

1 **Rax homeoprotein regulates photoreceptor cell maturation and survival in**
2 **association with Crx in the postnatal mouse retina**

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5 Shoichi Irie^{1,2,3}, Rikako Sanuki^{1,2}, Yuki Muranishi^{1,2}, Kimiko Kato^{1,2}, Taro Chaya^{1,2},
6 Takahisa Furukawa^{1,2,*}

7
8 ¹Laboratory for Molecular and Developmental Biology, Institute for Protein Research,
9 Osaka University, 3-2 Yamadaoka, Suita, Osaka, 565-0871, Japan.

10 ²JST, CREST, 3-2 Yamadaoka, Suita, Osaka, 565-0871, Japan.

11 ³Kyoto University Graduate School of Medicine, Yoshida Konoe-cho, Sakyo-ku, Kyoto,
12 606-8501, Japan.

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15 Rax regulates photoreceptor maturation and survival

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17 *** Author for correspondence:**

18 E-mail: takahisa.furukawa@protein.osaka-u.ac.jp

19 Laboratory for Molecular and Developmental Biology, Institute for Protein Research,
20 Osaka University, 3-2 Yamadaoka, Suita, Osaka, 565-0871, Japan.

21 Phone: +81-6-6879-8631

22 Fax: +81-6-6879-8633

23 **Abstract**

24 The *Rax* homeobox gene plays essential roles in multiple processes of vertebrate retina
25 development. Many vertebrate species possess *Rax* and *Rax2* genes, and different
26 functions have been suggested. In contrast, mice contain a single *Rax* gene and its
27 functional roles in late retinal development are still unclear. To clarify mouse *Rax*
28 function in postnatal photoreceptor development and maintenance, we generated
29 conditional knock-out mice in which *Rax* is inactivated in maturing or mature
30 photoreceptor cells by tamoxifen treatment (*Rax* iCKO mice). When *Rax* is
31 inactivated in postnatal *Rax* iCKO mice, developing photoreceptor cells showed a
32 significant decrease of the expression of rod and cone photoreceptor genes and mature
33 adult photoreceptors exhibited a specific decrease in cone cell numbers. In luciferase
34 assays, we found that *Rax* and *Crx* cooperatively transactivate *Rhodopsin* and *Cone*
35 *opsin* promoters and that an optimum *Rax* expression level to transactivate
36 photoreceptor gene expression exists. Furthermore, *Rax* and *Crx* co-localized in
37 maturing photoreceptor cells and their co-immunoprecipitation was observed in cultured
38 cells. Taken together, these results suggest that *Rax* plays essential roles in the
39 maturation of both cones and rods and in the survival of cones by regulating
40 photoreceptor gene expression with *Crx* in the postnatal mouse retina.

41 Introduction

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

55 *Rax* paralog genes have been identified in various vertebrate species. (20-22). In
56 *Xenopus laevis*, two *Rax* genes (*xRx*, *xRx-L/xRx2*) have been identified (7, 21), and in
57 Zebra fish, three *Rax* genes have been isolated (*zRx1-3*) (7). Interestingly, zebrafish
58 *Rx3* showed more similarity in expression pattern to frog and mouse *Rax* genes than to
59 zebrafish *Rx1* and *Rx2* (23). In chicks, two *Rax* genes (*cRax*, *cRaxL/cRax2*) have been
60 identified (20). The chick *Rax2* is expressed in both retinal progenitor cells and early
61 developing photoreceptors, while chick *Rax* is predominantly expressed in retinal
62 progenitor cells. It was also reported that chick *Rax2* is implicated in cone
63 photoreceptor differentiation and that the expression of a putative dominant negative
64 allele of a chick *Rax2* caused a significant reduction in expression of cone photoreceptor

65 genes (20). Human *RAX2/QRX*, which is expressed in the outer nuclear layer (ONL)
66 and inner nuclear layer (INL) of the adult human retina, was identified as a
67 PCE-1-binding protein by acting synergistically with CRX and NRL to modulate the
68 expression of photoreceptor genes. Monkey, cow, and dog genomes also contain two
69 *Rax* genes. On the other hand, the *Rax2* gene is absent from mouse and rat genomes
70 (22). This raises the question of whether mouse *Rax* plays an essential role in
71 photoreceptor development during postnatal stages as human *Rax2* does.

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

79 **Materials and Methods**

80

81 **Animal care**

82 All procedures conformed to the ARVO statement for the Use of Animals in Ophthalmic
83 and Vision Research, and these procedures were approved by the Institutional Safety
84 Committee on Recombinant DNA Experiments (approval ID 3380-3) and Animal
85 Experimental Committees of the Institute for Protein Research (approval ID 24-05-1),
86 Osaka University, and were performed in compliance with the Institutional guidelines.
87 Mice were housed in a temperature-controlled room at 22 °C with a 12 h light/dark
88 cycle. Fresh water and rodent diet were available at all times.

89

90 ***Rax^{fllox/fllox};Crx-CreERT2* mouse and tamoxifen**

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

96

97 **Plasmid constructs**

98 We subcloned a 3.8 kb upstream genomic fragment of the human *S-opsin* gene (−3769
99 to +1 bp) into the pGL3-Basic vector (Promega), generating a pS-opsin-Luc reporter
100 plasmid. Mutations of PCE-1 sites in the human Rhodopsin, S-opsin and M-opsin
101 promoters, previously described (15), were introduced by PCR with mutated PCR
102 primers. The resulting constructs were named hRhodopin-PCE-1-mut1,

103 hS-opsin-PCE-1-mut6, or hM-opsin-PCE-1-mut1. We constructed the expression
104 vectors by subcloning a full-length mouse *Rax* cDNA into the pME18S (pMIK)
105 expression vector (a gift by Dr. K. Maruyama, Tokyo Medical and Dental University,
106 School of Medicine, Tokyo, Japan). An open reading frame fragment of the mouse
107 *Crx* cDNA was amplified by PCR and cloned into the pBSKS-3xFlag vector. The
108 obtained mouse *Crx-3xFlag* fragment was cloned into the modified pCAGGS
109 expression vector, generating the pCAGGS-3xFlag-Crx expression plasmid. To obtain
110 the pCAGGS-2xHA-Rax expression plasmid, an open reading frame fragment of the
111 mouse *Rax* cDNA was amplified by PCR, and cloned into the pCAGGS-2xHA vector.

112

113 **Immunohistochemistry**

114 For immunohistochemistry, 20 μ m retinal sections were washed twice in
115 phosphate-buffered saline (PBS), and boiled in 10 mM sodium citrate buffer (pH 6.0)
116 for 10 min for antigen retrieval. Sections were rinsed twice using PBS and incubated
117 with blocking solution (4% normal donkey serum and 0.1% Triton X-100 in PBS) for 1
118 h at room temperature. The samples were incubated with a primary antibody at 4 °C
119 overnight. After PBS-washing, these samples were incubated with secondary
120 antibodies for 1 h at room temperature. Rhodamine-labeled Peanut Agglutinin (PNA)
121 (Vector Laboratories) was used for staining cone pedicles. DAPI (4',
122 6-diamino-2-phenylindole) and Hoechst 33342 were used for staining nuclear DNA.
123 For whole-mount immunostaining of the retina, each retina was gently peeled off from
124 the sclera, rinsed in PBS and fixed with 4% paraformaldehyde (wt/vol) in PBS for 2 h.
125 After washing in PBS, samples were blocked with 4% normal donkey serum and 0.05%
126 Triton X-100 (wt/vol) in PBS for 2 h. The retinas were then immunostained with

127 primary antibodies to S-opsin, M-opsin and Rhodopsin at 4 °C overnight. Reactions
128 with secondary antibodies were performed for 2 h at room temperature. The
129 specimens were observed under a laser confocal microscope (LSM700; Carl Zeiss).

130

131 **Antibody production**

132 “By PCR, a cDNA fragment encoding a center portion of mouse Nrl (residues 62-130;
133 Nrl-center) was amplified and subcloned into pGEX4T-2 (Amersham Biosciences). The
134 fusion protein was expressed in *Escherichia coli* strain BL21 and purified with
135 glutathione Sepharose 4B (Amersham Biosciences) according to the manufacturer’s
136 instructions. An antibody against Nrl was obtained by immunizing guinea pigs with
137 the purified GST-Nrl. The guinea pig antisera against Nrl-center was pre-absorbed
138 with GST-Sepharose and affinity purified with an immunizing fusion protein-bound
139 Sepharose column.

140

141 **Antibodies**

142 For immunostaining, anti-Rax antibody (guinea pig, 1:1000) (16), anti-S-opsin antibody
143 (goat, 1:500, Santa Cruz), anti-M-opsin antibody (Rabbit, 1:300, Millipore),
144 anti-Rhodopsin antibody (mouse, 1:5000, Sigma), anti-TR β 2 antibody (rabbit, 1:3000, a
145 gift from Douglas Forrest, NIDDK), anti-TR β 2 antibody (guinea pig, 1:50) (24),
146 anti-Lhx2 antibody (goat, 1:1000, Santa Cruz), and anti-Pikachurin antibody (rabbit,
147 1:500) (25) were used as primary antibodies. We used Cy3-conjugated secondary
148 antibodies (1:500, Jackson), Alexa Fluor 488-conjugated secondary antibodies (1:1000,
149 Sigma) and Dylight 649-conjugated secondary antibodies (1:500, Jackson). For
150 Western blot analysis, anti-Rax antibody (guinea pig, 1:1000) (16), anti-Crx antibody

151 (rabbit, 1:3000) (24), anti-Rhodopsin antibody (mouse, 1:5000, Sigma), anti- β -actin
152 antibody (mouse, 1:5000, Sigma), anti-Nrl antibody (guinea pig, 1:500), anti-FLAG
153 antibody (mouse, 1:15000, F1804, Sigma), and anti-HA antibody (rat, 1:5000, Roche)
154 were used as primary antibodies. We used a horseradish peroxidase-conjugated goat
155 antibody against mouse IgG (1:20000, Zymed), a horseradish peroxidase-conjugated
156 goat antibody against rabbit IgG (1:20000, Molecular probes), a horseradish
157 peroxidase-conjugated goat antibody against rat IgG (1:20000, Jackson), and a
158 horseradish peroxidase-conjugated donkey against guinea pig IgG (1:20000, Jackson) as
159 secondary antibodies.

160

161 **Western blot analysis**

162 Mouse retinas were dissected at P8 and lysed in a SDS-sample buffer. HEK293 cells
163 were washed by PBS twice and lysed in a SDS-sample buffer. Samples were resolved
164 by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for 1
165 h in 5% milk containing tris-buffered saline with 0.1% Tween 20 (TBST) and incubated
166 overnight with a primary antibody. The membrane was then incubated for 1 h with a
167 secondary antibody. The signals were measured using Image J (US National Institutes
168 of Health).

169

170 **TUNEL assay**

171 Fresh frozen retinas were sectioned to a thickness of 20 μ m and fixed with 4%
172 paraformaldehyde in PBS for 1 min. TUNEL assays were performed using the
173 Click-iT TUNEL Alexa Fluor 488 Imaging Assay Kit (Invitrogen) according to the
174 manufacture's protocols.

175

176 **ERG recordings**

177 ERGs were recorded with a white LED luminescent electrode placed on the cornea
178 (PuREC; Mayo, Japan). Two-month old mice were anesthetized with an
179 intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. Pupils were
180 dilated with topical 0.5% tropicamide and 0.5% phenylephrine HCl. The mice were
181 stimulated with four levels of stroboscopic stimuli ranging from -4.0 to $1.0 \log \text{cd-s/m}^2$
182 to elicit scotopic ERGs, and four levels of stimuli ranging from -0.5 to $1.0 \log \text{cd-s/m}^2$
183 for the photopic ERGs. The photopic ERGs were recorded on a rod-suppressing white
184 background of $1.3 \log \text{cd/m}^2$.

185

186 **In situ hybridization**

187 Digoxigenin (DIG)-labeled riboprobes were synthesized by T7 and T3 RNA polymerase
188 using the *Rax* cDNA or the *Cre* recombinase cDNA as a template in the presence of
189 11-digoxigenin UTPs (Roche Molecular Diagnostics, Mannheim, Germany). Postnatal
190 eyeballs from *129Sv/Ev* background *Rax*^{*flax/flax*} mice and/or *Rax*^{*flax/flax*}; *Crx-CreERT2*
191 mice were fixed with 4% paraformaldehyde in PBS overnight at 4 °C. The samples
192 were cryoprotected with 30% sucrose in PBS and embedded in OCT compound (Sakura
193 Finetechnical, Tokyo, Japan). These tissues were sliced with a Microm HM 560
194 cryostat microtome (Microm Laborgeräte GmbH, Walldorf, Germany) into 20 μm
195 sections. The sliced sections were fixed with 4% paraformaldehyde in PBS containing
196 0.1% Tween 20 (PBST), treated with 6% H_2O_2 , 5 $\mu\text{g/mL}$ proteinase K, and subsequently
197 2 mg/mL glycine in PBST. The samples were then post-fixed with 4%
198 paraformaldehyde in PBST. Hybridization was carried out overnight at 70 °C. After

199 post-hybridization washing, they were blocked with 5% sheep serum in 2.5 mM
200 Tris-HCl (pH7.5), 0.8% NaCl, 0.02% KCl, and 0.1% Tween 20 (TBST), and incubated
201 with an alkaline phosphatase-conjugated anti-DIG antibody (1:2,000, Roche) in TBST
202 with 1% sheep serum. The samples were visualized with nitroblue tetrazolium
203 chloride (Sigma, St. Louis, MO) and 5-bromo-4-chloro- 3-indolyl-phosphate, toluidine
204 salt (Sigma).

205

206 **Luciferase assay**

207 We transfected 0.4 µg of the luciferase reporter plasmid DNAs (pGL3b-human
208 Rhodopsin promoter-luc, pGL3b-human S-opsin promoter-luc, pGL3b-human M-opsin
209 promoter-luc, pGL3b-human Rhodopin-PCE-1-mut1, pGL3b-human S-opsin-PCE-1-
210 mut6, or pGL3b-human M-opsin-PCE-1-mut1) (26) and 0.25 - 4 µg of mouse *Rax* or
211 0.25 - 0.5 µg of mouse *Crx* or *Nrl* expression vector DNAs (pME18S, pME18S-Rax,
212 pME18S-Crx, and pME18S-Nrl) per well into NIH3T3 cells in a 6-well plate using
213 FuGene (Promega). β-galactosidase expression vector (β-SV) (Promega) was
214 co-transfected for normalization of transfection efficiency. After transfection, the cells
215 were incubated for 48 h and lysed with Reporter Lysis Buffer (Promega). Luciferase
216 activity was measured with Firefly Luciferase Assay System (Promega) according to the
217 manufacturer's protocol. Luminescence signal was detected using GloMax
218 Multi+Detection System (Promega).

219

220 **Quantitative PCR (qPCR)**

221 The mouse retinas were harvested and dissected at P20. Total RNA (1 µg) from the
222 retina was isolated using TRIzol reagent (Invitrogen) and converted to cDNA using

223 Superscript II Reverse Transcriptase (Invitrogen). Real time PCR was performed
224 using SYBR GreenER Q-PCR Super Mix (Invitrogen) and Thermal Cycler Dice Real
225 Time System Single MRQ TP870 (Takara) according to the manufacturer's instructions.
226 Quantification was performed by Thermal Cycler Dice Real Time System software
227 version 2.0 (Takara). The primer sequences used for qPCR are listed in Table 1.

228

229 **Immunoprecipitation**

230 We transfected pCAGGS-3xFlag-Crx and pCAGGS-2xHA-Rax expression plasmids
231 into HEK293T cells in a 35 mm dish using the calcium phosphate method. HEK293T
232 cells were co-transfected with the pCAGGS-3xFlag-Crx or pCAGGS-3xFlag expression
233 plasmid together with the pCAGGS-2xHA-Rax or pCAGGS-2xHA expression plasmid.
234 Two days after transfection, the cells were lysed with lysis buffer (50 mM Tris (pH 8.0),
235 150 mM NaCl, 1% NP-40 (wt/vol), 0.1% Triton X-100 (wt/vol) and Complete protease
236 inhibitor cocktail (Roche)), and centrifuged for 10 min at 15,100 x g. The supernatants
237 were incubated with an anti-FLAG M2 affinity gel (Sigma) or monoclonal
238 anti-HA-Agarose antibody (Sigma), and then eluted with 0.1 M Glycine buffer (pH 1.5).
239 After neutralization by 1 M Tris-HCl (pH 9.0), immunoprecipitation samples were
240 analyzed by Western blots.

241

242 **Statistical analysis**

243 Data are presented as means $\pm$ SD. Statistical comparisons of datasets were
244 performed with Student's t-test. For multiple comparisons, we performed one-way
245 ANOVA with Tukey-Kramer test, and $P < 0.05$ was taken to be statistically significant.

246 Results

247

248 Mouse Rax is expressed in postnatal photoreceptors and Müller glial cells

249 In order to examine Rax protein expression in the mouse retina during development,
250 we immunostained developing and adult mouse retinas with an anti-Rax antibody that
251 we previously generated (16) (Fig. 1A to D). Strong Rax signals were observed in the
252 neuroblastic layer (NBL) and the presumptive photoreceptor layer (PPL) at embryonic
253 day 15 (E15.5) (Fig. 1A). At postnatal day 6 (P6), Rax protein expression was
254 detected in the ONL, which corresponds to photoreceptor precursors, and the INL,
255 which corresponds to Müller glial cells (14) (Fig. 1B). Rax protein expression in the
256 ONL decreased at P14 and was weakly detected at 2 months (2 M) (Fig. 1C and D).
257 Although the expression level of Rax in the INL was unaltered at P14 compared with
258 that at P6, Rax signals in the INL became substantially fainter at 2M (Fig. 1D). These
259 results showed that Rax protein is expressed at high levels both in maturing
260 photoreceptors and Müller glial cells and significantly decreases by maturity.

261

262 Generation of maturing photoreceptor-specific Rax iCKO mice

263 To examine the possible role of Rax in maturing postnatal photoreceptor cells, we
264 ablated *Rax* in postnatal photoreceptor cells by using *Rax^{fllox/fllox};Crx-CreERT2* mice,
265 which we previously reported (16). We inactivated *Rax* in postmitotic maturing
266 photoreceptor cells by treating these mice with tamoxifen at P4 when the number of
267 retinal progenitors markedly decreases and many photoreceptor precursors begin their
268 maturation (27-30) (Fig. 1E). We also injected tamoxifen into *Rax^{fllox/fllox}* littermate
269 mice of the *Rax^{fllox/fllox};Crx-CreERT2* mice for use as controls in the current study. To

270 assess whether induced *Rax* inactivation occurs, we analyzed P8 control and *Rax* iCKO
271 retinas by immunostaining using the anti-*Rax* antibody. We address the *Rax* iCKO
272 mouse that were treated with tamoxifen at P4 and harvested at P8 as *Rax* iCKO (P4→
273 P8) mouse. The immunoreactivity to *Rax* in the ONL, which was strongly detected in
274 the control retina, was undetectable in the *Rax* iCKO (P4→P8) retina, while *Rax*
275 expression in the INL was almost unaffected (Fig. 1F and G). Furthermore, no
276 substantial reduction of Müller glial marker *Lhx2* was observed in the *Rax* iCKO (P4→
277 P8) mouse retina compared with that in the control retina (Fig. 1H and I). These
278 results showed that *Rax* was specifically deleted in postnatal maturing photoreceptor
279 cells in the *Rax* iCKO retina.

280

281 ***Rax* is required for the expression of opsin proteins in the maturing retina**

282 To investigate retinal phenotypes caused by *Rax* deficiency in postnatal maturing
283 photoreceptors, we performed flat-mount immunostaining of *Rax* iCKO (P4→P8)
284 retinas. We observed that the expression of cone- and rod-specific proteins (S-opsin,
285 M-opsin, and Rhodopsin) was slightly downregulated (Fig. 2A to F). Next, we
286 quantified the expression level of Rhodopsin and *Crx* proteins in control and *Rax* iCKO
287 (P4→P8) retinas by Western blot analysis. In the *Rax* iCKO (P4→P8) retina, the *Rax*
288 protein level decreased to ~40% of that in the control retina (Fig. 2G and H). The
289 Rhodopsin protein level in the *Rax* iCKO (P4→P8) retina was also reduced by ~40%
290 compared to that in the control retina (Fig. 2G and I). In contrast, *Crx* protein
291 expression was unaffected in the *Rax* iCKO (P4→P8) retina (Fig. 2G and J). To
292 examine whether *Rax* inactivation affects cone photoreceptors, we performed
293 immunostaining of control and *Rax* iCKO (P4→P8) retinas with an anti-TRβ2 antibody

294 that we generated, and counted TR β 2-positive cells on retinal sections (Fig. 2K to M).
295 The numbers of TR β 2-positive cells was unaltered between control and *Rax* iCKO
296 retinas (P4→P8) (number of TR β 2-positive cells/image, 32.0 $\pm$ 6.0 in control, 41.2
297 $\pm$ 6.4 in *Rax* iCKO, $p>0.05$) (Fig. 2M). These results suggest that *Rax* is not required
298 for *Crx* expression but essential for normal expression of opsins in postnatal maturing
299 photoreceptors.

300

301 **A defect of *Rax* in photoreceptor cells affects the expression of photoreceptor genes**

302 In order to examine whether *Rax* regulates photoreceptor-specific genes in postnatal
303 maturing photoreceptors, we selected twelve genes involved in photoreceptor
304 development and function, and measured expression levels of these genes in control and
305 *Rax* iCKO retinas by quantitative PCR (qPCR) analysis. For qPCR, we used *Rax*
306 iCKO (P4→P20) retinas when retinal neural circuit formation is almost completed (Fig.
307 3A) (30). We observed that *CreERT2* mRNA is predominantly expressed in the ONL
308 of the *Rax* iCKO (P4→P20) retina (see Fig. S1 in the supplemental material). We
309 found that expression levels of cone-specific genes (*M-opsin*, *S-opsin*, *Gnat2*, and
310 *Pde6c*) decreased by 80 to 90% in *Rax* iCKO retinas compared with those in control
311 retinas (Fig. 3B). Furthermore, the expression levels of the genes essential for light
312 sensing in rod photoreceptors (*Rhodopsin*, *Gnat1*, and *Pde6g*) also decreases by 20 to
313 40% from those in control retinas, whereas transcription factors (*Crx*, *Nrl* and *Nr2e3*),
314 which are essential for photoreceptor development *in vivo* (31, 32), were unaffected (Fig.
315 3B). Consistent with the qPCR results, we observed a reduced Rhodopsin
316 immunostaining signal in the *Rax* iCKO (P4→P20) retina (Fig. 3C and D). We also
317 found that the expression of cone photoreceptor proteins (S-opsin and M-opsin)

318 considerably decreased in *Rax* iCKO (P4→P20) retinas (Fig. 3E to H). We carried out
319 flat-mount immunostaining of control and *Rax* iCKO (P4→P20) retinas using
320 antibodies against S-opsin and M-opsin, and observed a significant reduction of the
321 cone cell numbers in the *Rax* iCKO (P4→P20) retina (Fig. 3I to L). To confirm these
322 results, we further immunostained control and *Rax* iCKO (P4→P20) retinas with
323 photoreceptor synaptic markers (anti-Pikachurin antibody and Rhodamine-labeled
324 peanut agglutinin (PNA)). Immunostaining with Pikachurin and PNA revealed that the
325 number of cone pedicles dramatically decreased in the *Rax* iCKO (P4→P20) retina
326 (number of cone pedicles/100 μm , 15.2 ± 2.7 in control, 1.8 ± 1.5 in *Rax* iCKO,
327 $p<0.01$) (Fig. 3M to S).

328 To examine whether *Rax* ablation affects the survival of maturing cone
329 photoreceptors, we immunostained cone photoreceptor cells in control and *Rax* iCKO
330 (P4→P14) retinas using the anti-TR β 2 antibody (Fig. 3T to V). In addition, we
331 detected apoptotic cell death by TUNEL assay in *Rax* iCKO (P4→P14) and the control
332 retinas (Fig. 3W and X). We found that the number of TR β 2-positive cells
333 significantly decreased in the *Rax* iCKO (P4→P14) retina compared to that of control
334 retinas (number of TR β 2+ cells/section, 45.4 ± 3.3 in control, 26.7 ± 7.0 in *Rax*
335 iCKO; $p<0.01$) (Fig. 3Y). We counted the apoptotic cell numbers in the ONL, INL,
336 and ganglion cell layer (GCL) separately, and found that apoptotic cells significantly
337 increased in the ONL (Fig. 3Z). Mislocalization of cone photoreceptor cells in the
338 retina has been shown to be associated with a disturbance of cone photoreceptor
339 maturation and consequent degeneration (33, 34). Furthermore we observed that
340 *CreERT2* mRNA is predominantly expressed in the ONL of the *Rax* iCKO (P4→P14)
341 retina (see Fig. S1 in the supplemental material). These results suggest that *Rax* is

342 essential for cone photoreceptors survival in the postnatal maturing mouse retina.

343

344 ***Rax* iCKO mice exhibit abnormal ERGs**

345 To test visual function in *Rax* iCKO mice, we performed electroretinogram (ERG)
346 recordings in scotopic and photopic conditions with several light intensities in control
347 and *Rax* iCKO (P4→2M) mice (Fig. 4A to F). In control mice, the negative a-wave,
348 which originates mainly from the activity of rod photoreceptors, appeared at higher
349 stimulus intensities of -1.0 to $1.0 \log \text{cd-s/m}^2$ under the scotopic condition (Fig. 4A).
350 A positive b-wave, which originates from rod bipolar cells (35), was seen even at a
351 lower stimulus intensity of $-3.0 \log \text{cd-s/m}^2$. In contrast, the amplitude of the
352 dark-adapted ERG a-wave in *Rax* iCKO (P4→2M) mice was reduced at higher stimulus
353 intensities of -1.0 to $1.0 \log \text{cd-s/m}^2$, compared with those in control mice (Fig. 4A and
354 B). The amplitude of the scotopic b-wave in *Rax* iCKO (P4→2M) mice was
355 significantly smaller than those of control mice at lower and higher stimulus intensities
356 of -3.0 to $1.0 \log \text{cd-s/m}^2$ (Fig. 4A and C). These results suggest an essential function
357 of *Rax* in rod phototransduction in the postnatal mouse retina. Under photopic
358 conditions, *Rax* iCKO (P4→2M) mice showed markedly decreased amplitudes in
359 a-waves, which originate from cone photoreceptors, at a high stimulus intensity of 1.0
360 $\log \text{cd-s/m}^2$, compared with those of control mice (Fig. 4D and E). The amplitude of
361 the photopic ERG b-wave in *Rax* iCKO (P4→2M) mice, which originates from cone
362 ON-bipolar cells, was significantly smaller than those of control mice at high stimulus
363 intensities of 0 to $1.0 \log \text{cd-s/m}^2$ (Fig. 4D and F), suggesting that *Rax* is also required
364 for cone phototransduction. These results showed that phototransduction in both rod
365 and cone photoreceptor cells is impaired in *Rax* iCKO (P4→2M) mice.

366

367 ***Rax* regulates the maintenance of mature cone photoreceptors**

368 To examine *Rax* expression in the adult mouse retina, we carried out *in situ*
369 hybridization of the 1M retina using *Rax* anti-sense and sense probes. *Rax* expression
370 was weakly detected with the anti-sense probe in the ONL of the retina, while the *Rax*
371 sense probe did not give any significant signal (Fig. 5A to B'). To investigate the *in*
372 *vivo* function of *Rax* in mature photoreceptors, we treated *Rax*^{flax/flax};*Crx-CreERT2* mice
373 with tamoxifen at 1M to inactivate *Rax* in mature photoreceptors. We observed that
374 *CreERT2* mRNA is predominantly expressed in the ONL of the *Rax* iCKO (1M→2M)
375 retina (see Fig. S1 in the supplemental material). We examined expressions of S-opsin
376 and M-opsin by whole-mount co-immunostaining of control and *Rax* iCKO (1M→2M)
377 retinas using anti-S-opsin and anti-M-opsin antibodies (Fig. 5C to J). In mice, S-opsin
378 is expressed predominantly in cones in the ventral retina, and M-opsin is predominantly
379 expressed in cones in the dorsal retina (36, 37). The number of S-opsin-positive cells
380 slightly decreased in both ventral and dorsal *Rax* iCKO (1M→2M) retinas (Fig. 5C and
381 D, G and H), while the number of M-opsin-positive cells notably decreased in both
382 dorsal and ventral *Rax* iCKO (1M→2M) retinas (Fig. 5E and F, I and J). On the other
383 hand, we analyzed Rhodopsin expression using retinal sections by immunostaining, and
384 observed that the Rhodopsin expression level was unaltered between control and *Rax*
385 iCKO (1M→2M) retinas (data not shown). To evaluate the physiological function of
386 the *Rax* iCKO retina, we recorded scotopic and photopic ERGs in control and *Rax*
387 iCKO (1M→2M) mice. Both waveforms and amplitudes for a- and b-waves in
388 scotopic ERGs were very similar between control and *Rax* iCKO (1M→2M) mice (data
389 not shown), indicating that rod function in *Rax* iCKO (1M→2M) mice is unaffected.

390 In contrast, the amplitudes of the photopic ERGs in *Rax* iCKO (1M→2M) mice were
391 significantly smaller than those in control mice (Fig. 5K to M). These results showed
392 that *Rax* is necessary for normal expression of cone opsins but not for Rhodopsin in
393 mature photoreceptors.

394

395 ***Rax* promotes *Crx* transactivation on *Rhodopsin*, *S-opsin* and *M-opsin* promoters**

396 It was reported that *Crx*, *Nrl* and *Nr2e3* synergistically regulate the expression of
397 rod-specific genes (31, 38-42). In the current study, we found that although expression
398 levels of *Crx*, *Nrl* and *Nr2e3* were similar between control and *Rax* iCKO (P4→P20)
399 retinas, transcripts of rod-specific genes significantly decreased in the *Rax* iCKO (P4→
400 P20) retina (Fig. 3B). *Nr2e3* functions downstream of *Nrl*. *Crx* and *Nrl* are major
401 transcription factors regulating *Rhodopsin* expression, and *Crx* is the major factor
402 regulating *Cone opsin* expression. Therefore, in the current study we investigated the
403 physiological relevance of *Rax* with *Crx* and/or *Nrl* for photoreceptor gene
404 transactivation. To test whether or not *Rax* can transactivate opsin genes, we carried
405 out luciferase assays using human *Rhodopsin*, *S-opsin*, and *M-opsin* promoters that have
406 been better characterized than the mouse promoters. These promoters contain both
407 PCE-1 (Ret1) and OTX elements, which are the binding sites for *Rax* and *Crx*,
408 respectively. These sites are known to be necessary for photoreceptor-specific gene
409 expression (15). We tested the effect of *Rax* expression on the promoter activity in
410 transient transfection experiments using NIH3T3 cells. The human *Rhodopsin*
411 promoter region (−181 to +49) contains a single PCE-1 site and two OTX sites. The
412 human *S-opsin* promoter region (−3768 to +1) and *M-opsin* promoter region (−212 to
413 +79) possess six PCE-1 sites and six OTX sites, and a single PCE-1 site and a single

414 OTX site, respectively (Fig. 6A). Although Rax alone showed no significant effect on
415 the activity of the human *Rhodopsin* promoter, Crx alone strongly transactivated the
416 human *Rhodopsin* promoter (Fig. 6B). Co-transfection of the *Rax* plasmid together
417 with the *Crx* plasmid exhibited a slight synergic effect on the transactivation activity by
418 the *Crx* plasmid alone (Fig. 6C). By contrast, we observed a prominent effect on the
419 activation of the human *Rhodopsin* promoter when the *Nrl* plasmid was co-transfected
420 with the *Crx* plasmid. Addition of the *Rax* plasmid to the *Crx* and *Nrl* plasmids
421 significantly increases the transactivation activity by *Crx* plus *Nrl* plasmids (Fig. 6C).

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

428 We revealed that the Rax protein expression level decreases during mouse
429 photoreceptor maturation (Fig. 1A to D and F). To examine whether the decrease of
430 the Rax expression level during photoreceptor maturation is biologically meaningful,
431 we examined the dose dependency of Rax transactivation activity on opsin promoters.
432 NIH3T3 cells were co-transfected with fixed amounts of the *Crx* and *Nrl* expression
433 plasmids (0.25 μ g, respectively) and four different amounts of the *Rax* expression
434 plasmid (0, 0.25, 1 and 4 μ g) together with the human *Rhodopsin* promoter-luc reporter
435 construct. Compared with the luciferase activity by co-expression of *Crx* and *Nrl*,
436 about 1.5-fold or 2-fold activation was observed upon 0.25 μ g or 1 μ g of *Rax* plasmid
437 was additionally transfected, respectively (Fig. 6F). Interestingly, a higher amount of

438 *Rax* plasmid (4 μ g) markedly suppressed the Rhodopsin transactivation by Crx and Nrl
439 (Fig. 6F). Next, we performed a similar assay on human *S-opsin* and *M-opsin*
440 promoters, which are mainly regulated by Crx. Compared with the activation by *Crx*
441 alone, an almost 1.4-fold activation of the human *S-opsin* promoter was observed when
442 the *Rax* plasmid (0.25 μ g) was co-transfected (Fig. 6G). However, this activation was
443 dose-dependently suppressed when higher amounts of *Rax* plasmids (1 and 4 μ g) were
444 co-transfected (Fig. 6G). Similarly, compared with *Crx* alone, the human *M-opsin*
445 promoter showed an about 1.5- or 2-fold activation when *Rax* (0.25 and 1 μ g,
446 respectively) was co-expressed, while transfection of a higher amount of the *Rax*
447 plasmid (4 μ g) did not promote the Crx transactivation activity (Fig. 6H). Next, to test
448 whether transactivation of the *Rhodopsin*, *S-opsin* and *M-opsin* promoters by *Rax*
449 depends on the PCE-1 sites, we altered the human PCE-1 core 5'-AATTA-3' to
450 5'-AACCA-3' (*hRhodopsin-PCE-1-mut1*, *hS-opsin-PCE-1-mut6* and *hM-opsin-PCE-1-*
451 *mut1*) as previously described (Kimura et al., 2000, J Biol Chem., 275;1152-1150). In
452 this study, neither *Rax* nor *Crx* bound detectably to this mutant oligonucleotide. When
453 we used *hRhodopsin-PCE-1-mut1* as a promoter, co-transfection with *Rax* and *Crx*
454 together did not further activate the promoter (Fig. 6I). Intriguingly, a higher amount of
455 *Rax* plasmid (4 μ g) markedly suppressed Rhodopsin transactivation by Crx and Nrl (Fig.
456 6I). Next, we performed a similar assay on *hS-opsin-PCE-1-mut6* and
457 *hM-opsin-PCE-1-mut1* promoters. When we used the *hS-opsin-PCE-1-mut6* promoter,
458 the activation was suppressed in a dose dependent manner (Fig. 6J). Interestingly,
459 compared with *hRhodopsin-PCE-1-mut1* and *hS-opsin-PCE-1-mut6*, the
460 *hM-opsin-PCE-1-mut1* promoter showed about a 1.4-fold activation when *Rax* (0.25 μ g)
461 was co-expressed, while transfection of a higher amount of the *Rax* plasmid (4 μ g)

462 suppressed M-opsin transactivation by Crx (Fig. 6K). Furthermore we analyzed the
463 protein levels of Rax, Crx and Nrl in the reporter assays to examine whether higher
464 expression of Rax affects the protein levels of the other factors, Nrl and Crx, which
465 could lead to the activation or suppression of transactivation. We observed that
466 suppression was not due to changes in the Crx and Nrl expression levels (Fig. 6F to K).
467 These results suggested and demonstrated that proper levels of Rax protein and Rax
468 binding to the PCE-1 elements are important for photoreceptor gene transcription.

469

470 **Rax protein interacts with Crx protein**

471 Since the luciferase assay suggested that Rax is functionally associated with Crx, we
472 then tested whether Rax protein co-localizes with Crx protein in the maturing postnatal
473 mouse retina. We immunostained P4 wild-type mouse retinas using anti-Rax and
474 anti-Crx antibodies. We observed co-localization of these two proteins in almost all
475 nuclei of photoreceptor cells (Fig. 7A to C). In order to assess whether the Rax protein
476 interacts with the Crx protein, we carried out a co-immunoprecipitation assay. We
477 expressed FLAG-tagged full length Crx and/or HA-tagged full length Rax in HEK293
478 cells, and performed an immunoprecipitation assay using anti-FLAG and anti-HA
479 antibodies (Fig. 7D and E). The HEK293 cell lysates were immunoprecipitated using
480 the anti-FLAG antibody, and the HA-tagged Rax was detected in the
481 immunoprecipitates by Western blot analysis (Fig. 7D, lane 4). To further confirm the
482 interaction between Rax and Crx proteins, a reciprocal experiment was performed.
483 The HEK293 cell lysates were immunoprecipitated using the anti-HA antibody, and the
484 FLAG-tagged Crx was detected in the immunoprecipitates by Western blot analysis
485 (Fig. 7E, lane 4). These results suggested that the Rax protein interacts with the Crx

486 protein.

487 Discussion

488

489 Functional roles of Rax in immature and mature photoreceptor cells

490 In the current study, we investigated expression patterns and functional roles of Rax
491 in immature and mature postnatal photoreceptors in mice, which possess a single *Rax*
492 gene in the genome. In order to investigate the *in vivo* function of *Rax* in the postnatal
493 maturing retina, we treated *Rax^{flax/flax};Crx-CreERT2* mice with tamoxifen at P4 and
494 harvested at P8 or P20 (P4→P8 or P20) (Fig. 2 and 3). In the *Rax* iCKO (P4→P8)
495 retina, expressions of Rhodopsin, S-opsin, and M-opsin proteins were slightly decreased,
496 while Crx was unaffected (Fig. 2A to J). We observed that TRβ2-positive cells
497 survived in the *Rax* iCKO (P4→P8) retina (Fig. 2K to M). In the *Rax* iCKO (P4→
498 P20) retina, expressions of cone photoreceptor-specific genes (*M-opsin*, *S-opsin*, *Gnat2*,
499 and *Pde6c*) markedly decreased compared to those in the control retina, and that
500 expressions of rod photoreceptor-specific genes (*Rhodopsin*, *Gnat1*, and *Pde6g*), which
501 are involved in rod phototransduction, moderately decreased (Fig. 3B). By *in situ*
502 hybridization of *Rax*, we observed that *Rax* mRNA expression continues in the ONL
503 until the adult stage (1M) (Fig. 5A and A'). We, therefore, investigated the *in vivo*
504 function of *Rax* in mature photoreceptors by inactivating *Rax* after 1M. We found that
505 S-opsin and M-opsin expressions were remarkably reduced (Fig. 5C to J), but
506 Rhodopsin expression was unaffected in the *Rax* iCKO (1M→2M) retina (data not
507 shown). These histological observations match well with the results obtained by ERG
508 recordings (Fig. 5K to M). Taken together, these *in vivo* analyses showed that Rax has
509 an essential role in cone photoreceptor gene expression and survival and is also partially
510 required for rod photoreceptor gene expression, but not essential for rod survival in the

511 postnatal mouse retina.

512

513 **Functional difference between Rax and Rax2**

514 It is known that various vertebrates contain at least two *Rax* genes, *Rax* and *Rax2*.
515 On the other hand, mice and rats encode only a single *Rax* gene in their genomes (22).
516 Several studies reported *in vivo* functions of *Rax* and *Rax2* in photoreceptor
517 development at maturing stages. In chick, it was reported that RaxL/Rax2 is not
518 sufficient to promote photoreceptor cell fate choice, but is required for photoreceptor
519 cell development (20). In this study, the engrailed repressor domain-fused
520 dominant-negative Rax and Rax2 expression vectors were electroporated into optic
521 vesicles of chick embryos to knock-down the function of Rax or Rax2, respectively,
522 suggesting that chick Rax2, but not Rax, is required for photoreceptor cell development.
523 It was also reported that Rhodopsin expression and PNA staining were decreased in a
524 *Rax* shRNA-expressing transgenic tadpoles in which both *Rax* and *Rax2* were
525 downregulated (18). Interestingly, the expression of mouse Rax can restore reduced
526 Rhodopsin expression and PNA staining. In humans, three heterozygous *QRX/RAX2*
527 mutant alleles were identified in genomes of degenerative retinal disease patients (22).
528 R87Q was found in a patient with age-related macular degeneration (AMD), and G137R
529 and 140PGlns were identified in cone-rod dystrophy (CRD) patients. This study also
530 reported that these mutant RAX2 proteins exhibited alterations in transactivation
531 activities and interaction strength with CRX, however, it seems that these mutants are
532 heterozygous and missense mutations which did not show drastic biochemical
533 abnormalities, so there might not be sufficient evidence to conclude that these mutations
534 are responsible for AMD or CRD. Moreover, a recent study reported that interspecific

535 variation in *Rax* expression controls opsin expression and causes visual system diversity
536 in African cichlid fishes (43).

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

542

543 ***Rax* modulates photoreceptor gene expression in cooperation with *Crx***

544 In the present study, we examined the transactivation activity of mouse *Rax* using
545 human photoreceptor gene promoters by luciferase assay. In luciferase assays, we
546 observed that the addition of *Rax* significantly increases the transactivation activities of
547 *Crx* plus *Nrl* on the human *Rhodopsin* promoter or *Crx* alone on the human *S-opsin* and
548 *M-opsin* promoters (Fig. 6A to E). This result matches well with significant reductions
549 of photoreceptor protein and gene expression in *Rax* iCKO (P4→P8 and P20) retinas
550 (Fig. 2A to I, 3B to L). We observed that a specific amount of *Rax* shows the strongest
551 transactivation effect to the photoreceptor gene promoters in luciferase assays (Fig. 6F
552 to H). Furthermore, our studies also showed that PCE-1 elements are necessary for
553 high levels of photoreceptor-specific gene expression as described (15), while PCE-1
554 elements are not necessary for transactivation of *Rax* on the human *M-opsin* promoter at
555 low levels of *Rax* (Fig. 6I to K). The human *QRX/RAX2* study also showed that
556 transactivation effects by *RAX2* peak depending on the amount of *RAX2* plasmids used
557 in the assays. In addition, it was also reported that human *RAX* binds to PCE-1 sites
558 in the mouse rod *Arrestin* promoter and activates it with an appropriate proportion of

559 *RAX* and *CRX* expression plasmids in the CAT assay (15). These results suggest that
560 there exists an appropriate level of Rax protein for proper Rax function in photoreceptor
561 gene transactivation. In addition, our observation that *Rax* ablation at the adult stage
562 resulted in cone photoreceptor loss suggests that even a relatively small amount of Rax
563 has an important *in vivo* function (Fig. 5).

564 We found that Rax and Crx proteins colocalized in the photoreceptor nuclei at P4
565 and were co-immunoprecipitated using cultured cells (Fig. 7A to C). Similar results
566 were observed in the study on the human RAX2 (22). In co-immunoprecipitation
567 analyses, the G137R and 140PGIns RAX2 proteins showed a reduced interaction with
568 CRX compared to wild-type RAX2. However, this study did not describe any
569 interaction between RAX and CRX. Since both Rax and Crx proteins are highly
570 conserved through evolution, our result that mouse Rax protein interacts with mouse
571 Crx protein suggests a possible interaction between Rax and Crx in other species as well
572 as in mice. Clarification of this hypothesis awaits future studies.

573

574 **Rax has a more critical role in cone maturation than in rod maturation**

575 We detected a significant increase of apoptosis in the *Rax* iCKO (P4→P14) retina
576 (Fig. 3T to Z). Various studies reported that a deficiency of a rod photoreceptor gene
577 causes rod photoreceptor degeneration including *Rhodopsin*, *Gnat1* and *Pde6g* (44-46).
578 The cone photoreceptor function loss 1 (*cpfl1*) mutation in *Pde6c* causes a lack of cone
579 function and cone photoreceptor degeneration that are associated with achromatopsia
580 (47). Although we observed a significant reduction of cone photoreceptors, the
581 number of rod photoreceptor cells were unchanged or only very slightly decreased.
582 This cone-specific cell death in the *Rax* iCKO (P4→P14) retina may be due to a drastic

583 reduction of cone photoreceptor proteins and a moderate reduction of rod photoreceptor
584 genes. Our results indicate that *Rax* has a modulatory function in rod gene expression
585 but a crucial function in cone gene expression in postnatal photoreceptors. While *Nrl*
586 together with other transcription factors including *Crx* transactivates rod genes, *Nrl* does
587 not participate in cone gene transactivation. Absence of *Nrl* in cone gene
588 transactivation might explain why *Rax* has a more essential role in cone maturation than
589 in rod maturation.

590

591 **Prospective studies on *Rax* and *Rax2* in photoreceptor maturation**

592 Our findings in the current study demonstrated that mouse *Rax* plays an essential
593 role not only in the proliferation of retinal progenitor cells, cone cell fate determination,
594 and Müller glia development, but also in the maturation and survival of photoreceptor
595 cells (12-14, 16). Previous studies seemed to propose that *Rax* mainly regulates
596 progenitor proliferation and *Rax2* mainly functions in photoreceptor maturation. This
597 idea may be supported partly because human *QRX/RAX2* protein (184 amino acid
598 residues) is poorly conserved with human *RAX* protein (346 amino acid residues)
599 except for the homeodomain. However, it should be noted that *Xenopus Rax1* and
600 Zebrafish *Rax (Rx1)* continues to be expressed in mature photoreceptors during
601 development (11, 17, 18). Thus, together with the current results on the *Rax* function
602 in mice containing a single *Rax* gene, the possibility that *Rax* and *Rax2* have a
603 redundant function in photoreceptor maturation rather than a separate function can be
604 proposed. Further studies in various species are needed to clarify and conclude
605 whether *Rax* and *Rax2* are functionally redundant or separate for photoreceptor
606 maturation. Identification of many more mutations in human *QRX/RAX2* and *RAX* will

607 be especially useful. For a future study, it is also important to elucidate how
608 expression level balance among multiple transcription factors is controlled in
609 photoreceptor development. We previously showed that *Rax* promotes the
610 differentiation of Müller glial cells (5, 14). In the present study, we observed that *Rax*
611 is expressed in Müller glial cells until 2M. What the role of *Rax* is in maturing Müller
612 glial cells is another interesting question to be addressed in the future.

613 **Author contributions**

614 S.I., R.S. and T.F. designed the project. T.F. and Y.M. generated the $Rax^{flox/flox}$ and
615 $Crx-CreERT2$ mice, and S.I. and Y.M. produced the Rax iCKO mice. S.I., K.K., R.S.
616 and T.C. carried out molecular and biochemical experiments. S.I., Y.M. and K.K.
617 performed histological and cell biological analysis. S.I. carried out ERG experiments.
618 S.I., R.S. and T.F. wrote the manuscript. T.F. supervised the project.

619

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800 **Figure legends**

801

802 **FIG 1** Expression and conditional inactivation of *Rax* in postnatal mouse retinas.

803 (A to D) Mouse retinal sections were immunostained with an anti-*Rax* antibody (red) at

804 E15.5 (A), P6 (B), P14 (C), and 2M (D).

805 (E) Schematic diagram of schedule for tamoxifen administration and harvest of retinas.

806 To conditionally inactivate *Rax* in postnatal photoreceptors, we treated *Rax*^{flox/flox};

807 *Crx-CreERT2* mice with tamoxifen at P4 and harvested them at P8.

808 (F to I) Retinal sections from *Rax* iCKO (P4 → P8) and control mice were

809 immunostained with antibodies against *Rax* (red, F and G) and *Lhx2* (Müller glia

810 marker, red, H and I). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer

811 nuclear layer; NBL, neuroblastic layer; PPL, presumptive photoreceptor layer; RPE,

812 retinal pigment epithelium. Scale bar represents 50 μ m.

813

814 **FIG 2** Expression of opsin genes decreases in the *Rax* iCKO retina.

815 (A to F) Whole-mount retinas from control and *Rax* iCKO (P4 → P8) mice were

816 immunostained with antibodies against S-opsin (magenta, A and B), M-opsin (green, C

817 and D) and Rhodopsin (blue, E and F). The inset shows S-opsin-, M-opsin- and

818 Rhodopsin-positive cells at high magnification. Scale bar represents 50 μ m.

819 (G to J) Comparison of photoreceptor protein levels in control and *Rax* iCKO (P4 → P8)

820 retinas. Western blots of *Rax*, Rhodopsin and *Crx* in the retina are shown (G).

821 β -actin was used as a loading control. The signal intensities of *Rax*, Rhodopsin and

822 *Crx* proteins are shown, respectively (H to J). Data are means $\pm$ S.D. (n=3). **P <

823 0.01, n.s., not significant. Values of *p* by Student's t-test.

(K to M) The number of cone photoreceptor cells was unaltered in the *Rax* iCKO (P4→P8) retina. Retinal sections from control (K) and *Rax* iCKO (P4→P8) (L) mice were immunostained with an anti-TRβ2 antibody (red, rabbit), a marker for developing cone photoreceptor cells. Scale bar represents 50 μm. The number of TRβ2-positive cells is shown (M). Data are means ± S.D. (n=4). n.s., not significant. Values of *p* by Student's t-test. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

831

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 them at P20.

(B) Expression levels of photoreceptor genes in *Rax* iCKO (P4→P20) retinas were analyzed by qPCR. Each expression level was normalized to the expression level of a housekeeping gene, *Gapdh*. The mean value of each control was set as 1.0. Error bars show ±S.D. (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). Scale bar represents 50 μm.

(I to S) Reduction of cone photoreceptor cells in the *Rax* iCKO (P4→P20) retina. Whole-mount retina from control and *Rax* iCKO (P4 → P20) retinas were immunostained with the antibody against S-opsin (magenta, I and J) or M-opsin (green, K and L). Retinal sections were immunostained with an anti-Pikachurin antibody (a

848 synaptic marker, green, M and N). Cone pedicles were stained with PNA (magenta, O
849 and P). (Q), Merge of (M) and (O). (R), Merge of (N) and (P).
850 Higher-magnification views of (Q) and (R) are shown in (Q') and (R'), respectively.
851 Arrowheads indicate cone pedicles. Scale bar represents 50 μ m. The number of cone
852 pedicles decreased in the *Rax* iCKO (P4→P20) retina (S). Data are means $\pm$ S.D.
853 (n=3). **P < 0.01. Values of *p* by Student's t-test.
854 (T) Schematic diagram of schedule for tamoxifen administration and harvest of retinas.
855 We treated *Rax*^{flax/flax}; *Crx-CreERT2* mice with tamoxifen at P4 and harvested them at
856 P14.
857 (U to Z) Retinal sections from control (U) and *Rax* iCKO (P4→P14) (V) mice were
858 immunostained with an anti-TR β 2 antibody (magenta, guinea pig), a marker for
859 developing cone photoreceptor cells. Scale bar represents 50 μ m.
860 (W and X) TUNEL staining of retinas from control (W) and *Rax* iCKO (P4→P14) (X)
861 mice. The number of TR β 2-positive cells is shown (Y). Data are means $\pm$ S.D.
862 (n=3). *P < 0.05. TUNEL-positive cells in control and *Rax* iCKO (P4→P14)
863 retinas were counted (Z). Data are means $\pm$ S.D. (n=3). **P < 0.01, n.s., not
864 significant. Values of *p* by Student's t-test. INL, inner nuclear layer; ONL, outer
865 nuclear layer; OPL, outer plexiform layer.

866

867 **FIG 4** ERG analysis of *Rax* iCKO and control mice.

868 (A to F) ERGs were recorded from *Rax* iCKO (P4→2M) mice. Scotopic ERGs
869 elicited by four different stimulus intensities are shown (A). The amplitudes of the
870 scotopic ERG a-wave (B) and the b-wave (C) are shown as a function of the stimulus
871 intensity. Photopic ERGs elicited by four different stimulus intensities are shown (D).

872 The amplitude of the photopic ERG a-wave (E) and b-wave (F) are shown as a function
873 of the stimulus intensity. Blue lines indicate control (n=3) and red lines indicate *Rax*
874 iCKO (n=3). Data are means $\pm$ S.D.. **P < 0.01, *P < 0.05. Values of *p* by
875 Student's t-test.

876

877 **FIG 5** *Rax* is required for maintenance of cone photoreceptors.

878 (A to B') Expression of *Rax* in the *Rax*^{flax/flax} mouse retina at 1M was detected by *in situ*
879 hybridization using an antisense probe (A) or a sense probe (B) for *Rax*. The white
880 box in the left panels was magnified in right panels (A', B', respectively). Scale bar
881 represents 100 μ m.

882 (C to J) whole-mount retinas from control and *Rax* iCKO (1M $\rightarrow$ 2M) mice were
883 immunostained with antibody against S-opsin (magenta, C and D, G and H) or M-opsin
884 (green, E and F, I and J). Ventral (C to F) and dorsal regions (G to J) are shown.
885 Scale bar represents 100 μ m.

886 (K to M) ERGs were recorded from *Rax* iCKO (1M $\rightarrow$ 2M) mice. Photopic ERGs
887 elicited by four different stimulus intensities are shown (K). The amplitude of the
888 photopic ERG a-wave (L) and b-wave (M) are shown as a function of the stimulus
889 intensity. Blue lines indicate control (n=5) and red lines indicate *Rax* iCKO (n=3).
890 Data are means $\pm$ S.D.. **P < 0.01, *P < 0.05. Values of *p* by Student's t-test.
891 GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE,
892 retinal pigment epithelium.

893

894 **FIG 6** Transactivation of *Rhodopsin*, *S-opsin*, and *M-opsin* promoters by *Rax*, *Crx*
895 and/or *Nrl*.

896 (A to H) Luciferase reporter assay using human *Rhodopsin*, *S-opsin* and *M-opsin*
897 promoter-luc constructs. Schematics of luciferase assay promoter construct are shown
898 (A). NIH3T3 cells were co-transfected with 0.4 μ g of the human *Rhodopsin* promoter
899 (–181 to +49)-luc construct together with 0.5 μ g *Rax*, *Crx* and *Nrl* expression plasmids
900 (B and C). Luciferase activity was corrected for transfection efficiency using a
901 β -galactosidase internal control (0.3 μ g) and shown as fold change, which was
902 calculated as the ratio of each combination to the reporter plasmid with an empty vector.
903 Data are means $\pm$ S.D. (n=4). Same as (B and C), except that the reporter plasmid
904 was human *S-opsin* promoter (–3768 to +1)-luc construct with 0.5 μ g *Rax* expression
905 plasmid (D). Data are means $\pm$ S.D. (n=3). Same as (D), except that the reporter
906 plasmid was human *M-opsin* promoter (–212 to +79)-luc construct (E). Data are
907 means $\pm$ S.D. (n=3). NIH3T3 cells were co-transfected with the human *Rhodopsin*
908 promoter-luc reporter construct plus the *Crx* and *Nrl* expression plasmids (0.25 μ g) and
909 the *Rax* expression plasmid (0, 0.25, 1 and 4 μ g) (F), or the human *S-opsin* promoter-luc
910 reporter construct plus the *Crx* expression plasmid (0.25 μ g) and the *Rax* expression
911 plasmid (0, 0.25, 1 and 4 μ g) (G), or the human *M-opsin* promoter-luc reporter construct
912 plus the *Crx* expression plasmid (0.25 μ g) and the *Rax* expression plasmid (0, 0.25, 1
913 and 4 μ g) (H). NIH3T3 cells were co-transfected with the human
914 *Rhodopsin-PCE-1-mut1* promoter-luc reporter construct plus the *Crx* and *Nrl* expression
915 plasmids (0.25 μ g) and the *Rax* expression plasmid (0, 0.25, 1 and 4 μ g) (I), or the
916 human *S-opsin-PCE-1-mut6* promoter-luc reporter construct plus the *Crx* expression
917 plasmid (0.25 μ g) and the *Rax* expression plasmid (0, 0.25, 1 and 4 μ g) (J), or the
918 human *M-opsin-PCE-1-mut1* promoter-luc reporter construct plus the *Crx* expression
919 plasmid (0.25 μ g) and the *Rax* expression plasmid (0, 0.25, 1 and 4 μ g) (K). *Rax*, *Crx*,

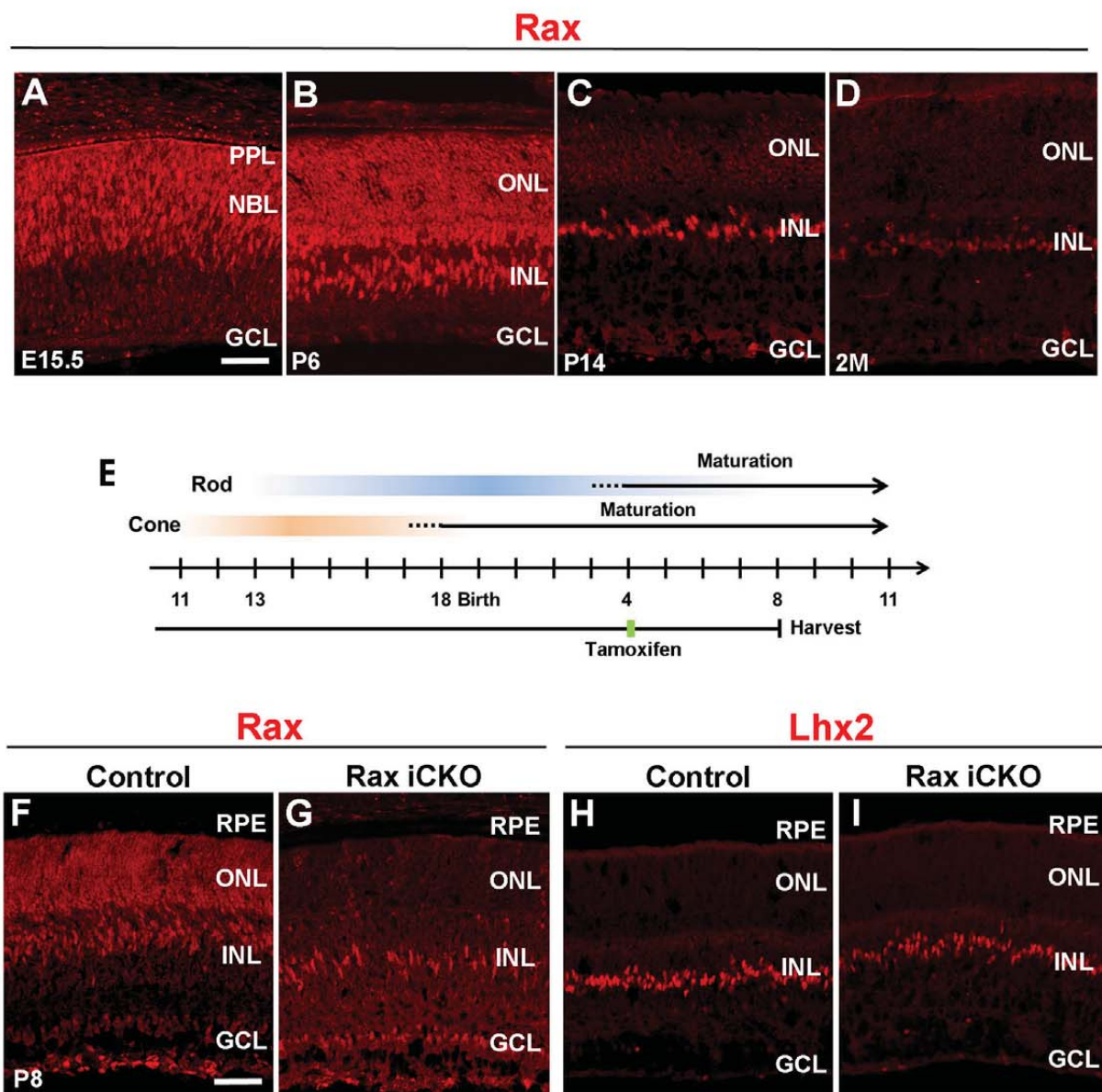
920 and Nrl expression levels were determined by Western blotting after normalization
921 using a β -galactosidase internal control. Data are means $\pm$ S.D. (n=3). **P < 0.01,
922 *P < 0.05 and n.s., not significant were calculated by Tukey-Kramer multiple
923 comparison test. ^{##}P < 0.01 and [#]P < 0.05 were calculated by Student's t-test.

924

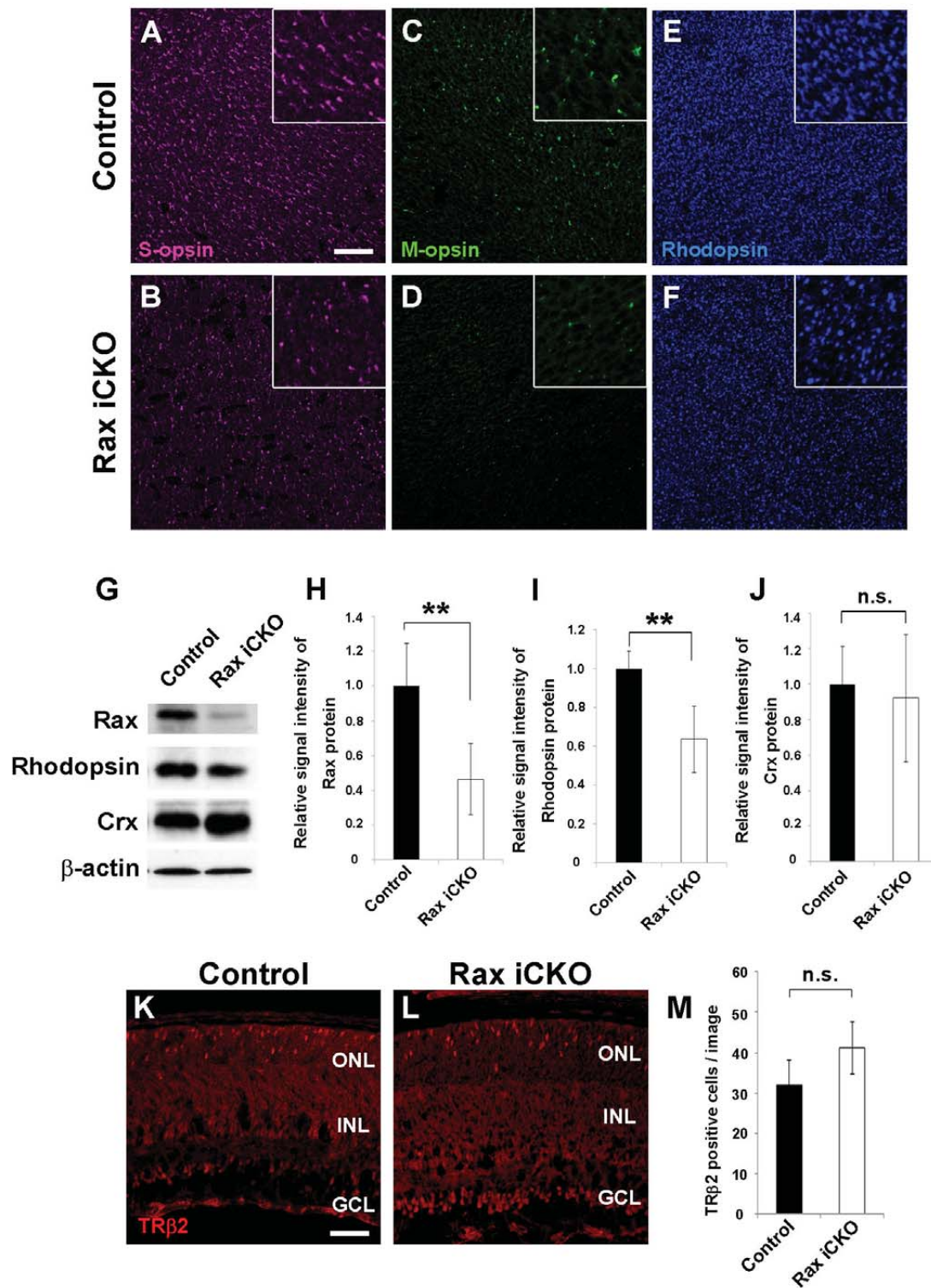
925 **FIG 7** Rax protein physically associates with Crx protein.

926 (A to C) Rax co-localizes with Crx in the postnatal retina. Retinal sections at P4 were
927 immunostained with the anti-Rax (magenta, A) and the anti-Crx antibodies (green, B).
928 (C), Merge of (A) and (B). The inset shows Rax- and Crx-double-positive cells at high
929 magnification. Scale bar represents 50 μ m.

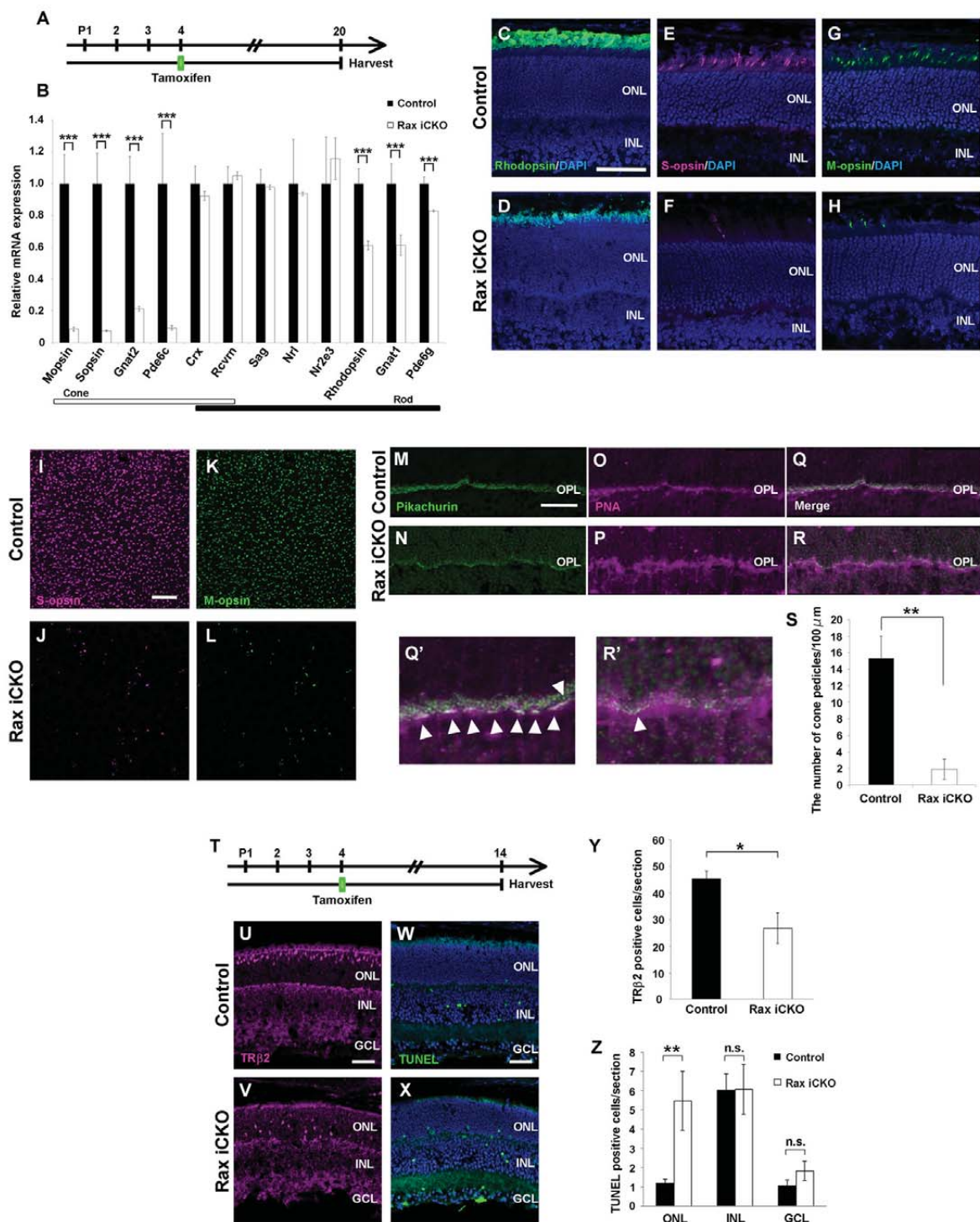
930 (D and E) Rax protein interacts with Crx protein. The HA-tagged Rax expression
931 plasmid was transfected with the FLAG-tagged Crx expression plasmid into HEK293T
932 cells. Cell lysates were incubated with either an anti-FLAG antibody (D) or an
933 anti-HA antibody (E). Immunoprecipitated proteins were analyzed by Western blot
934 analysis. Lanes 5-8 are input proteins without immunoprecipitation (IP). An arrow
935 indicates immunoprecipitated protein by the anti-FLAG antibody, and an arrowhead
936 indicates immunoprecipitated protein by the anti-HA antibody. IB, immunoblot.
937 NBL, neuroblastic layer.



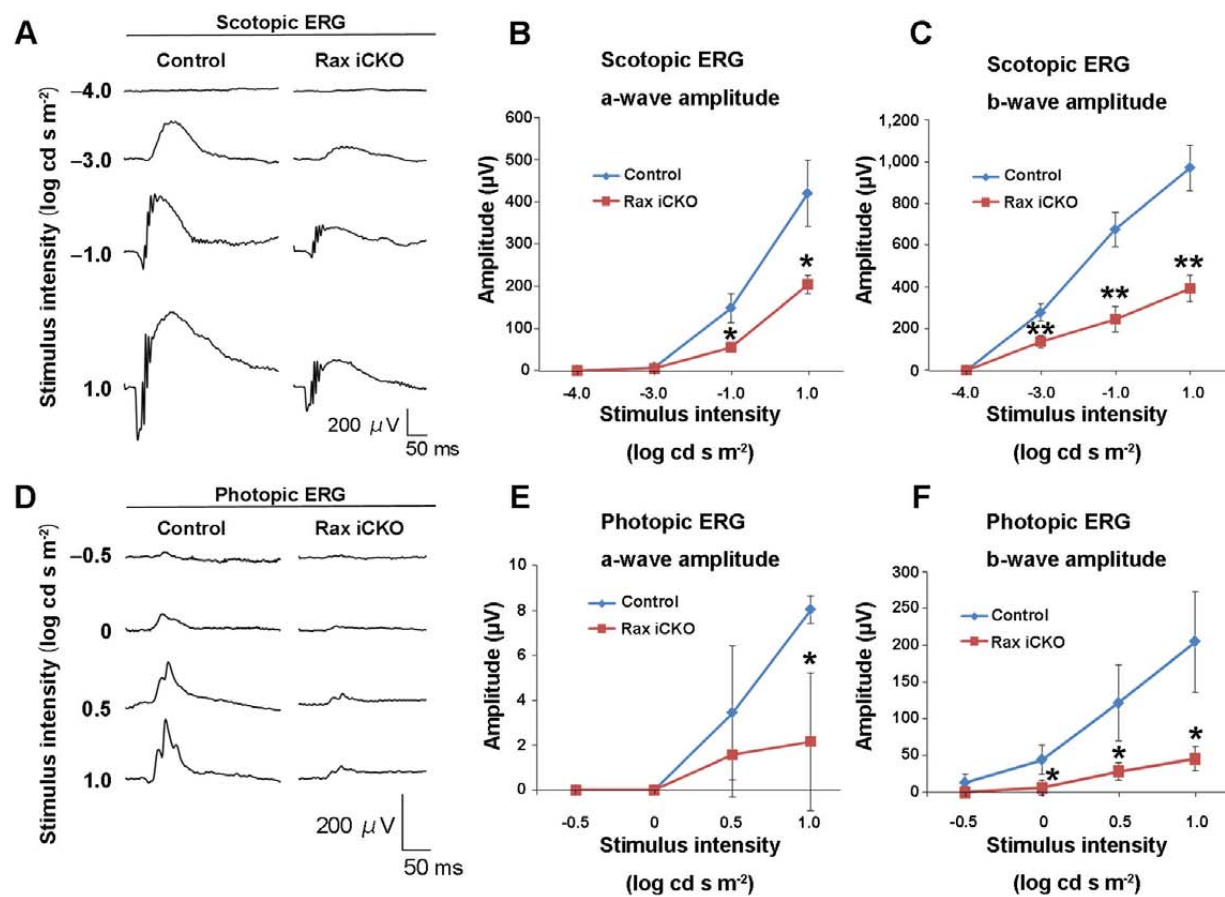
Irie et al., Figure 1



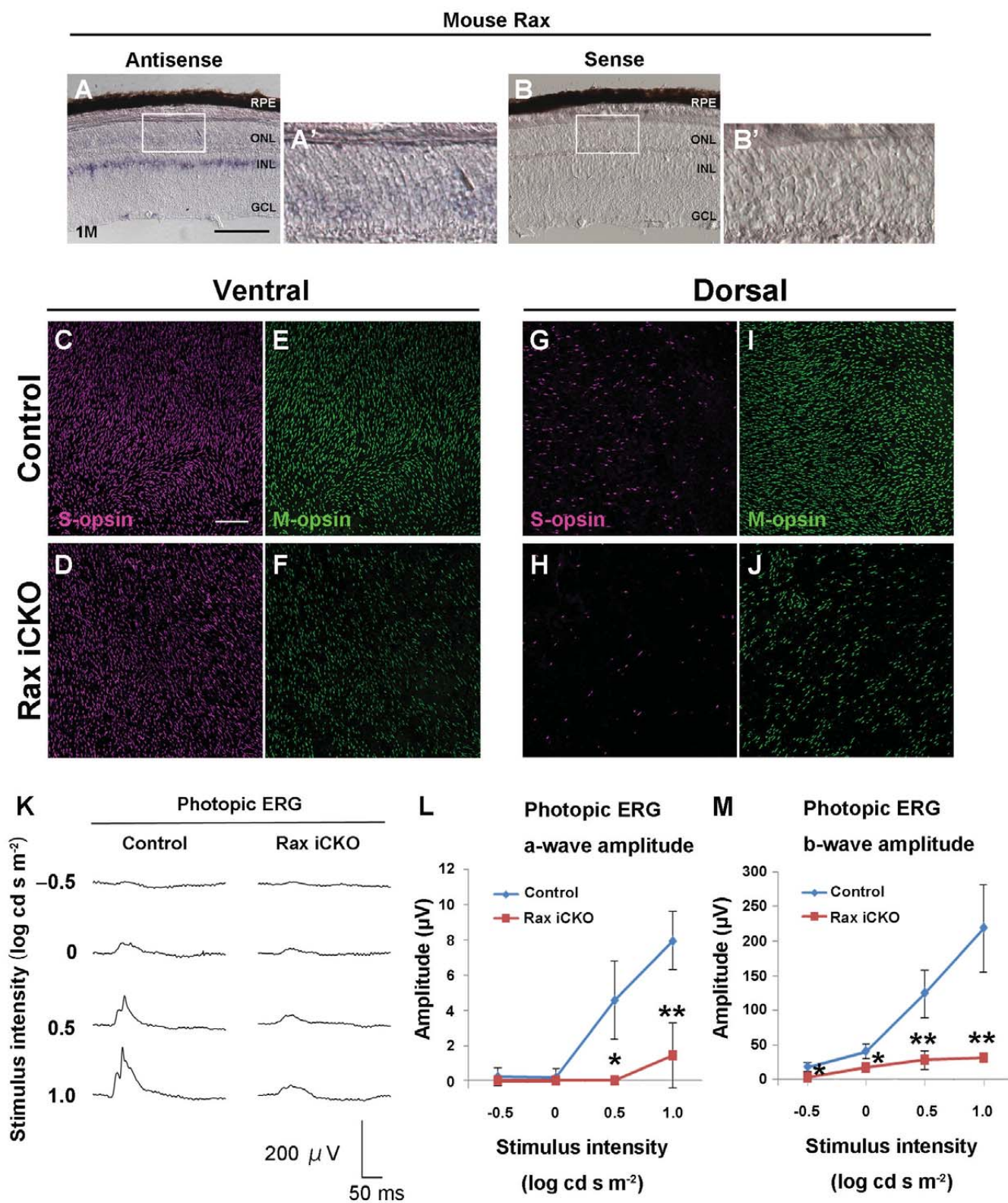
Irie et al., Figure 2



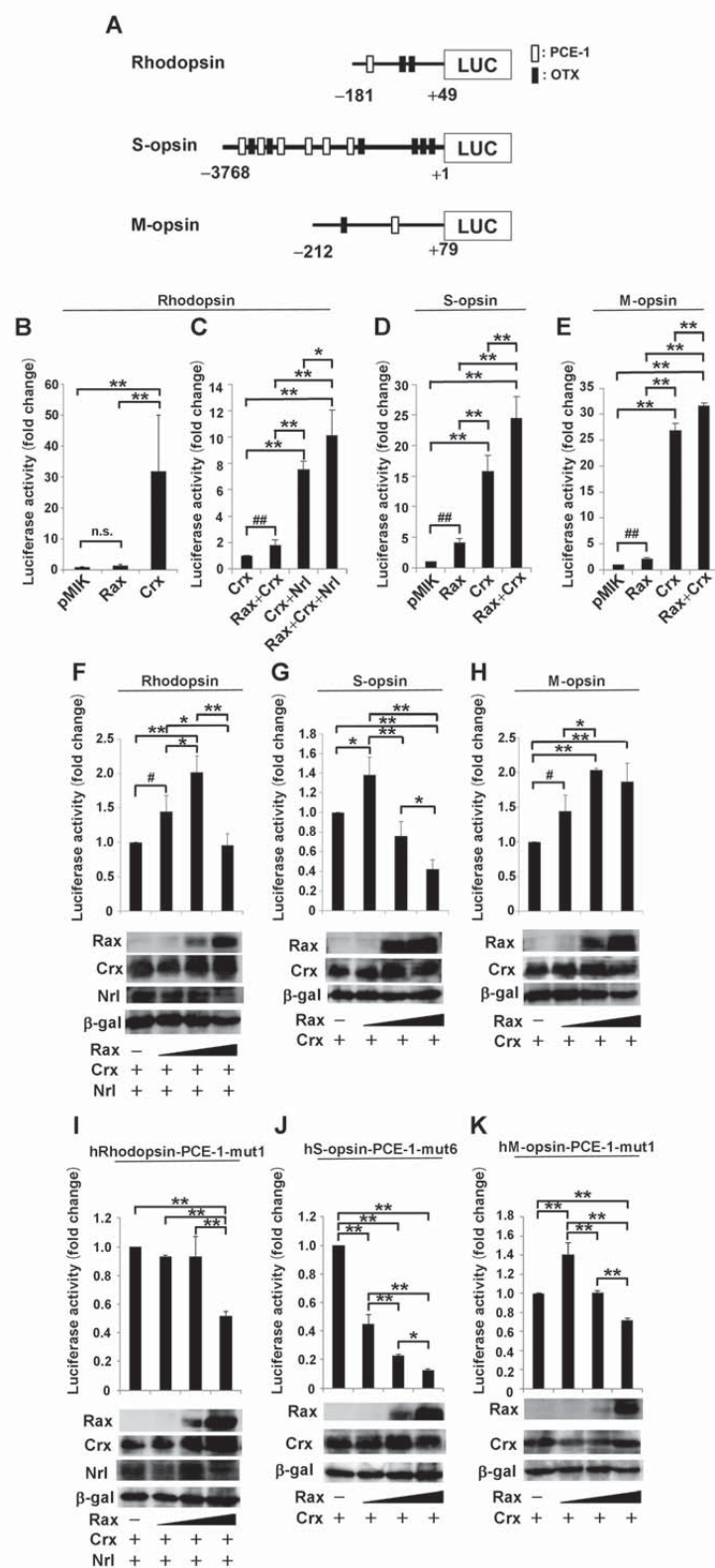
Irie et al., Figure 3



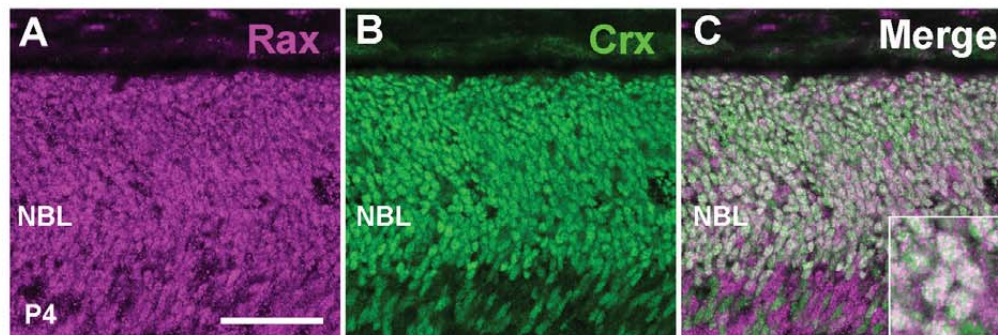
Irie et al., Figure 4



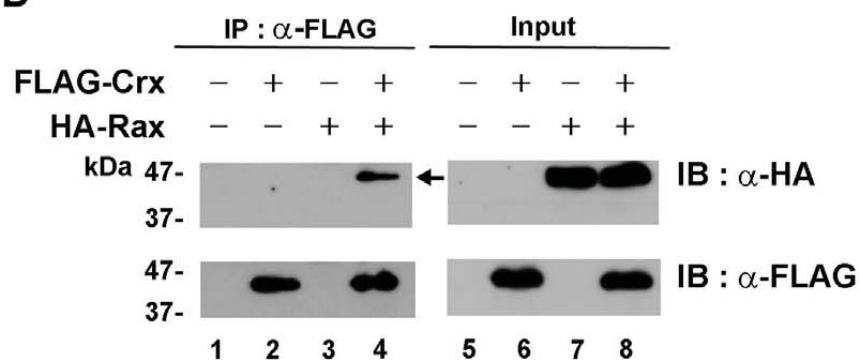
Irie et al., Figure 5



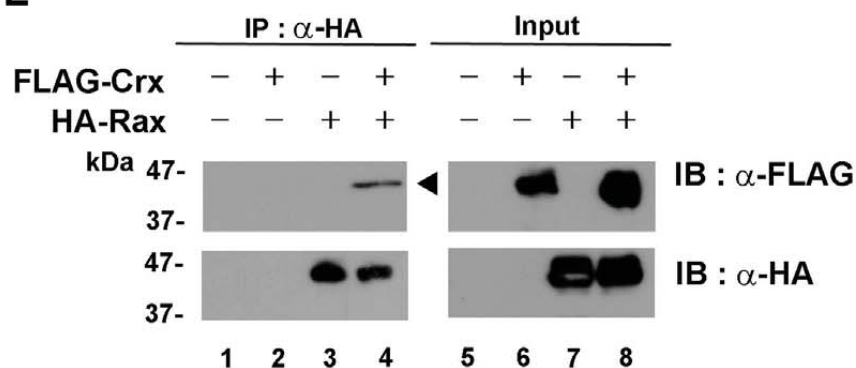
Irie et al., Figure 6



D



E



Irie et al., Figure 7

Table 1. Primer sequences used for q-PCR analysis

Gene		Primer sequence (5'-3')
M-opsin	sense	TTGCTTTGCCACTGCTCG
	anti-sense	CTGTGGCCCAGACGTGTTC
S-opsin	sense	GCTGGACTTACGGCTTGTCAAC
	anti-sense	TGTGGCGTTGTGTTTGCTGC
Gnat2	sense	ATGCTGACAAGGAAGCCAAGACTG
	anti-sense	GACAGACTTGAACCTAGGCACTC
Pde6c	sense	GATCAACGTGATTCCGTCACCTCC
	anti-sense	AGTCTCATCTACCGGTCCTTTCTG
Crx	sense	CACCAGGCTGTCCCACTC
	anti-sense	TCGCCCTACGATTCTTGAAC
Revrn	sense	GAGTACGTGATTGCTCTGCACATG
	anti-sense	AGGAGTTTCACATCCTCAGGCTTG
Sag	sense	AGCCTGCTCAAGAACTGGGAGACA
	anti-sense	AGCTGGCTGCAACATCACTGAACA
Nrl	sense	GCTGTGCCTTTCTGGTTCTGA
	anti-sense	GCTCCCGCTTTATTTGAACT
Nr2e3	sense	TTGATGTCACCAGCAATGACC
	anti-sense	TTTTGGCCCATTTGACAGC
Rhodopsin	sense	GCTTCCCTACGCCAGTGTG
	anti-sense	CAGTGGATTCTTGCCGAG
Gnat1	sense	CGCTCAACATTCAATATGGAGA
	anti-sense	CGAAGTAGCGATGATTGCAGATG
Pde6g	sense	CAAACAAGGCAGTTCAAGAGCAAG
	anti-sense	CAGGGATCTGACTAAATGATGCCA
GAPDH	sense	ACTGGCATGGCCTTCCGTGTTCTA
	anti-sense	TCAGTGTAGCCCAAGATGCCCTTC