

# Ceramide Kinase-Like (CERKL) Interacts with Neuronal Calcium Sensor Proteins in the Retina in a Cation-Dependent Manner

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**PURPOSE.** *CERKL* encodes for a ceramide kinase (CERK)-like protein. *CERKL* mutations are associated with severe retinal degeneration. Several studies have been conducted to prove a biochemical similarity between CERK and CERKL enzymatic activities. However, so far there has been no evidence that CERKL phosphorylates ceramide or any other lipid substrate in vitro or in vivo. The purpose of this work was to characterize CERKL's function by identification of CERKL-interacting proteins in the mammalian retina.

**METHODS.** CERKL-interacting proteins were identified implementing the Ras-recruitment system (RRS) on a bovine retina cDNA library. Co-immunoprecipitation (co-IP) in transfected cells and in photoreceptor outer segments was used to verify the identified interactions. Serial deletion constructs were used to map the interacting sites. CERKL's kinase activity was tested by a CERK activity assay.

**RESULTS.** We identified an interaction between CERKL and several neuronal calcium sensor (NCS) proteins, including guanylate cyclase activating protein 1 (GCAP1), GCAP2, and recoverin. These interactions were confirmed by co-IP experiments in transfected mammalian cells. Moreover, the interaction between endogenous CERKL and GCAP2 was confirmed by co-IP in photoreceptor outer segments. We found that CERKL-GCAP interaction is cation dependent and is mediated by CERKL's N-terminal region and by GCAPs cation-binding domains (EF-hands 2–4).

**CONCLUSIONS.** This study, which is the first to describe the interactions of CERKL with other retinal proteins, links CERKL to proteins involved in the photoresponse and Ca<sup>2+</sup> signaling, providing important clues for future research required in this direction. (*Invest Ophthalmol Vis Sci.* 2012;53:4565–4574) DOI:10.1167/iovs.12-9770

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Hereditary retinal dystrophies (HRDs) are a heterogeneous group of diseases, which cause visual loss owing to the death of rod and cone photoreceptors in the retina.<sup>1</sup> The increasing list of known genes underlying various forms of HRD includes a large group of proteins of unknown function (provided in the public domain by Retnet: Retinal Information Network, <http://www.sph.uth.tmc.edu/Retnet/>). *CERKL* (ceramide kinase-like) was originally identified as the gene underlying autosomal recessive HRD in several Spanish families. To date, eight *CERKL* mutations have been reported.<sup>2–7</sup> Although the *CERKL*-associated phenotype was originally characterized as RP,<sup>7,8</sup> most patients are diagnosed with cone-rod degeneration (CRD).<sup>2–4,6,9</sup> This phenotype is in correlation with the observation that in the mouse retina, the CERKL protein is located mainly in cone photoreceptors.<sup>10</sup>

CERKL is a homolog of the ceramide kinase (CERK) protein. Both proteins harbor a kinase domain related to the diacylglycerol kinases (DAGK) and a Pleckstrin homology (PH) domain. In addition, a C-terminal CERK-specific region is also present in CERKL.<sup>11,12</sup> Ceramide is the metabolic product of sphingolipids, which are part of the cell membrane, especially in neuronal cells. Generation of ceramide by hydrolysis of sphingolipids (e.g., sphingomyelin) is associated with the induction of apoptotic cell death and other stress-related responses.<sup>13</sup> Aberrant sphingolipid metabolism is associated with several neurodegenerative disorders; many of them, such as Tay-Sachs disease, Fabry's disease, and Neimann-Pick disease, are associated with blindness resulting from retinal degeneration.<sup>14</sup> Recent evidence suggests a strong correlation between sphingolipid signaling and survival and homeostasis of photoreceptor and retinal pigment epithelium cells.<sup>15–17</sup> The possibility that CERKL belongs to the CERK family and has a similar function was therefore very intriguing. However, various ceramides, known substrates for CERK and for other sphingolipids (including C8-, C2-, C6-, and C18-ceramide, dihydroceramide, phytoceramide, glucosylceramide, diacylglycerols, sphingosine, and complex sphingolipids such as sulfatides, cerebroside, and gangliosides) were not phosphorylated by CERKL in vitro. Moreover, even using total lipid extracts from brain or kidney as a source of substrates, or providing the assay with crude tissue extracts to test for potential CERKL cofactors, did not stimulate CERKL activity.<sup>11</sup> Generation of *Cerkl*-mutant mice did not help in clarifying the role of CERKL in the retina, as wild type (wt) levels of ceramide and ceramide-1-phosphate were found in the retinae of these mice.<sup>18</sup> Therefore, the kinase activity of CERKL remains questionable.

In the current article, we describe the use of the Ras-recruitment system (RRS),<sup>19</sup> a cytoplasmic-based yeast two-hybrid system, to identify CERKL-interacting proteins. We identified an interaction between CERKL and several neuronal

calcium sensor (NCS) proteins, including guanylate cyclase activating proteins (GCAPs).

NCS proteins are key components in a number of neuronal calcium signaling regulatory pathways. Their main signature is the existence of several helix-loop-helix  $\text{Ca}^{2+}$ -binding structures known as EF hands.<sup>20</sup> GCAPs are retina-specific NCS proteins. In many mammalian species, including humans, three forms of GCAPs are known (GCAP1–3), but only GCAP1 and GCAP2 are encoded in the mouse genome.<sup>21</sup> Both GCAP1 and GCAP2 are present in photoreceptors, with some interspecies variability in their cone versus rod specificities.<sup>22–24</sup> GCAPs contain four EF-hands typically numbered as EF1 through EF4 from N- to C-terminus in the primary protein structure. EF2 through EF4 bind both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , while EF1 does not bind metal and is required for recognition of retinal-specific guanylate cyclase (RetGC).<sup>25,26</sup> Stimulation of the phototransduction cascade by a photon of light leads to a decrease of intracellular cGMP concentration, thereby closing cGMP-gated cation channels located in photoreceptor plasma membranes and initiating a neural response.<sup>27</sup> RetGCs and their associated proteins are responsible for the  $\text{Ca}^{2+}$ -sensitive restoration of cGMP levels after the light activation of phototransduction. In the dark, when intracellular  $\text{Ca}^{2+}$  levels are high, GCAPs act as RetGC inhibitors, while in the light, when intracellular  $\text{Ca}^{2+}$  levels are low, GCAPs bind  $\text{Mg}^{2+}$  and act as RetGC activators.<sup>25</sup>

Here, we characterize the interaction between CERKL and GCAPs and demonstrate that it is cation dependent. This study, which is the first to describe the interactions of CERKL with other retinal proteins, provides important clues regarding CERKL's possible retinal function.

## MATERIALS AND METHODS

### Ras-Recruitment System

For the RRS,<sup>19</sup> two bait plasmids were constructed expressing partially overlapping residues of the murine CERKL protein (amino acids [aa] 1–358 and 272–525) as C-terminal fusion to Myc-Ras in the plasmid pMet425<sup>28</sup> (Fig. 1A). The bait expression is induced in media lacking methionine. Each of these plasmids was cotransformed with a commercial bovine retinal cDNA library cloned into the CytoTrap pMyr XR vector (library was obtained from Stratagene, Agilent Technologies, Santa Clara, CA) into the temperature-sensitive yeast strain *cdc25-2*. The expression of the library plasmid fused to Src myristoylated sequences occurs in the presence of galactose in the medium. Approximately 200,000 colonies were screened with each bait. Transformed yeast were plated on solid synthetic media containing glucose and lacking leucine and uracil (GLU-LU), left to recover at 24°C for 4 to 5 days, and then replicated to solid synthetic media containing galactose and lacking leucine, uracil, and methionine (GAL-LUM). Yeast colonies that grew on this media at the restrictive temperature (36°C) were considered putative positives. Positive colonies were excluded from the screen if they were able to grow at 36°C on GAL-LU plates (on which the bait protein is not expressed). After this selection, library plasmids were isolated from positive clones, sequenced, and retransformed into *cdc25-2* yeast with either CERKL or nonspecific bait plasmids to confirm the interactions and bait specificity.

### Antibodies

Primary antibodies used were as follows: mouse monoclonal antibody against HA tag (ab18181), rabbit polyclonal antibody against Myc tag (ab9106; Abcam, Cambridge, MA); mouse monoclonal antibody against Myc tag (clone 4A6; a gift from Ami Aronheim); rabbit polyclonal antibodies against GCAP1 and GCAP2 (UW-101 and UW-50; a gift from Wolfgang Baehr<sup>29,30</sup>); mouse monoclonal antibody against GCAP2 (G-

10; Santa Cruz Biotechnology, Santa Cruz, CA); and rabbit polyclonal antibody against CERKL.<sup>10</sup> The following secondary antibodies were used: peroxidase-conjugated affiniPure goat anti-mouse IgG, peroxidase-conjugated affiniPure goat anti-rabbit IgG, and peroxidase-conjugated IgG fraction monoclonal mouse anti-rabbit IgG light-chain specific (Jackson ImmunoResearch Laboratories, West Grove, PA); FITC-conjugated goat anti-rabbit IgG (MP Biomedicals, Santa Ana, CA); and protein A-peroxidase (Invitrogen, Grand Island, NY).

### Cell Culture

COS-7 and HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% glutamine (Biological Industries, Beit Ha'Emek, Israel) at 37°C and 5%  $\text{CO}_2$ . Cells were transfected with the desired constructs using the jetPEI transfection reagent (Polyplus-transfection SA, Illkirch, France). Cells were harvested 48 hours post transfection.

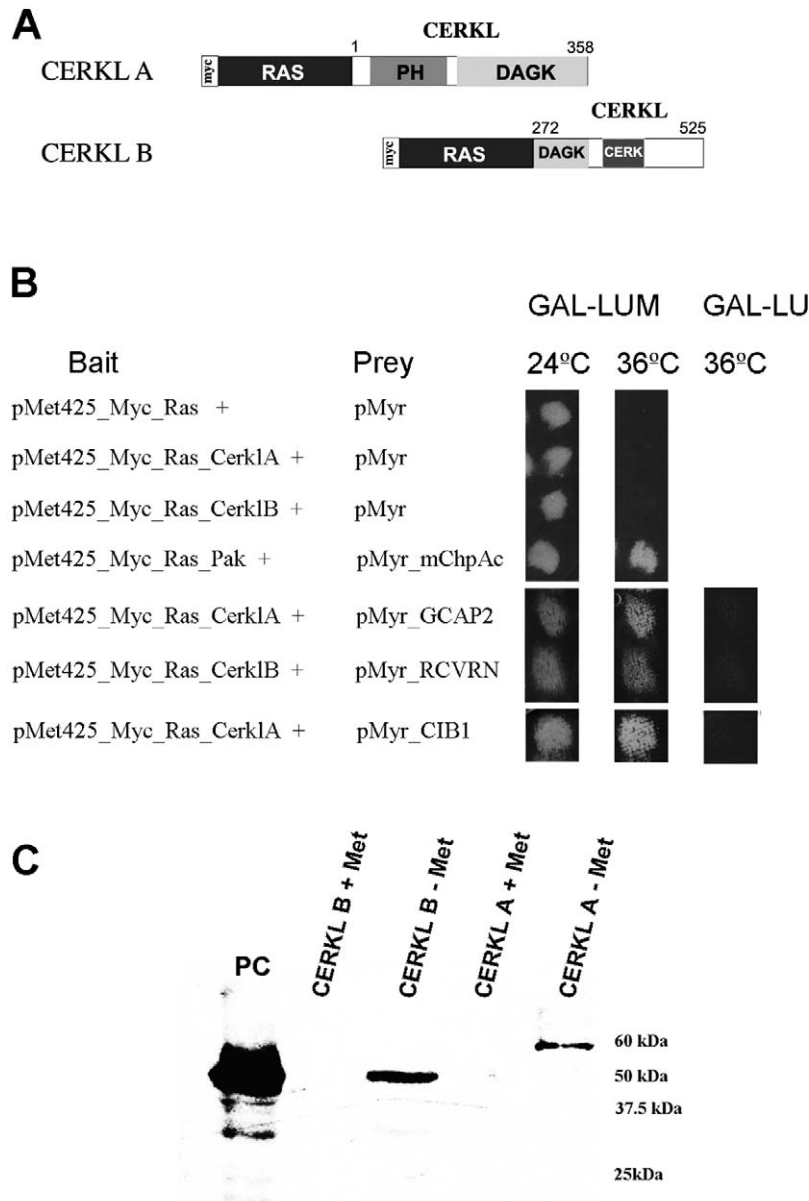
### Co-Immunoprecipitation (co-IP)

*CERKL* cDNA fragments were cloned into the pCS2+MT expression vector, in frame with six C-terminal Myc tags.<sup>31</sup> *GCAP1*, *GCAP2*, *Neurocalcin D*, *CIB1*, and *calmodulin* cDNAs were cloned into the pcDNA-3HA expression vector (a gift from Ami Aronheim). Partial *GCAP1/2* cDNAs were cloned into the Airap expression vector (Addgene, Cambridge, MA). Various mutations were inserted using the Quick Change II Site-Directed Mutagenesis Kit (Stratagene). Transfected cells were lysed with WCE buffer (25 mM HEPES pH 7.7, 0.3 M NaCl, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM  $\beta$ -glycerolphosphate, 0.1 mM  $\text{Na}_2\text{VO}_4$ , 100  $\mu\text{g}/\text{mL}$  PMSE protease inhibitor cocktail 1:100 [P8340; Sigma-Aldrich, St. Louis, MO]). Antibodies against Myc tag were incubated overnight at 4°C with COS-7 protein extracts. Protein A sepharose beads (Sigma-Aldrich) were added to the extracts for 1 hour at 4°C. After four washes with WCE buffer, the precipitated proteins were eluted using SDS-PAGE sample buffer. To test the effect of cations on CERKL-GCAP binding, co-IP was performed in a similar manner; however, protein extraction and washes were performed in a modified WCE solution (containing 5 mM  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ , or EGTA and EDTA). For Western blot analysis, samples were boiled for 5 minutes and then subjected to denaturing SDS-PAGE using 12.5% polyacrylamide gels, followed by transfer to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). Membranes were incubated with the primary antibody, followed by a peroxidase-conjugated secondary antibody. The results were visualized by chemiluminescence using the Amersham ECL Western blotting analysis system (GE Healthcare).

For preparation of bovine photoreceptor outer segments (POS), fresh bovine eyes were obtained from a local slaughterhouse and transported in the dark on ice. Retinae were dissected and collected into homogenization buffer (10 mM Tris-HCl pH 7.4, 2 mM  $\text{MgCl}_2$ , 65 mM NaCl, 1 mM DTT, 0.5 mM PMSE, 34% sucrose). Selective breaking of the OS was achieved by 3 minutes of vigorous shaking. The resulting crude fraction was homogenized with a 26-gauge needle and further purified by ultracentrifugation in sucrose density gradient (26.3%, 29%, 35%, and 40% sucrose steps). POS disks were collected at the 29% to 35% sucrose interface, washed three times and stored at  $-80^\circ\text{C}$  until use. For each co-IP experiment, half a retina was used (800  $\mu\text{g}$  protein). In order to extract the protein, POS were centrifuged for 5 minutes at 13,000 rpm and then resuspended in homogenization buffer (20 mM HEPES, 1 mM  $\text{MgCl}_2$ , 10.8% sucrose, 50 mM  $\beta$ -mercaptoethanol, protease inhibitor cocktail 1:100, and 0.5% Nonidet P40 (NP-40), 1 mL for each 0.1 g tissue).

### Immunofluorescence

The research was performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice euthanized by lethal injection of ketamine/zyllasine were perfused with



**FIGURE 1.** Identification of CERKL interaction with neuronal calcium sensor proteins in yeast. **(A)** pMet-Myc-Ras-CERKL bait constructs used for RRS. These constructs encode for chimeric proteins, composed of mouse CERKL amino acids 1 to 358 (CERKL A) or 272 to 525 (CERKL B), fused to a cytoplasmic Ras mutant, with a myc tag at the N-terminus. CERKL domains included in each construct are indicated. **(B)** *cdc25-2* yeast were cotransformed with the indicated bait and prey combinations and grown on GAL-LUM or on GAL-LU plates incubated in the permissive (24°C) and the restrictive (36°C) temperatures. Cotransformation of yeast with empty bait and prey vectors (*first row*) or with CERKL-bait vectors and an empty prey vector (*second and third rows*) served as a negative control. Pak and ChpAc are two proteins known to interact with each other,<sup>60</sup> and thus served as a positive control (*fourth row*). Yeast transformed with a combination of CERKL and GCAP2, recoverin (RCVRN), or CIB1 grew on GAL-LUM plates (on which both bait and prey proteins are expressed) at both the permissive and the restrictive temperatures, therefore indicating an interaction between these proteins. The same plasmid combinations did not allow growth of *cdc25-2* yeast when grown at 36°C on GAL-LU plates (on which the bait protein is not expressed). **(C)** Protein extracts from *cdc25-2* yeast transformed with CERKL A or CERKL B bait constructs were subjected to Western blot analysis with an anti-Myc tag antibody. Both constructs yielded proteins of the expected size (58 kDa for CERKL A and 50 kDa for CERKL B) in media lacking methionine (–Met), but not in methionine-rich media (+Met), indicating inducibility of the pMET425 promoter. The PAK protein (50 kDa) served as a positive control (PC).

PBS and then with freshly prepared sodium phosphate-buffered 4% paraformaldehyde, pH 7.4. Eyes were enucleated and immediately fixed in 4% paraformaldehyde on ice for 5 hours, washed with PBS, impregnated with 30% sucrose for 48 hours at 4°C, and frozen embedded in optimum cutting temperature (OCT) medium. Retina cross-sections were taken using a Hacker-Bright cryomicrotome (Hacker Instruments & Industries, Winnsboro, SC) and pretreated for 1 hour in citrate buffer at 85°C for epitope unmasking. The sections were probed with anti-CERKL rabbit polyclonal antibody and developed

using secondary FITC-labeled goat anti-rabbit antibody (Cappel/MP Biomedicals). Images were recorded using an Olympus Spectral FV1000 confocal system (Olympus Imaging America, Center Valley, PA).

### CERK Activity Assay

HEK293T cells were transfected with various combinations of CERK, CERKL, GCAP1, GCAP2, and recoverin expression vectors. After 48 hours, the cells were collected and sonicated. The reaction was

TABLE. Candidate CERKL-Interacting Proteins Identified by RRS

Clone	Protein	Amino Acids Included in Prey
Bait: CERKL A		
H3	Recoverin	40–191
H1-2		20–191
H4, 17	GCAP2	50–179
H6		46–179
H5	Proteasome 26S ATPase subunit 1 (PSMC1)	52–301
H7	Tumor protein translationally-controlled 1 (TPT1)	1–172
DE18	Neural epidermal growth factor-like 1 (NELL1)	372–563
Bait: CERKL B		
B2, B6, G9	CIB1	1–191
F1	Adenosylmethionine decarboxylase 1 (AMD1)	145–334
A4, G7	Fumarylacetoacetate hydrolase domain containing 1 (FAHD1)	57–221
B7	G2/M-phase specific E3 ubiquitin ligase (G2E3)	185–250

performed in a total volume of 250  $\mu$ L. Homogenates (250  $\mu$ g protein per reaction) were incubated in homogenization buffer (20 mM MOPS pH 7.2, 50 mM NaCl, 1 mM DTT, 2 mM EGTA, 3 mM CaCl<sub>2</sub>, protease inhibitor cocktail 1:500) with 1 mM adenosine triphosphate (ATP), 5  $\mu$ M defatted BSA, and 20  $\mu$ M C6-NBD-Ceramide (Avanti Polar Lipids, Alabaster, AL) for 20 minutes at 37°C. The reaction was stopped using chloroform:methanol (1:2). Lipids were extracted and separated by thin layer chromatography (Merck, Whitehouse Station, NJ) using chloroform/methanol/acetic acid/5 mM CaCl<sub>2</sub> (vol:vol:vol:vol, 60/35/4/2) as the developing solvent. Fluorescence was quantified using Typhoon 9410 (GE Healthcare) and ImageQuant analysis software (GE Healthcare).

## RESULTS

### CERKL Interacts with NCS Proteins

To identify potential binding partners of CERKL, we made two bait constructs (CERKL A and CERKL B), harboring partially overlapping fragments covering the entire CERKL coding sequence (Fig. 1A). These proteins were successfully expressed in yeast in media lacking methionine (Fig. 1C), and by themselves did not allow growth of *cdc25-2* yeast at 36°C (Fig. 1B). We used both constructs as baits to screen a bovine retinal cDNA expression library using the RRS. In this system, Ras membrane localization and activation is achieved through interaction between two hybrid proteins, resulting in complementation of the *cdc25-2* strain.<sup>19</sup>

A total of 15 clones, representing nine different putative binding partners of CERKL, were identified (the Table). Of interest, three of these proteins, GCAP2, recoverin, and calcium and integrin binding 1 (CIB1), are calcium-binding proteins belonging to the NCS extended family. The combination of each of these three proteins as prey with a CERKL bait construct enabled growth of *cdc25-2* yeast on GAL-LUM media, at both the permissive (24°C) and the restrictive (36°C) temperatures. In this media, both bait and prey proteins are expressed. However, the same plasmid combination did not allow growth of *cdc25-2* yeast at 36°C on GAL-LU plates (on which the bait protein is not expressed) (Figs. 1B, 1C). These results indicate that each of the identified prey proteins does not complement the *cdc25-2* mutation by itself, and that complementation was achieved only due to their interaction with CERKL.

The interaction of full-length CERKL with each of the three identified NCS proteins was further tested by co-IP, using lysates from COS-7 cells expressing Myc-tagged CERKL and HA-tagged GCAP2, recoverin, or CIB1. The presence of each of the three proteins in the anti-Myc (CERKL) immunocomplexes supported their interaction with CERKL in mammalian cells

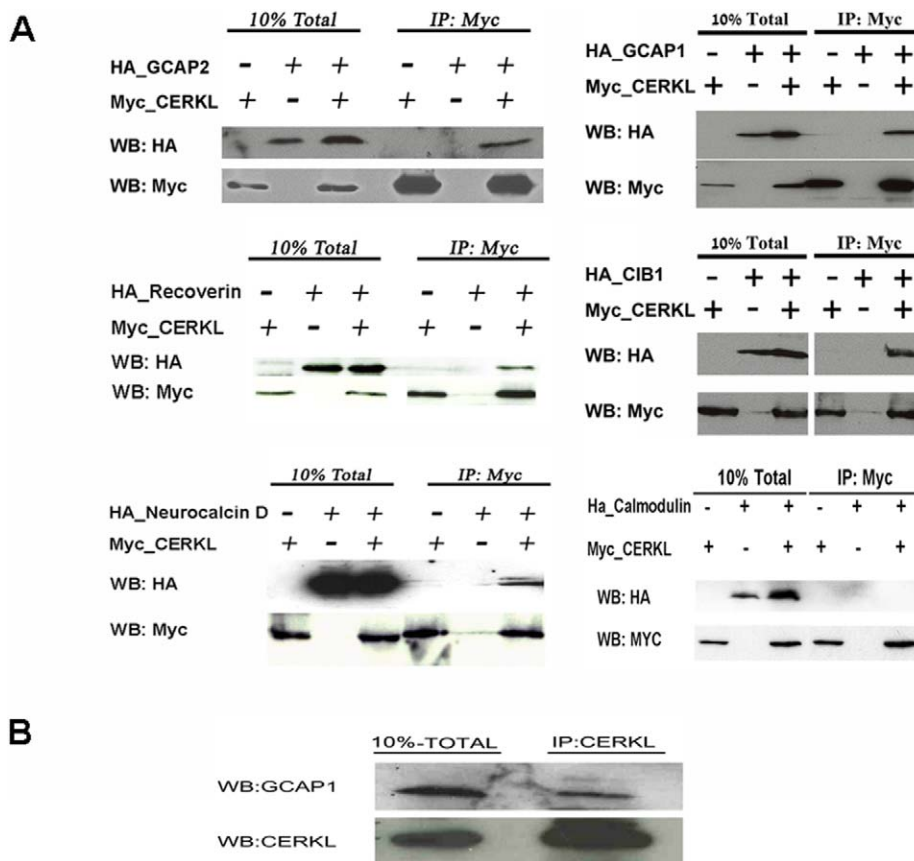
(Fig. 2A). We then used co-IP to test for possible interaction between CERKL and two additional NCS proteins: GCAP1, a close homolog of GCAP2; and neurocalcin delta, another prominent NCS protein, which is highly expressed in the retina.<sup>32</sup> Positive results were obtained for both proteins (Fig. 2A).

Notably, the enzymatic activity of CERK was found to be calcium dependent through its interaction with the calcium sensor protein calmodulin. A characteristic type 1-8-14B calmodulin-binding motif is located in CERK's C-terminal domain.<sup>33,34</sup> This binding site is not fully conserved in both human and mouse CERKL orthologues. Nevertheless, we used co-IP to test for possible interaction between CERKL and calmodulin and found that the two proteins do not interact (Fig. 2A).

For further analysis of putative CERKL interactors, we chose to focus on GCAP1 and GCAP2 owing to their well-established role in the photoresponse,<sup>29</sup> their retinal localization pattern, which overlaps CERKL,<sup>10,22–24,30,35</sup> and the similarity between GCAPs and CERKL-related retinal phenotypes.<sup>2,4,36</sup> To further confirm a physiologic interaction between endogenous CERKL and GCAPs, co-IP was performed using bovine POS lysates, in which both CERKL and GCAP2 are clearly expressed. Reproducibly detectable levels of GCAP2 were present in the anti-CERKL immunocomplexes, demonstrating an endogenous interaction between these two proteins in photoreceptors (Fig. 2B). These results were obtained for both dark- and light-adapted POS. A similar experiment could not be performed with GCAP1, since its detection level in bovine POS was very low.

### CERKL-GCAP Interaction Is Mediated by CERKL's N-Terminal Region and GCAP's EF Hands 2 through 4

To map the interacting regions in each protein, we performed co-IP using lysates from COS-7 cells expressing different combinations of Myc-tagged CERKL and HA-tagged GCAP1/2-truncated constructs (Fig. 3A). Within CERKL, the interacting region was mapped to the N-terminal part, between aa 1 and 287. This region includes the PH domain (aa 48–160) and part of the DAGK domain (aa 165–287). While both domains can separately bind CERKL, a stronger binding was achieved with the PH domain, and especially with aa 1–88 (Fig. 1A). In GCAPs, we found that each one of EF hands 2, 3, and 4 can independently bind CERKL, while EF1 does not (Figs. 3A, 3B, and data not shown). These results are in agreement with our findings in the RRS, in which the identified interaction was between the CERKL A bait construct, which harbors the N-terminal part of CERKL (Fig. 1A), and a partial bovine GCAP2



**FIGURE 2.** Verification of the interaction between CERKL and NCS proteins by co-IP. (A) COS-7 cells were transiently cotransfected with a combination of any of six different HA-tagged NCS proteins (GCAP1, GCAP2, Recoverin, CIB1, Neurocalcin D, or calmodulin) and Myc-tagged CERKL. Cell lysates were subjected to co-IP with an anti-Myc antibody, and eluted proteins as well as total cell lysates were separated by SDS-PAGE, followed by Western blotting (WB) with anti-HA and anti-Myc antibodies. The presence of each of the NCS proteins GCAP1, GCAP2, Recoverin, CIB1, and Neurocalcin D in the anti-Myc (CERKL) immunocomplexes supported their interaction with CERKL in mammalian cells. Calmodulin did not interact with CERKL. (B) Bovine POS extracts were subjected to IP with an anti-CERKL antibody, and eluted proteins as well as the total extract were separated by SDS-PAGE, followed by WB with anti-CERKL and anti-GCAP2 antibodies. The presence of GCAP2 in the anti-CERKL immunocomplexes demonstrates an endogenous interaction between these two proteins in photoreceptors.

cDNA encoding a region homologous to human GCAP2 aa 48-167 (harboring EF hands 2-4).

We next used co-IP to test the effect of the following missense mutations detected in human HRD patients on CERKL-GCAP interaction: two CERKL missense mutations, R106S and C125W,<sup>3,5</sup> both located within the PH domain; the only GCAP2 mutation reported to date, G157R, located within EF4<sup>37</sup>; and the GCAP1 mutations P50L (located near EF2), E89K, C99Y, N104K, and D100E (located within EF3),<sup>38-41</sup> I143NT and E155G (both located within EF4).<sup>42,43</sup> CERKL-GCAP interaction was not affected by any of these mutations (Fig. 4).

**CERKL-GCAP Interaction Is Cation Dependent**

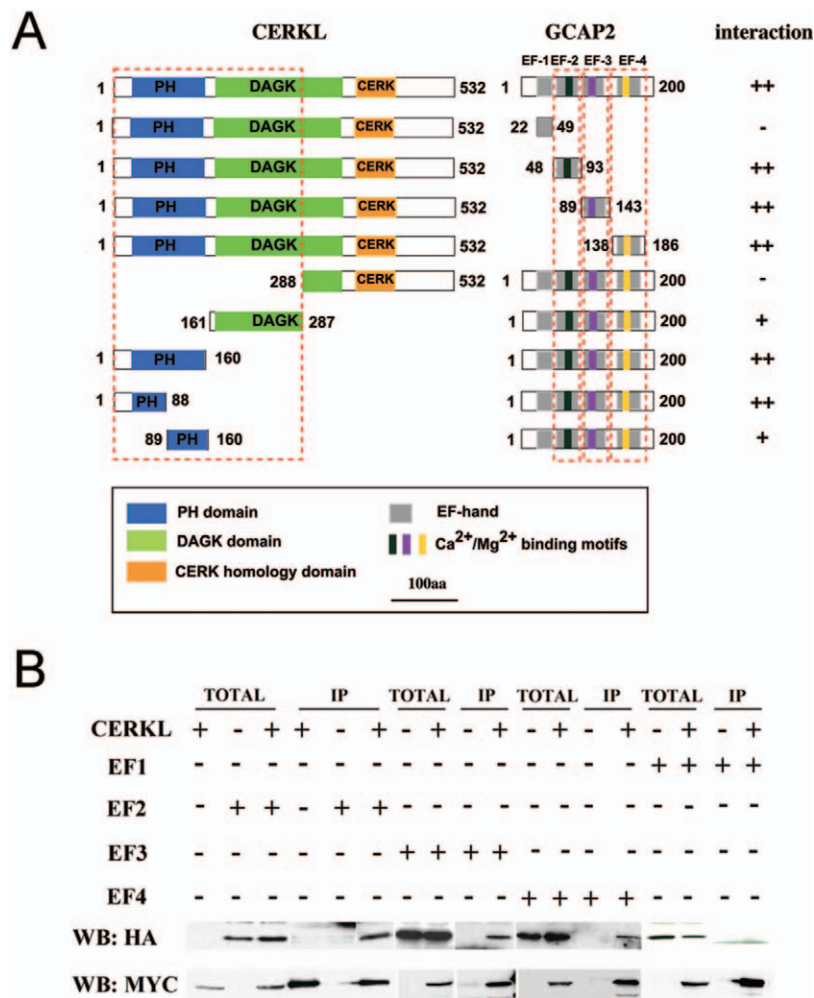
As indicated before, CERKL is bound by GCAPs EF2 through EF4, which also bind both Ca<sup>2+</sup> and Mg<sup>2+</sup>, while it is not bound by EF1, which does not bind cations (Fig. 3).<sup>25,26</sup> We therefore set up to determine whether the ability of EF2 through EF4 to bind CERKL correlates with their ability to bind cations. For that purpose, we generated two mutations in the EF2 of GCAP2: E77Q, which was shown to eliminate binding of Ca<sup>2+</sup> but does not affect binding of Mg<sup>2+</sup>, and D66N, which eliminates binding of both Ca<sup>2+</sup> and Mg<sup>2+</sup> in corresponding homologous GCAP1 EF hands (Fig. 5A).<sup>44-46</sup> Co-IP demonstrat-

ed that while the E77Q mutation did not affect CERKL binding, the D66N mutation eliminated it (Figs. 4, 5A, 5B).

To further establish whether CERKL-GCAP binding is cation dependent, we performed co-IP with the full-length wt proteins in the presence of Ca<sup>2+</sup>, Mg<sup>2+</sup>, or cation-chelating agents (EDTA and EGTA). While binding was observed in the presence of either Ca<sup>2+</sup> or Mg<sup>2+</sup>, it was eliminated in the presence of cation-chelating agents (Fig. 5C).

**GCAPs Are Not Required for Proper Localization of CERKL in Cone Photoreceptors**

To test whether CERKL-GCAP interaction affects CERKL's localization in photoreceptors we performed immunostaining of retinal sections obtained from GCAP1/GCAP2 double knockout mice<sup>47</sup> and wt controls, with a specific anti-CERKL antibody. As can be seen in Figure 6, CERKL localized to cone OS in both mutant and wt animals. If CERKL localization in cones depends on NCS proteins, GCAPs are clearly not the unique type of NCS proteins required for that, and in their absence, any other NCS protein may take over the interaction required for proper localization. Provided that CERKL is able to bind other NCS proteins, this observation is not surprising, but the question, whether or not CERKL localization depends on its interaction with NCS proteins remains open.



**FIGURE 3.** Mapping the interacting regions of CERKL and GCAP2. (A) Schematic representation of the different co-IP experiments performed to map the interacting regions. The *scattered line* represents the detected interacting parts of the two proteins. (B) COS-7 cells were transiently cotransfected with various combinations of partial GCAP2\_HA constructs and full-length CERKL\_MYC. Cell lysates were subjected to co-IP with an anti-Myc antibody, and eluted proteins as well as total cell lysates were separated by SDS-PAGE, followed by WB with anti-HA and anti-Myc antibodies. Each one of GCAP2 EF-hands 2, 3, and 4 was found to independently bind CERKL, while EF1 did not.

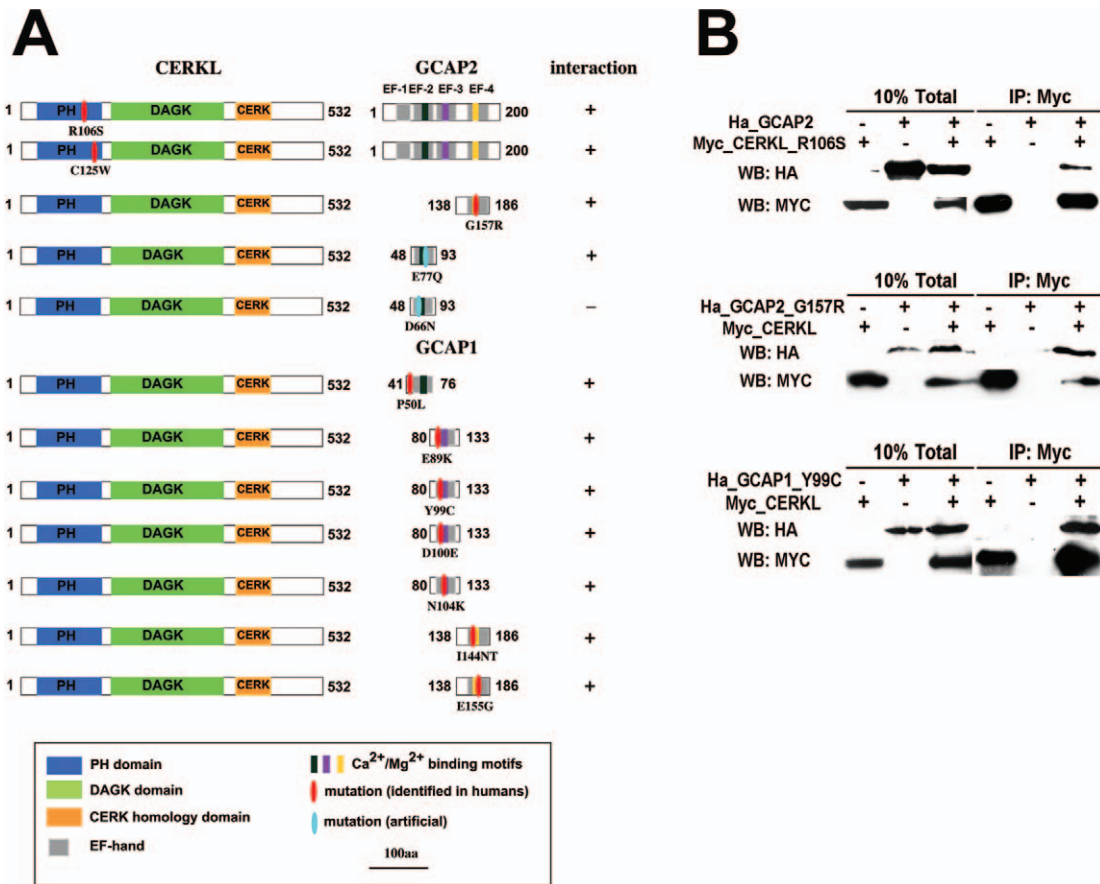
**Enzymatic Activity of CERKL with GCAPs and Recoverin**

As previously mentioned, CERKL has sequence homology to CERK and harbors a DAGK domain. However, CERKL kinase activity could not be shown.<sup>11,18,48</sup> Of interest, it was previously reported that members of the type-I DAGK family have EF-hand domains at their N-termini, making them calcium responsive. The N-termini of these DAGKs also have a domain with homology to the recoverin family of NCS. This domain is required for calcium-dependent activation of DAGK $\alpha$  and thus has an important regulatory role in its enzymatic activity.<sup>49,50</sup> We therefore hypothesized that CERKL, which does not contain EF-hand motifs, may depend on binding to an NCS family member for its kinase activity. To test this hypothesis, we transfected HEK293T cells with either CERKL alone, or a combination of CERKL and GCAP1, GCAP2, or recoverin, and performed a ceramide-kinase assay. The assay was performed in the presence of either a standard buffer (containing 1 mM CaCl<sub>2</sub>) or in modified buffers containing 5 mM of either CaCl<sub>2</sub> or MgCl<sub>2</sub>. Kinase activity could be clearly demonstrated in CERK-transfected cells, but no activity was demonstrated for CERKL under any of the tested conditions (Fig. 7).

**DISCUSSION**

The increasing list of known genes underlying HRD includes a large group of genes with unknown function. One of these genes is *CERKL*. Characterization of such genes will provide significant insights into mechanisms of normal retinal function and the types of cellular defects that lead to retinal degeneration. Protein-protein interactions play a key role in many biological systems and are the basis for formation of complexes and pathways that carry out different biological processes. Hence, correctly identifying interacting proteins is useful for assigning functions to unknown proteins based on their interacting partners. We therefore aimed to identify CERKL-interacting proteins in the retina. Of interest, we identified an interaction between CERKL and several NCS proteins.

Calcium is a second messenger that controls a large variety of signaling pathways. Very often these pathways involve Ca<sup>2+</sup>-binding proteins with a Ca<sup>2+</sup>-sensor function. The EF-hand superfamily is the largest group of Ca<sup>2+</sup>-sensor proteins.<sup>51,52</sup> Of interest, the enzymatic activity of CERK was found to be calcium dependent, through its interaction with the calcium sensor calmodulin.<sup>33,34</sup> However, based on our data, CERKL does not bind calmodulin (Fig. 2A).



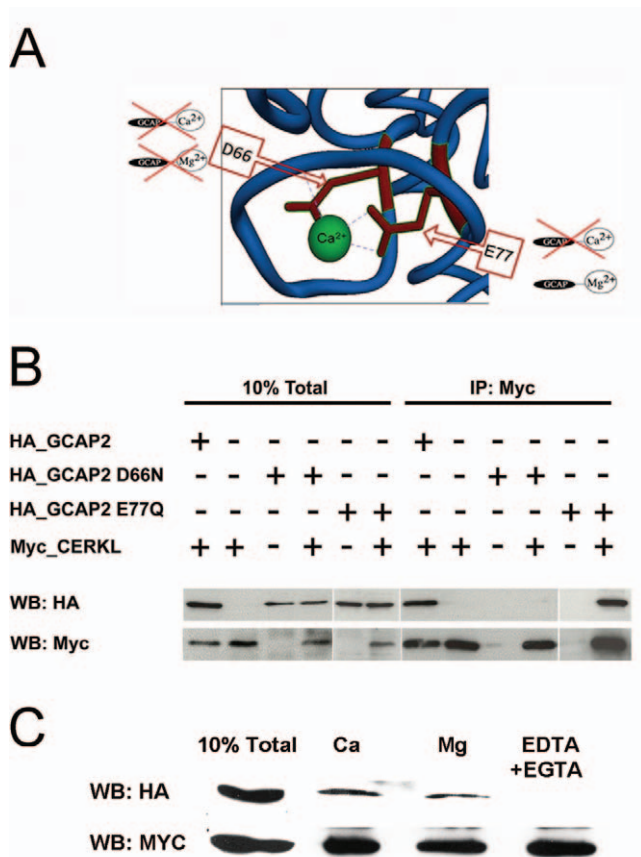
**FIGURE 4.** The effect of mutations in CERKL and GCAPs on their mutual interaction. (A) Schematic representations of the different GCAP1, GCAP2, and CERKL mutated constructs tested. Each line represents a co-IP experiment. None of the disease-causing mutations tested (marked in red) affected the interaction. However, the D66N mutation in EF2 of GCAP2 (marked in blue), which was shown to affect binding of both Ca<sup>2+</sup> and Mg<sup>2+</sup>, eliminated the interaction between the two proteins. (B) Examples of experimental data for the results summarized in panel A. COS-7 cells were transiently cotransfected with various combinations of wt or mutant CERKL\_MYC, GCAP1\_HA, and GCAP2\_HA. Cell lysates were subjected to co-IP with an anti-Myc antibody, and eluted proteins as well as total cell lysates were separated by SDS-PAGE, followed by WB with anti-HA and anti-Myc antibodies.

Calcium plays a crucial role in the regulation of phototransduction in photoreceptors. The concentration of free Ca<sup>2+</sup> in the cytoplasm drops from ~250 to 50 nM in the dark to below 50 nM in the light, because closing of cGMP-gated cation channels blocks the influx of Ca<sup>2+</sup>, and the Na<sup>+</sup>/Ca<sup>2+</sup>-K<sup>+</sup> exchanger extrudes Ca<sup>2+</sup> from the cell.<sup>53,54</sup> Retina-specific NCS proteins typically bind Ca<sup>2+</sup> at the higher concentrations characteristic of the dark state but not at the lower concentrations achieved after prolonged activation, and link phototransduction to Ca<sup>2+</sup> signaling (reviewed in Koch<sup>55</sup> and Stephen et al.<sup>56</sup>). Two classes of NCS proteins are expressed in photoreceptors and are active in phototransduction: GCAPs, that regulate RetGC in response to Ca<sup>2+</sup> and mediate the restoration of dark levels of cGMP<sup>36</sup>; and recoverin, which plays a role in prolonging the photoresponse, likely via interaction with rhodopsin kinase.<sup>57</sup> Both GCAPs and recoverin are therefore involved in the adaptation and recovery stages of the photoresponse. Of interest, we found that both classes bind CERKL.

In the current work, we chose to focus on the interaction between CERKL and GCAPs for two reasons: GCAPs have a well-established role in the photoresponse<sup>29</sup>; and within the retina, there is an overlap between GCAPs and CERKL localization. In the mouse retina, CERKL localizes to the OS of cone photoreceptors, as well as to amacrine cells of the inner nuclear layer and to the ganglion cell layer.<sup>10,58</sup> CERKL's localization in the human retina is currently unknown,

although the CERKL-associated phenotype in humans implicates marked cone involvement.<sup>2,4</sup> Both GCAP1 and GCAP2 are also found mainly in photoreceptor OS. GCAP1 is most strongly expressed in cones (human, monkey, mouse, and bovine). GCAP2 is predominantly expressed in rods in mouse and bovine and predominantly expressed in cones in humans and monkeys. GCAP2 was also reportedly detected in amacrine and ganglion cells in the mammalian retina.<sup>22-24,30,35</sup> And last, although the phenotypes associated with mutations in GCAPs and CERKL differ in their inheritance pattern (autosomal dominant versus autosomal recessive, respectively), they are highly similar in nature. In both cases, affected individuals present with a spectrum of retinal phenotypes, including RP with macular involvement, CRD, and CD.<sup>2,4,36</sup>

GCAPs contain four EF hands of which EF2 through EF4 can bind both Ca<sup>2+</sup> and Mg<sup>2+</sup>. The state of RetGC regulation by GCAP (activation versus inhibition) depends on the relative intracellular levels of Ca<sup>2+</sup> versus Mg<sup>2+</sup>.<sup>25,26,59</sup> Our results indicate that CERKL binding to GCAP is also cation dependent and is similar in the presence of either Ca<sup>2+</sup> or Mg<sup>2+</sup> (Fig. 5), which may correlate to interaction in both the dark and the light states of phototransduction. Therefore, CERKL is unlikely to be involved in modulation of RetGC activity through its interaction with GCAP. Nevertheless, based on our findings and on previous reports,<sup>49,50</sup> we hypothesized that CERKL may depend on binding to an NCS family member for its kinase activity. Currently, there are no direct data to prove this

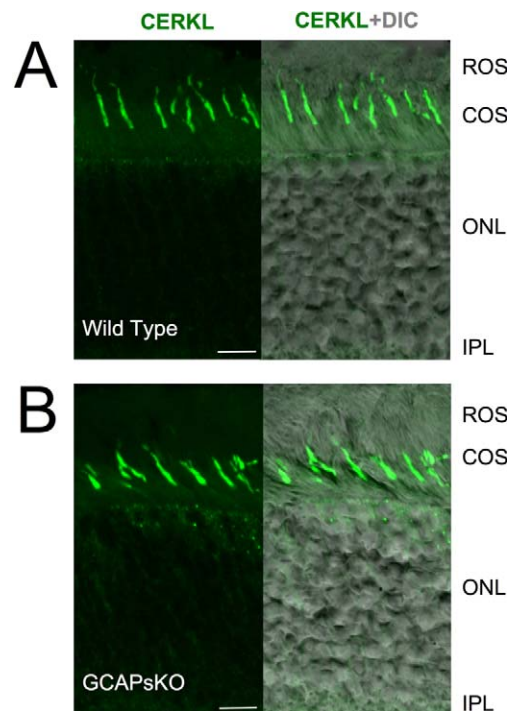


**FIGURE 5.** CERKL-GCAP interaction is cation dependent. **(A)** Schematic representation (produced with the UCSF Chimera software provided in the public domain at <http://www.cgl.ucsf.edu/chimera/>) of  $\text{Ca}^{2+}$ -bound EF2 hand of GCAP1 (PDB ID 2R2I; resolution: 2.00Å). The two critical residues for cation binding (D66 and E77) are colored *red*. The  $\text{Ca}^{2+}$  ion is colored *green*. **(B)** COS-7 cells were transiently transfected with wt CERKL\_MYC and either wt or mutant GCAP2\_HA. Cell lysates were subjected to co-IP with an anti-Myc antibody, and eluted proteins as well as total cell lysates were separated by SDS-PAGE, followed by WB with anti-HA and anti-Myc antibodies. Note that while the E77Q mutation did not affect CERKL-binding, the D66N mutation eliminated it. **(C)** COS-7 cells were transiently transfected with full-length wt GCAP2\_HA and CERKL\_MYC. Cell lysates were subjected to co-IP with an anti-Myc antibody. Lysates were incubated and washed with a modified lysis buffer containing either 5 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgCl}_2$ , or 5 mM of both EGTA and EDTA. Eluted proteins as well as total cell lysates were separated by SDS-PAGE, followed by WB with anti-HA and anti-Myc antibodies. Note that while binding was observed in the presence of either  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , it was eliminated in the presence of EDTA+EGTA.

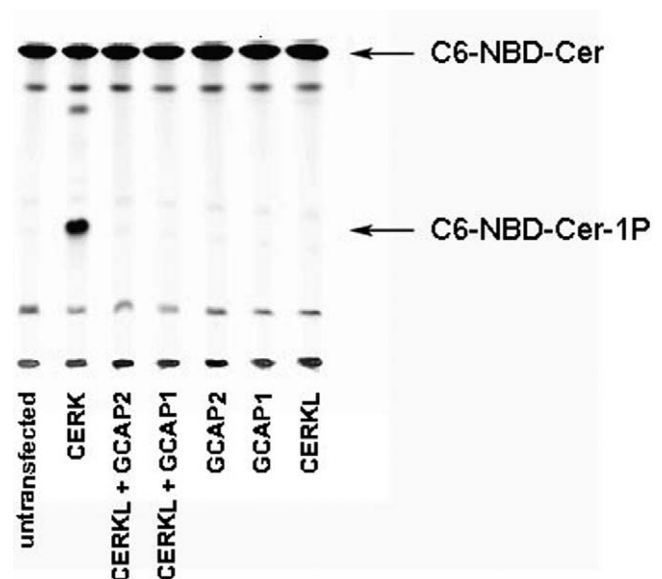
hypothesis. However, it remains possible that CERKL's kinase activity toward specific substrate(s) depends on NCS proteins. Although the exact mechanism of CERKL's function within the retina is still unknown, our findings link CERKL to proteins involved in the photoresponse and  $\text{Ca}^{2+}$  signaling and provide important clues for future research required in this direction.

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**FIGURE 6.** GCAPs are not required for proper localization of CERKL in cone photoreceptors. Immunofluorescence of retinal sections from wt **(A)** and GCAP1/GCAP2 double knockout mice **(B)** labeled with a CERKL-specific antibody (*left panels*). The anti-CERKL immunofluorescence (*right panels*) is superimposed on differential contrast image (DIC). ROS, rod outer segments; COS, cone outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; scale bar, 10  $\mu\text{m}$ . Note that the anti-CERKL immunofluorescence is localized to cone outer segments in both wt and GCAP double knockout retinas.



**FIGURE 7.** Ceramide kinase assay. Cells were transiently transfected with the indicated expression vectors and incubated with C6-NBD-ceramide. C6-NBD-Cer-1P was detected in cells transfected with CERK, but not with CERKL. The experiment was performed in duplicate and repeated twice.



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