Supporting Information

Kolesnikov et al. 10.1073/pnas.1712405114

SI Materials and Methods

Generation of PP2A Conditional Knockout Mouse Lines. All experiments were approved by the Washington University Animal Studies Committee and the Case Western Reserve University Animal Care Committee. Unless otherwise specified, all control and experimental mice of either sex were used at 2–5 mo of age. Animals were fed with standard chow (LabDiet 5053; Purina Mills) and kept under standard 12-h dark/light cyclic conditions.

Ppp2ca-floxed mice were generated by the Molecular Genetics Core at Washington University. The *Ppp2ca* gene contains seven exons and six introns (78, 79). The targeting vector for the *Ppp2ca* gene encoding the major α-isoform of the catalytic subunit of PP2A (PP2A-Cα) was constructed by recombination methodology (80) using the strategy shown in Fig. 1. The targeting construct was designed to replace the first *Ppp2ca* exon with an excisable *Neo* cassette to eliminate the coding region of PP2A-Cα. The first step was the retrieval of the entire length of the construct from the BAC (RP23-46N3) plasmid. Next, the lone *LoxP* site was inserted 624 bp upstream of the *Ppp2ca* gene exon 1. The last step was the insertion of the second *LoxP* site and *Frt*-flanked *Neo* cassette with a bGH polyadenylation signal in intron 1, 551 bp downstream of exon 1. Thus, the construct contained a 5′ homology arm, a conditional arm with a single *LoxP* site upstream of it, a PGK promoter-driven *Frt-Neo-Frt* cassette with a *LoxP* site downstream of it, and finally a 3′ homology arm (Fig. 1*A*). The 5′ arm started at 5,247 bp upstream of exon 1 and was 4,623 bp in length. The conditional arm was 1,645 bp long and contained exon 1. The 3′ arm started 551 bp downstream of exon 1 and was 2,479 bp in length. The linearized targeting vector was electroporated into 129 × 1Sv/J ES cells (SCC10 line; Siteman Cancer Center Embryonic Stem Cell Core at Washington University) and the recombination event in selected G418-resistant clones was confirmed by Southern blotting and PCR. Positive ES cells were microinjected into C57BL/6J mouse embryos that were then implanted into surrogate mothers (The Jackson Laboratory). The resulting chimeras were bred with C57BL/6J mice to generate heterozygous $Ppp2ca^{+}Ppp2ca(+Neo)^{f}$ mice.

Germline transmission in F_1 and all subsequent generations was confirmed by PCR using primers P1 (5′-TTTCCAGCCAGGAACATTCAAGCTCCACCA-3′) and P2 (5′-TGGCCGTTGCA-GGCTCCTATAATTAGGTCA-3′) for the *Ppp2ca-LoxP* allele (PCR product of 619 bp) and the *Ppp2ca-WT* allele (PCR product of 524 bp). To remove the *Frt*-flanked *Neo* cassette, heterozygous $Ppp2ca^{+}Ppp2ca(+Neo)^{f}$ mice were bred with a highly efficient germline *Neo*-deleter strain B6(C3)-Tg(Pgk1-FLPo)10Sykr/J mice expressing *FLPo* recombinase (81). Genotyping for determination of the presence of *FLPo* transgene in these mice was performed according to The Jackson Laboratory web protocol. Excision of the *Frt-Neo-Frt* cassette was confirmed by PCR with primers P3 (5′-TATGGAAAGGTCACCTCGCTTTCCTGTCGGT-3′) and P4 (5′-AATGGCT-CCACGCCAAGTACGTTACACACA-3′), resulting in the appearance of a PCR product of 410 bp (vs. a 309-bp product for the WT allele).

For rod-specific elimination of the PP2A-Cα, $Ppp2ca^{+}Ppp2ca(-Neo)^{f}$ mice were bred with *rhodopsin-Cre* ($iCre75^{+}$) mice (30) and finally inbred to produce homozygous $Ppp2ca^{f/f}iCre75^{+}$ animals. For all rod-related experiments, littermate $Ppp2ca^{+/+}iCre75^{+}$ mice were used as controls. To delete PP2A-Cα exclusively in M-cones, $Ppp2ca(-Neo)^{f/f}$ mice were bred with human red/green pigment gene promoter *Cre* ($HRGP\text{-}Cre^{+}$) mice that express *Cre* recombinase selectively in cones (41). These animals were further bred with rod transducin α-subunit knockout ($Gnat1^{-/-}$) mice, which lack functional rod phototransduction (42). The resulting $Ppp2ca^{f/f}Gnat1^{-/-}HRGP\text{-}Cre^{+}$ mice were used for all cone-related electrophysiological experiments, with $Ppp2ca^{f/f}Gnat1^{-/-}HRGP\text{-}Cre^{-}$ or $Ppp2ca^{+/+}Gnat1^{-/-}HRGP\text{-}Cre^{+}$ mice employed as controls. Genotyping for the presence of *iCre75* or *HRGP-Cre* transgenes in each generation was performed according to protocols designed by Transnetyx.

All PP2A mice were homozygous for the Met-450 isoform of RPE65 (71) and were free of the *Crb1*/*rd8* mutation (72). $Nrl^{-/-}$ mice used to detect PP2A-Cα mRNA in cones have been described earlier (73).

Light Microscopy. Five-month-old mice were killed by CO_2 asphyxiation, and their eyes were enucleated and immersion-fixed for 24 h in PBS (pH 7.4) containing 2% glutaraldehyde and 2% paraformaldehyde, at 4 °C. After dehydration, eyecups were embedded in an EPON-Araldite mixture, and 1-μm sections were cut dorsal to ventral through the optic nerve and stained with H&E. Images were acquired from the central retina near the optic nerve head.

Antibodies and Immunohistochemistry. After removal of the cornea and lens, the remaining mouse eyecup was fixed in freshly prepared 4% paraformaldehyde in 0.1 M PBS at pH 7.4 for 2 h at 4 °C. The eyecup was then washed once in PBS for 10 min and dehydrated with 30% sucrose buffered with PBS overnight at 4 °C. Next, the eyecup was embedded in Optimal Cutting Temperature compound (Ted Pella), flash-frozen in 2-methylbutane (Millipore Sigma) on dry ice, and cut with a cryo-microtome (CM1850; Leica) to produce 12-μm sections from the central retinal region immediately ventral to the optic nerve head. Sections were dried for 30 min at room temperature (RT), gently washed in deionized water for 10 min, dried again for 10 min at RT, and blocked for 1 h at RT with a solution containing either 1% BSA, 1% donkey serum or 1.5% goat serum, and 0.1–0.25% Triton X-100 (all from Millipore Sigma) in PBS. Sections were then incubated overnight at 4 °C with the appropriate primary antibodies (PP2A-Ca, ABIN319372, antibodies-online.com; or cone arrestin, AB15282; EMD Millipore) diluted in a solution containing 0.1% Triton X-100 in PBS. Next, sections were washed once in PBS and then incubated with secondary antibodies A11070 and A11072 (Thermo Fisher Scientific), respectively, diluted in PBS-containing DAPI for 2 h at RT, washed twice with PBS (10 min at RT), and mounted on coverslips with Fluoromount-G. Fluorescent images from the central region of the retina near the optic nerve head were captured with a CCD camera (Retiga Exi Fast 1394; QImaging) attached to a fluorescence microscope (CTR 6000; Leica).

In Situ mRNA Hybridization. Expression of PP2A-Cα mRNA in mouse rods and cones was visualized by in situ mRNA hybridization in control and $Nrl^{-/-}$ retinas, respectively. Mouse *Ppp2ca* target oligonucleotide probes for manual assays were designed and produced by Advanced Cell Diagnostics (ACD). The RNAscope 2.5 HD Brown Assay Kit (ACD) was used for in situ mRNA hybridization, and all procedures were carried out according to the manufacturer's RNAscope Technology protocol (31).

qRT-PCR. Mouse retinas were promptly homogenized and passed through a QIAShredder column (Qiagen) to further homogenize the eye tissues. Total RNA was then purified with the RNeasy Mini Kit (Qiagen) along with on-column DNase treatment (Qiagen) as per the manufacturer's directions. Total RNA from

mouse retinas (~1 μg after DNase treatment) was reverse-transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems) in a total volume of 20 μL. Then, 1% of this reaction mixture was used as a template for a qRT-PCR with TaqMan Gene Expression Assays (Applied Biosystems) following the manufacturer's instructions. The Mm00479816_m1 probe set was used to probe *Ppp2ca* and the Mm00479549_m1 set was used to probe *Ppp2cb*, with the 18S rRNA (Mm03928990_g1) probe set (Applied Biosystems) employed as an endogenous control. All real-time experiments were done with a Step-One Plus qRT-PCR machine (Applied Biosystems).

Rhodopsin Phosphorylation Analysis. This method for separation of rhodopsin phospho- and nonphosphopeptides by reversed-phase HPLC in combination with tandem mass spectrometry (MS/MS) has been described previously (37, 63). Briefly, dark-adapted or light-exposed (5,000-lx white light, 2 min) mice were killed by cervical dislocation at specified postbleach times, and their right eyes were immediately removed, flash-frozen in liquid N_2, and homogenized in 700 μL of 7 M urea in 10 mM Tris·HCl, pH 7.4. Eyes were homogenized with a biomasher tube and pestle with three rotations followed by sonication (QSonica).

Each sample was centrifuged at 55,000 × *g* for 1 h at 4 °C, the supernatant was discarded, and the precipitate was washed three times with 700 μL of deionized H_2O (Milli-Q). The sample was then suspended in 80 μL of deionized H_2O and digested with 20 μL of 20 μg/mL Asp-N enzyme (Promega) for 17 h in the dark at RT. Next, the sample was spun at 55,000 × *g* for 1 h at 4 °C, and supernatant was removed. After acidification with 10 μL of 0.1% formic acid, 10 μL of the supernatant was loaded onto an Onyx Monolithic C18 100 × 3.0-mm reverse-phase column (Phenomenex), and rhodopsin peptides were eluted using a 2–98% acetonitrile gradient in 0.1% formic acid for 45 min. Eluents were directed into a LTQ Velos mass spectrometer (Thermo Scientific) operated in positive ionization mode. Ionization was achieved using a temperature-controlled electrospray.

Selected ion monitoring was set up for the unphosphorylated C terminus peptide of mouse rhodopsin obtained by cleavage (identical to systemic peptide DDDASATASKTETSQVAPA) together with its monophosphorylated, double-phosphorylated, and triple-phosphorylated counterparts, with *m/z* of 933.95, 973.95, 1,013.95, and 1,053.95, respectively. The percentage of rhodopsin phosphorylation was evaluated using the following equation:

$$\%P = \frac{\sum_{i=1}^{3} P_i}{\sum_{i=0}^{3} P_i} \times 100\%,$$

where $\%P$ is the percentage of rhodopsin phosphorylation, and P_i (i = 0, 3) are areas of peaks corresponding to unphosphorylated, monophosphorylated, double-phosphorylated, and triple-phosphorylated rhodopsin peptides, respectively.

Quantification of Visual Cycle Retinoids. After pigment bleaching and the killing of animals, as described in a previous section, left eyes of the same dark-adapted mice were flash-frozen in liquid N_2 and thoroughly homogenized in 1 mL of ice-cold buffer containing 50 mM Mops, pH 7.0, 10 mM NH_2OH, and 50% ethanol. Each eye was homogenized until no visible sign of tissue remained, incubated for 20 min at RT, and gently vortexed with 4 mL of ice-cold hexane for 1 min to extract the retinoids. The sample was centrifuged for 10 min until phase separation occurred. Capped tubes wrapped in aluminum foil were used to protect retinoids from light exposure. The sample was subjected to centrifugation at 4,000 × *g* for 5 min using an Eppendorf centrifuge, and the upper organic phase was collected. The extraction steps were repeated three times. The resulting hexane fractions were pooled and dried in a SpeedVac for 1 h at RT. The dried retinoids were dissolved in 300 μL of hexane/ethyl acetate (90:10) solvent, separated by normal-phase HPLC (1100 series; Agilent Technologies) on a 5-μm, 4.6 × 250-mm Zorbax Sil column (Agilent Technologies) at a flow rate of 1.4 mL/min, using the same solvent as a mobile phase, and analyzed by monitoring their absorbance at both 325 and 360 nm. Retinoids were identified and quantified by comparison with authentic standards, as described previously (74).

Single-Cell Rod Suction Electrode Recordings. Control and rod-specific PP2A mutant animals were dark-adapted overnight and killed by CO_2 asphyxiation, and their retinas were removed under infrared illumination, chopped into small pieces, and transferred into a perfusion chamber located on the stage of an inverted microscope. A single rod outer segment was drawn into a glass microelectrode filled with Locke's solution containing 140 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 3 mM Hepes (pH 7.4), 0.02 mM EDTA, and 10 mM glucose. The perfusion solution contained 112.5 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM Hepes (pH 7.4), 20 mM $NaHCO_3$, 3 mM Na succinate, 0.5 mM Na glutamate, 0.02 mM EDTA, and 10 mM glucose. The solution was bubbled with a 95% O_2/5% CO_2 mixture and heated to 37–38 °C.

Test flashes (20 ms) of calibrated 500-nm light were delivered by an optical bench. The stimulating light intensity was controlled by neutral density filters in 0.5 log unit steps. Intensity–response relationships were fitted with Naka–Rushton hyperbolic functions, as follows:

$$R = \frac{R_{\max} * I^n}{I^n + I_{1/2}^n},$$

where R is the transient-peak amplitude of the response, $R_{\max}$ is the maximal response amplitude, I is the flash intensity, n is the Hill coefficient, and $I_{1/2}$ is the half-saturating light intensity.

Photoresponses were amplified, low-pass filtered (30 Hz, eight-pole Bessel), and digitized (1 kHz). Normalized rod dim flash fractional sensitivity (S_f) was calculated from the linear region of the intensity–response curve as the ratio of the response amplitude to a given flash strength and then normalized by the amplitude of the saturated response. Half-saturating light intensity ($I_{1/2}$) was calculated from the intensity–response relationship as the test flash intensity required to produce a response with an amplitude equal to one-half of the amplitude of the corresponding saturated response. Integration time ($T_{\text{integr.}}$) was calculated as the integral of the dim flash response with the transient peak amplitude normalized to unity. The time constant for the dim flash response recovery (τ_{rec}) was derived from the best single-exponential fit to the declining phase of the response. The dominant recovery time constant (τ_D) was determined from supersaturating flashes (82), by using a 10% criterion for photocurrent recovery from saturation. Data were analyzed with Clampfit 10.4 and Origin 8.5 software.

Ex Vivo Cone Recordings from Isolated Mouse Retinas. Control and rod-specific PP2A-mutant mice were dark-adapted overnight and killed by CO_2 asphyxiation, and the whole retina was removed from each mouse eyecup under infrared illumination and stored in oxygenated aqueous L15 (13.6 mg/mL, pH 7.4) solution (Millipore Sigma) containing 0.1% BSA, at RT. The retina was oriented with its photoreceptor side up and placed into a perfusion chamber (75) between two electrodes connected to a differential amplifier. The specimen was perfused with Locke's solution supplemented with 1.5 mM L-glutamate and 40 μM DL-2-amino-4-phosphonobutyric acid to block postsynaptic components of the photoresponse (76),

and with 70 μM $BaCl_2$ to suppress the slow glial PIII component (77). The perfusion solution was continuously bubbled with a 95% O_2/5% CO_2 mixture and heated to 36–37 °C.

Light stimulation was applied in 20-ms test flashes of calibrated 505-nm LED light. To maintain light uniformity, a glass optical diffuser was placed between the LED and the retina. Stimulating light intensity was controlled by a computer in 0.5 log unit steps. Intensity–response relationships were fitted with Naka–Rushton hyperbolic functions, as noted in a previous section. Photoresponses were amplified by a differential amplifier (DP-311; Warner Instruments), low-pass filtered at 300 Hz (eight-pole Bessel), and digitized at 1 kHz. Normalized cone dim flash fractional sensitivity (S_f) and other relevant cone response parameters were determined similarly to those described above. Data were analyzed with Clampfit 10.4 and Origin 8.5 software.

In Vivo ERG. Dark-adapted mice were anesthetized with an i.p. injection of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg). Pupils were dilated with a drop of 1% atropine sulfate. Mouse body temperature was maintained at 37 °C with a heating pad. ERG responses were measured from both eyes by contact corneal electrodes held in place by a drop of Gonak solution (Akorn). Full-field ERGs were recorded with a UTAS BigShot apparatus (LKC Technologies) using Ganzfeld-derived test flashes of calibrated green 530-nm LED light.

Rod a-wave flash sensitivity (S_f, calculated as described for suction recordings) in control and rod-specific PP2A-mutant animals was first determined in the dark (from an average of eight dim flash responses). In this case, normalization was done to the maximal rod-driven ERG a-wave response (A_{max}) determined at 23.5 cd·s·m^{-2}. Similarly, cone b-wave flash sensitivity (S_f) in dark-adapted control and rod-specific PP2A mutant mice was determined from the average of up to 20 dim flash responses and normalized to the maximal b-wave amplitude obtained with the brightest white light stimulus from the xenon flash tube (700 cd•s·m^{-2}). To monitor the postbleach recovery of either rod ERG A_{max} and rod S_f or cone S_f, >90% of the relevant visual pigment was bleached with a 30-s exposure to 520-nm LED light focused at the surface of the cornea. The bleached fraction was then estimated from the following equation:

$$F = 1 - \exp(-I \cdot P \cdot t),$$

where F is the fraction of pigment bleached, t is the duration of the light exposure (in seconds), I is the bleaching light intensity of 520-nm LED light (1.3×10^8 photons·μm^{-2}·s^{-1}), and P is the photosensitivity of mouse photoreceptors at the wavelength of peak absorbance [5.7×10^{-9} μm^2 for mouse rods (83) and 7.5×10^{-9} μm^2 for mouse cones (84)]. Mice were reanesthetized every 30–40 min with a lower dose of ketamine (approximately one-half of the initial dose), and a 1:1 mixture of PBS and Gonak solutions was gently applied to their eyes with a plastic syringe to protect them from drying and maintain electrode contacts.

Statistics. For all experiments, data were expressed as mean ± SEM and analyzed with the independent two-tailed Student's t test (using an accepted significance level of $P < 0.05$) or two-way repeated-measures ANOVA (with genotype as main factor and time as repeated-measures factor). In the latter case, pairwise comparisons were performed using the Bonferroni post hoc test, and $P < 0.05$ was considered significant.