Supplementary Material and Methods

Animals

All animal work was in strict accordance with the NIH Guide for the Care Use of Laboratory Animals, and the Association for Research in Vision and Ophthalmology on the Use of Animals in Vision Research. All the protocols were approved by the IACUC of the University of Oklahoma Health Sciences Center and the Dean A. McGee Eye Institute. The generation of Grb14 knockout mice was reported previously (Cooney et al, 2004). A breeding colony of Albino Sprague-Dawley rats is maintained in our vivarium in cyclic light (12 h on; 12 h off; ~ 300 lux). Experiments were carried out on both male and female rats (150-200 g). Rats or mice were dark-adapted overnight and sacrificed either under dim red light or following 30 min of light exposure (300 lux).

Plasmids and vectors

X-press–tagged mouse Grb14 and Myc-tagged bovine Grb14 constructs were cloned as described previously (Rajala et al, 2009). The RA (residues 105-195) and RA-PH (residues 105-355) domains of Grb14 were amplified from full-length Grb14 and cloned into Myc-tagged pCDNA3 vector with *BamH1* and *Xho1* sites. pCDNA3-CNGA1, pCDNA3-CNGB1 and respective channel antibodies were a kind gift from Dr. Robert Molday, University of British Columbia (Canada). Myc-tagged CNGA1 construct was generated from the amplification of pCDNA3-CNGA1 with sense (GAA TTC ACC ATG GAG CAA AAA CTC ATC TCA GAA GAG GAG GAT CTG ATG AAG AAA GTG ATT ATC AAT ACA TGG CAC) and antisense (CTC GAG TCA GTC CTG TGT AGA GTC TGT GGG CCC ACT TTC) primers. The NH_2-terminal FLAG-tagged C-terminal region of CNGA1 (CTR-CNGA1) (residues 483-690) was cloned into pCDNA3

using sense (GAA TTC ACC ATG GAT TAC AAG GAT GAC GAC GAT AAG GCT TGG TCT GTT GGT GGAG) and antisense (CTC GAG TCA GTC CTG TGT AGA GTC TGT GGG CCC ACT TTC) primers. The cDNA encoding the full-length CNGA1 was cloned into the *NcoI* site of pDHB1 membrane yeast two-hybrid bait vector and full-length Grb14 was cloned as *BamH1/Sal1* into pDL2-Nx prey vector. The positive (pMBV-Alg5 and pAlg5-NubI) and negative (pMBV-Alg5 and pAlg5-NubG) plasmids were obtained from Dualsystems Biotech AG (Switzerland). The membrane yeast two-hybrid assay (Stagljar et al, 1998) was carried out using the Dual hybrid kit (Dualsystems Biotech AG, Switzerland). The yeast two-hybrid screen was used in the *S. cerevisiae* strains L40 [MATa his3D200 trp1-901 leu2-3112 ade2 LYS2:: (lexA-*HIS3*) (URA3::lexA-*lacZ*) GAL4] and DSY-1. The cDNA encoding the CTR region of CNGA1 (amino acids 483-690) was cloned into pLexA yeast two-hybrid vector. The generation of full-length Grb14, and the RA, BPS, PH, BPS-SH2 and SH2 domains carrying the activation domain have been reported previously (Rajala et al, 2005).

Assessment of the Channel Activity by Ratiometric Measurement of $[Ca^{2+}]_i$

The fluorescent indicator Indo-1/AM was used to monitor Ca^{2+} influx through the CNGA1 channels in cell suspensions. The assays were performed as described (Grynkiewicz et al, 1985, Ma et al, 2005) using a spectrofluorometer (Fluostar Omega, BMG lab tech GmBH, Offenburg, Germany). This assay was designed to determine CNG channel activity in cell populations (2×10^6) in response to 8-pCPT-cGMP stimulation. Briefly, cells (36–48 h post-transfection) were harvested with cell dissociation medium (Invitrogen, Carlsbad, CA), washed with the extracellular solution (ECS; 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, 15 mM HEPES, pH 7.4), and

incubated with 2 μM Indo-1/AM (Sigma-Aldrich) in ECS in the presence of 0.05% Pluronic F-127 (Invitrogen, Carlsbad, CA) for 40 min at room temperature. Then, the cells were washed three times with ECS and resuspended in ECS (1×10^6/mL). Ca^{2+} influx in response to 8-pCPT-cGMP was determined by ratiometric measurement, which represents the free intracellular Ca^{2+} concentration. Changes of intracellular Ca^{2+} concentration were expressed as a Δ405/485 ratio.

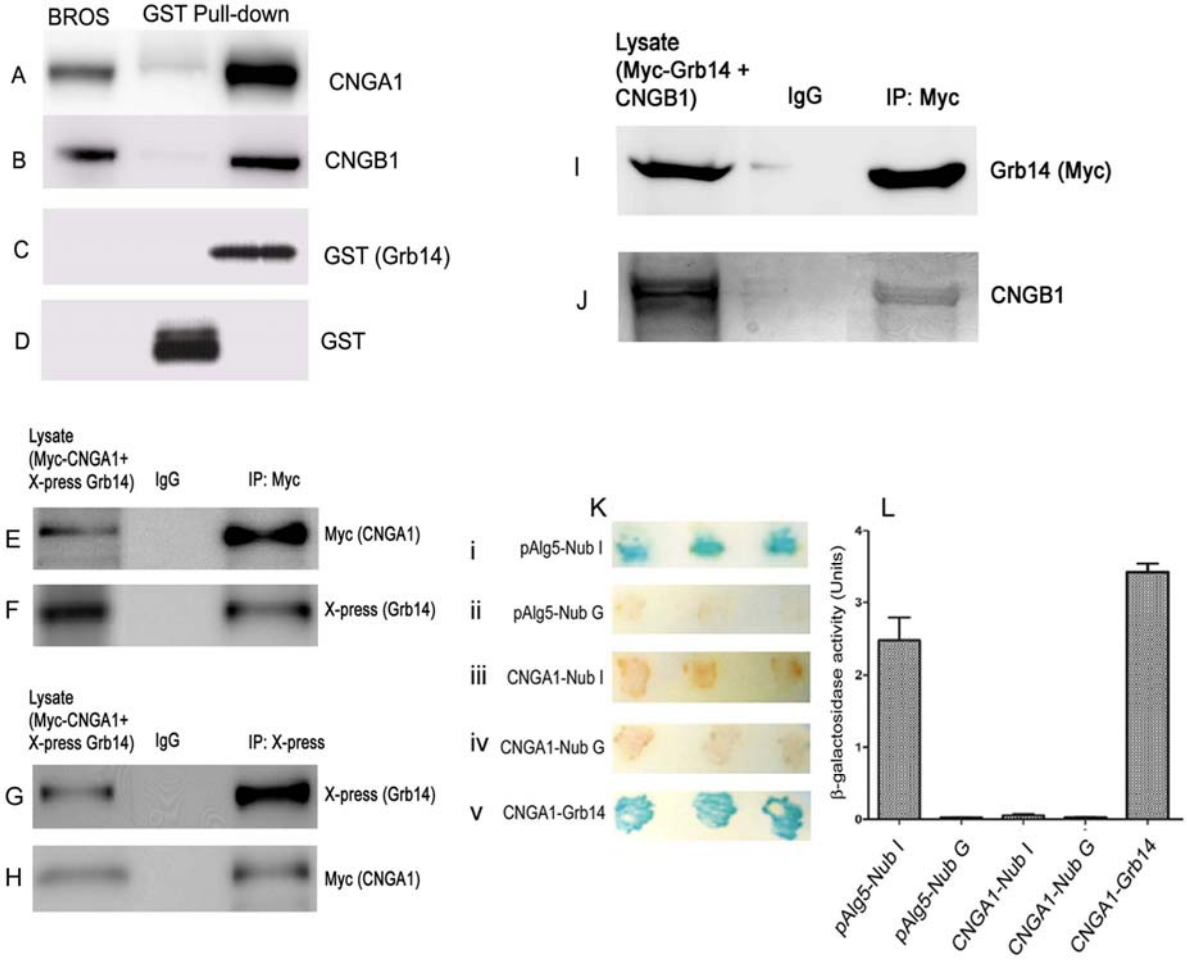
Immunoprecipitation

Immunoprecipitation was carried out according to the method described earlier (Li et al, 2007). ROS were solubilized for 30 min at 4°C in a lysis buffer containing 1% Triton X-100, 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM Na_3VO_4, 10 μg/ml leupeptin, and 1 μg/ml aprotinin. Retinas or proteins expressed in HEK293T cells were lysed in the lysis buffer. Insoluble material was removed by centrifugation at 17,000 x g for 20 min at 4°C, and the solubilized proteins were pre-cleared by incubation with 40 μl of protein A-Sepharose for 1 h at 4°C with mixing. The supernatant was incubated with primary antibodies or normal IgG (control) overnight at 4°C and subsequently with 40 μl of protein A-Sepharose for 2 h at 4°C. Following centrifugation at 17,000 x g for 1 min at 4°C, immune complexes were washed three times with ice-cold wash buffer [50 mM HEPES (pH 7.4) 118 mM NaCl, 100 mM NaF, 2 mM $NaVO_3$, 0.1% (w/v) SDS and 1% (v/v) Triton X-100]. The immunoprecipitates were subjected to immunoblot analysis with indicated antibodies in the respective figures.

Statistical Methods

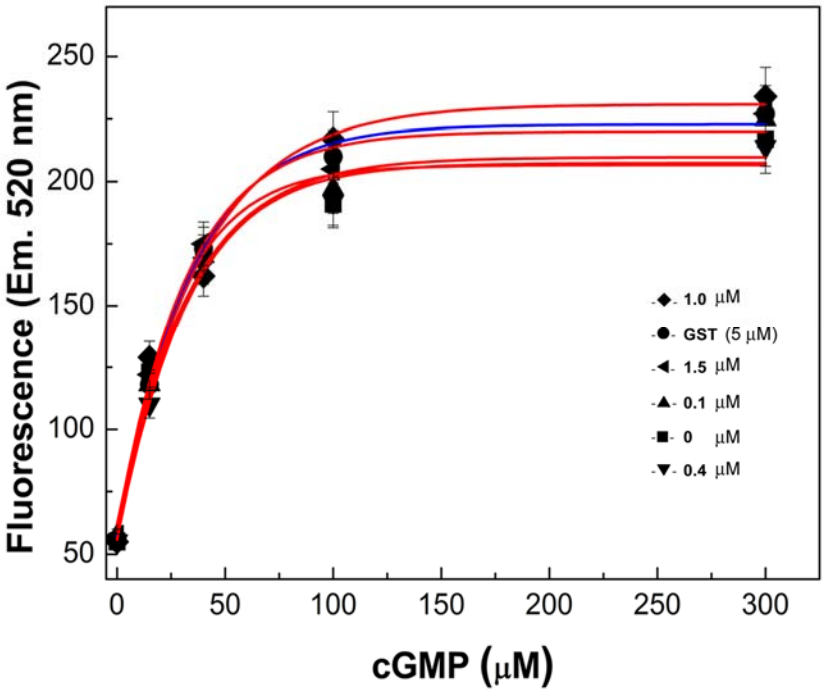
Data were analyzed and graphed using Graphpad Prism software (GraphPad Software, San Diego, CA). The data were expressed as the mean ± S.D. and compared by Student's *t* test for unpaired data. The significance was set at $p < 0.05$.

Supplementary Figures



Supplementary Figure 1. Grb14 interacts with rod photoreceptor CNG channel. Solubilized bovine ROS were incubated with either GST or GST-Grb14 fusion proteins and subjected to GST pull-down (Rajala et al, 2007). Bound proteins were subjected to Western blot analysis with anti-CNGA1 (A) and anti-CNGB1 (B) antibodies. To ensure the equal protein loading we reprobed the blot with anti-GST antibody (C, D). Myc-tagged CNGA1 was co-expressed with X-press–tagged Grb14 and Myc-Grb14 was similarly expressed along with CNGB1 in HEK293T cells. Protein extracts expressing Myc-CNGA1 and X-press-Grb14 were subjected to reciprocal co-immunoprecipitation

using anti-Myc-antibody and then immunoblotted with either Myc (E) or X-press (F) antibodies or immunoprecipitated with anti-X-press antibodies and immunoblotted with either X-press (G) or Myc (H) antibodies. Protein extracts expressing Myc-Grb14 and CNGB1 were subjected to co-immunoprecipitation using anti-Myc-antibody and immunoblotted with either anti-Myc (I) or anti-CNGB1 (J) antibodies. CNGA1 interacts with Grb14 in split ubiquitin-based membrane yeast two-hybrid system (Stagljar *et al.* 1998). DSY-1 yeast cells co-expressing (i) pMBV-Agl5 and pAlg5-NubI as positive control (ii) pMBV-Agl5 and pAlg5-NubG as negative control (iii) pDHB1-CNGA1 and pAlg5-NubI as negative control, (iv) pDHB1-CNGA1 and pAlg5-NubG as negative control and (v) pDHB1-CNGA1 and pDL2-Nx-Grb14. Transformants were assayed for β-galactosidase by colony color by filter lift assay (K) or β-galactosidase activity was quantitatively determined by the solution assay (L) according to the method described earlier (Rajala et al, 2004).



Supplementary Figure 2. GST-RA domain does not modulate the Ca^{2+} flux across the photoreceptor membrane micelles. Micelles were prepared from bovine rod outer segments with trapped fluo-3 dye. The ROS micelles were incubated with different concentrations of GST-RA-Grb14 as indicated and followed for the effect of cGMP-induced alteration of fluorescence at 520 nm as a function of Ca^{2+} flux across the membrane. GST is the blue trace. The experiment was done in triplicate and data plotted as mean ± SD.

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