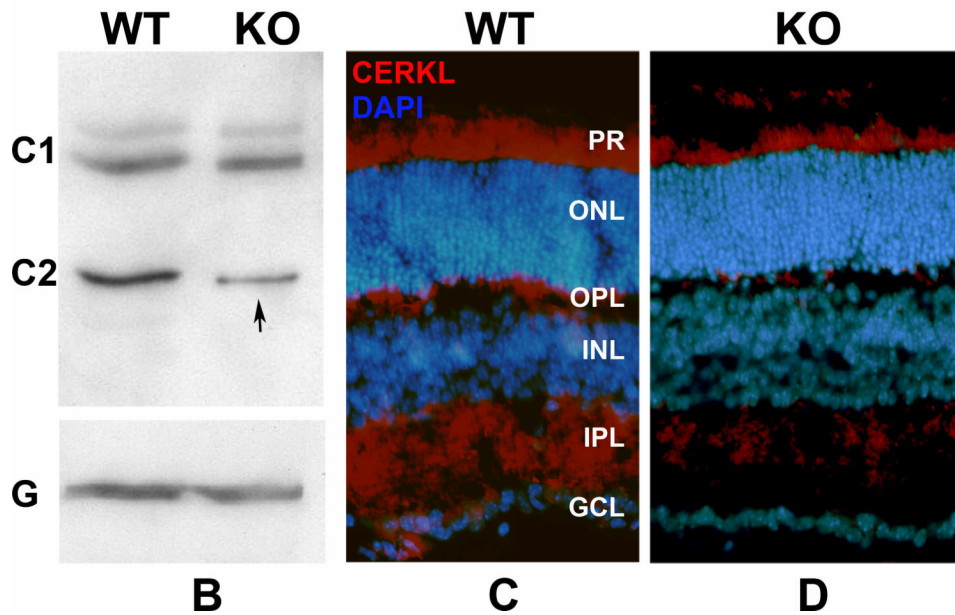
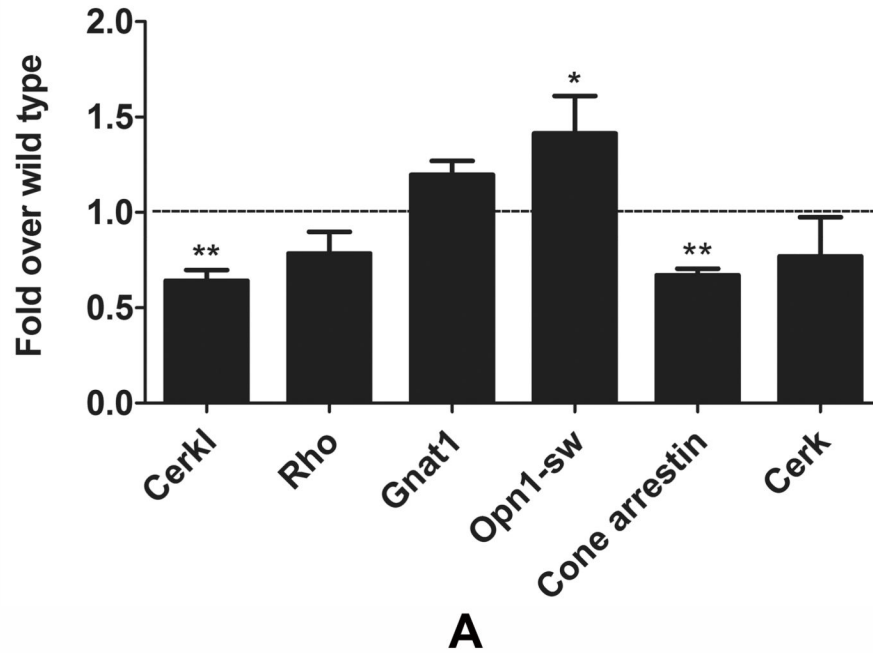


FIGURE 6. Expression of CERKL in *NeuroDI* knock-out retina

A. Quantitative RT-PCR was used to measure *Cerkl* mRNA in the retina obtained from 1-month-old *NeuroDI* knock-out (KO) mice and from their wild-type littermate (WT). *Cerkl* expression was significantly lower in the KO retina. Other rod and cone photoreceptor markers tested for comparison. (** = $p < 0.001$; $n=4$; Student's *t* test). **B.** Western blot analysis was performed. Similar to the data from the rats, the anti-CERKL antibody recognized two principal polypeptides in the mouse retina, C1 and C2 with the approximate molecular weights of 59 and 37 kDa, respectively. Compared to the WT C2 band, the intensity of C2 band in the KO retina was significantly lower (arrow). **G.** GAPDH, was used

as a loading control. **C and D.** Immunohistochemical labeling was performed on 2-month-old WT (C) and KO retinas (D). The intensity of CERKL labeling was less in the KO retinas than in the WT retinas.

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