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2.1 Dicer CKO mice

*Immunocytochemistry*

The eyes of mice aged P2, P16, P30, P45 and 3, 5 and 7 months were harvested, quickly enucleated, and immersion fixed for 1 h in 4% PFA in 0.1 M phosphate buffer (PB). The eyes were then rinsed in buffer, infiltrated overnight in 30% sucrose in 0.1 M PB, embedded in OCT/Tissue Tek (Sakura Finetek, Zoeterwoude, The Netherlands), and frozen on a cryostat stage at -25/-30°C. Eyes were sectioned vertically in 12–16 μm serial sections with a Leica cryostat (Wetzlar, Germany). Sections were collected on Superfrost Plus slides and air dried for 5 min to 2 h. Slides were rinsed for 10 min with 0.01 M PBS and blocked for 2 h in a solution containing 5% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS. Primary antibodies were diluted in 1% BSA and 0.1% Triton X-100 in PBS. Slides were incubated in primary antibodies overnight at 4°C. Primary antibodies were washed three times for 10 min in PBS at room temperature. Sections were then incubated for 1–2 h in solutions containing appropriate secondary antibodies, diluted 1:400, and conjugated with Oregon Green 488, Alexa Fluor 568, Alexa Fluor 647 (Invitrogen), or Cy-3 (Sigma). Sections were then rinsed in PBS and counterstained with the fluorescent nuclear dyes BOBO-1 or TOTO-3 (Invitrogen). Slides were then mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA)

Primary antibodies and dilutions were as follows: mouse anti-rhodopsin (1:2500; Sigma); rabbit anti-recoverin (1:2000; Chemicon, Temecula, CA, USA); mouse and rabbit anti-protein kinase C-α [PKC-α; 1:1000, clone MC5 (Sigma); and sc-208 (Santa Cruz Biotechnology, Santa Cruz, CA, USA)]; mouse and rabbit anti-calbindin D-28k (1:1000, clone CB955; Sigma and 1:2000, Swant, Bellinzona, Switzerland); mouse anti-G0α (1:1000; MAB 3073; Chemicon); mouse anti-neurofilament 200 kDa (1:100, clone N52; Sigma); rabbit anti-mGluR6 (1:2000; from Dr. S. Nakanishi, Osaka University, Osaka, Japan); mouse anti-postsynaptic density protein
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95 (PSD95; 1:500; AbCam, Cambridge, UK); mouse anti-glutamine synthase (1:2000, MAB302; Chemicon); rabbit anti-glial fibrillary acidic protein (GFAP; 1:1000; Sigma); goat anti-choline acetyl transferase (1:500; Chemicon); mouse anti-Cre recombinase (1:1000, MAB3120; Chemicon); rabbit anti-green fluorescent protein (GFP–Alexa Fluor 488 conjugate (1:1000; Invitrogen); rabbit anti-phosphohistone H3 (1:400; Millipore, Billerica, MA, USA); rabbit anti-atypical PKC (C20, 1:500; Santa Cruz Biotechnology); rabbit anti-zonula occludens-1 (ZO-1; 1:200; Zymed, San Francisco, CA, USA); rabbit anti-laminin (1:400; Sigma); guinea pig anti-vesicular glutamate transporter (vGLUT1, 1:500; Chemicon); and rabbit anti-vesicular GABA transporter (vGAT; 1:500; Synaptic System, Göettingen, Germany). Rabbit anti-S-cone opsin (1:1000; Chemicon) and Alexa Fluor 488-PNA lectins (1:400; Invitrogen) were used to label cone photoreceptors.

Retinal preparations were examined with a Leica TCS-NT confocal microscope equipped with an argon–krypton laser or with a Leica TCS-SL spectral confocal microscope using high numerical-aperture (over 1.00) oil immersion objectives. Images acquired at a resolution of 1024 x 1024 pixel were saved as TIFF files and exported on a workstation for offline analysis.

Retinas from at least one wild-type, one heterozygote, and three CKO littermates for each age group (2, 16, 30, and 45 days and 3–5 and 7 months) were used for ICCH and screened with the full panel of antibodies listed above. A total of 57 animals were analyzed for ICCH. Except for P2 and P16 animals, eyes used for morphological analysis were from animals previously used for ERG recordings described above.

In situ hybridization

Whole mouse eyes were fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS), infiltrated with 30% sucrose, frozen, and sectioned as described below. A 260-nt riboprobe (Harris et al., 2006), specific to the floxed Dicer exon 23, was used to assess the distribution of the wild-type transcript.

After a brief wash in PBS, retinal sections were treated twice for 10 min with radioimmunoprecipitation assay (RIPA) buffer, post-fixed 15 min in 4% PFA/PBS, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine. Slides were then
prehybridized for 2 h in a solution of 50% formamide, 5x SSC, 5x Denhart’s, 500 μg/ml salmon sperm DNA, and 250 μg/ml yeast RNA and hybridized overnight at 48 °C, with 100–300 ng of digoxigenin-labeled probe in a plastic chamber humidified with 50% formamide in 5x SSC. Post-hybridization washes were performed in 50% formamide and 0.1% Tween-20 in 2x SSC for 2 h at the same temperature of hybridization.

Sections were then re-equilibrated in MABT (0.1% Tween 20 in 0.1 M maleic acid buffer), blocked for 1–2 h in 10% sheep serum in MABT, and incubated overnight at 4°C with an anti-digoxigenin polyclonal antibody conjugated with alkaline phosphatase (anti-digoxigenin-AP; 1:2000 in blocking buffer; Roche, Basel, Switzerland). After MABT washing, the pH of the specimens was adjusted with AP buffer (100 mM Tris, pH 9.5, 50 mM MgCl₂, and 100 mM NaCl).

Retinal sections were subsequently incubated for 1–2 h with NBT-BCIP (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) solution (Sigma) for chromogenic reaction of alkaline phosphatase.

Finally, they were washed two or three times for 10 minutes in PBS, rinsed in distilled water, let air-dried and mounted in DPX, and acquired with a Zeiss Axioplan microscope (Jena, Germany), equipped with a Nomarsky filter and an AxioCam HRC color camera with dedicated AxioVision software.

For in situ hybridization (ISH) on retinal specific miRNAs, we used the protocol described above, with minor modifications. Slides were processed using 5’-DIG labelled LNA modified probes (miRCURY LNA detection probes, Exiqon) against miR-182 and miR-183 (mmu-miR-182 and mmu-miR-183, respectively). As a control, ISH protocol was performed on slides by using Scrambled probes (miRCURY LNA control detection probes, Exiqon), as recommended by manufacturer. In addition, slides were hybridized ON at RT. Anti-DIG antibody reaction was performed O.N. at RT.

**Electron microscopy**

One litter composed of four mutants and five wild-type animals aged P2 was used for EM studies of immature retinas. After decapitation, the eyes were removed and immersion fixed in 2% PFA and 2.5% glutaraldehyde for 12 h. After dissections, retinal tissue was
postfixed in osmium tetroxide, bloc stained with 1% uranyl acetate, dehydrated in ethanol, and embedded in plastic. Semithin sections (1–2 μm thick) were stained with Epoxy Tissue stain (EMS, Hatfield, PA) and observed at the light microscope. Ultrathin sections from retinal blocs were counterstained with uranyl acetate and lead citrate and examined with a Jeol (Tokyo, Japan) 1200 EXII electron microscope. Photographs of retinal neuroblasts and differentiating photoreceptors were taken at 8000–20,000x.

*Bromodeoxyuridine administration*

Three intraperitoneal injections of 50 mg/kg bromodeoxyuridine (BrdU) were performed at 3 h intervals in postnatal day 35 (P35) mice (n = 5). Mice were then killed 3 h after the final injection, their eyes were prepared as described above, and BrdU was detected by immunocytochemistry (ICCH) on frozen retinal sections following the procedure of Close et al. (2005).

*Electrophysiology*

Mice were dark adapted overnight in a Faraday shielded room, and all subsequent procedures were performed under dim red light (over 650 nm). The corneas of the mice were given some drops of 1% atropine, 2.5% phenylephrine, and 0.5% proparacaine (Akorn, Buffalo Grove, IL) in order to obtain mydriasis and topical corneal anesthesia.

The mice were then anesthetized by intraperitoneal injection (6 μl/g) of a sterile mixture of 100 mg/ml ketamine, 20 mg/ml xylazine, and normal saline at a 1:1:5 ratio, respectively. When the mice were fully sedated, they were placed onto a heated electroretinogram (ERG) platform in ventral recumbency. Balanced salt solution (Alcon, Ft. Worth, TX) and Gonak (Akorn) were used to lubricate the cornea before electrode placement. Custom platinum loop electrodes were positioned on the cornea using a three-axis micro-positioning system (Narishige, Tokyo, Japan). Grass (West Warwick, RI) platinum subdermal needle electrodes were used for ground and reference. All ERGs were performed using a Multiliner Vision (Jaeger/Toennies, Hochberg, Germany) system equipped with a Ganzfeld stimulator (Jaeger/Toennies). The Multiliner Vision apparatus complies with and exceeds the relevant International Society for Clinical Electrophysiology
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of Vision standards. Unless otherwise indicated, all mice were tested for dark-adapted ERG responses followed by light-adapted ERG responses. White light from a xenon source was used as the stimulus. For the scotopic (dark-adapted) ERG series, mice were exposed to a series of 10 flashes at 0.1 cd s/m2. For the photopic (light-adapted) ERG series, mice were exposed to background light at 100 cd/m2 for 1 min, and ERG recordings were done in the presence of a constant 100 cd/m2 background light. Mice were then exposed to a series of 50 flashes at 10 cd · s/m2. Right and left eyes were tested simultaneously, and the data were recorded. Analysis of the ERG waveforms for a- and b-wave maxima was performed using the Multiliner Vision software. Statistical analysis of data was performed calculating significance with a Student’s t test by using SigmaPlot software.

Northern blot

Retinas from 1 month (n = 3 mutant; n = 6 wild type), 3 month (n = 3 mutant; n = 2 wild type) and 24 month (n = 2 mutant; n = 3 heterozygous; n = 2 wild type) animals were pooled. RNA was isolated from fresh homogenized pooled retinas with TRI-Reagent (Sigma, St. Louis, MO). Approximately 12 µg of total RNA for the 1 and 3 month time points and about 8 µg of total RNA from 24 month animals was resolved on 10% urea/polyacrylamide gels and electroblotted to Hybond N+ membranes (GE Healthcare, Little Chalfont, UK) at 200 mA for 3 h. Blots were cross-linked using a Stratalinker (Stratagene, La Jolla, CA) and prehybridized for at least 1 h at 37 °C in ULTRAhyb-Oligo (Ambion, Austin, TX) hybridization buffer before overnight incubation at 37 °C in hybridization buffer containing the [32P]-end-labeled probe. Probes were generated by end-labeling 20 pmol of DNA oligonucleotide (Invitrogen, Carlsbad, CA) complementary to a specific miRNA or U6 with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) and 250 Ci of [32P] ATP (PerkinElmer, Waltham, MA) followed by purification with MicroSpin G-25 columns (GE Healthcare). Blots were washed (2x SSC and 0.1% SDS) at 37°C for 30 min followed by two 30 min washes at room temperature. Blots were exposed to Kodak (Rochester, NY) BioMax film, and quantification was determined by densitometry of autoradiographs using ImageQuant TL version 2003.02 software. For
subsequent probing, blots were stripped by incubating twice in boiling 1% SDS for 15 min each and were exposed to film to confirm that probes were removed.

In vivo microRNA knockdown

Wild type C57BL/6J animals aged P23 were anesthetized with intraperitoneal injections of Avertin (0.1 ml/5 g). Mice were intravitreally injected in one eye with 1 µM 5’-fluorescein labelled LNA-modified antisense probes against miR-182 or miR-183 (miRCURY LNA Knock-Down probes; Exiqon). As a negative control, contralateral eyes were injected with the same amount of probes with similar length and composition and no homology to any known microRNA or mRNA sequences in mouse (control, miRCURY LNA Knock-Down probes, Exiqon). Total injecting volume was 500 nl per eye. After a week, mice were sacrificed and eyes were processed as previously described for immunocytochemistry for retinal specific markers. The following antibodies were used: PKCα (rod bipolars), GS (Muller glia), calbindin (horizontal and some amacrine cells). Slides were successively counterstained with TOTO-3 nuclear staining (Invitrogen) and mounted in Vectashield (Vector Laboratories) for confocal microscopy.
2.2 Retinal ganglion cells in rd1/Thy-1 GFP

Animals

Experimental procedures were in accordance with institutional guidelines and with the Association for Research in Vision and Ophthalmology statement for the use of animals in research. All mice were kept in a local facility with water and food ad libitum, in a 12 h light/dark cycle, with illumination levels <60 lux. C3H/HeJ mutants (from here on, rd1 mice) and C57BL/6J wild-type controls (wt) were obtained from Charles River Laboratories. Mice of the B6.Cg-Tg(Thy1-GFP-M)JRS/RHM strain were a kind gift from R. H. Masland (Harvard Medical School, Boston, MA) and were homozygous for the Thy1-GFP allele. The last were derived by breeding from the B6.Cg-Tg(thy1-GFP)/J strain originally devised by J. R. Sanes (Feng et al., 2000) and will be referred to as Thy1-GFP-M from here on. In these homozygous animals (Thy1-GFP/Thy1-GFP), a small number of were used for this study. Transgenic mice of the Thy1-GFP-M strain, aged 3–9 months, were used as a set of founders. A new line of mice, C3H/HeJ /Tg(Thy1-GFP-M)JRS/RHM, from here on named rd1/Thy1-GFP-M, was obtained by crossing rd1 with Thy1-GFP-M mice. Thy1-GFP-M mice were first crossed with homozygous rd1/rd1 animals. Individuals obtained from the first generation (F1) were backcrossed with rd1/rd1 animals obtaining the F2. Genotyping was performed by PCR on tail-extracted DNA of F2 individuals to identify Thy1-GFP-positive animals. The following primers were used:

- Thy1-GFP forward (F) (AAGTTTCATCTGCACCACCG) and
- Thy2-GFP reverse (R) (TCCTTGAAGAAGATGGTGCG),

following a protocol recommended by The Jackson Laboratories. The PCR amplification of the corresponding 173 bp fragment was performed in 35 cycles by denaturation at 94°C for 1.5 min; annealing at 94, 61, and 72°C, respectively, for 30 s, 1 min, and 1 min; and elongation at 72°C for 2 min.

To identify mice homozygous for the rd1 mutation among Thy1-GFP-M-positive individuals, a second PCR was performed. In this case, the primers were as follows:

- rd 81 (F) (ACCTGCATGTGAACCCAGTATT);
- rd2 (F) (AGAAGAAGTAGAATCAGTGAAATAGAT);
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rd 82 (R) (ACCCATGTCCTACAGCCCCTCTCCAA).

The corresponding PCR amplification was performed in 30 cycles by denaturation at 94°C for 3 min; annealing at 94, 60, and 72°C, respectively, for 1 min, 30 s, and 1 min, and elongation at 72°C for 7 min.

The homozygous rd1 mutation is revealed by the presence of a single band having a size of 650 bp, while in rd1/wt heterozygous mice a second 247 bp band is amplified too.

**GFP immunocytochemistry**

rd1/Thy1-GFP-M mice, aged 1, 4, 5, 9 and 12 months were anesthetized with intraperitoneal injections of Avertin (0.1 ml/5 g) and killed by cervical dislocation on eye removal. Eyes were quickly enucleated, a reference on the dorsal pole was taken with a lab marker, and a small cut was made at the corneal margin before immersion in fixative [4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB), pH7.4] for 1 h, at 4°C. Subsequently, the anterior segment of the eye was removed and the retina separated from the pigment epithelium (still maintaining a reference at the dorsal pole) and flattened by making four radial cuts toward the optic nerve head. Routinely, retinas were infiltrated for several hours or O.N. in 30% sucrose in 0.1 M PB, frozen in OCT TissueTek, and stored at -20°C. On use, retinal samples were brought to room temperature, washed extensively in 0.1 M PB, and blocked overnight in a solution with 0.5% Triton X-100, 10% rabbit serum, 5% BSA in PBS 0.01M, pH 7.4 (Sigma-Aldrich) at 4°C. Then, retinas were incubated in a 1:500 solution of rabbit anti-GFP-Alexa Fluor 488 (Invitrogen), with 0.1% TritonX-100, 1% rabbit serum, 1% BSA in PBS, for 2 days at 4°C, to enhance the GFP signal. Retinal specimens were then rinsed three times for 15 min each time in PBS and incubated in a solution of RNase A (Invitrogen; 1:1000 in PBS) at 37°C for 1 h. After rinsing in PBS, retinas were stained with 2 µM ethidium homodimer-1 (Invitrogen) for 1 h at room temperature on a rotary shaker. This allowed fluorescent staining of nuclei necessary to locate the boundaries between retinal layers. Finally, retinas were rinsed extensively in PBS and mounted on glass slides with Vectashield (H1000; Vector Laboratories), “ganglion cells up.” Retinal preparations were coverslipped, sealed with nail polish, and inspected.
with a Zeiss Axioplan fluorescence microscope (Carl Zeiss), using 5 or 10X objectives. GFP-positive RGCs were localized; when necessary for cell retrieval, low-power images of the whole retinas were taken with a Zeiss Axiocam color camera. Subsequently, well isolated RGCs were scanned with a Leica TCS-SL spectral confocal microscope (Leica Microsystems) equipped with an helium-neon laser, at resolutions of 1024 x 1024 pixels. Images were obtained using a 40x HCX PL APO 1.25 oil objective. Z-stacks were obtained encompassing the optic fiber, ganglion cell (GC), inner plexiform and innermost part of the inner nuclear layers. The distance between adjacent focal planes was set at a constant value (1.0042 μm). Image files were saved in export format and analyzed off-line with MetaMorph (version 5.0r1 MetaMorph; Molecular Devices), to perform three-dimensional reconstructions of single cells and to measure their dendritic tree and body areas. A total number of 50 retinas from different animals was analyzed (Table 1).

**Classification of RGCs**

RGCs were classified following Sun et al. (2002). Accordingly, the parameters used were (1) the diameter of the dendritic tree; this was obtained measuring with MetaMorph the area of the smallest two-dimensional convex polygon traced joining the outermost dendritic tip on a projection of the dendritic arborization when collapsed along the z-axis; this measure was repeated three times for each cell; the average was taken as the area of the cell dendritic tree and then used to calculate the diameter, assuming a circular shape of the tree; (2) the diameter of the RGC body, measured after tracing the contour of the projection of the cell body, obtained from optimal, non-saturated confocal images, usually separate from those used for three-dimensional reconstruction of the cell; (3) the mean stratification depth of the GC dendritic arborization within the inner plexiform layer (IPL), measured on orthogonal projections of the cells obtained from confocal z series, as reported by Badea and Nathans (2004); (4) the shape of the dendritic arbor, according to the description of Sun et al. (2002), as well as following Lin et al. (2004) and Kong et al. (2005); this feature constitutes a blueprint of a cell type and allows the distinction among cells sharing some morphometric parameters. A total of 224 GFP-labelled RGCs were classified; of these, 82
belonging to the most represented types (B3 inner and outer, C2 inner and outer) were selected for statistical analysis. They cover 4 different types, of the 17 totally identified by Sun et al. (2002). A summary of the RGC types examined is reported in Table 1.

GFP-labelled cells with regular and abnormal morphology were grouped separately. We compared data on RGCs from one-month aged retinas with data from older retinas (4-12 months). As a wt control, we took data from previously analyzed cells from 7 months aged wild type Thy-1 GFP-M (Mazzoni et al., 2008).
<table>
<thead>
<tr>
<th>Analyzed Type</th>
<th>Characteristic Size</th>
<th>Dendritic Features</th>
</tr>
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<tbody>
<tr>
<td>B3 inner</td>
<td>It is the smallest ON type GC; Small to medium sized soma (14 μm diameter average); The average dendritic field diameter is 173 μm.</td>
<td>Curvy, recursive dendrites forming a relatively sparse dendritic field; Discrete asymmetry between soma and dendrites; Stratifies in the inner IPL (67 ± 10%).</td>
</tr>
<tr>
<td>C2 inner</td>
<td>Abundant in mouse; Medium sized soma (16 μm diameter average); The average dendritic field diameter is 240 μm.</td>
<td>Smooth, recursive, medium caliber dendrites arising from large primary ones; No asymmetry between soma and dendrites; Stratifies in the inner IPL (75 ± 7%).</td>
</tr>
<tr>
<td>B3 outer</td>
<td>It is the OFF counterpart of B3 inner; Small to medium sized soma (14 μm diameter average); The average dendritic field diameter is 173 μm.</td>
<td>Curvy, recursive dendrites forming a relatively sparse dendritic field; Discrete asymmetry between soma and dendrites; Stratifies in the outer IPL (25 ± 16%).</td>
</tr>
<tr>
<td>C2 outer</td>
<td>It is the OFF counterpart of C2 inner; Medium sized soma (16 μm diameter average); The average dendritic field diameter is 240 μm.</td>
<td>Smooth, recursive, medium caliber dendrites arising from large primary ones; Discrete asymmetry between soma and dendrites; Stratifies in the outer IPL (32 ± 18%).</td>
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</table>
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**Table 1: Characteristics of RGC types analyzed in this work** (modified from Mazzoni et al., 2008)

*Survival in the GCL*

Retinal whole mounts obtained as above and counterstained with ethidium homodimer-1 were used to estimate survival of cells in the GC layer (GCL) of rd1 mice (not the GFP line) aged 4.5 months and 1 year. Three and four retinas from different animals were used respectively for 4.5 month and one year aged mice. Confocal microscopy was used to obtain serial optical sections at 1,0042 µm intervals encompassing the entire thickness of GCL, using a 40x objective as above. Sampling areas were 32 fields (250 x 250 µm) per retina, regularly spaced along the dorsal–ventral and nasal–temporal retinal meridians. Counts of cells were performed on extended-focus images of the GCL, covering an average thickness of 20 µm on the z plane. Astrocytes, endothelial and perivascular cells were excluded from the counts on the basis of their characteristic shape and high intensity of staining. Astrocytes were excluded also for their localization in the optic fiber layer (OFL), where axons of RGCs reside.

Total number of cells per retina was obtained multiplying average cellular densities by corresponding retinal areas. These were measured on low magnification images obtained at the Zeiss light microscope with a Zeiss Axiocam camera. Statistical analysis (one-way ANOVA) on cell counts was performed with Origin 7.0 (version 7SR1; OriginLab Corporation).

Condensed nuclei were also counted in the same GCL fields used for cell survival. A nucleus was considered condensed when no internal structure were visible due to saturation. For this reason, ethidium-stained fields were acquired setting the confocal photomultiplier gain at a value at which the naturally dense astrocyte nuclei could clearly be resolved. The total number of condensed nuclei per retina was obtained as previously described for survival in the GCL.

Some rd1 retinas were incubated with goat polyclonal antibodies against Brn3b (Santa Cruz Biotechnology) and ChAT (Millipore). The first is a transcription factor selectively expressed in a large fraction of adult RGCs. The second is a crucial enzyme in the
production of acetylcholine, and is expressed in the retina only by cholinergic, starburst amacrine cells. Subsequently, retinas were counterstained with ethidium as explained above. Images of the GCL were obtained at the confocal microscope at peripheral and central retinal locations, using a 40 x oil-immersion objective. Merged images were screened for possible co-localization of Brn-3b and ChAT staining in cells exhibiting condensed nuclei.