Supporting Information

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SI Materials and Methods

Animals. All procedures conformed to the Association for Research in Vision and Ophthalmology statement on the use of animals in ophthalmic and vision research, and these procedures were approved by the Institutional Safety Committee on Recombinant DNA Experiments and the Animal Research Committee of Osaka Bioscience Institute. Mice were housed in a temperature-controlled room with a 12-h light/dark cycle. Fresh water and rodent diet were available at all times.

Antibodies. The following primary antibodies were used for immunostaining: mouse monoclonal anti–FLAG-M2 (F1804, 1:1,000; Sigma), anti-ROM1 (1:100; a gift from R. S. Molday, The University of British Columbia, Canada) (1), anti-rhodopsin (O-4886, 1:5,000; Sigma), anti-acetylated α-tubulin (6–11B-1, 1:1,000; Sigma), anti–γ-tubulin (GTU-88, 1:300; Sigma); rabbit polyclonal anti-FLAG (F-7425, 1:1,000; Sigma), anti-Rpgr (1:1,000; a gift from T. Li, National Institutes of Health) (2), anti-IFT88 (1:1,000; a gift from G. J. Pazour, University of Massachusetts Medical School), anti-IFT57 (1:250; a gift from G. J. Pazour) (3), anti-Kif3a (ab11259, 1:250; Abcam) (4), anti-HA (sc-805, 1:3,000; Santa Cruz); guinea pig polyclonal anti-Mak (1:1,000); goat polyclonal anti-IFT88 (1:500; a gift from J. C. Besharse, Medical College of Wisconsin) (3); and chicken polyclonal anti–RP1-C (1:2,000; a gift from E. A. Pierce, University of Pennsylvania School of Medicine) (5).

Immunohistochemistry and in Situ Hybridization. Mouse eyes were fixed in 4% paraformaldehyde in PBS for 30 s (for ciliary staining) or 10 min (for outer-segment staining), embedded in TissueTec optimum cutting temperature (OCT) compound 4583 (Sakura), frozen, and sectioned. Frozen 20-μm sections on slides were dried for 30 min at room temperature, rehydrated in PBS for 5 min, incubated with blocking solution (5% normal goat serum and 0.5% Triton X-100 in PBS) for 1 h, and then incubated with the antibodies for 4 h at room temperature. Slides were washed three times with PBS for 10 min and incubated with the secondary antibodies for 2 h at room temperature. The specimens were observed under a laser confocal microscope (LSM510, Carl Zeiss). Alexa Fluor 488 (1:500) or Cy3 (1:500) conjugated IgG (Jackson ImmunoResearch Laboratories) was used as a secondary antibody. Dissociation of photoreceptor cells was performed as described previously (6). In brief, a dissected retina was collected in a tube with 0.5 mL of PBS and shaken vigorously. Dissociated photoreceptors in PBS were transferred and spread on slides and dried for 4 h at room temperature. Cells were fixed with 4% paraformaldehyde in PBS for 2 min and used for immunostaining analysis. In situ hybridization was performed as described previously (7) with a probe containing an 891-bp fragment of male germ cell-associated kinase (*Mak*) cDNA amplified using primers 5′-ATGAAACCTGAGAACTTGCTCTGC-3′ (*Mak-P3*) and 5′- TGGCTTCTTGGAGTGGGAGGC-3′ (*Mak-P8*).

Cell Culture and Transfection. A 2.0-kb cDNA fragment containing an N-terminal portion of retinitis pigmentosa 1 (RP1-N) (residues 1–661) was amplified using the RIKEN full-length enriched library clone (A930011G17; GenBank accession no. AK044413) as a template and inserted into a pCAGGSII expression vector to produce pCAG-RP1-N. To obtain a full-length cDNA of RP1, a 4.8-kb cDNA fragment encoding a C-terminal portion of RP1 (residues 481–2095) was amplified from cDNA reverse-transcribed from mouse retinal total RNA at postnatal day 12 (P12) by PCR and given a NotI site on the downstream primer end using a high-fidelity DNA polymerase (KOD Plus; Toyobo). It was digested with NheI, an internal site in the cDNA, and NotI, and inserted into the NheI and NotI sites of pCAG-RP1-N. The DNA sequences were confirmed using an ABI310 genetic analyzer. NIH 3T3 cells were grown in DMEM (Sigma) with 10% FCS and 2 mg/L L-glutamine. Transfection was performed using Lipofectamine-LTX (Invitrogen) for NIH 3T3 cells according to the manufacturer's instructions. At 24 h after transfection, the medium was replaced by serum-free medium. Cells were cultured for 24 h in serum-free medium to develop cilia. For immunostaining, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 3 min at room temperature, and subsequently incubated with blocking solution for 30 min. Cells were immunostained with a primary antibody in the blocking solution for 4 h at room temperature and subsequently incubated with the secondary antibody solution for 2 h at room temperature.

Electroretinograms. Electroretinographic (ERG) recordings were performed as described in detail (8). In brief, mice were dark-adapted overnight and then were anesthetized with an i.p. injection of ketamine and xylazine. ERGs were picked up with a gold-wire loop electrode placed on the cornea. The mice were placed in a Ganzfeld dome and stimulated with stroboscopic stimuli ranging from −5.0 to 1.0 log cd-s/m^2 to elicit the scotopic ERGs and with four levels of stimuli ranging from −0.5 to 1.0 log cd-s/m^2 for the photopic ERGs. The photopic ERGs were recorded on a rod-suppressing white background of 1.3 log cd-s/m^2.

RT-PCR Analysis. Retinal total RNA (1 μg) was isolated using TRIzol reagent (Invitrogen) and converted to cDNA using SuperScript II RTase (Invitrogen). The following sets of PCR primers were used for the amplification of full-length *Mak*: 5′-TGGACAAGGAAGAGACCTCCGTCA-3′ (*Mak-P1*) and 5′-AGCAATGAGTCAGGGCTTCCACCT-3′ (*Mak-P11*).

Quantitative Real-Time PCR. Quantitative PCR (qPCR) was performed using SYBR Green ER Q-PCR Super Mix (Invitrogen) and Thermal Cycler Dice Real Time System Single MRQ TP870 (Takara) according to the manufacturers' instructions. Quantification was performed by Thermal Cycler Dice Real Time System software v. 2.0 (Takara). The following sets of PCR primers were used: 5′-TATCCAGATGGTGTGCAGAAGAGCC-3′ (*Mak-Q2*) and 5′-GAGATGCTGAACTGGGATCCAAAG-3′ (*Mak-P5*).

Antibody Production. The cDNA fragment encoding the C-terminal portion of mouse Mak (residues 296–622) was amplified by PCR and subcloned into *pET28b*. The His-tagged fusion protein of Mak was expressed in *Escherichia coli* and purified with a HisTrap FF column (GE Healthcare) according to the manufacturer's instructions. An antibody against Mak was obtained by immunizing guinea pigs with the purified fusion protein (Medical and Biological Laboratories). The guinea pig antiserum against Mak-C was purified with an immunizing fusion protein-bound Sepharose 4B column.

Transmission Electron Microscope Analysis. Transmission electron microscope analysis was performed as described previously with some modifications (9). Eye cups were fixed with 2% glutaraldehyde, 2% paraformaldehyde, and 0.1 M phosphate buffer. After fixation with 2% osmium tetraoxide for 3 h, the retinas were dehydrated through a graded series of ethanol (30–100%)

and propylene oxide. Finally, the retinas were embedded in Spurr's resin. Sections were cut on an ultramicrotome (Ultracut E; Reichert-Jung) and stained with uranyl acetate and lead citrate. Retinas were observed by transmission electron microscope (H-9500SD; Hitachi). A cross-section view of the connecting cilia was obtained by electron tomography. The images were taken at 15,000× from −60° to +60° at 1° intervals around a single axis and collected with a 2 k × 2 k F224HD slow scan charge-coupled device camera (TVIPS). The effective magnification was 19,500×, and the pixel size on the specimen corresponds to 1.23 nm.

Immunoprecipitation and Western Blot Analysis. Transfected HEK293 cells were lysed in a lysis buffer [1% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA] supplemented with protease inhibitor mixture (Roche) and Phospho-Stop (Roche). Lysates and antibodies were incubated for 6 h at 4 °C and then were incubated for 4 h with Protein G-Sepharose (GE Healthcare), washed four times with wash buffer [1% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA], and resolved by SDS/PAGE. Western blot analysis was performed using a semidry transfer cell (iBlot system; Invitrogen) with iBlot Gel Transfer Stack PVDF (Invitrogen). Signals were detected using Can Get Signal (Toyobo) and ECL Plus Western Blotting Detection System (GE Healthcare).

In Vitro Kinase Assay. The cDNA fragments encoding partial sequences of mouse retinitis pigmentosa 1 (RP1) (RP1-N, residues 1–661; RP1-C1, residues 662–1,380; RP1-C2, residues 1,381–2,095) were amplified by PCR and subcloned into *pGEX4T-1* (Amersham Biosciences). The fusion protein was expressed in *E. coli* strain *DH5α* and purified with glutathione Sepharose 4B (Amersham Biosciences) as previously described (10). Purified recombinant GST-human Mak (residues 1–458; Abnova) containing the entire kinase domain of Mak was used for the kinase assay. The kinase reaction was performed with kinase buffer [50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 20 μM ATP, and 10 μCi of [γ-^{32}P] ATP] supplemented with protease inhibitor mixture (Roche) and Phospho-Stop (Roche) at 30 °C for 60 min. Reaction products were resolved by SDS/PAGE. The gels were dried and exposed to X-ray film. Quantification of the radioactivity was performed with a BAS5000 imaging analyzer (Fuji Film).

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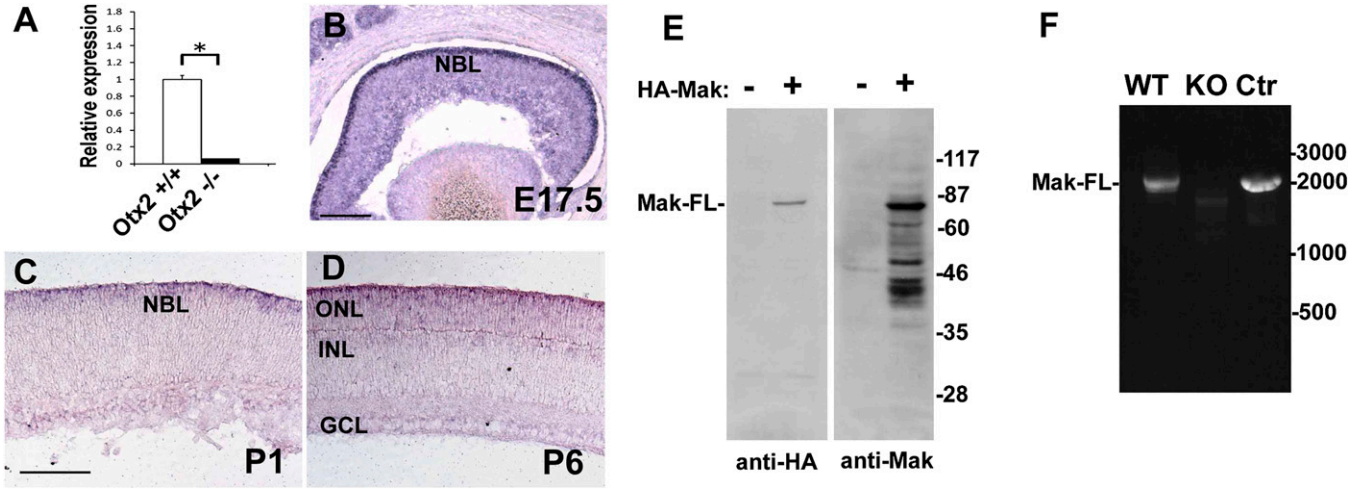

Fig. S1. Expression of Mak in the developing and mature retina. (*A*) Quantitative PCR analysis of *Mak* expression in the *Otx2* CKO retina. Expression of *Mak* was markedly decreased in the *Otx2* CKO retina at P12. Error bars show the SD. *$P < 0.03$. (*B–D*) In situ hybridization analysis of retinal sections at embryonic day 17.5 (E17.5) (*B*), postnatal day 1 (P1) (*C*), and postnatal day 6 (P6) (*D*). *Mak* mRNA is expressed in both photoreceptor precursors and developing photoreceptors in the retina. (Scale bars: 100 μm.) GCL, ganglion cell layer; INL, inner nuclear layer; NBL, neuroblastic layer; ONL, outer nuclear layer. (*E*) Western blot analysis of HEK293 cell extract expressing the HA-tagged full-length Mak (Mak-FL) using the anti-Mak and anti-HA antibodies. The anti-Mak antibody recognizes the full-length Mak. (*F*) Expression of the *Mak* variant (+75 bp) in the wild-type and *Mak*-KO retinas. RT-PCR analysis was performed using a cDNA library prepared from the retinas of 2-mo-old wild-type and *Mak*-KO mice. A plasmid containing the *Mak* variant (+75 bp) cDNA was used for a template as a positive control (Ctr).

Fig. S2. Amino acid sequence alignment of Mak, intestinal cell kinase (ICK), and dye-filling defective 5 (Dyf-5). The predicted amino acid sequences of mouse Mak (NP_001139275.1), ICK (NP_064371.1), and *C. elegans* Dyf-5 (NP_001129786.1) were aligned by the ClustalW program (http://clustalw.ddbj.nig.ac.jp/). Asterisks indicate identical amino acids. Colons and periods indicate similar amino acids. Three Mak, ICK, and Dyf-5 (MID) homology domains are shown in red. Alternative splice amino acids (+25 a.a.) are represented in blue.

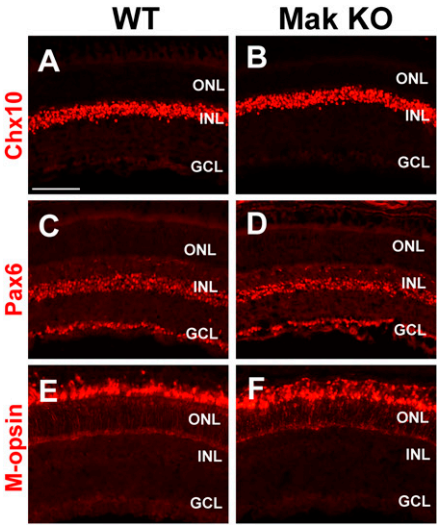

Fig. S3. Normal cell composition in the *Mak*-KO retina at postnatal day 14 (P14). (*A*–*F*) Retinal sections from wild-type mice (*A*, *C*, and *E*) and *Mak*-KO mice (*B*, *D*, and *F*) at P14 were stained with anti-ceh-10 homeo domain containing homolog (Chx10) (a marker for bipolar cells; *A* and *B*), anti-paired box gene 6 (Pax6) (a marker for both amacrine and ganglion cells in this stage; *C* and *D*) and anti–M-opsin (a marker for M-cone photoreceptor cells; *E* and *F*) antibodies. (Scale bar: 100 μm.)

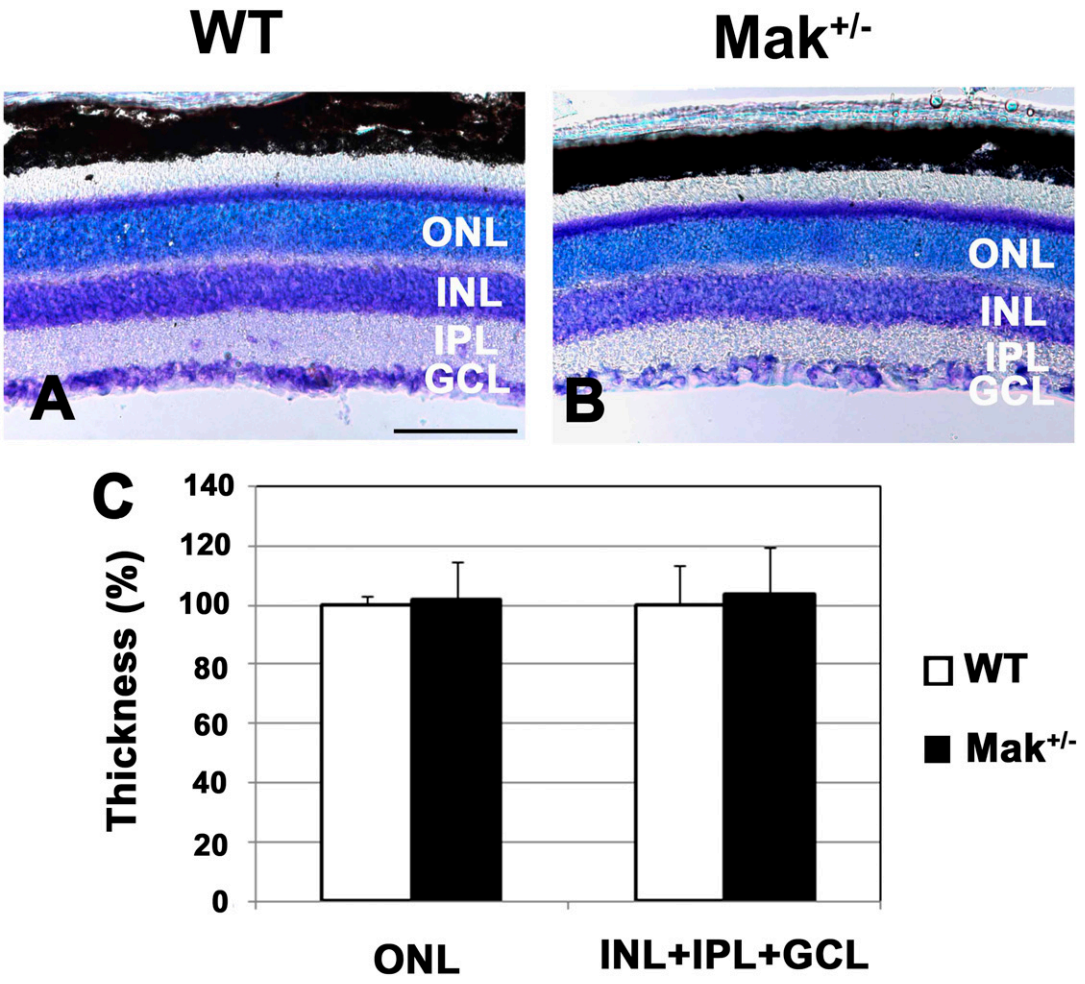

Fig. S4. No photoreceptor degeneration in the *Mak*$^{+/-}$ retina. (*A* and *B*) Retinal sections from 6-mo-old *Mak*$^{+/-}$ mice (*B*) and wild-type mice (*A*) were stained with toluidine blue. IPL, inner plexiform layer. (Scale bar In A: 100 μm.) (*C*) The thickness of retinal layers was measured. No obvious change was observed between the *Mak*$^{+/-}$ and wild-type retinas. Average of layer thickness in the wild-type retina was set to 100%. Error bars show SD.

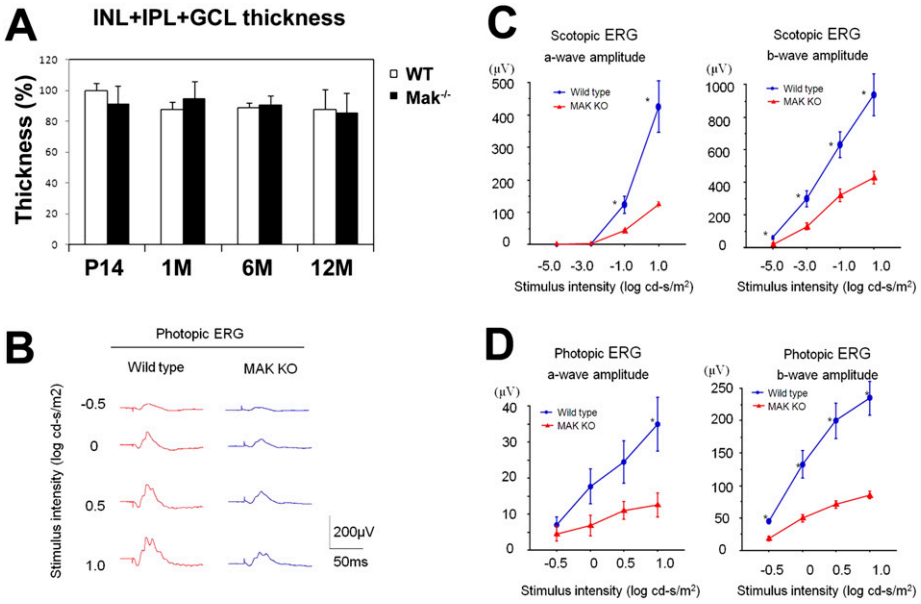

Fig. S5. Thickness of retinal layers and ERGs recorded from *Mak*-KO mice. (*A*) Thickness of retinal layers was measured at age P14, 1 mo, 6 mo, and 12 mo. The thickness of retinal layers (INL+IPL+GCL) shows no significant change at all stages. Average of layer thickness in the wild-type retina at P14 was set to 100%. (*B*) ERGs recorded from *Mak*-KO mice. Photopic ERGs elicited by four different stimulus intensities (−0.5 to 1.0 log cd-s/m^2). (*C*) Scotopic ERGs elicited by four different stimulus intensities (−5.0 to 1.0 log cd-s/m^2). Amplitude of the dark-adapted a-wave and b-wave as a function of the stimulus intensity (n = 5). (*D*) Photopic ERGs elicited by four different stimulus intensities (−-5.0 to 1.0 log cd-s/m^2). Amplitude of the light-adapted ERG a-wave and b-wave as a function of the stimulus intensities (n = 5). Error bars represent SE. *$P < 0.05$.

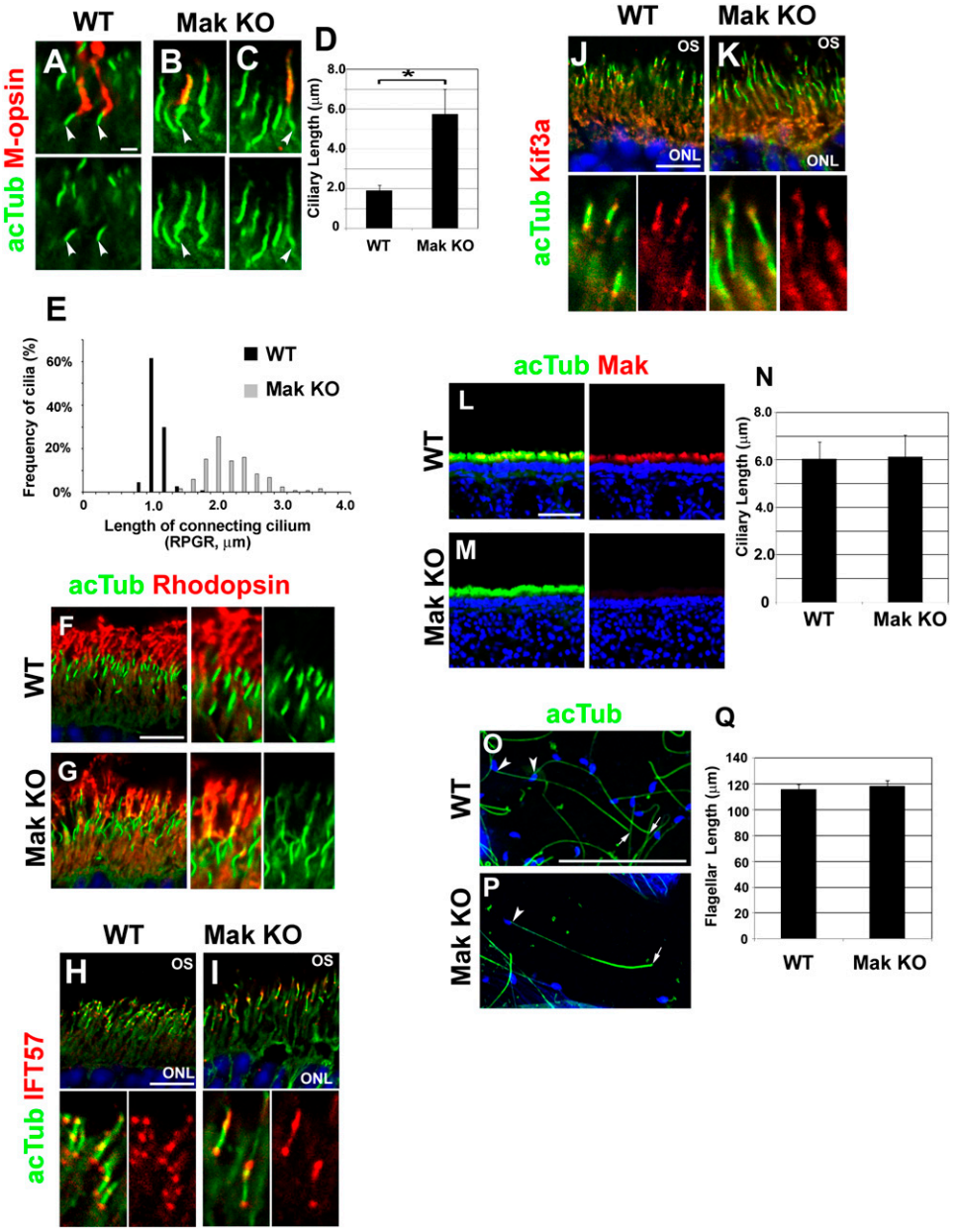

Fig. S6. Immunofluorescent analysis of the cilia in the *Mak*-KO mice. (*A*–*D*) Cone photoreceptor cilia are elongated in the *Mak*-KO retina. Outer segments of M-cone photoreceptor cells were stained with an anti-M opsin antibody (red). Both rod and cone photoreceptor cilia were stained with an anti-acetylated α-tubulin antibody (green). Arrowheads indicate basal body-connecting cilium junctions. Cone photoreceptor cilia are elongated in the *Mak*-KO retina (*B* and *C*) compared with wild-type retina (*A*). (*D*) The lengths of the cone cilia were measured ($n \geq 25$). *$P < 0.03$. (*E*) Lengths of the connecting cilia stained with an anti-retinitis pigmentosa GTPase regulator (RPGR) antibody in the wild-type photoreceptors (black bars) and *Mak*-KO photoreceptors (gray bars) were measured. (*F* and *G*) Acetylated α-tubulin staining (green) did not reach the top of the outer segments stained with the anti-Rhodopsin antibody (red) in both wild-type (*F*) and *Mak*-KO (*G*) photoreceptor cells. (*H*–*K*) Distribution of Kif3a and intraflagellar transport 57 (IFT57) in the *Mak*-KO photoreceptor cilia. The retinal sections of wild-type retinas (*H* and *J*) and *Mak*-KO retinas (*I* and *K*) were stained with anti-acetylated–α-tubulin (green in *H*–*K*), anti-Kif3a (red in *J* and *K*), or anti-IFT57 (red in *H* and *I*) antibodies. (*L*–*N*) Ciliary length of nasal respiratory epithelia is not affected in *Mak*-KO mice. Nasal respiratory epithelia of wild-type mice (*L*) and *Mak*-KO mice (*M*) were stained with anti-acetylated α-tubulin (green) and anti-Mak (red) antibodies. (*N*) Length of the cilia in wild-type and *Mak*-KO mice ($n \geq 27$) was measured. (*O*–*Q*) Length of epididymal sperm axonemes is not affected in the *Mak*-KO mice. Epididymal sperm axonemes from wild-type mice (*O*) and *Mak*-KO mice (*P*) were stained with the anti-acetylated α-tubulin antibody (green). (*Q*) Length of the axonemes between the proximal tips (arrowheads) and distal tips (arrows) was measured ($n \geq 17$). [Scale bars: 2 μm (*A*), 10 μm (*F*, *H*, *J*), and 100 μm (*L* and *O*).] Error bars show the SD.

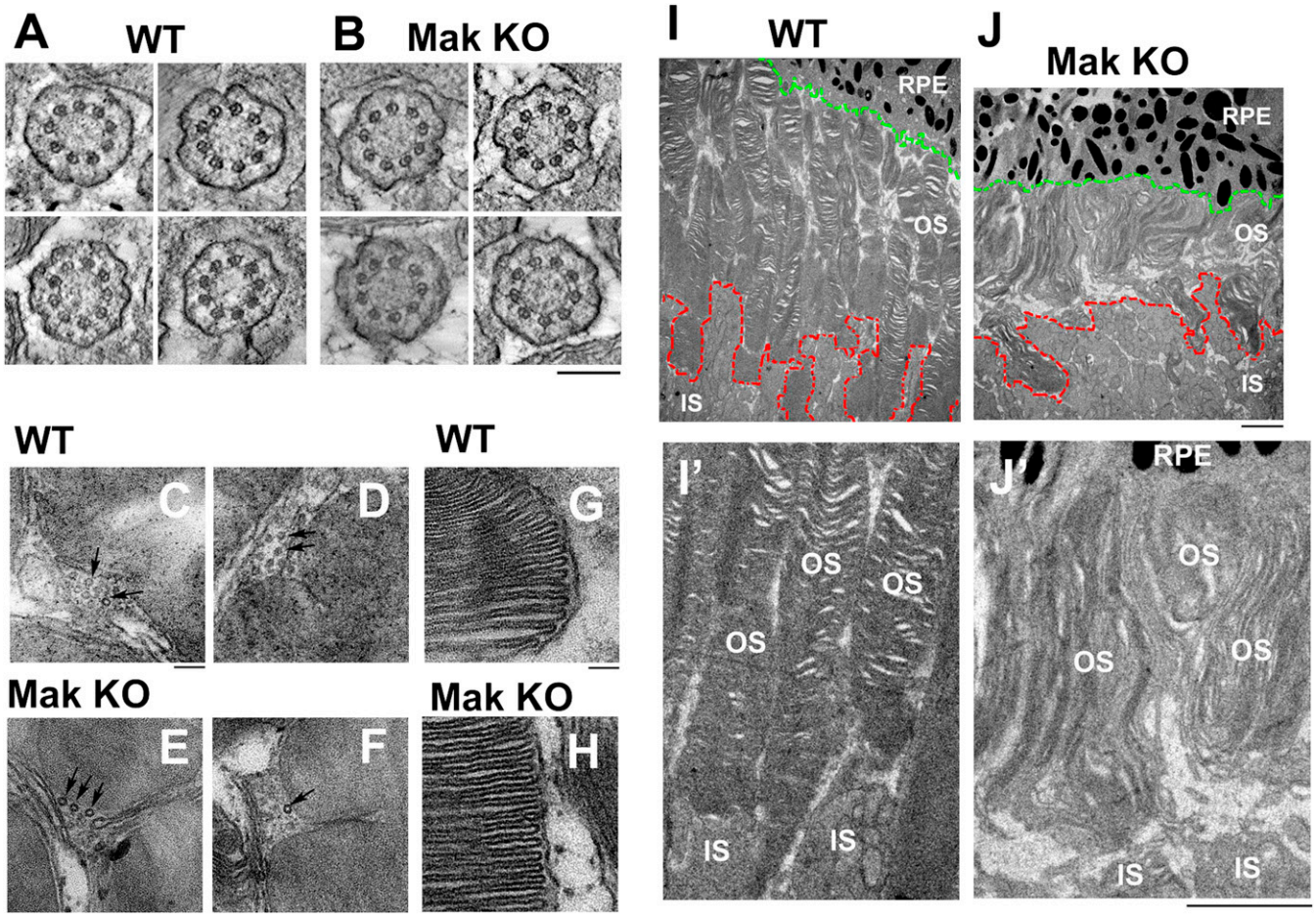

Fig. S7. Ultrastructural analysis of the photoreceptor cilia and outer segments in the *Mak*-KO retina. (*A* and *B*) Transverse profile of the connecting cilia in wild-type (*A*) and *Mak*-KO (*B*) photoreceptors at age P14 (*A* and *B*. *Lower*) and 1 mo (*A* and *B*, *Upper*). (*C*–*F*) Singlet microtubules of outer-segment axonemes, which are positioned near the disk clefts, were observed in both wild-type (*C* and *D*) and *Mak*-KO retinas (*E* and *F*). Arrows indicate singlet microtubules in outer segments. (*G* and *H*) Disk rim structure was normal in *Mak*-null outer segments (*H*) compared with wild-type (*G*). (*I* and *J*) Orientation of outer-segment disks is altered in *Mak*-KO photoreceptors. The disk diameters in the *Mak*-KO outer segments are two to four times larger (*J* and *J*′) than in wild-type outer segments (*I* and *I*′). Green broken lines indicate boundaries between the retinal pigment epithelia and the outer segments; red broken lines indicate boundaries between the outer segments and inner segments. RPE, retinal pigment epithelium. [Scale bars represent 0.2 μm (*B*), 0.1 μm (*C and G*), and 2 μm (*J*).]

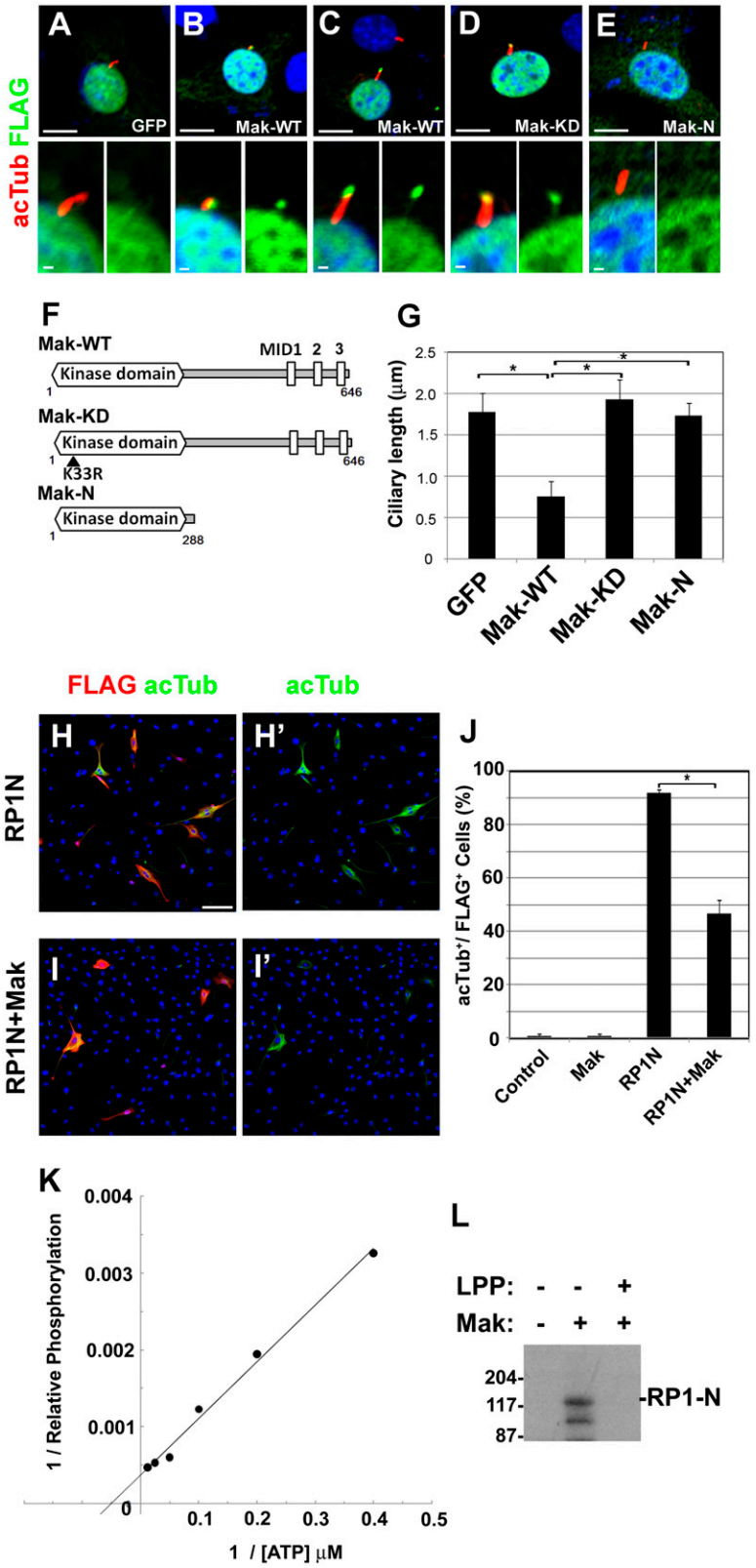

Fig. S8. Functional analysis of Mak using ciliated NIH 3T3 cells and in vitro kinase assay. (*A–F*) Kinase activity of Mak is required for the regulation of ciliary length but not for its localization in the cilia. (*A–E*) FLAG-tagged constructs expressing GFP (*A*), wild-type Mak (*Mak-WT*; *B* and *C*), kinase dead-Mak K33R (*Mak-KD*; *D*) or Mak lacking a C-terminal portion (*Mak-N*; *E*) were transfected into NIH 3T3 cells. Localization of FLAG-tagged proteins was observed using anti-FLAG (green) and anti-acetylated α-tubulin (red) antibodies and DAPI (blue). (*F*) Schematic diagrams of Mak constructs. (*G*) The length of cilia stained with anti-acetylated α-tubulin antibody ($n > 30$ in each construct). (*H* and *I*) Increased acetylated α-tubulin signal in the cells expressing RP1-N was partially rescued by Mak. The FLAG-tagged RP1-N- or GFP- (control) expressing plasmid was transfected with or without the Mak expression plasmid into NIH 3T3 cells. Cells were

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stained with the anti-FLAG (red) and anti-acetylated α-tubulin (green) antibodies. (*J*) Percentages of acetylated α-tubulin–positive cells in FLAG-positive cells were calculated. (*K*) K_m value of Mak for ATP was measured using ^{32}P-labeled ATP by in vitro kinase assay. K_m= 19 μM. (*L*) RP1-N phosphorylated by Mak was dephosphorylated by λ phosphatase (LPP) in vitro. [Scale bars: 10 μm (*A–E*, *Upper*); 1 μm (*A–E*, *Lower*); 100 μm (*H*).] Error bars show the SE. *$P < 0.03$.

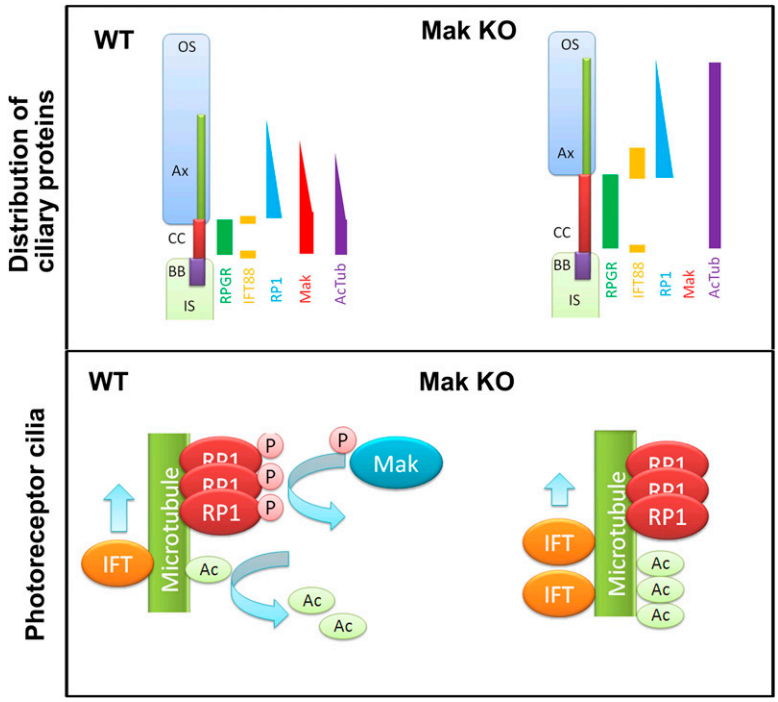

Fig. S9. Summary of ciliary protein distribution and a hypothetical model for Mak function in photoreceptor cilia. (*Upper*) Distribution of ciliary proteins (RPGR, IFT88, RP1, Mak, acetylated α-tubulin) analyzed by immunofluorescent staining in wild-type photoreceptors (*Upper Left*) and *Mak*-KO photoreceptors (*Upper Right*). Ax, axoneme; BB, basal body; CC connecting cilium. (*Lower*) A hypothetical model of Mak function in photoreceptor cilia. In this model, Mak controls ciliary length by regulation of RP1. Mak may phosphorylate RP1 and regulate the stability of photoreceptor cilia (*Lower Left*). Mak may be involved in the regulation of anterograde transport through controlling intraflagellar transport (IFT) and kinesin motors and acetylation of microtubules in the cilia. In *Mak*-KO photoreceptors, loss of Mak leads to increased intensity of acetylated α-tubulin labeling and accumulation of IFT (*Lower Right*).