Supplementary Information

Spata7 is a retinal ciliopathy gene critical for correct RPGRIP1 localization and protein trafficking in the retina

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Materials and Methods:

Generation of Spata7 Mutant Mice and Transgenic Mice

To generate *Spata7* knockout mice, AB 2.2 embryonic stem cells (Mouse ES Cell Core Facility at Baylor College of Medicine) derived from the 129 SvEv strain were electroporated with linearized targeting vector. DNA from embryonic stem cell lines was digested with HindIII and analyzed by Southern blot using 5′ and 3′ probes (flanking the recombination arms and not included in the targeting vector) and genomic PCR. Three independently targeted cell lines were selected and microinjected into the C57BL6 blastocysts to generate chimeras. One chimera underwent germline transmission. Total RNA was extracted from mouse retinas using Trizol reagent (Invitrogen) and first-strand cDNA was synthesized using the SuperScript^{TM}III First-Strand Synthesis System for RT-PCR (Invitrogen).

To generate the Flag tagged *Spata7* transgenic mice, three Flag-encoding fragments were inserted into a Bacterial artificial chromosome (BAC) containg the entire *Spata7* gene immediately after the start codon (ATG), Linearized vector was injected into C57 mouse embryos in the GEM core facility at Baylor College of Medicine. Transgene expression was characterized by immunoblotting and immunohistochemistry. The *Spata7-Flag* transgene appears to be expressed at physiological levels with the same expression pattern as the endogenous *Spata7* gene. All animals were handled in accordance with the policies on the treatment of laboratory animals by Baylor College of Medicine.

Transmission Electron Microscopic Analysis of Retinal Sections

Eye cups were fixed in 2% glutaraldehyde, 2% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 48 hrs. The tissue was then rinsed in buffer and post-fixed in 1% osmium tetroxide/1N sodium cacodylate buffer solution for 2 hrs. Tissue was rinsed in several changes of buffer and dehydrated through a series of ethanol solutions. Tissue was infiltrated with a 2:1 mixture of Epon/propylene oxide for 1 hr, followed by 1:2 Epon/propylene oxide for 1 hr and then infiltrated in pure Epon resin overnight. Finally, eyecups were embedded in molds. Thick (700 nm) and ultrathin (70 nm) sections were obtained using a Leica Ultracut R microtome. Thick sections were stained with Toluidine Blue and images captured on a Zeiss AxioImager. Ultrathin sections were stained with uranyl acetate and lead citrate salts and images were captured on a Zeiss 902EM.

Measurements of Outer Nuclear Layer (ONL) Thickness

Mouse eyes were dissected, embedded in paraffin, and sectioned along the vertical meridian. The thickness of the outer nuclear layer (ONL) was measured at 20 positions equally spaced along the retina (10 positions each in the superior and inferior hemispheres). For each position, three measurements were taken and the average value of these three measurements was calculated. Measurements were made using a camera lucida connected to a light microscope, a WACOM graphics tablet (WACOM, Vancouver, WA), and AxioVision LE Rel. 4.1. software (Zeiss, Goettingen, Germany). Before each measurement session, the setup was calibrated using a stage micrometer (Klarmann Rulings, Litchfield, NH).

Electroretinographic Analysis

Prior to ERG testing, mice were allowed to dark-adapt overnight. Under dim red illumination, mice were anesthetized with an intraperitoneal injection of ketamine (70 mg/kg), xylazine (14 mg/kg), and acepromazine (1.2 mg/kg). A single drop of 0.5% proparacaine was applied to each eye for corneal anesthesia and drops of 1% tropicamide and 2.5% phenylephrine were used to dilate the pupils. Mice were placed on a heating pad maintained at 39^{o}C inside a Ganzfeld dome. Platinum electrodes mounted on micromanipulators were positioned on each cornea and a small amount of 2.5% methylcellulose was applied to each eye. Platinum subdermal needle electrodes were inserted into the tail and forehead to serve as ground and reference, respectively. Signals were bandpass filtered from 0.1 – 1,000 Hz and digitally sampled at 10 kHz. Flashes in the scotopic range were generated by a pair of cyan Luxeon K2 LEDs (λ_{peak} = 505 nm, $\Delta\lambda_{1/2}$ = 30 nm; Phillips Lumileds, San Jose, CA) wired in series. Square pulses of 0.5 ms duration and varying currents were driven through the LEDs to create flashes of different intensities. Bright flashes were generated with 1500 W xenon flash bulbs (Novatron, Dallas, TX). At the lowest intensity, 25 responses were averaged with a delay of 4 seconds between each flash. As flash intensity increased, fewer responses were averaged with a longer delay between flashes. For analysis of ERG waveforms, the a-wave was measured from baseline to the trough of the initial negative deflection (unfiltered) and the b-wave was measured from the a-wave trough to the peak of the subsequent positive deflection (low-pass filtered, f_c = 60 Hz).

Western Blot Analysis

Whole retinas from wild-type and mutant mice were homogenized in 100 µl of 1xNETN buffer (20% glycerol, 50mM Tris-HCI pH8.0, 150mM NaCI, 1mM EDTA, 0.5% NP-40) and centrifuged for 20 min at 13,000 rpm at 4˚C to harvest supernatant. Proteins were quantified by BCA protein assay and heated at 95˚C in sample application buffer (5% SDS,15% sucrose, 50mM Na_2CO_3,

50 mM DTT, 1% 2-mercaptoethanol, bromophenol blue) before being separated on a 10% SDS-PAGE gel (pH 8.8) with a 4% stacking gel (pH 6.8) and then transferred to nitrocellulose membranes at 40 mA overnight at 4˚C. Membranes were first blocked with 5% dry milk in Tris-buffered saline Tween 20 (TBST) (0.05M Tris–HCl, pH 8.0, 0.15M NaCl, 0.05% Tween 20) at room temperature for 1 hr and then incubated overnight at 4˚C with primary antibodies in 5% dry milk in TBST: 1/1000 rabbit anti-Spata7 (Proteintech Group, 12020-1-AP), and 1/5000 mouse anti-β-Actin (Sigma). Membranes were further incubated with 1/5000 anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Santa Cruz Biotechnology) in 5% dry milk in TBST for 1 h at room temperature. Bands were detected with the enhanced chemiluminescence (ECL) plus system (GE Healthcare).

Immunohistochemistry

Eyes were enucleated from knockout and wild-type mice. Corneas were pierced with a surgical blade tip before the eyes were immersed in 4% paraformaldehyde in PBS buffer for 5 to 10 minutes. The eyes were incubated in 10% sucrose for 1 hr, followed by 20% sucrose for 1 hr, and then transferred to 30% sucrose in 1X PBS to soak overnight. Eyes were then transferred through a 1:1 mixture of OCT and 30% sucrose for 1 hr, then OCT for at least 1 hr before being placed in a cryomold, overlayed with OCT and frozen on dry ice. Sixteen micrometer sections were cut (Cryostat; Leica) and placed on glass slides, and stored at -80˚C until use.

For evaluation of RPGRIP1 localization in photoreceptor cells of wild-type and *Spata7* mutant mice, unfixed eyes of 14 day-old (P14) mice were harvested and frozen in melting isopentane. 5-8 µm cryosections were cut and treated with 0.1% Triton X100 in PBS for 10 minutes and subsequently blocked in 5% normal goat serum in PBS (blocking buffer) for 30

minutes. Cryosections were then incubated overnight at 4°C with corresponding primary antibodies diluted in blocking buffer.

Modified Davidson's Fixative was used to fix eyes overnight for paraffin embedding. Seven micrometer eye sections were cut (Microtome, Leica). Slides were deparaffinized and antigen retrieval was performed by boiling sections in 0.01 M Tris, EDTA buffer (pH 9.0) for 30 min, followed by cooling for 30 min at room temperature. Slides were washed in PBS, incubated for 1 hr at room temperature in hybridization buffer (10% normal goat serum, 0.1% Triton X-100, PBS), then incubated overnight in primary antibody diluted in hybridization buffer. Slides were then washed in PBS, incubated with secondary antibody diluted in hybridization buffer at room temperature for 2 hr, washed in PBS, mounted with anti-fade medium (Prolong; Invitrogen) to reduce bleaching, and coverslipped.

Fluorescent images were captured with a Zeiss LSM 510 confocal microscope and processed with Image J and Adobe Photoshop software. Images were processed using Axiovision 4.3 (Zeiss) and Adobe Photoshop CS4. Analysis of signal distribution was performed using 3D Surface Plot analysis of FIJI software.

Detection of Apoptotic Cells in the Retina

Apoptotic cell death was detected by the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay according to the manufactory's manual (Roche, In Situ Cell Death Detection it, 11684795910, Roche Diagnostics, Indianapolis, IN). Paraffin embedded retinal sections were used for the TUNEL assay.

Staining of Retinal Whole Mounts

Quantification of cones in retinal whole mount staining was carried out according to previously published methods(1) with a slight modification. Briefly, eyes from adult mice were removed and dropped into fresh 4% paraformaldehyde (PFA) in PBS, pH 7.2. After 10 min, the cornea and lens were removed and the retina carefully removed from the eyecup. Retinas were incubated in 4% PFA for a total of 3 hr at 4˚C and transferred to PBS, and small cuts were made in the retina to facilitate flat mounting. Retinas were rinsed in 1x PBS (pH 7.2) three times for 5 min each, blocked with 10% NGS in PBS with 0.1% Triton-x100 for 1-2 hr, and incubated with PNA (1:200) overnight at 4˚C in blocking buffer. Retinas were washed in an Eppendorf tube three times for 5 min each, then incubated with secondary antibodies. Binding was detected by incubating slides with Cy3-streptavidin (1:100) at RT for 2 hr. Retinas were rinsed in PBS three times for 5 min each, and retinal whole-mounts were mounted with the photoreceptor side up. Cone cells stained with PNA were counted using a square reticule inserted into the microscope with a 20x objective lens. Each quadrant of the retina was further subdivided into 35 squares (0.25 mm x 0.25 mm, 0.0625 mm^2), the size of one square of the reticule. The total number of cone cells from all 35 squares in one retina was calculated, and *Spata7*$^{+/+}$ and *Spata7*$^{-/-}$ were compared.

Immunocytochemistry

hTERT-RPE1 cells were cultured as previously described (2). To induce ciliogenesis, cells were seeded on coverslips and serum starved for 48 hrs to detect endogenous SPATA7 expression or 24 hrs prior to transfection. Subsequently, PEI (0.1mg/ml) was used to transfect cells with the appropriate vector. 24 hrs post-transfection, ciliated cells were fixed in 2% PFA for 20 min, treated with 1% Triton X-100 in PBS for 5 min, and blocked in 2% bovine serum albumin (BSA)

in PBS for 30 minutes. Fixed cells were stained for 1 hr with the corresponding primary antibodies: rabbit polyclonal α-Flag (1:1000, Sigma-Aldrich), rabbit polyclonal α-RPGRIP1 (1:5000, kindly provided by Dr. P. Ferreira, Duke Eye Center, Duke University School of Medicine, USA), guinea pig α-RPGRIP1L (1:500, affinity purified antibody was previously described (3), mouse monoclonal α-acetylated tubulin (1:1000, Sigma-Aldrich). Coverslips were then washed in PBS and stained for 45 min with Alexa Fluor 405-, 488-, 568-, or 647-conjugated secondary antibodies (1:500, *Invitrogen*/Molecular Probes). Coverslips were washed again with PBS and briefly with mQ water before mounting in Vectashield with or without DAPI (Vector Laboratories, Burlingame, CA, USA). Samples for immunocytochemistry were analysed on a Zeiss Axio Imager ZI fluorescence microscope (Zeiss), equipped with a 63x objective oil lens. Optical sections were generated through structured processed using Axiovision 4.3 (Zeiss), Adobe Photoshop CS4, and FIJI software.

References:

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Figure legends

Supplementary Fig.1. Subcellular localization of SPATA7 and SPATA7 fragments in hTERT RPE-1 cells.

(A) Overexpressed mRFP-SPATA7 (red) associates with the microtubule network and localizes to the ciliary axoneme (arrow), marked by an antibody against acetylated alpha tubulin (magenta). (B) The N-terminal fragment (aa 2-310) of SPATA7 (red) maintained the microtubular and ciliary axoneme localization, but also localizes to the nucleus of some cells. (C) The C-terminal fragment (aa 311-568) of SPATA7 diffusely localizes to the cytoplasm. Scale bar: 5 µm.

Supplementary Fig. 2. **SPATA7 is readily detected as early as at P4**. (A-P) Paraffin embedded mouse retinal sections were stained for SPATA7 (red) and DAPI (blue) beginning at P2. SPATA7 protein is first weakly detected at P2 (A,B) with protein levels increasing as time progresses (E,I,M). The location of SPATA7 coincides with developing photoreceptors. At P15, strong SPATA7 is observed specifically in the photoreceptor cell layer (M,N) and no significant signal is observed in any other cell type in the retina. Scale bar is 20 µm.

Supplementary Fig. 3 SPATA7 is expressed in cone photoreceptors and enriched in basal body of hTERT RPE-1 cells

Protein subcellular localization of SPATA7 was examined at P29. Paraffin embedded wild-type retinal sections were double labeled for PNA (cone marker) (A) and SPATA7 (B). Panel B is a merge of PNA (red) and SPATA7 (green); yellow arrows indicate double-labeled cones. Scale bar is 10 µm. Paraffin embedded *Spata7-Flag* transgenic retinal section was labeled for mouse

anti-Flag(red) and rabbit anti-Opsin blue(green) antibodies to further confirm SPATA7 expression in cones (C) at P25, yellow arrows indicate double-labeled cones. Scale bar is 5µm., CC is connecting cilium, IS is inner segment. Panel D is staining of endogenous SPATA7 in hTERT RPE-1 cells, endogenous SPATA7 is detected at the basal body of the primary cilium in cultured hTERT-RPE1 cells. Acetylated a- tubulin (red) is distal to SPATA7 (green), scale bar is 10um.

Supplementary Fig. 4. Colocalization of SPATA7 and RPGRIP1 in hTERT RPE1 cells. (A) Overexpressed 3xFlag-RPGRIP1 full length is diffusely localized in the cytoplasm. (B) Co-expressed mRFP-SPATA7 (red) recruits 3xFlag tagged RPGRIP1 (green) to the microtubule network. Microtubules of the ciliary axoneme and part of the microtubule network were stained with anti-acetylated alpha tubulin (magenta); nuclei were stained with DAPI (blue). Scale bar: 5 µm.

Supplementary Fig. 5. Immunostaining of retinal sections with a panel of cell-type specific markers. (A) Frozen sections of wild-type and *Spata7* mutant retinas at P26 labeled with BRN3B(ganglion cells), CHX10 (bipolar cells), PAX6 (amacrine cells and ganglion cells), and Calbindin (horizontal cells), respectively. (B) Frozen sections of P26 wild-type and *Spata7* mutant retinas were labeled for cone Opsins. All antibodies are in green and propidium iodide (PI) is in red. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer;

Supplementary Fig. 6. Aged *Spata7* mice exhibit significant defects in response to light. Electroretinograms (ERG) were performed on *Spata7* mutant mice at the ages of 6 and 12 months. Fractional scotopic a-wave (A) and b-wave amplitudes (B) of ERG recordings as a

function of increasing light intensities at 6 and 12 months. n=5 or more for each group. Each point represents mean ±SEM obtained for each group.

Supplementary Fig. 7. Peripherin and ROM-1 are properly localized in *Spata7* mutant retinas. Subcellular localization of PRPH (Peripherin) and Rom-1 were examined at P15 and P29. Paraffin embedded wild-type and *Spata7* mutant retina sections were stained for PRPH (A-H) and Rom-1 (I-P). Antibodies are in green, nuclei are labeled with propidium iodide (PI, red). Scale bar is 20 µm.

Supplementary Fig. 8. The majority of Transducin properly translocates in *Spata7* mutants

Immunofluorescence staining of GAT1 (rod Transducin-α) in paraffin embedded retinal sections was performed on retinas obtained from mice either reared under a normal light/dark cycle (A, B) or constant dark (C, D) conditions. Mice used in this experiment are litter mates and are either wild-type (A, C) or *Spata7* homozygous mutant (B, D). Scale bar is 10 µm.

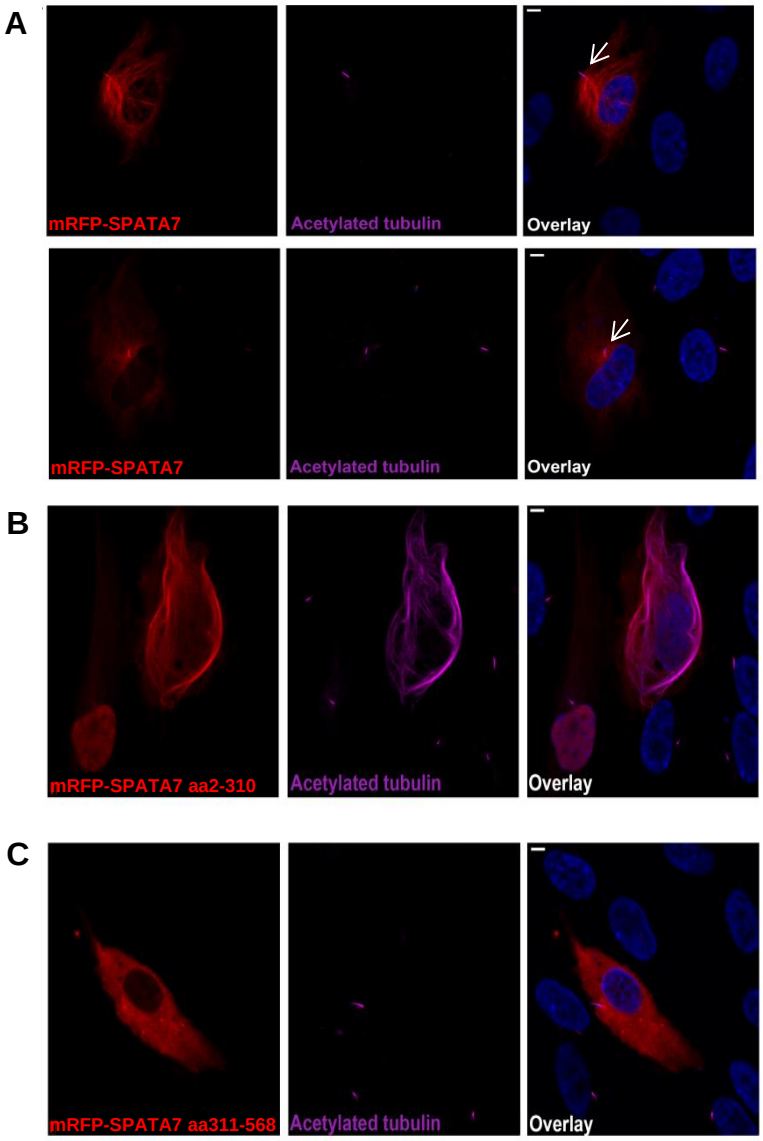
Supplementary Fig. 9. Mislocalized Rhodopsin is glycosylated and triggers ER stress.

(A,A') WT retinas of P15 mice show no CHOP (ER stress marker) staining while mutant *Spata7* photoreceptors (B, B') are positive for CHOP (green). While CHOP staining is not suppressed in the *Spata7* mutant retina removing one copy of *Rho* (C, C'), no CHOP positive cells are detected when both copies of *Rho* are removed (D, D'). Scale bar: 20 µm. Similarly, to visualize if mislocalized RHO, lectin Concanavalin A (ConA) staining that labels glycosylated protein is

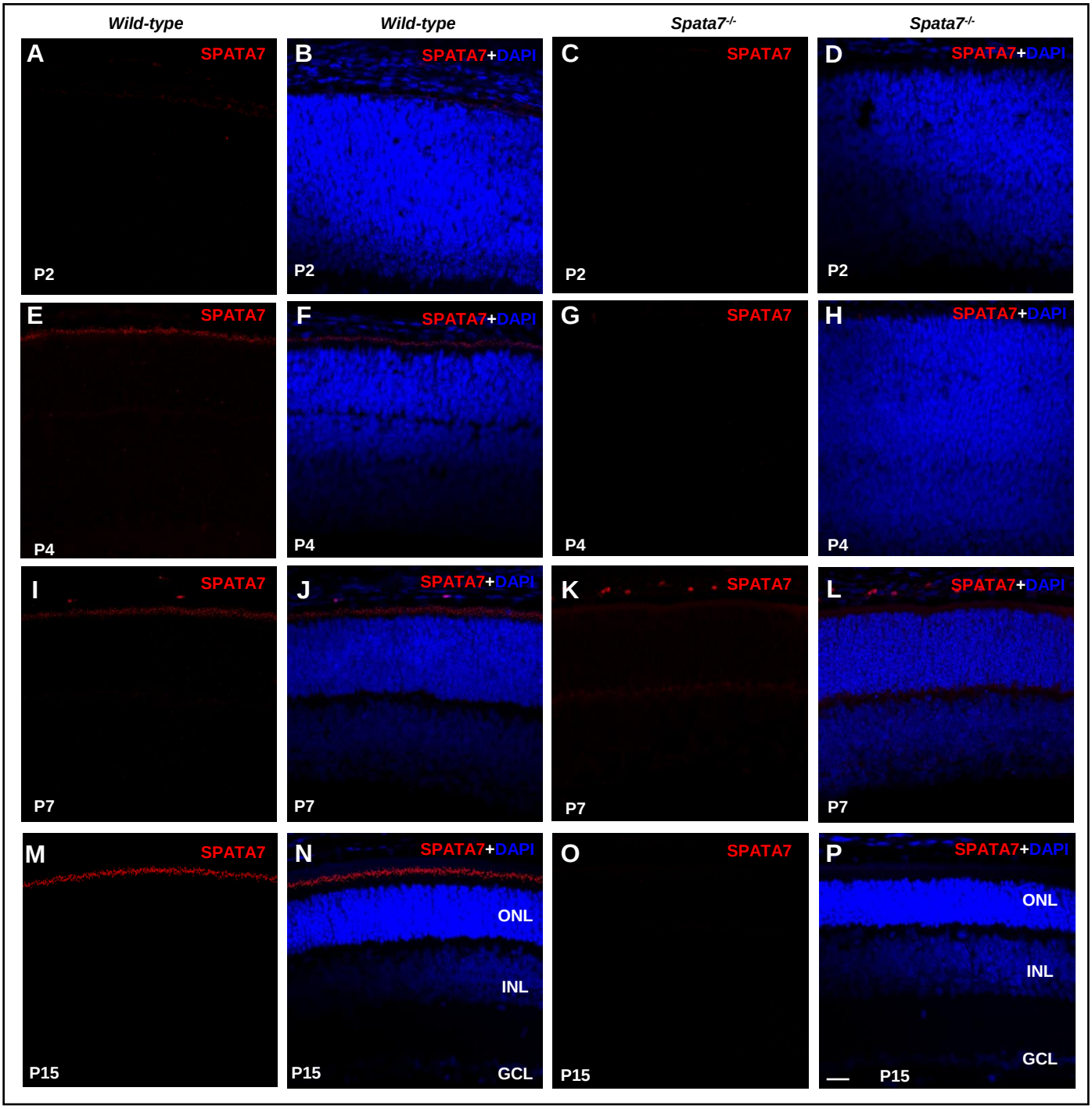
performed. Immunofluorescence labeling of paraffin embedded retinas shows proper localization of Rhodopsin (red) in a P25 wild-type mouse (E, E', E''). Note that RHO is restricted to the outer segment (OS), where phototransduction occurs. In a *Spata7* mutant litter mate, there is evidence of considerable mislocalization of Rhodopsin (red) and increasing ConA stating in the IS and ONL (G, G', G''). Consistent with the idea that ConA staining is largely due to accumulation of glycosylated Opsin in the IS and ONL, reduced ConA staining is observed when both copies of *Rho* is removed in the *Spata7* mutant mice (F,F'). Scale bar: 10 µm.

Supplementary Fig. 10. Rhodopsin mislocalization is observed prior to photoreceptor cell apoptosis. (A-H) Subcellular localization of RHO was examined at multiple time points, including P7 and P11. Mislocalization of RHO (green) was observed at both time points in paraffin embedded retinas. Rhodopsin is labeled in green and propidium iodide (PI) in red. (I-P) Cell apoptosis was examined in frozen retinal sections by Caspase3 staining (green) at P7 and P11. No difference was found for the number of Caspase3 positive cells in the ONL between wild-type and *Spata7* mutant retinas at P7 or P11. ONL-outer nuclear layer, INL-inner nuclear layer**.** Scale bar: 20 µm.

Supplementary Fig. 11. **Deletion of both copies of *Rho* partially rescues the *Spata7* mutant phenotype.** Panels B-E are magnified images of photoreceptors in panels of A-D of Figure 10 that represents photoreceptor rescue experiments. Panel A is an additional wild-type control. Scale bar is 20 µm.



Supplementary Fig.1 . Eblimit et al.



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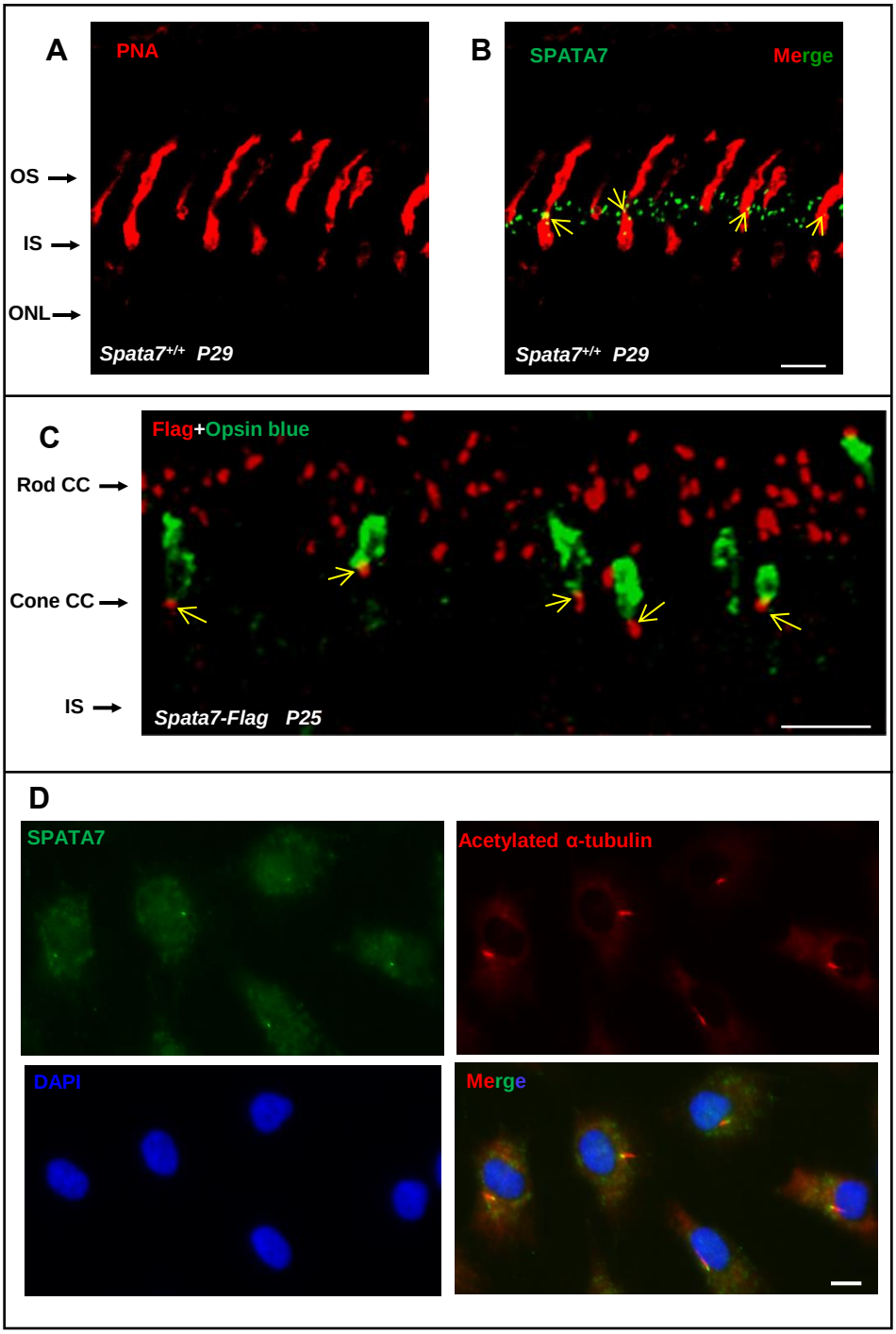

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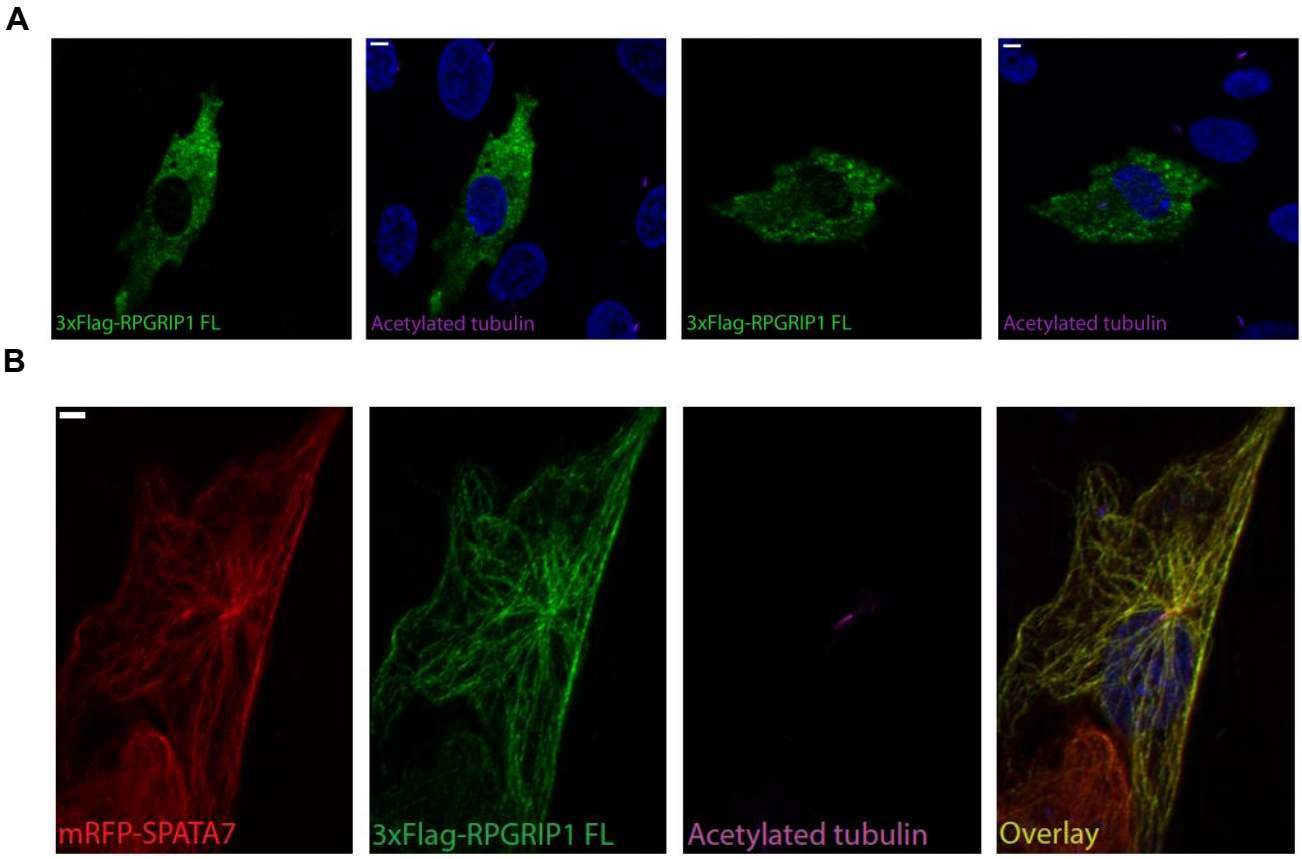

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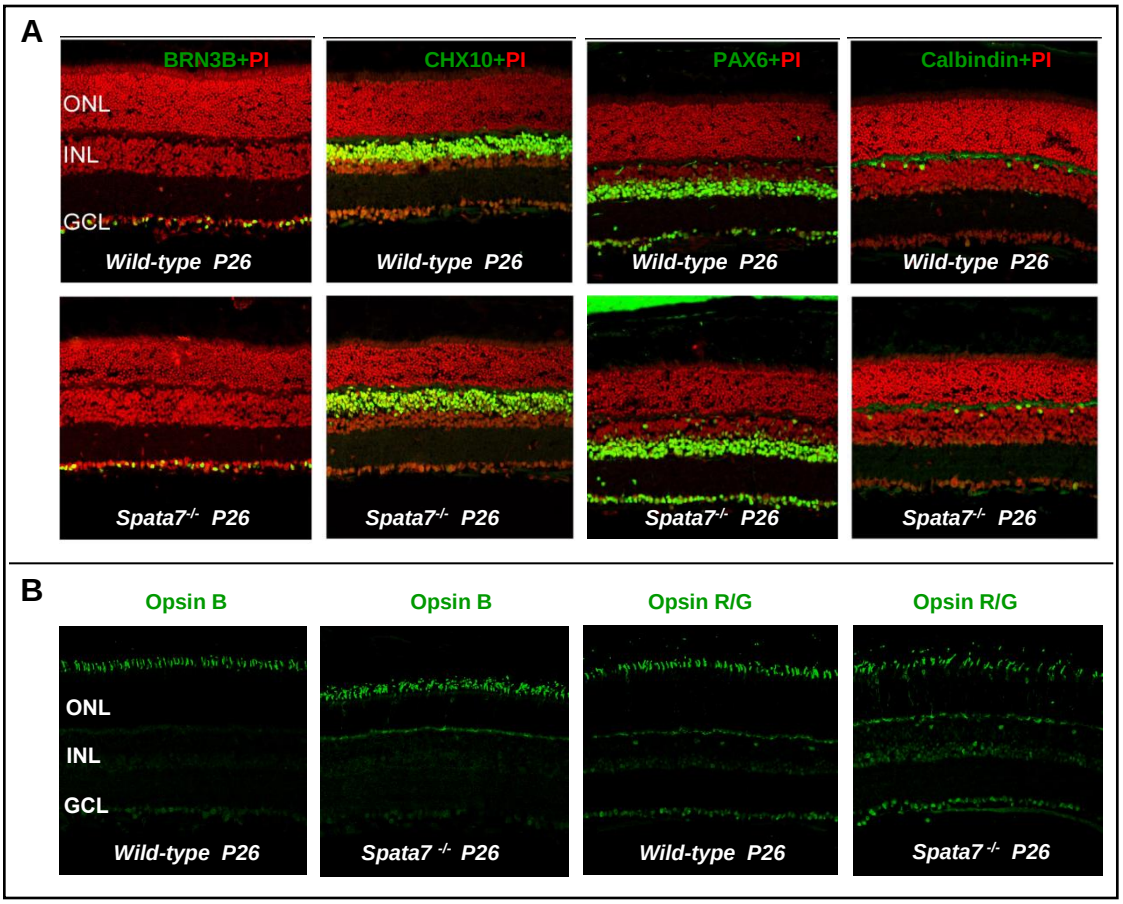

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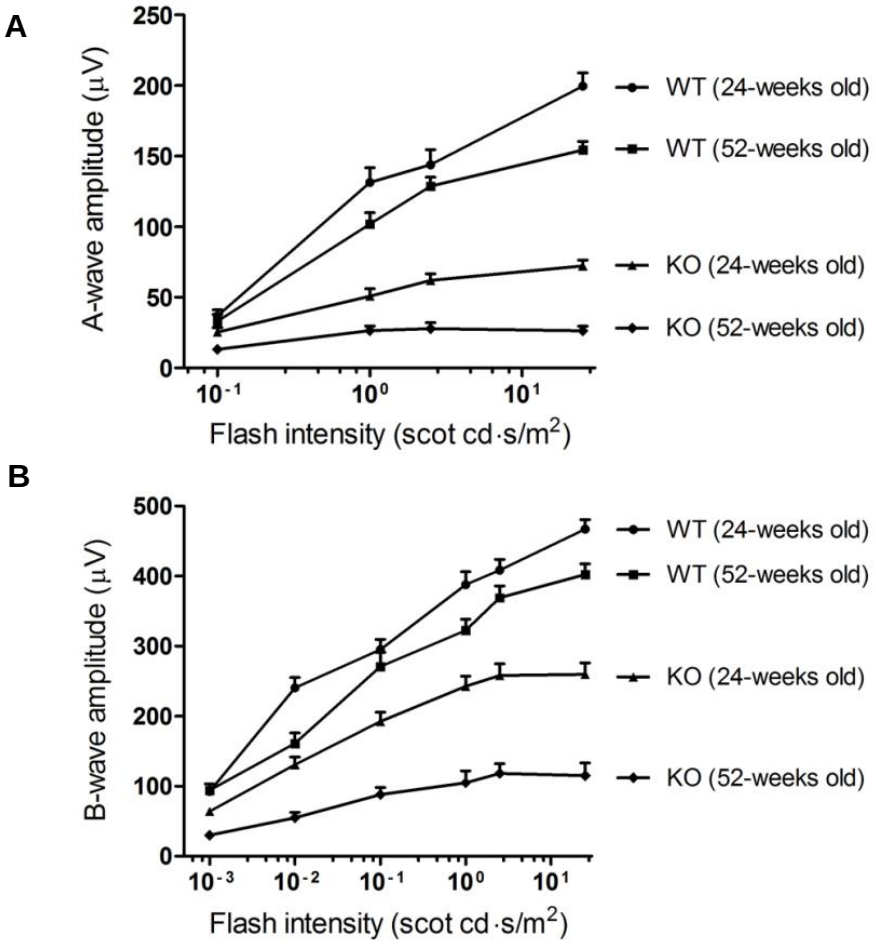

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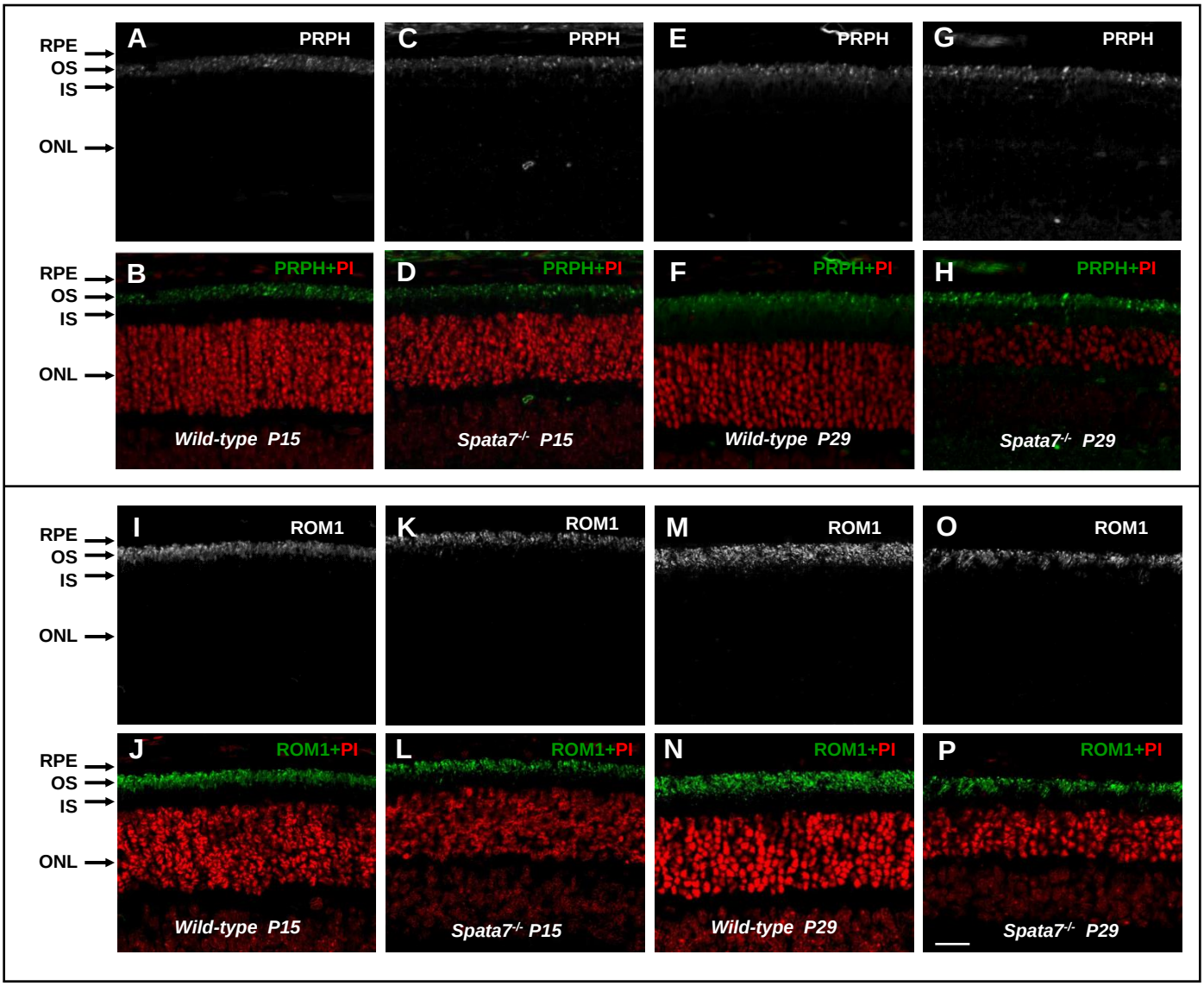

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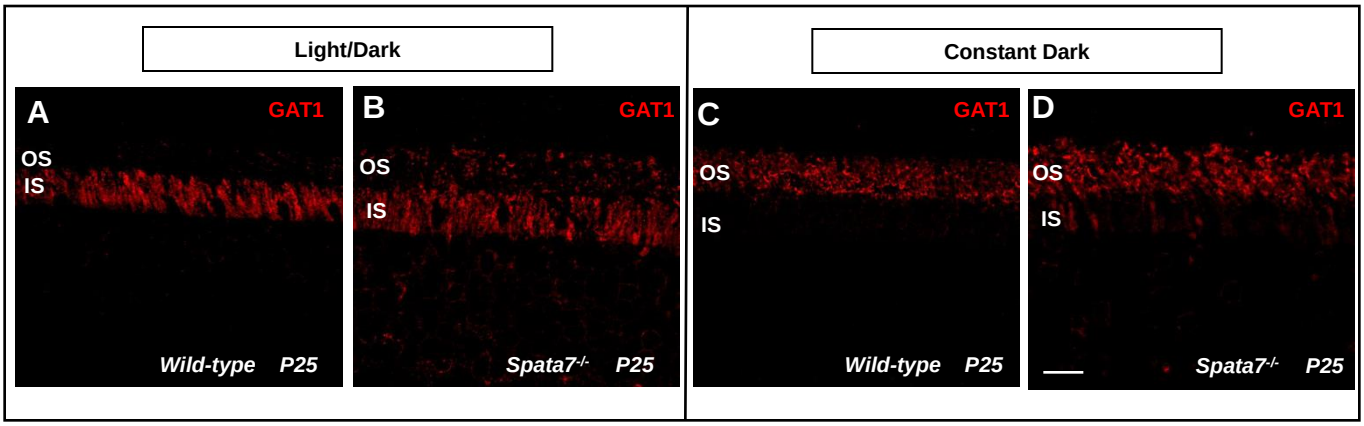

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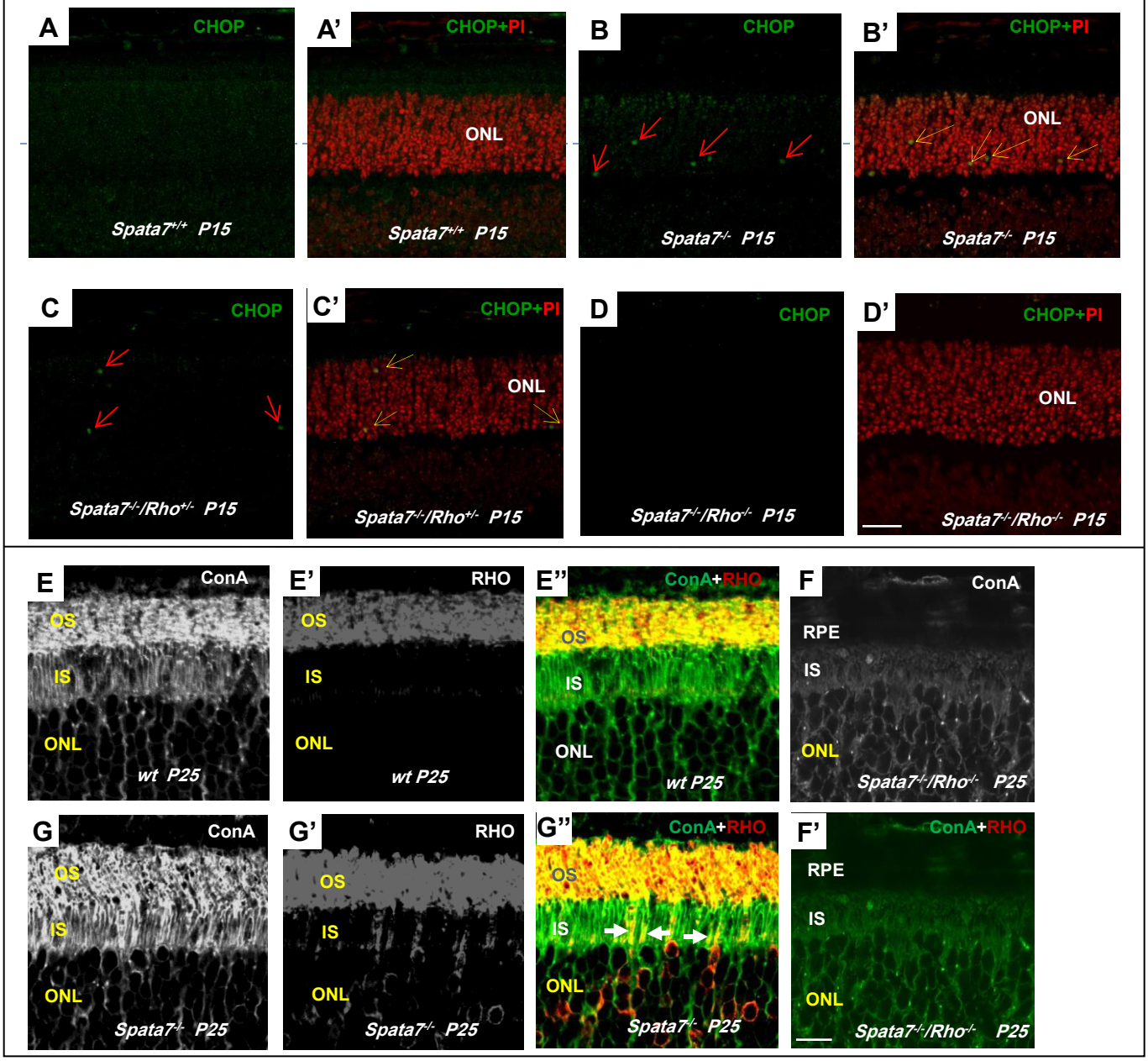

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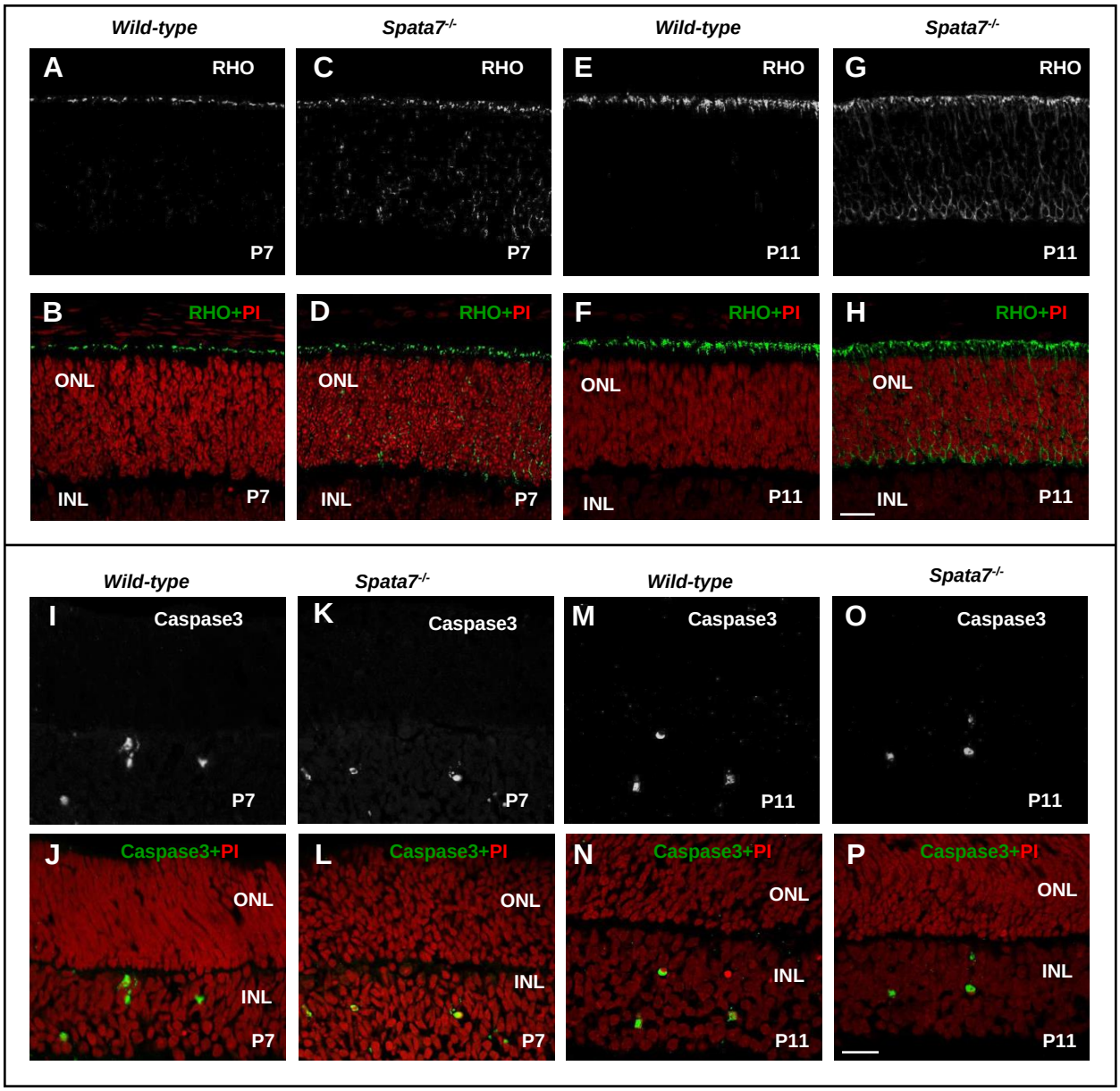

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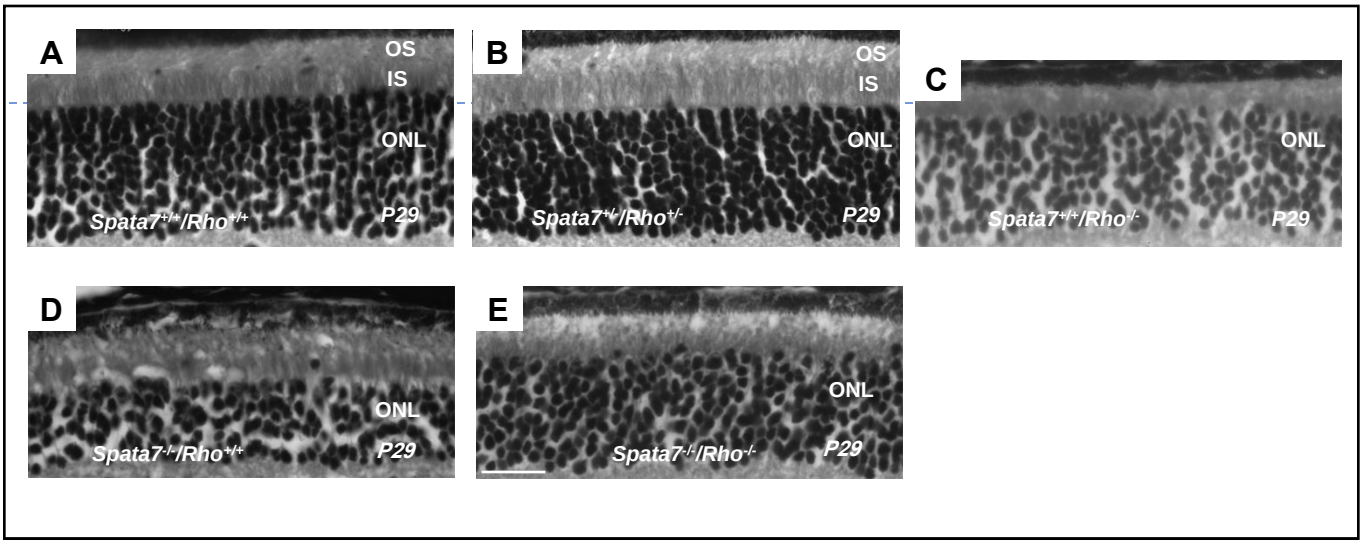

Supplementary Fig.11 . Eblimit et al.