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Rax Homeoprotein Regulates Photoreceptor Cell Maturation and Survival in Association with Crx in the Postnatal Mouse Retina

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The *Rax* homeobox gene plays essential roles in multiple processes of vertebrate retina development. Many vertebrate species possess *Rax* and *Rax2* genes, and different functions have been suggested. In contrast, mice contain a single *Rax* gene, and its functional roles in late retinal development are still unclear. To clarify mouse Rax function in postnatal photoreceptor development and maintenance, we generated conditional knockout mice in which *Rax* in maturing or mature photoreceptor cells was inactivated by tamoxifen treatment (*Rax* iCKO mice). When *Rax* was inactivated in postnatal *Rax* iCKO mice, developing photoreceptor cells showed a significant decrease in the level of the expression of rod and cone photoreceptor genes and mature adult photoreceptors exhibited a specific decrease in cone cell numbers. In luciferase assays, we found that Rax and Crx cooperatively transactivate *Rhodopsin* and *cone opsin* promoters and that an optimum Rax expression level to transactivate photoreceptor gene expression exists. Furthermore, Rax and Crx colocalized in maturing photoreceptor cells, and their coimmunoprecipitation was observed in cultured cells. Taken together, these results suggest that Rax plays essential roles in the maturation of both cones and rods and in the survival of cones by regulating photoreceptor gene expression with Crx in the postnatal mouse retina.

A number of homeodomain transcription factors, which play significant roles in retinal development, have been identified in vertebrates (1–4). Rax is a homeodomain transcription factor that is essential for various processes in vertebrate retinal development (5). The *Rax* gene was first identified as a paired-type homeobox gene expressed in the optic vesicle and the presumptive diencephalon area in the early mouse embryo (6, 7). *Rax* is evolutionarily well conserved from *Drosophila melanogaster* to humans. Rax is highly expressed in retinal progenitor cells (RPCs), and its expression in the retina gradually decreases as RPCs become postmitotic and begin to differentiate. *Rax*-null mutant mice exhibit a reduction of brain size and an absence of the optic vesicle (5, 7). Mutations in human *RAX* are associated with anophthalmia and microphthalmia (8, 9). Rax overexpression promotes the proliferation of RPCs in frogs and zebra fish (7, 10–13). In addition to the function in RPCs, Rax plays significant roles in the development of photoreceptor cells and Müller glial cells (14–19).

Rax paralog genes have been identified in various vertebrate species (20–22). In *Xenopus laevis*, two *Rax* genes (*xRx* and *xRx-L/xRx2*) have been identified (7, 21), and in zebra fish, three *Rax* genes (*zRx1* to *zRx3*) have been isolated (7). Interestingly, the expression pattern of zebra fish *Rx3* showed more similarity to that of frog and mouse *Rax* genes than to that of the zebra fish *Rx1* and *Rx2* genes (23). In chicks, two *Rax* genes (*cRax* and *cRaxL/cRax2*) have been identified (20). The chick *Rax2* gene is expressed in both retinal progenitor cells and early-developing photoreceptors, while chick *Rax* is predominantly expressed in retinal progenitor cells. It was also reported that chick *Rax2* is implicated in cone photoreceptor differentiation and that the expression of a putative dominant negative allele of a chick *Rax2* gene caused a significant reduction in the level of expression of cone photoreceptor genes (20). Human *RAX2/QRX*, which is expressed in the outer nuclear layer (ONL) and inner nuclear layer (INL) of the adult human retina, was identified to be a PCE-1-binding protein by acting synergistically with CRX and NRL to modulate the expression of photoreceptor genes. Monkey, cow, and dog genomes also contain two *Rax* genes. On the other hand, the *Rax2* gene is absent from mouse and rat genomes (22). This raises the question of whether mouse *Rax* plays an essential role in photoreceptor development during postnatal stages like human *Rax2* does.

In the current study, we investigated a functional role for *Rax* in postnatal mouse retinas, which contain a single *Rax* gene. We report that mouse Rax modulates the expression of photoreceptor genes in the postnatal retina by interacting with Crx. Conditional ablation of *Rax* in postnatal photoreceptors led to a significant decrease in the level of expression of rod and cone genes and to cone photoreceptor cell death, suggesting that Rax is essential for the maturation of rods and cones as well as for the survival of cones.

MATERIALS AND METHODS

Animal care. All procedures conformed to the ARVO statement for the use of animals in ophthalmic and vision research, and these procedures were approved by the Institutional Safety Committee on Recombinant DNA Experiments (approval 3380-3) and the Animal Experimental Committees of the Institute for Protein Research (approval 24-05-1), Osaka University, and were performed in compliance with institutional guidelines. Mice were housed in a temperature-controlled room at 22°C with a

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12-h light/12-h dark cycle. Fresh water and rodent diet were available at all times.

***Rax*$^{flox/flox}$; *Crx-CreERT2* mice and tamoxifen.** The *Rax*$^{flox/flox}$; *Crx-CreERT2* mice (129Sv/Ev background) were generated as described in our previous study (16). Tamoxifen (Sigma, St. Louis, MO) was dissolved in a sunflower oil (Sigma) at 4 mg/ml, and 0.2 mg or 1 mg of tamoxifen was injected intraperitoneally into *Rax*$^{flox/flox}$; *Crx-CreERT2* mice at postnatal day 4 (P4) or 1 month of age, respectively.

Plasmid constructs. We subcloned a 3.8-kb upstream genomic fragment of the human *S-opsin* gene (bp −3769 to +1) into the pGL3-Basic vector (Promega), generating the pS-opsin-Luc reporter plasmid. Mutations of PCE-1 sites in the human rhodopsin, S-opsin, and M-opsin promoters, previously described (15), were introduced by PCR with mutated PCR primers. The resulting constructs were named hRhodopsin-PCE-1-mut1, hS-opsin-PCE-1-mut6, and hM-opsin-PCE-1-mut1. We constructed the expression vectors by subcloning a full-length mouse *Rax* cDNA into the pME18S (pMIK) expression vector (a gift from K. Maruyama, Tokyo Medical and Dental University, School of Medicine, Tokyo, Japan). An open reading frame fragment of mouse *Crx* cDNA was amplified by PCR and cloned into the pBSKS-3×FLAG vector containing a 3× FLAG tag. The mouse *Crx-3×FLAG* fragment obtained was cloned into the modified pCAGGS expression vector, generating the pCAGGS-3×FLAG-Crx expression plasmid. To obtain the pCAGGS-2×HA-Rax expression plasmid containing a 2× hemagglutinin (HA) tag, an open reading frame fragment of the mouse *Rax* cDNA was amplified by PCR and cloned into the pCAGGS-2×HA vector.

Immunohistochemistry. For immunohistochemistry, 20-μm retinal tissue sections were washed twice in phosphate-buffered saline (PBS) and boiled in 10 mM sodium citrate buffer (pH 6.0) for 10 min for antigen retrieval. Sections were rinsed twice using PBS and incubated with blocking solution (4% normal donkey serum and 0.1% Triton X-100 in PBS) for 1 h at room temperature. The samples were incubated with a primary antibody at 4°C overnight. After washing with PBS, these samples were incubated with secondary antibodies for 1 h at room temperature. Rhodamine-labeled peanut agglutinin (PNA; Vector Laboratories) was used for staining cone pedicles. DAPI (4′,6-diamidino-2-phenylindole) and Hoechst 33342 were used for staining nuclear DNA.

For whole-mount immunostaining of the retina, each retina was gently peeled off from the sclera, rinsed in PBS, and fixed with 4% (wt/vol) paraformaldehyde in PBS for 2 h. After washing in PBS, samples were blocked with 4% normal donkey serum and 0.05% (wt/vol) Triton X-100 in PBS for 2 h. The retinas were then immunostained with primary antibodies to S-opsin, M-opsin, and rhodopsin at 4°C overnight. Reactions with secondary antibodies were performed for 2 h at room temperature. The specimens were observed under a laser confocal microscope (LSM700; Carl Zeiss).

Antibody production. By PCR, a cDNA fragment encoding a center portion of mouse Nrl (residues 62 to 130; Nrl-center) was amplified and subcloned into pGEX4T-2 (Amersham Biosciences). The fusion protein was expressed in *Escherichia coli* strain BL21 and purified with glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions. An antibody against Nrl was obtained by immunizing guinea pigs with purified glutathione *S*-transferase (GST)-tagged Nrl (GST-Nrl). The guinea pig antiserum against Nrl-center was preabsorbed with GST-Sepharose and affinity purified with an immunizing fusion protein-bound Sepharose column.

Antibodies. For immunostaining, anti-Rax antibody (guinea pig, 1:1,000) (16), anti-S-opsin antibody (goat, 1:500; Santa Cruz), anti-M-opsin antibody (rabbit, 1:300; Millipore), antirhodopsin antibody (mouse, 1:5,000; Sigma), anti-TRβ2 antibody (rabbit, 1:3,000; a gift from Douglas Forrest, NIDDK), anti-TRβ2 antibody (guinea pig, 1:50) (24), anti-Lhx2 antibody (goat, 1:1,000; Santa Cruz), and antipikachurin antibody (rabbit, 1:500) (25) were used as primary antibodies. We used Cy3-conjugated secondary antibodies (1:500; Jackson), Alexa Fluor 488-conjugated secondary antibodies (1:1,000; Sigma), and Dylight 649-conjugated secondary antibodies (1:500; Jackson). For Western blot analysis, anti-Rax antibody (guinea pig, 1:1,000) (16), anti-Crx antibody (rabbit, 1:3,000) (24), antirhodopsin antibody (mouse, 1:5,000; Sigma), anti-β-actin antibody (mouse, 1:5,000; Sigma), anti-Nrl antibody (guinea pig, 1:500), anti-FLAG antibody (mouse, 1:15,000; catalog number F1804; Sigma), and anti-HA antibody (rat, 1:5,000; Roche) were used as primary antibodies. We used a horseradish peroxidase-conjugated goat antibody against mouse IgG (1:20,000; Zymed), a horseradish peroxidase-conjugated goat antibody against rabbit IgG (1:20,000; Molecular Probes), a horseradish peroxidase-conjugated goat antibody against rat IgG (1:20,000; Jackson), and a horseradish peroxidase-conjugated donkey antibody against guinea pig IgG (1:20,000; Jackson) as secondary antibodies.

Western blot analysis. Mouse retinas were dissected at P8 and lysed in an SDS-sample buffer. HEK293T cells were washed with PBS twice and lysed in an SDS-sample buffer. Samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h in 5% milk containing Tris-buffered saline with 0.1% Tween 20 and incubated overnight with a primary antibody. The membrane was then incubated for 1 h with a secondary antibody. The signals were measured using ImageJ software (U.S. National Institutes of Health).

TUNEL assay. Fresh frozen retinas were sectioned to a thickness of 20 μm and fixed with 4% paraformaldehyde in PBS for 1 min. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays were performed using a Click-iT TUNEL Alexa Fluor 488 imaging assay kit (Invitrogen) according to the manufacturer's protocols.

ERG recordings. Electroretinograms (ERGs) were recorded with a white light-emitting diode luminescent electrode placed on the cornea (PuREC; Mayo, Japan). Two-month-old mice were anesthetized with an intraperitoneal injection of 100 mg ketamine per kg of body weight and 10 mg/kg xylazine. The pupils were dilated with topical 0.5% tropicamide and 0.5% phenylephrine HCl. The mice were stimulated with four levels of stroboscopic stimuli ranging from −4.0 to 1.0 log cd-s/m^2 to elicit scotopic ERGs and 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/m^2.

***In situ* hybridization.** Digoxigenin (DIG)-labeled riboprobes were synthesized by T7 and T3 RNA polymerase using *Rax* cDNA or *Cre* recombinase cDNA as a template in the presence of 11-digoxigenin UTPs (Roche Molecular Diagnostics, Mannheim, Germany). Postnatal eyeballs from 129Sv/Ev background *Rax*$^{flox/flox}$ mice and/or *Rax*$^{flox/flox}$; *Crx-CreERT2* mice were fixed with 4% paraformaldehyde in PBS overnight at 4°C. The samples were cryoprotected with 30% sucrose in PBS and embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan). These tissues were sliced into 20-μm sections with a Microm HM 560 cryostat microtome (Microm Laborgeräte GmbH, Walldorf, Germany). The sliced sections were fixed with 4% paraformaldehyde in PBS containing 0.1% Tween 20 (PBST), treated with 6% H_2O_2, 5 μg/ml proteinase K, and subsequently treated with 2 mg/ml glycine in PBST. The samples were then postfixed with 4% paraformaldehyde in PBST. Hybridization was carried out overnight at 70°C. After posthybridization washing, they were blocked with 5% sheep serum in 2.5 mM Tris-HCl (pH 7.5), 0.8% NaCl, 0.02% KCl, and 0.1% Tween 20 (TBST) and incubated with an alkaline phosphatase-conjugated anti-DIG antibody (1:2,000; Roche) in TBST with 1% sheep serum. The samples were visualized with nitroblue tetrazolium chloride (Sigma, St. Louis, MO) and 5-bromo-4-chloro-3-indolylphosphate, toluidine salt (Sigma).

Luciferase assay. We transfected 0.4 μg of the luciferase reporter plasmid DNAs (pGL3b-human rhodopsin promoter-luc, pGL3b-human S-opsin promoter-luc, pGL3b-human M-opsin promoter-luc, pGL3b-human rhodopsin-PCE-1-mut1, pGL3b-human S-opsin-PCE-1-mut6, or pGL3b-human M-opsin-PCE-1-mut1) (26) and 0.25 to 4 μg of mouse *Rax* or 0.25 to 0.5 μg of mouse *Crx* or *Nrl* expression vector DNAs (pME18S, pME18S-Rax, pME18S-Crx, and pME18S-Nrl) per well into NIH 3T3 cells in a 6-well plate using the FuGene reagent (Promega). A β-galactosidase expression vector (β-SV; Promega) was cotransfected for normalization of transfection efficiency. After transfection, the cells were incubated for 48 h and lysed with reporter lysis buffer (Promega). Lucif-

TABLE 1 Primer sequences used for qPCR analysis

Gene	Orientation	Primer sequence (5′-3′)
M-opsin	Sense	TTGCTTTGCCACTGCTCG
	Antisense	CTGTGGCCCAGACGTGTTC
S-opsin	Sense	GCTGGACTTACGGCTTGTCACC
	Antisense	TGTGGCGTTGTGTTTGCTGC
Gnat2	Sense	ATGCTGACAAGGAAGCCAAGACTG
	Antisense	GACAGACTTGAACTCTAGGCACTC
Pde6c	Sense	GATCAACGTGATTCCGTCACCTCC
	Antisense	AGTCTCATCTACCGGTCCTTTCTG
Crx	Sense	CACCAGGCTGTCCCATACTC
	Antisense	TCGCCCTACGATTCTTGAAC
Rcvrn	Sense	GAGTACGTGATTGCTCTGCACATG
	Antisense	AGGAGTTTCACATCCTCAGGCTTG
Sag	Sense	AGCCTGCTCAAGAAACTGGGAGACA
	Antisense	AGCTGGCTGCAACATCACTGAACA
Nrl	Sense	GCTGTGCCTTTCTGGTTCTGA
	Antisense	GCTCCCGCTTTATTTCGAACT
Nr2e3	Sense	TTGATGTCACCAGCAATGACC
	Antisense	TTTTGGCCCATTTGACAGC
Rhodopsin	Sense	GCTTCCCTACGCCAGTGTG
	Antisense	CAGTGGATTCTTGCCGCAG
Gnat1	Sense	CGCTCAACATTCAGTATGGAGA
	Antisense	CGAAGTAGCGATGATTGCAGATG
Pde6g	Sense	CAAACAAGGCAGTTCAAGAGCAAG
	Antisense	CAGGGATCTGACTAAATGATGCCA
Gapdh[a]	Sense	ACTGGCATGGCCTTCCGTGTTCCTA
	Antisense	TCAGTGTAGCCCAAGATGCCCTTC

[a] *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase gene.

erase activity was measured with a firefly luciferase assay system (Promega) according to the manufacturer's protocol. The luminescence signal was detected using a GloMax Multi+ detection system (Promega).

qPCR. The mouse retinas were harvested and dissected at P20. Total RNA (1 μg) was isolated from the retina using the TRIzol reagent (Invitrogen) and converted to cDNA using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was performed using SYBR GreenER qPCR SuperMix (Invitrogen) and thermal cycler Dice real-time system single MRQ TP870 (TaKaRa) according to the manufacturer's instructions. Quantification was performed by thermal cycler Dice real-time system software (version 2.0; TaKaRa). The primer sequences used for quantitative PCR (qPCR) are listed in Table 1.

Immunoprecipitation. We transfected pCAGGS-3×FLAG-Crx and pCAGGS-2×HA-Rax expression plasmids into HEK293T cells in a 35-mm dish using the calcium phosphate method. HEK293T cells were cotransfected with the pCAGGS-3×FLAG-Crx or pCAGGS-3×FLAG expression plasmid together with the pCAGGS-2×HA-Rax or pCAGGS-2×HA expression plasmid. Two days after transfection, the cells were lysed with lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% [wt/vol] NP-40, 0.1% [wt/vol] Triton X-100, cOmplete protease inhibitor cocktail [Roche]), and centrifuged for 10 min at 15,100 × *g*. The supernatants were incubated with an anti-FLAG M2 affinity gel (Sigma) or monoclonal anti-HA-agarose antibody (Sigma) and then eluted with 0.1 M glycine buffer (pH 1.5). After neutralization with 1 M Tris-HCl (pH 9.0), immunoprecipitated samples were analyzed by Western blotting.

Statistical analysis. Data are presented as means ± standard deviations (SDs). Statistical comparisons of data sets were performed with Student's *t* test. For multiple comparisons, we performed one-way analysis of variance by the Tukey-Kramer test, and a *P* value of <0.05 was taken to be statistically significant.

RESULTS

Mouse Rax is expressed in postnatal photoreceptors and Müller glial cells. In order to examine Rax protein expression in the mouse retina during development, we immunostained developing and adult mouse retinas with an anti-Rax antibody that we previously generated (16) (Fig. 1A to D). Strong Rax signals were observed in the neuroblastic layer (NBL) and the presumptive photoreceptor layer (PPL) at embryonic day 15.5 (E15.5) (Fig. 1A). At postnatal day 6 (P6), Rax protein expression was detected in the ONL, which corresponds to photoreceptor precursors, and the INL, which corresponds to Müller glial cells (14) (Fig. 1B). Rax protein expression in the ONL decreased at P14 and was weakly detected at age 2 months (Fig. 1C and D). Although the expression level of Rax in the INL was unaltered at P14 compared with that at P6, Rax signals in the INL became substantially fainter at age 2 months (Fig. 1D). These results show that the Rax protein is expressed at high levels in both maturing photoreceptors and Müller glial cells and significantly decreases by maturity.

Generation of maturing photoreceptor-specific *Rax* iCKO mice. To examine the possible role of Rax in maturing postnatal photoreceptor cells, we ablated *Rax* in postnatal photoreceptor cells by using $Rax^{flox/flox}$; *Crx-CreERT2* mice, which we previously reported (16). We inactivated *Rax* in postmitotic maturing photoreceptor cells by treating these mice with tamoxifen at P4, when the number of retinal progenitors markedly decreases and many photoreceptor precursors begin their maturation (27–30) (Fig. 1E). We also injected tamoxifen into $Rax^{flox/flox}$ littermate mice of the $Rax^{flox/flox}$; *Crx-CreERT2* mice for use as controls in the current study. To assess whether induced *Rax* inactivation occurs, we analyzed the retinas of P8 control mice and conditional knockout mice in which *Rax* in maturing or mature photoreceptor cells was inactivated by tamoxifen treatment (*Rax* iCKO) by immunostaining using the anti-Rax antibody. We refer to the *Rax* iCKO mice that were treated with tamoxifen at P4 and whose retinas were harvested at P8 as *Rax* iCKO (P4 → P8) mice. The immunoreactivity to Rax in the ONL, which was strongly detected in the control retina, was undetectable in the *Rax* iCKO (P4 → P8) mouse retina, while Rax expression in the INL was almost unaffected (Fig. 1F and G). Furthermore, no substantial reduction of the Müller glial cell marker Lhx2 was observed in the *Rax* iCKO (P4 → P8) mouse retina compared with that in the control mouse retina (Fig. 1H and I). These results show that *Rax* is specifically deleted in postnatal maturing photoreceptor cells in the *Rax* iCKO mouse retina.

Rax is required for the expression of opsin proteins in the maturing retina. To investigate the retinal phenotypes caused by *Rax* deficiency in postnatal maturing photoreceptors, we performed flat-mount immunostaining of *Rax* iCKO (P4 → P8) mouse retinas. We observed that the expression of cone- and rod-specific proteins (S-opsin, M-opsin, and rhodopsin) was slightly downregulated (Fig. 2A to F). Next, we quantified the expression levels of the rhodopsin and Crx proteins in control and *Rax* iCKO

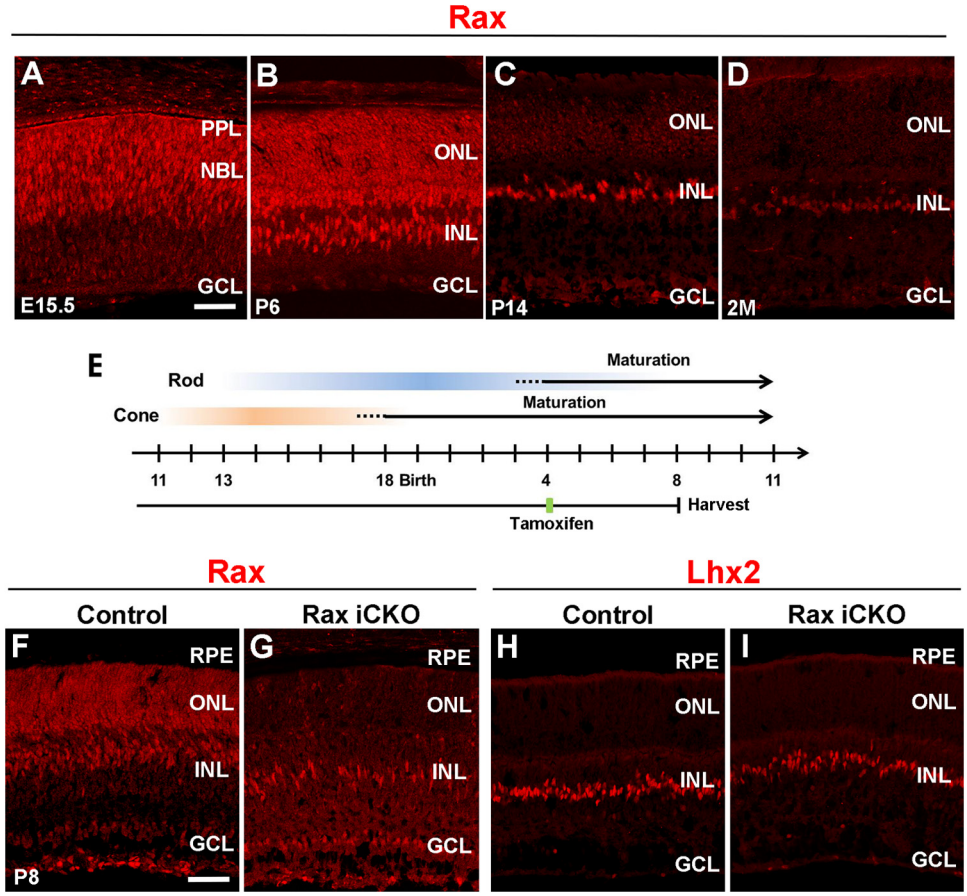


FIG 1 Expression and conditional inactivation of *Rax* in postnatal mouse retinas. (A to D) Mouse retinal sections were immunostained with an anti-Rax antibody (red) at E15.5 (A), P6 (B), P14 (C), and age 2 months (2M) (D). (E) Schematic diagram of schedule for tamoxifen administration and harvest of retinas. To conditionally inactivate *Rax* in postnatal photoreceptors, we treated $Rax^{flox/flox}$; *Crx-CreERT2* mice with tamoxifen at P4 and harvested the retinas at P8. (F to I) Retinal sections from *Rax* iCKO (P4 → P8) and control mice were immunostained with antibodies against Rax (red) (F and G) and Lhx2 (a Müller glia marker, red) (H and I). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; NBL, neuroblastic layer; PPL, presumptive photoreceptor layer; RPE, retinal pigment epithelium. Bars, 50 μm.

(P4 → P8) mouse retinas by Western blotting. In the *Rax* iCKO (P4 → P8) mouse retina, the Rax protein level decreased to ~40% of that in the control retina (Fig. 2G and H). The rhodopsin protein level in the *Rax* iCKO (P4 → P8) mouse retina was also reduced by ~40% compared to that in the control retina (Fig. 2G and I). In contrast, Crx protein expression was unaffected in the *Rax* iCKO (P4 → P8) mouse retina (Fig. 2G and J). To examine whether Rax inactivation affects cone photoreceptors, we performed immunostaining of control and *Rax* iCKO (P4 → P8) mouse retinas with an anti-TRβ2 antibody that we generated and counted the TRβ2-positive cells on retinal sections (Fig. 2K to M). The numbers of TRβ2-positive cells was unaltered between control and *Rax* iCKO (P4 → P8) mouse retinas (number of TRβ2-positive cells per image, 32.0 ± 6.0 for control mice and 41.2 ± 6.4 for *Rax* iCKO mice; $P > 0.05$) (Fig. 2M). These results suggest that Rax is not required for Crx expression but is essential for the normal expression of opsins in postnatal maturing photoreceptors.

A defect of *Rax* in photoreceptor cells affects the expression of photoreceptor genes. In order to examine whether Rax regulates photoreceptor-specific genes in postnatal maturing photoreceptors, we selected 12 genes involved in photoreceptor development and function and measured the expression levels of these genes in control and *Rax* iCKO mouse retinas by quantitative PCR (qPCR) analysis. For qPCR, we used *Rax* iCKO (P4 → P20) mouse retinas, in which retinal neural circuit formation is almost completed (Fig. 3A) (30). We observed that *CreERT2* mRNA is predominantly expressed in the ONL of the *Rax* iCKO (P4 → P20) mouse retina (see Fig. S1 in the supplemental material). We found that the expression levels of cone-specific genes (*M-opsin*, *S-opsin*, *Gnat2*, and *Pde6c*) decreased by 80 to 90% in *Rax* iCKO retinas compared with those in control retinas (Fig. 3B). Furthermore, the expression levels of the genes essential for light sensing in rod photoreceptors (*Rhodopsin*, *Gnat1*, and *Pde6g*) also decreased by 20 to 40% from those in control retinas, whereas transcription factors (*Crx*, *Nrl* and *Nr2e3*), which are essential for photoreceptor development *in vivo* (31, 32), were unaffected (Fig. 3B). Consistent with the qPCR results, we observed a reduced rhodopsin immunostaining signal in the *Rax* iCKO (P4 → P20) mouse retina (Fig. 3C and D). We also found that the expression of cone photoreceptor proteins (S-opsin and M-opsin) considerably decreased in *Rax* iCKO (P4 → P20) mouse retinas (Fig. 3E to H). We carried out flat-mount immunostaining of control and *Rax* iCKO (P4 → P20) mouse retinas using antibodies against S-opsin and

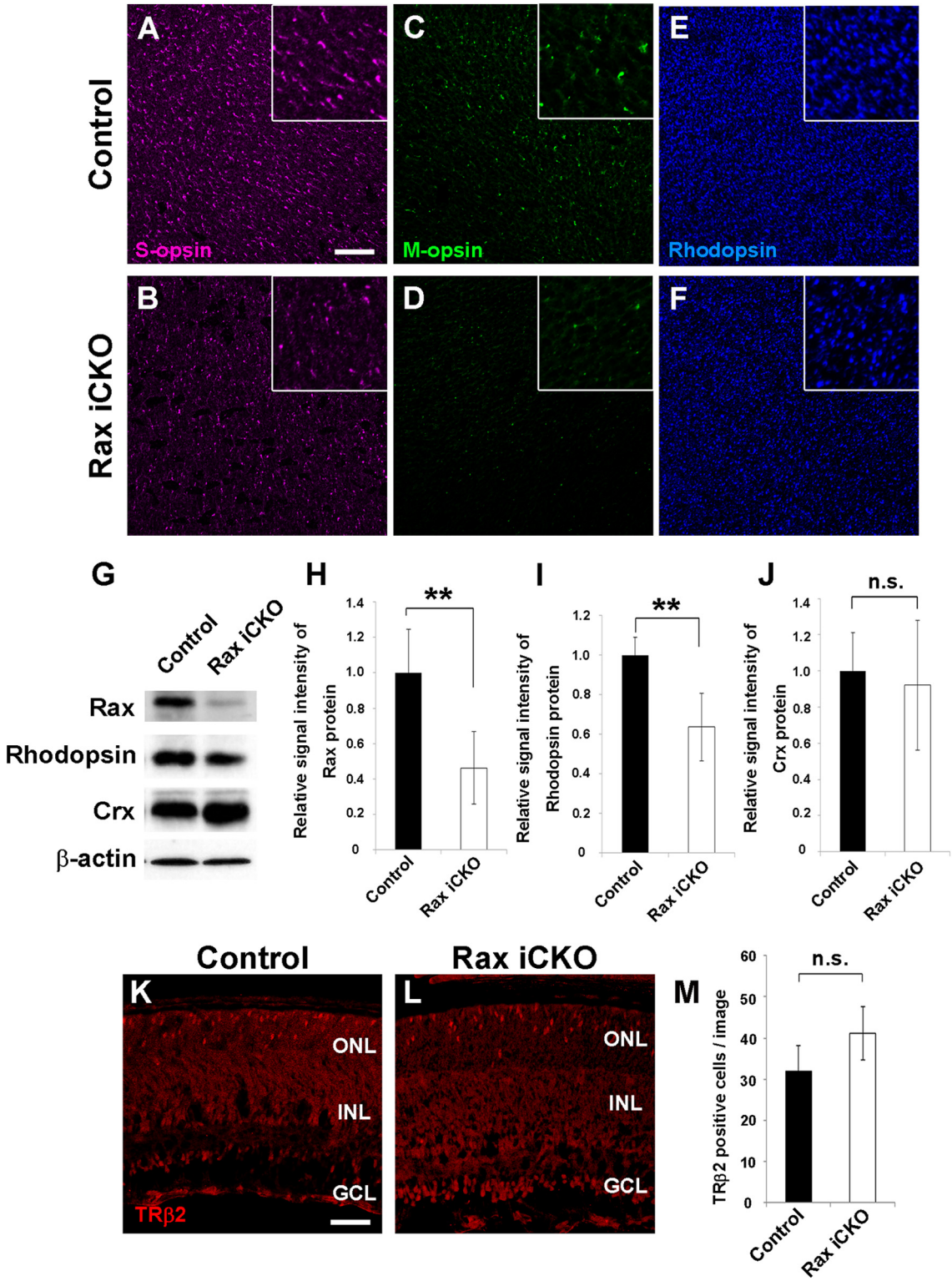
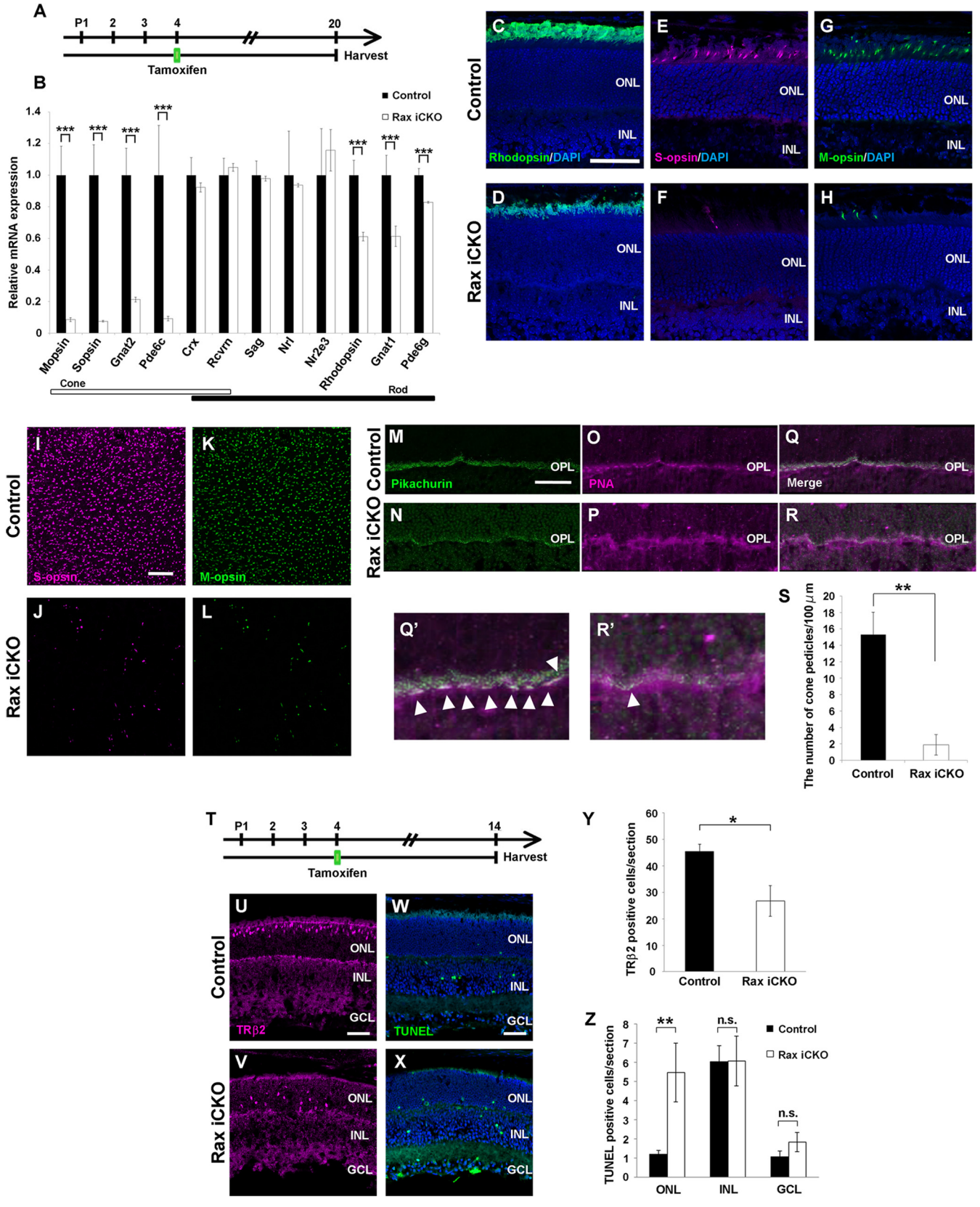


FIG 2 Expression of opsin genes decreases in the *Rax* iCKO mouse retina. (A to F) Whole-mount retinas from control and *Rax* iCKO (P4 → P8) mice were immunostained with antibodies against S-opsin (magenta) (A and B), M-opsin (green) (C and D), and rhodopsin (blue) (E and F). (Insets) S-opsin-, M-opsin-, and rhodopsin-positive cells at high magnification. Bar, 50 μm. (G to J) Comparison of photoreceptor protein levels in control and *Rax* iCKO (P4 → P8) mouse retinas. (G) Western blots of Rax, rhodopsin, and Crx in the retina are shown. β-Actin was used as a loading control. (H to J) The signal intensities of the Rax, rhodopsin, and Crx proteins, respectively, are shown. Data are means ± SDs ($n = 3$). **, $P < 0.01$ by Student's *t* test; n.s., not significant by Student's *t* test. (K to M) The number of cone photoreceptor cells was unaltered in the *Rax* iCKO (P4 → P8) mouse retina. Retinal sections from control (K) and *Rax* iCKO (P4 → P8) (L) mice were immunostained with an anti-TRβ2 antibody (rabbit; red), a marker for developing cone photoreceptor cells. Bar, 50 μm. (M) The number of TRβ2-positive cells is shown. Data are means ± SDs ($n = 4$). n.s., not significant by Student's *t* test. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer;.


A
P1
2
3
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20
Harvest
Tamoxifen
B
Control
Rax iCKO
Relative mRNA expression
Mopsin
Sopsin
Gnat2
Pde6c
Crx
Rcvrn
Sag
Nrl
Nr2e3
Rhodopsin
Gnat1
Pde6g
Cone
Rod
C
D
E
F
G
H
Control
Rax iCKO
ONL
INL
Rhodopsin/DAPI
S-opsin/DAPI
M-opsin/DAPI
I
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M-opsin
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INL
GCL

M-opsin and observed a significant reduction of the cone cell numbers in the *Rax* iCKO (P4 → P20) mouse retinas (Fig. 3I to L). To confirm these results, we further immunostained control and *Rax* iCKO (P4 → P20) mouse retinas with photoreceptor synaptic markers (antipikachurin antibody and rhodamine-labeled peanut agglutinin [PNA]). Immunostaining with pikachurin and PNA revealed that the number of cone pedicles dramatically decreased in the *Rax* iCKO (P4 → P20) mouse retinas (number of cone pedicles per 100 μm, 15.2 ± 2.7 in control mice and 1.8 ± 1.5 in *Rax* iCKO mice; $P < 0.01$) (Fig. 3M to S).

To examine whether Rax ablation affects the survival of maturing cone photoreceptors, we immunostained cone photoreceptor cells in control and *Rax* iCKO (P4 → P14) mouse retinas using the anti-TRβ2 antibody (Fig. 3T to V). In addition, we detected apoptotic cell death in *Rax* iCKO (P4 → P14) and control mouse retinas by the TUNEL assay (Fig. 3W and X). We found that the number of TRβ2-positive cells significantly decreased in the *Rax* iCKO (P4 → P14) mouse retinas compared to that in the control retinas (number of TRβ2-positive cells per section, 45.4 ± 3.3 in control mice and 26.7 ± 7.0 in *Rax* iCKO mice; $P < 0.01$) (Fig. 3Y). We counted the apoptotic cell numbers in the ONL, INL, and ganglion cell layer (GCL) separately and found that apoptotic cell numbers significantly increased in the ONL (Fig. 3Z). Mislocalization of cone photoreceptor cells in the retina has been shown to be associated with a disturbance of cone photoreceptor maturation and consequent degeneration (33, 34). Furthermore, we observed that *CreERT2* mRNA was predominantly expressed in the ONL of the *Rax* iCKO (P4 → P14) mouse retinas (see Fig. S1 in the supplemental material). These results suggest that Rax is essential for cone photoreceptor survival in the postnatal maturing mouse retina.

***Rax* iCKO mice exhibit abnormal ERGs.** To test visual function in *Rax* iCKO mice, we performed electroretinogram (ERG) recordings under scotopic and photopic conditions with several light intensities in control mice and *Rax* iCKO mice that were treated with tamoxifen at P4 and whose retinas were harvested at age 2 months [*Rax* iCKO (P4 → 2 months) mice] (Fig. 4). In control mice, the negative a-wave, which originates mainly from the activity of rod photoreceptors, appeared at higher stimulus intensities of −1.0 to 1.0 log cd-s/m^2 under the scotopic condition (Fig. 4A). A positive b-wave, which originates from rod bipolar cells (35), was seen at an even lower stimulus intensity of −3.0 log cd-s/m^2. In contrast, the amplitude of the dark-adapted ERG a-wave in *Rax* iCKO (P4 → 2 months) mice was reduced at higher stimulus intensities of −1.0 to 1.0 log cd-s/m^2 compared with those in control mice (Fig. 4A and B). The amplitude of the scotopic b-wave in *Rax* iCKO (P4 → 2 months) mice was significantly less than that in control mice at lower and higher stimulus intensities of −3.0 to 1.0 log cd-s/m^2 (Fig. 4A and C). These results suggest an essential function of Rax in rod phototransduction in the postnatal mouse retina. Under photopic conditions, *Rax* iCKO (P4 → 2 months) mice showed markedly decreased amplitudes in a-waves, which originate from cone photoreceptors, at a high stimulus intensity of 1.0 log cd-s/m^2 compared with those in control mice (Fig. 4D and E). The amplitude of the photopic ERG b-wave in *Rax* iCKO (P4 → 2 months) mice, which originates from cone ON-bipolar cells, was significantly smaller than those of control mice at high stimulus intensities of 0 to 1.0 log cd-s/m^2 (Fig. 4D and F), suggesting that Rax is also required for cone phototransduction. These results showed that phototransduction in both rod and cone photoreceptor cells is impaired in *Rax* iCKO (P4 → 2 months) mice.

Rax regulates the maintenance of mature cone photoreceptors. To examine *Rax* expression in the adult mouse retina, we carried out *in situ* hybridization of the retina harvested from mice at age 1 month using *Rax* antisense and sense probes. With the antisense probe, *Rax* expression was weakly detected in the ONL of the retina, while the *Rax* sense probe did not give any significant signal (Fig. 5A to B′). To investigate the *in vivo* function of *Rax* in mature photoreceptors, we treated *Rax*$^{flox/flox}$; *Crx-CreERT2* mice with tamoxifen at 1 month to inactivate *Rax* in mature photoreceptors. We observed that *CreERT2* mRNA is predominantly expressed in the ONL of the retina of *Rax* iCKO mice that were treated with tamoxifen at age 1 month and whose retinas were harvested at age 2 months [*Rax* iCKO (1 month → 2 months) mice] (see Fig. S1 in the supplemental material). We examined the expressions of S-opsin and M-opsin by whole-mount coimmunostaining of control and *Rax* iCKO (1 month → 2 months) mouse retinas using anti-S-opsin and anti-M-opsin antibodies (Fig. 5C to J). In mice, S-opsin is expressed predominantly in cones in the ventral retina, and M-opsin is predominantly expressed in cones in the dorsal retina (36, 37). The number of S-opsin-positive cells slightly decreased in both the ventral and dorsal retinas of *Rax* iCKO (1 month → 2 months) mice (Fig. 5C, D, G, and H), while the number of M-opsin-positive cells notably decreased in both the dorsal and ventral retinas of *Rax* iCKO (1 month → 2 months) mice (Fig. 5E, F, I, and J). On the other hand, we analyzed rhodopsin expression using retinal sections by im-

FIG 3 Rax is required for cone photoreceptor cell survival during maturation. (A) Schematic diagram of schedule for tamoxifen administration and harvest of retinas. We treated *Rax*$^{flox/flox}$; *Crx-CreERT2* mice with tamoxifen at P4 and harvested the retinas at P20. (B) The expression levels of photoreceptor genes in *Rax* iCKO (P4 → P20) mouse retinas were analyzed by qPCR. The expression level of each was normalized to the expression level of a housekeeping gene, *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase). The mean value for each control was set equal to 1.0. Error bars show ±SDs ($n = 3$). ***, $P < 0.001$. (C to H) *Rax* iCKO (P4 → P20) and control retinal sections were immunostained with the antibody against rhodopsin (green) (C and D), S-opsin (magenta) (E and F), or M-opsin (green) (G and H). The nuclei were stained with DAPI (blue). Bar, 50 μm. (I to S) Reduction of cone photoreceptor cells in the *Rax* iCKO (P4 → P20) mouse retina. (I to L) Whole-mount retinas from control and *Rax* iCKO (P4 → P20) mice were immunostained with the antibody against S-opsin (magenta) (I and J) or M-opsin (green) (K and L). (M and N) Retinal sections were immunostained with an antipikachurin antibody (a synaptic marker, green). (O and P) Cone pedicles were stained with PNA (magenta). (Q) Merge of panels M and O. (R) Merge of panels N and P. (Q′) and R′) Higher-magnification views of panels Q and R, respectively. Arrowheads, cone pedicles. Bars, 50 μm. (S) The number of cone pedicles decreased in the *Rax* iCKO (P4 → P20) mouse retina. Data are means ± SDs ($n = 3$). **, $P < 0.01$ by Student's *t* test. (T) Schematic diagram of schedule for tamoxifen administration and harvest of retinas. We treated *Rax*$^{flox/flox}$; *Crx-CreERT2* mice with tamoxifen at P4 and harvested their retinas at P14. (U to Z) Retinal sections from control (U) and *Rax* iCKO (P4 → P14) (V) mice were immunostained with an anti-TRβ2 antibody (guinea pig; magenta), a marker for developing cone photoreceptor cells. (W and X) TUNEL staining of retinas from control (W) and *Rax* iCKO (P4 → P14) (X) mice. Bars, 50 μm. (Y) The number of TRβ2-positive cells is shown. Data are means ± SDs ($n = 3$). *, $P < 0.05$. (Z) TUNEL-positive cells in control and *Rax* iCKO (P4 → P14) mouse retinas were counted. Data are means ± SDs ($n = 3$). **, $P < 0.01$ by Student's *t* test; n.s., not significant by Student's *t* test. INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer; GCL, ganglion cell layer.

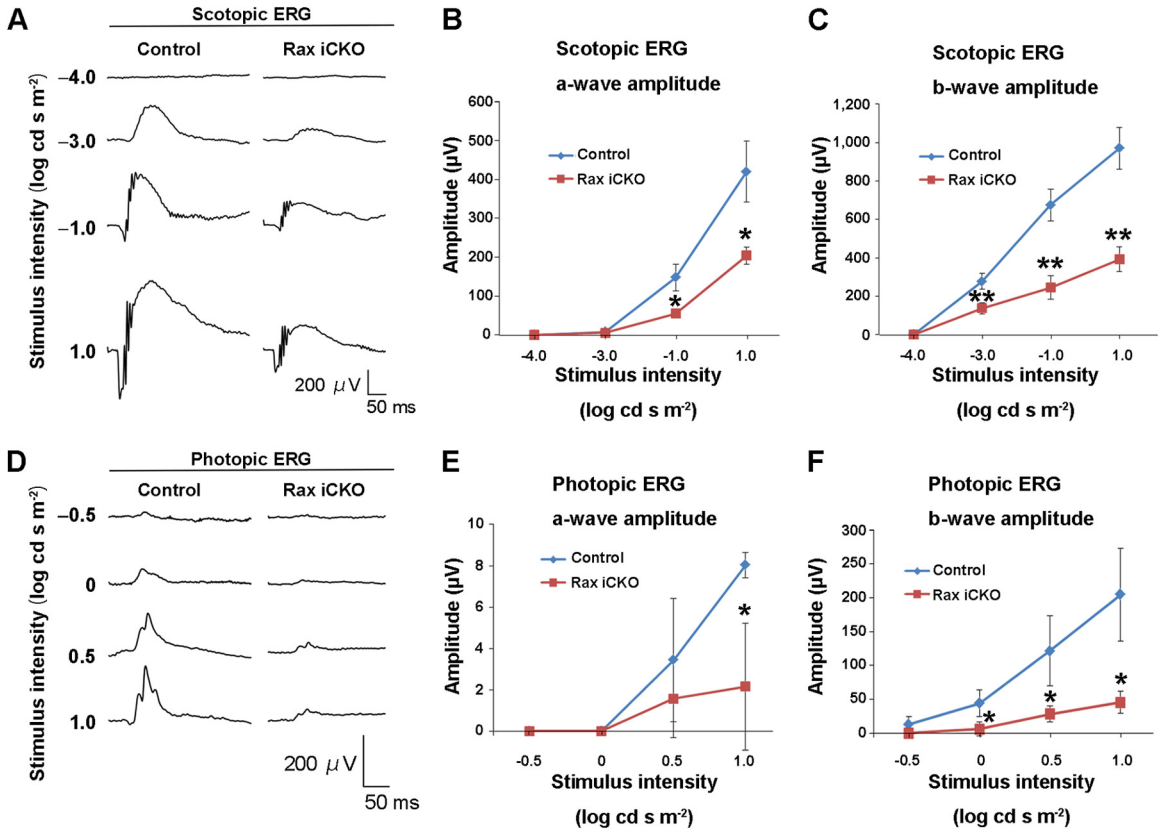

FIG 4 ERG analysis of *Rax* iCKO and control mice. (A to F) ERGs from *Rax* iCKO (P4 → 2 months) mice were recorded. (A) Scotopic ERGs elicited by four different stimulus intensities are shown. (B and C) The amplitudes of the scotopic ERG a-wave (B) and the b-wave (C) are shown as a function of the stimulus intensity. (D) Photopic ERGs elicited by four different stimulus intensities are shown. (E and F) The amplitudes of the photopic ERG a-wave (E) and b-wave (F) are shown as a function of the stimulus intensity. Data are means ± SDs ($n = 3$). **, $P < 0.01$ by Student's t test; *, $P < 0.05$ by Student's t test.

munostaining and observed that the rhodopsin expression level was unaltered between control and *Rax* iCKO (1 month → 2 months) mouse retinas (data not shown). To evaluate the physiological function of the *Rax* iCKO mouse retina, we recorded scotopic and photopic ERGs in control and *Rax* iCKO (1 month → 2 months) mice. Both the waveforms and amplitudes for a- and b-waves in scotopic ERGs were very similar between control and *Rax* iCKO (1 month → 2 months) mice (data not shown), indicating that rod function in *Rax* iCKO (1 month → 2 months) mice is unaffected. In contrast, the amplitudes of the photopic ERGs in *Rax* iCKO (1 month → 2 months) mice were significantly smaller than those in control mice (Fig. 5K to M). These results show that Rax is necessary for the normal expression of cone opsins but not for that of rhodopsin in mature photoreceptors.

Rax promotes Crx transactivation on *Rhodopsin*, *S-opsin*, and *M-opsin* promoters. It was reported that Crx, Nrl, and Nr2e3 synergistically regulate the expression of rod-specific genes (31, 38–42). In the current study, we found that although the expression levels of *Crx*, *Nrl*, and *Nr2e3* were similar between control and *Rax* iCKO (P4 → P20) mouse retinas, the levels of transcripts of rod-specific genes significantly decreased in the *Rax* iCKO (P4 → P20) mouse retina (Fig. 3B). Nr2e3 functions downstream of Nrl. Crx and Nrl are major transcription factors regulating *Rhodopsin* expression, and Crx is the major factor regulating *cone opsin* expression. Therefore, in the current study we investigated the physiological relevance of Rax with Crx and/or Nrl for photoreceptor gene transactivation. To test whether or not Rax can transactivate opsin genes, we carried out luciferase assays using human *Rhodopsin*, *S-opsin*, and *M-opsin* promoters, which have been better characterized than the mouse *Rhodopsin*, *S-opsin*, and *M-opsin* promoters. These promoters contain both PCE-1 (Ret1) and OTX elements, which are the binding sites for Rax and Crx, respectively. These sites are known to be necessary for photoreceptor-specific gene expression (15). We tested the effect of Rax expression on the promoter activity in transient-transfection experiments using NIH 3T3 cells. The human *Rhodopsin* promoter region (positions −181 to + 49) contains a single PCE-1 site and two OTX sites. The human *S-opsin* promoter region (positions −3768 to + 1) possesses six PCE-1 sites and six OTX sites, and the *M-opsin* promoter region (positions −212 to + 79) possesses a single PCE-1 site and a single OTX site (Fig. 6A). Although Rax alone showed no significant effect on the activity of the human *Rhodopsin* promoter, Crx alone strongly transactivated the human *Rhodopsin* promoter (Fig. 6B). Cotransfection of the *Rax* plasmid together with the *Crx* plasmid exhibited a slight synergic effect on transactivation activity compared the effect of the *Crx* plasmid alone (Fig. 6C). In contrast, we observed a prominent effect on the activation of the human *Rhodopsin* promoter when the *Nrl* plasmid was cotransfected with the *Crx* plasmid. Addition of the *Rax* plasmid to the *Crx* and *Nrl* plasmids significantly increased the transactivation

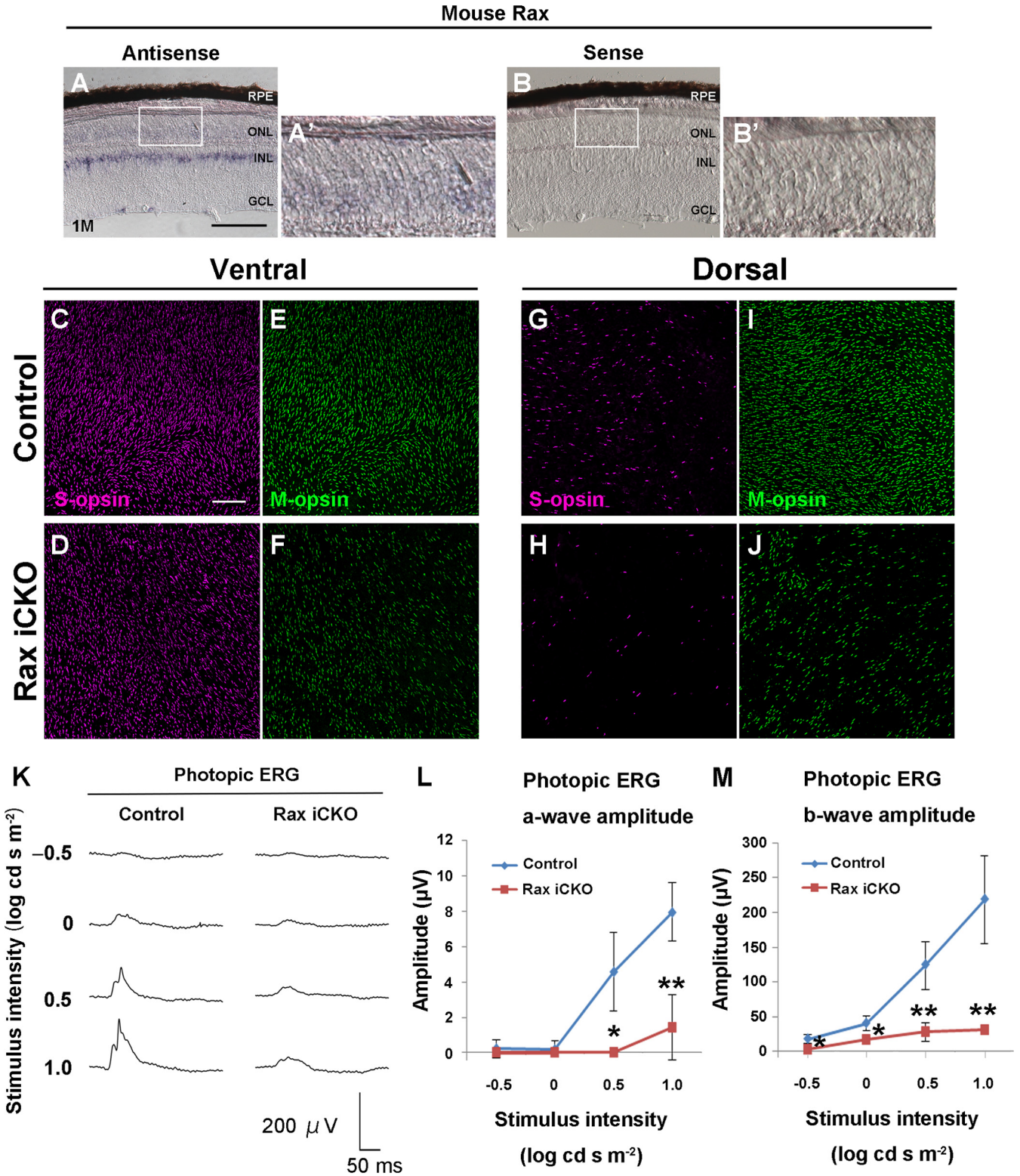

FIG 5 Rax is required for maintenance of cone photoreceptors. (A to B′) Expression of *Rax* in the $Rax^{flox/flox}$ mouse retina at 1 month was detected by *in situ* hybridization using an antisense probe (A) or a sense probe (B) for *Rax*. (A′ and B′, magnifications of boxes in panels A and B, respectively. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; 1M, harvest of retinas at age 1 month. Bar, 100 μm. (C to J) Whole-mount retinas from control and *Rax* iCKO (1 month → 2 months) mice were immunostained with antibody against S-opsin (magenta) (C, D, G, and H) or M-opsin (green) (E, F, I, and J). Ventral (C to F) and dorsal regions (G to J) are shown. Bar, 100 μm. (K to M) ERGs were recorded from *Rax* iCKO (1 month → 2 months) mice. (K) Photopic ERGs elicited by four different stimulus intensities are shown. (L and M) The amplitudes of the photopic ERG a-wave (L) and b-wave (M) are shown as a function of the stimulus intensity. For control mice, $n = 5$; for *Rax* iCKO mice, $n = 3$. Data are means ± SDs. **, $P < 0.01$ by Student's *t* test; *, $P < 0.05$ by Student's *t* test.

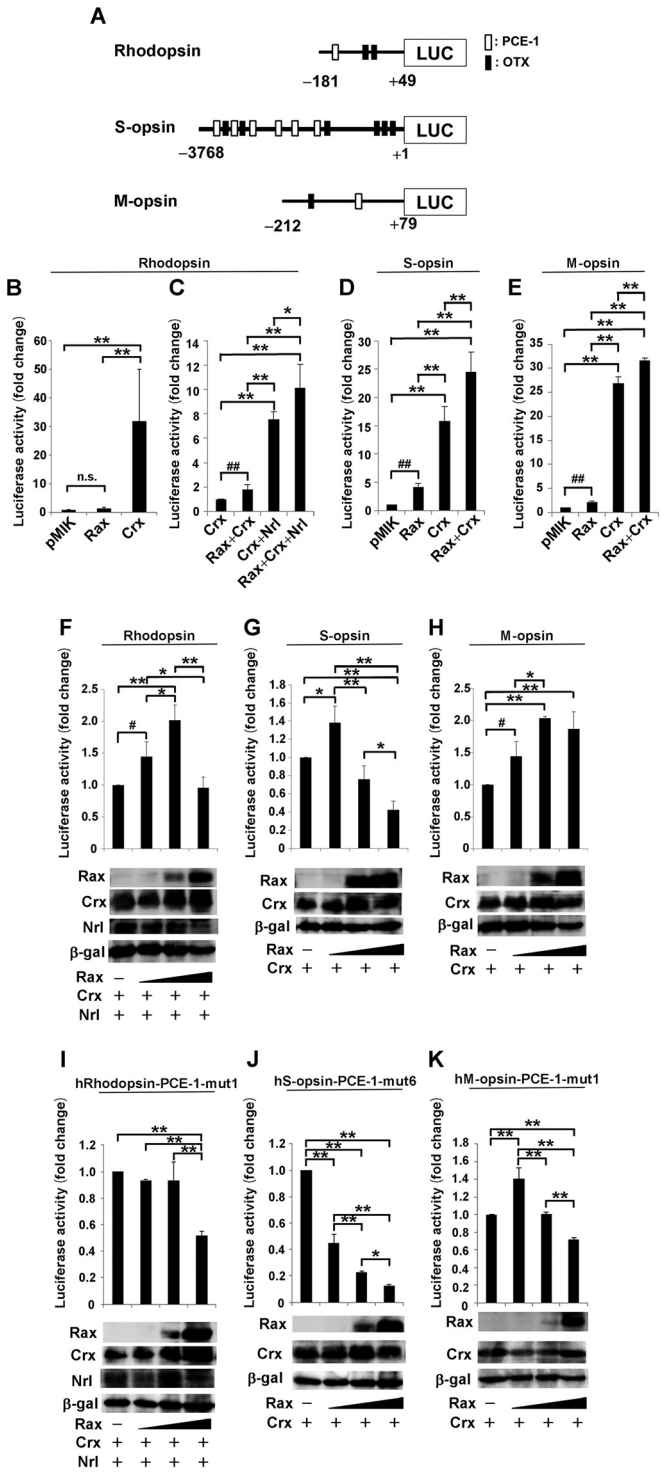

FIG 6 Transactivation of *Rhodopsin*, *S-opsin*, and *M-opsin* promoters by Rax, Crx, and/or Nrl. (A to H) Luciferase (LUC) reporter assay using human *Rhodopsin*, *S-opsin*, and *M-opsin* promoter-luciferase constructs. (A) Schematics of luciferase assay promoter construct. (B and C) NIH 3T3 cells were cotransfected with 0.4 μg of the human *Rhodopsin* promoter (positions −181 to + 49)-luciferase construct together with 0.5 μg *Rax*, *Crx*, and *Nrl* expression plasmids. Luciferase activity was corrected for transfection efficiency using a β-galactosidase internal control (0.3 μg) and is shown as the fold change, which was calculated as the ratio of the value for each combination to the value for the reporter plasmid with an empty vector. Data are means ± SDs ($n = 4$). (D) As for panels B and C, except that the reporter plasmid was the human *S-opsin* promoter (positions −3768 to + 1)-luciferase construct with 0.5 μg *Rax* expression plasmid. Data are means ± SDs ($n = 3$). (E) As for panel D, except that the reporter plasmid was the human *M-opsin* promoter (positions −212 to + 79)-luciferase construct. Data are means ± SDs ($n = 3$). (F to H) NIH 3T3 cells were cotransfected with the human *Rhodopsin* promoter-luciferase reporter construct plus the *Crx* and *Nrl* expression plasmids (0.25 μg) and the *Rax* expression plasmid (0, 0.25, 1, and 4 μg) (F), the human *S-opsin* promoter-luciferase reporter construct plus the *Crx* expression plasmid (0.25 μg) and the *Rax* expression plasmid (0, 0.25, 1, and 4 μg) (G), or the human *M-opsin* promoter-luciferase reporter construct plus the *Crx* expression plasmid (0.25 μg) and the *Rax* expression plasmid (0, 0.25, 1, and 4 μg) (H). (I to K) NIH 3T3 cells were cotransfected with the human *Rhodopsin-PCE-1-mut1* promoter-luciferase reporter construct plus the *Crx* and *Nrl* expression plasmids (0.25 μg) and the *Rax* expression plasmid (0, 0.25, 1, and 4 μg) (I), the human *S-opsin-PCE-1-mut6* promoter-luciferase reporter construct plus the *Crx* expression plasmid (0.25 μg) and the *Rax* expression plasmid (0, 0.25, 1, and 4 μg) (J), or the human *M-opsin-PCE-1-mut1* promoter-luciferase reporter construct plus the *Crx* expression plasmid (0.25 μg) and the *Rax* expression plasmid (0, 0.25, 1, and 4 μg) (K). Rax, Crx, and Nrl expression levels were determined by Western blotting after normalization using a β-galactosidase (β-gal) internal control. Data are means ± SDs ($n = 3$). **, $P < 0.01$ by the Tukey-Kramer multiple-comparison test; *, $P < 0.05$ by the Tukey-Kramer multiple-comparison test; n.s., not significant by the Tukey-Kramer multiple-comparison test; ##, $P < 0.01$ by Student's *t* test; #, $P < 0.05$ by Student's *t* test.

activity compared with that induced by the *Crx* plus *Nrl* plasmids alone (Fig. 6C).

We next tested whether the human *Cone opsin* promoters (*S-opsin* and *M-opsin*) are transactivated by Rax alone or only in the combination with Crx (Fig. 6D and E). While Rax alone weakly transactivated the *S-opsin* or *M-opsin* promoters, Crx alone exhibited a strong transactivation effect on both the *S-opsin* and *M-opsin* promoters (Fig. 6D and E). The addition of Rax significantly augmented the transactivation activities induced by Crx alone for both the *S-opsin* and *M-opsin* promoters (Fig. 6D and E).

We revealed that the Rax protein expression level decreases during mouse photoreceptor maturation (Fig. 1A to D and F). To examine whether the decrease of the Rax expression level during photoreceptor maturation is biologically meaningful, we examined the dose dependency of Rax transactivation activity on opsin promoters. NIH 3T3 cells were cotransfected with fixed amounts of the *Crx* and *Nrl* expression plasmids (0.25 μg each) and four different amounts of the *Rax* expression plasmid (0, 0.25, 1, and 4 μg), together with the human *Rhodopsin* promoter-luciferase reporter construct. Compared with the luciferase activity obtained by coexpression of *Crx* and *Nrl*, about 1.5-fold or 2-fold activation was observed when 0.25 μg or 1 μg of *Rax* plasmid was additionally transfected, respectively (Fig. 6F). Interestingly, a larger amount of the *Rax* plasmid (4 μg) markedly suppressed *Rhodopsin* transactivation by Crx and Nrl (Fig. 6F). Next, we performed a similar assay on the human *S-opsin* and *M-opsin* promoters, which are mainly regulated by Crx. Compared with the level of activation achieved by *Crx* alone, an almost 1.4-fold activation of the human *S-opsin* promoter was observed when the *Rax* plasmid (0.25 μg) was cotransfected (Fig. 6G). However, this activation was dose-dependently suppressed when larger amounts of *Rax* plasmids (1 and 4 μg) were cotransfected (Fig. 6G). Similarly, compared with the level of activation achieved with *Crx* alone, the human *M-opsin* promoter showed an approximately 1.5- or 2-fold activation when *Rax* (0.25 or 1 μg, respectively) was coexpressed, while transfection of a larger amount of the *Rax* plasmid (4 μg) did not promote the Crx transactivation activity (Fig. 6H).

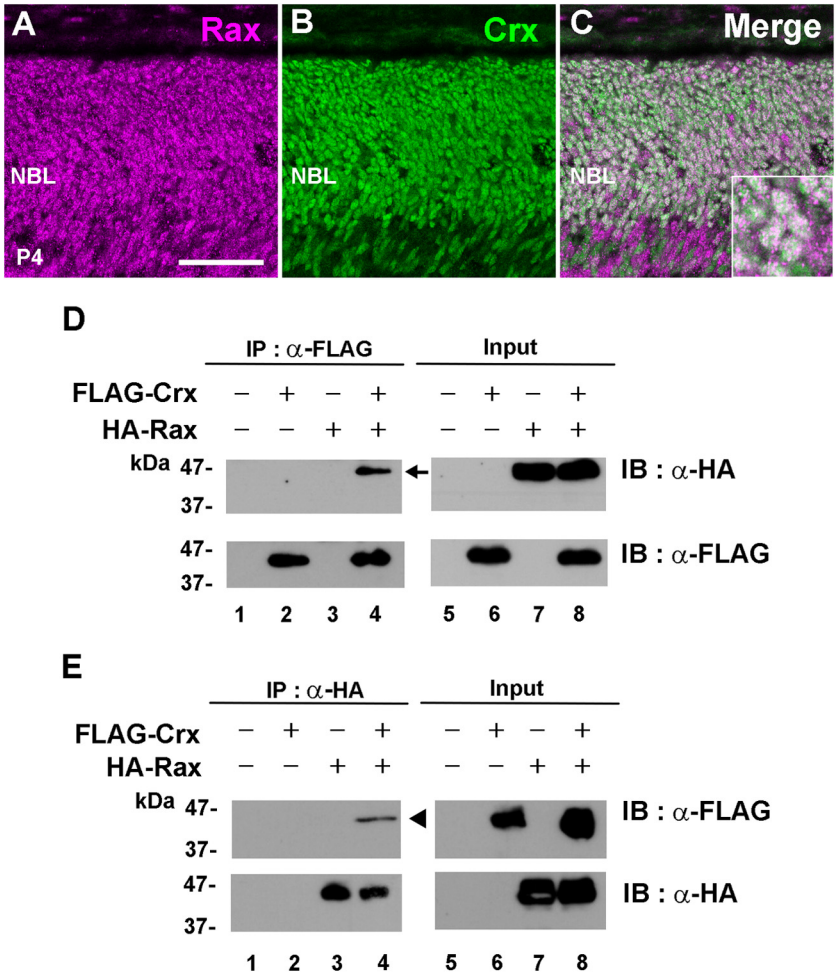

FIG 7 The Rax protein physically associates with the Crx protein. (A to C) Rax colocalizes with Crx in the postnatal retina. (A and B) At P4, retinal sections were immunostained with anti-Rax (magenta) (A) and anti-Crx (green) (B) antibodies. (C) Merge of panels A and B. (Inset) Rax- and Crx-double-positive cells at high magnification. Bar, 50 μm. (D and E) The Rax protein interacts with the Crx protein. The HA-tagged Rax expression plasmid was transfected with the FLAG-tagged Crx expression plasmid into HEK293T cells. Cell lysates were incubated with either an anti-FLAG antibody (D) or an anti-HA antibody (E). Immunoprecipitated proteins were analyzed by Western blotting. Lanes 5 to 8, input proteins without immunoprecipitation (IP). Arrow, protein immunoprecipitated by the anti-FLAG antibody; arrowhead, protein immunoprecipitated by the anti-HA antibody. IB, immunoblot; NBL, neuroblastic layer.

Next, to test whether transactivation of the *Rhodopsin*, *S-opsin*, and *M-opsin* promoters by Rax depends on the PCE-1 sites, we altered the human PCE-1 core from 5′-AATTA-3′ to 5′-AACCA-3′ (in *hRhodopsin-PCE-1-mut1*, *hS-opsin-PCE-1-mut6*, and *hM-opsin-PCE-1-mut1*) as previously described (15). In this study, neither Rax nor Crx bound detectably to this mutant oligonucleotide. When we used *hRhodopsin-PCE-1-mut1* as a promoter, cotransfection with *Rax* and *Crx* together did not further activate the promoter (Fig. 6I). Intriguingly, a larger amount of *Rax* plasmid (4 μg) markedly suppressed *Rhodopsin* transactivation by Crx and Nrl (Fig. 6I). Next, we performed a similar assay on the *hS-opsin-PCE-1-mut6* and *hM-opsin-PCE-1-mut1* promoters. When we used the *hS-opsin-PCE-1-mut6* promoter, the activation was suppressed in a dose-dependent manner (Fig. 6J). Interestingly, compared with the level of activation by the *hRhodopsin-PCE-1-mut1* and *hS-opsin-PCE-1-mut6* promoters, the *hM-opsin-PCE-1-mut1* promoter showed about a 1.4-fold activation when *Rax* (0.25 μg) was coexpressed, while transfection of a larger amount of the *Rax* plasmid (4 μg) suppressed *M-opsin* transactivation by Crx (Fig. 6K). Furthermore, we analyzed the levels of the Rax, Crx, and Nrl proteins in the reporter assays to examine whether a higher level of expression of Rax affects the protein levels of the other factors, Nrl and Crx, which could lead to the activation or suppression of transactivation. We observed that suppression was not due to changes in the Crx and Nrl expression levels (Fig. 6F to K). These results suggest and demonstrate that proper levels of Rax protein and Rax binding to the PCE-1 elements are important for photoreceptor gene transcription.

Rax protein interacts with Crx protein. Since the luciferase assay suggested that Rax is functionally associated with Crx, we then tested whether the Rax protein colocalizes with the Crx protein in the maturing postnatal mouse retina. At P4, we immunostained wild-type mouse retinas using anti-Rax and anti-Crx antibodies. We observed the colocalization of these two proteins in almost all nuclei of photoreceptor cells (Fig. 7A to C). In order to assess whether the Rax protein interacts with the Crx protein, we carried out a coimmunoprecipitation assay. We expressed FLAG-

tagged full-length Crx and/or HA-tagged full-length Rax in HEK293T cells and performed an immunoprecipitation assay using anti-FLAG and anti-HA antibodies (Fig. 7D and E). The HEK293T cell lysates were immunoprecipitated using the anti-FLAG antibody, and the HA-tagged Rax was detected in the immunoprecipitates by Western blotting (Fig. 7D, lane 4). To further confirm the interaction between the Rax and Crx proteins, a reciprocal experiment was performed. The HEK293T cell lysates were immunoprecipitated using the anti-HA antibody, and the FLAG-tagged Crx protein was detected in the immunoprecipitates by Western blotting (Fig. 7E, lane 4). These results suggest that the Rax protein interacts with the Crx protein.

DISCUSSION

Functional roles of Rax in immature and mature photoreceptor cells. In the current study, we investigated the expression patterns and functional roles of Rax in immature and mature postnatal photoreceptors in mice, which possess a single *Rax* gene in the genome. In order to investigate the *in vivo* function of *Rax* in the postnatal maturing retina, we treated $Rax^{flox/flox}$; *Crx-CreERT2* mice with tamoxifen at P4 and harvested the retinas at P8 or P20 (P4 → P8 or P20) (Fig. 2 and 3). In the *Rax* iCKO (P4 → P8) mouse retina, the levels of expression of the rhodopsin, S-opsin, and M-opsin proteins were slightly decreased, while the expression of Crx was unaffected (Fig. 2A to J). We observed that TRβ2-positive cells survived in the *Rax* iCKO (P4 → P8) mouse retina (Fig. 2K to M). In the *Rax* iCKO (P4 → P20) mouse retina, the levels of expression of cone photoreceptor-specific genes (*M-opsin*, *S-opsin*, *Gnat2*, and *Pde6c*) markedly decreased compared to those in the control retina, and the levels of expression of rod photoreceptor-specific genes (*Rhodopsin*, *Gnat1*, and *Pde6g*), which are involved in rod phototransduction, moderately decreased (Fig. 3B). By *in situ* hybridization of *Rax*, we observed that *Rax* mRNA expression continues in the ONL until the adult stage (1 month) (Fig. 5A and A′). We therefore investigated the *in vivo* function of *Rax* in mature photoreceptors by inactivating *Rax* after 1 month. We found that the levels of S-opsin and M-opsin expression were remarkably reduced (Fig. 5C to J) but that the level of rhodopsin expression was unaffected in the *Rax* iCKO (1 month → 2 months) mouse retina (data not shown). These histological observations match well with the results obtained by the use of ERG recordings (Fig. 5K to M). Taken together, these *in vivo* analyses show that Rax has an essential role in cone photoreceptor gene expression and survival and is also partially required for rod photoreceptor gene expression but is not essential for rod survival in the postnatal mouse retina.

Functional difference between Rax and Rax2. It is known that various vertebrates contain at least two *Rax* genes, *Rax* and *Rax2*. On the other hand, the genomes of mice and rats encode only a single *Rax* gene (22). Several studies reported on the *in vivo* functions of *Rax* and *Rax2* in photoreceptor development at maturing stages. In chicks, it was reported that RaxL/Rax2 is not sufficient to promote the photoreceptor cell fate choice but is required for photoreceptor cell development (20). In this study, the engrailed repressor domain-fused dominant negative Rax and Rax2 expression vectors were electroporated into the optic vesicles of chick embryos to knock down the function of Rax and Rax2, respectively, and the results suggested that chick Rax2, but not Rax, is required for photoreceptor cell development. It was also reported that rhodopsin expression and PNA staining were decreased in *Rax* short hairpin RNA-expressing transgenic tadpoles in which both *Rax* and *Rax2* were downregulated (18). Interestingly, the expression of mouse Rax can restore reduced rhodopsin expression and PNA staining. In humans, three heterozygous *QRX/RAX2* mutant alleles were identified in the genomes of degenerative retinal disease patients (22). R87Q was found in a patient with age-related macular degeneration (AMD), and G137R and 140P_141Gdup (140PGIns) were identified in cone-rod dystrophy (CRD) patients. This study also reported that these mutant RAX2 proteins exhibited alterations in their transactivation activities and the strength of their interaction with CRX; however, it seems that these mutants were heterozygous and had missense mutations which did not result in drastic biochemical abnormalities, so there might not be sufficient evidence to conclude that these mutations are responsible for AMD or CRD. Moreover, a recent study reported that interspecific variation in *Rax* expression controls opsin expression and causes visual system diversity in African cichlid fishes (43).

Taken together, although previous studies showed that *Rax* and/or *Rax2* is involved in the maturation of photoreceptors *in vivo*, it is still unclear whether one *Rax* gene or both of them play essential roles in photoreceptor maturation. In addition, to our best knowledge, there has been no study in which *Rax* and/or *Rax2* function is inactivated or knocked down specifically at the adult stage.

Rax modulates photoreceptor gene expression in cooperation with Crx. In the present study, we examined the transactivation activity of mouse Rax by luciferase assays using human photoreceptor gene promoters. In the luciferase assays, we observed that the addition of Rax significantly increases the transactivation activities of Crx plus Nrl on the human *Rhodopsin* promoter or Crx alone on the human *S-opsin* and *M-opsin* promoters (Fig. 6A to E). This result matches well with the significant reductions in the level of photoreceptor protein and gene expression in *Rax* iCKO (P4 → P8 and P20) mouse retinas (Fig. 2A to I and 3B to L). We observed that a specific amount of Rax shows the strongest transactivation effect on the photoreceptor gene promoters in luciferase assays (Fig. 6F to H). Furthermore, our studies also showed that PCE-1 elements are necessary for high levels of photoreceptor-specific gene expression, as described previously (15), while PCE-1 elements are not necessary for the transactivation of Rax on the human *M-opsin* promoter at low levels of Rax (Fig. 6I to K). The human *QRX/RAX2* study also showed that the transactivation effects of *RAX2* peak depending on the amount of *RAX2* plasmids used in the assays. In addition, it was also reported that human RAX binds to PCE-1 sites in the mouse rod *arrestin* promoter and activates it with an appropriate proportion of *RAX* and *CRX* expression plasmids in the chloramphenicol acetyltransferase assay (15). These results suggest that there exists an appropriate level of Rax protein for proper Rax function in photoreceptor gene transactivation. In addition, our observation that *Rax* ablation at the adult stage resulted in cone photoreceptor loss suggests that even a relatively small amount of Rax has an important *in vivo* function (Fig. 5).

We found that the Rax and Crx proteins colocalized in the photoreceptor nuclei at P4 and were coimmunoprecipitated using cultured cells (Fig. 7A to C). Similar results were observed in the study of human RAX2 (22). In coimmunoprecipitation analyses, the G137R and 140PGIns RAX2 proteins showed a reduced interaction with CRX compared to wild-type

RAX2. However, this study did not describe any interaction between RAX and CRX. Since both the Rax and Crx proteins have been highly conserved through evolution, our result that the mouse Rax protein interacts with the mouse Crx protein suggests a possible interaction between Rax and Crx in other species as well as in mice. Clarification of this hypothesis awaits future studies.

Rax has a more critical role in cone maturation than in rod maturation. We detected a significant increase in the level of apoptosis in the *Rax* iCKO (P4 → P14) mouse retina (Fig. 3T to Z). Various studies reported that a deficiency of a rod photoreceptor gene, including *Rhodopsin*, *Gnat1*, and *Pde6g*, causes rod photoreceptor degeneration (44–46). The cone photoreceptor function loss 1 (*cpfl1*) mutation in *Pde6c* causes a lack of cone function and cone photoreceptor degeneration, which are associated with achromatopsia (47). Although we observed a significant reduction in the number of cone photoreceptors, the number of rod photoreceptor cells was unchanged or only very slightly decreased. This cone-specific cell death in the *Rax* iCKO (P4 → P14) mouse retina may be due to a drastic reduction of cone photoreceptor proteins and a moderate reduction of rod photoreceptor genes. Our results indicate that Rax has a modulatory function in rod gene expression but a crucial function in cone gene expression in postnatal photoreceptors. While Nrl together with other transcription factors, including Crx, transactivates rod genes, Nrl does not participate in cone gene transactivation. The absence of Nrl in cone gene transactivation might explain why Rax has a more essential role in cone maturation than in rod maturation.

Prospective studies on Rax and Rax2 in photoreceptor maturation. Our findings in the current study demonstrate that mouse *Rax* plays an essential role not only in the proliferation of retinal progenitor cells, cone cell fate determination, and Müller glia cell development but also in the maturation and survival of photoreceptor cells (12–14, 16). Previous studies seemed to propose that Rax mainly regulates progenitor proliferation and Rax2 mainly functions in photoreceptor maturation. This idea may be supported partly because the human QRX/RAX2 protein (184 amino acid residues) is poorly conserved with the human RAX protein (346 amino acid residues) except in the homeodomain. However, it should be noted that *Xenopus Rax1* and zebra fish *Rax* (*Rx1*) continue to be expressed in mature photoreceptors during development (11, 17, 18). Thus, together with the current results on *Rax* function in mice, which contain a single *Rax* gene, the possibility that *Rax* and *Rax2* have a redundant function in photoreceptor maturation rather than a separate function can be proposed. Further studies in various species are needed to clarify and conclude whether Rax and Rax2 are functionally redundant or are used separately for photoreceptor maturation. Identification of many more mutations in human *QRX/RAX2* and *RAX* will be especially useful. For a future study, it is also important to elucidate how expression level balance among multiple transcription factors is controlled in photoreceptor development. We previously showed that *Rax* promotes the differentiation of Müller glial cells (5, 14). In the present study, we observed that Rax is expressed in Müller glial cells until age 2 months. What the role of Rax is in maturing Müller glial cells is another interesting question to be addressed in the future.

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S.I., R.S., and T.F. designed the project. T.F. and Y.M. generated the $Rax^{flox/flox}$ and *Crx-CreERT2* mice, and S.I. and Y.M. produced the *Rax* iCKO mice. S.I., K.K., R.S., and T.C. carried out the molecular and biochemical experiments. S.I., Y.M., and K.K. performed histological and cell biological analysis. S.I. carried out the ERG experiments. S.I., R.S., and T.F. wrote the manuscript. T.F. supervised the project.

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