CRB2 acts as a modifying factor of CRB1-related retinal dystrophies in mice

Lucie P. Pellissier1, Ditte M. S. Lundvig1, Naoyuki Tanimoto3, Jan Klooster2, Rogier M. Vos1, Fabrice Richard4, Vithiyanjali Sothilingam3, Marina Garcia Garrido3, André Le Bivic4, Mathias W. Seeliger3 and Jan Wijnholds1,

1Department of Neuromedical Genetics, 2Department of Retinal Signal Processing, The Netherlands Institute for Neuroscience, Royal Netherlands Academy of Arts and Sciences (KNAW), Amsterdam 1105 BA, The Netherlands, 3Division of Ocular Neurodegeneration, Institute for Ophthalmic Research, Centre for Ophthalmology, Eberhard Karls University of Tübingen, Tübingen D-72076, Germany and 4Aix-Marseille Université, CNRS, UMR 7288, Developmental Biology Institute of Marseille (IBDM), Case 907, Marseille, Cedex 09 13288, France

Mutations in the CRB1 gene lead to retinal dystrophies ranging from Leber congenital amaurosis (LCA) to early-onset retinitis pigmentosa (RP), due to developmental defects or loss of adhesion between photoreceptors and Müller glia cells, respectively. Whereas over 150 mutations have been found, no clear genotype–phenotype correlation has been established. Mouse Crb1 knockout retinas show a mild phenotype limited to the inferior quadrant, whereas Crb2 knockout retinas display a severe degeneration throughout the retina mimicking the phenotype observed in RP patients associated with CRB1 mutations. Crb1Crb2 double mutant retinas have severe developmental defects similar to the phenotype observed in LCA patients associated with CRB1 mutations. Therefore, CRB2 is a candidate modifying gene of human CRB1-related retinal dystrophy. In this study, we studied the cellular localization of CRB1 and CRB2 in human retina and tested the influence of the Crb2 gene allele on Crb1 mutant mice. We found that in contrast to mice, in the human retina CRB1 protein was expressed at the subapical region in photoreceptors and Müller glia cells, and CRB2 only in Müller glia cells. Genetic ablation of one allele of Crb2 in heterozygote Crb1+/− retinas induced a mild retinal phenotype, but in homozygote Crb1 knockout mice lead to an early and severe phenotype limited to the entire inferior retina. Our data provide mechanistic insight for CRB1-related LCA and RP.

INTRODUCTION

Inherited retinal dystrophies are chronic, disabling disorders of sight with a worldwide prevalence of 1/3000. The most common form is retinitis pigmentosa (RP), which affects the retina, causing the progressive loss of light-sensitive photoreceptor cells, ultimately leading to blindness in early adulthood (1). The earliest and most severe form is Leber congenital amaurosis (LCA) that causes blindness at birth or within the first year of life (2). Mutations in the Crumbs-homologue-1 (CRB1) gene lead to 10–15% of LCA cases and 1–4% of RP cases worldwide (2,3). Since 1999, over 150 mutations in the CRB1 gene have been identified but no genotype–phenotype correlation has been established yet (4,5). LCA retinas associated with CRB1 variants are remarkably thick and display an immature layering in contrast to other genes causing LCA (6,7).

The Crumbs protein was first identified in Drosophila as a key developmental regulator of apical polarity (8). In mammals, the family is composed of three genes, CRB1, CRB2 and CRB3. Prototypical CRB proteins have a large extracellular domain composed of epidermal growth factor (EGF) and laminin-globular domains, a single transmembrane domain, and a short intracellular domain, which interacts with PALS1/MPP5, PATJ and MUPP1 to form the core CRB complex (9). CRB3 lacks the extracellular domain and has two variants (CRB3A and CRB3B) due to alternative splicing. The apical CRB complex resides at the subapical region above the adherens junctions.

*To whom correspondence should be addressed at: Department of Neuromedical Genetics, The Netherlands Institute for Neuroscience, Meibergdreef 47, 1105 BA, Amsterdam, The Netherlands. Tel: +31 205664597; Fax: +31 205666121; Email: j.wijnholds@nin.knaw.nl

© The Author 2014. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
between photoreceptors and Müller glia cells in mouse and humans (10–13). In adult mouse retinas, CRB1 is expressed only in Müller glia cells and maintains adherens junctions between photoreceptors and Müller glia cells (10–13). CRb1–/–, CRb1C249W/–, and CRb1pDOS/– mutant mice show limited and mild retinal disorganization restricted to one inferior quadrant (11,14–16). As expected, the comparison of human and mouse retinal dystrophies due to lack of CRB1 function shows prominent differences (7).

In adult mouse retinas, CRB2, which is four EGF domains shorter than CRB1, is expressed in photoreceptors and Müller glia cells (10). In contrast to Crb1 mutant mice, Crb2 conditional knockout (cKO) retinas show a more severe phenotype throughout the retina with retinal activity impairment, mimicking the phenotype observed in RP patients associated with CRB1 variants (17). Selective ablation of CRB2 in either Müller glial cells or photoreceptors showed that CRB2 has redundant functions in Müller glial cells, while CRB2 function is essential in photoreceptors (18). Furthermore, Crb1Crb2 cKO retinas lacking both CRB1 and CRB2 display lamination defects in early retinal development leading to immature and thinner retina in adult mice and severe retinal activity impairment, mimicking several clinical characteristics of LCA patients associated with CRB1 variants (19). Whereas no mutations in the human CRB2 gene have been reported to cause retinal dystrophies (20), CRB2 might be a candidate modifying factor of retinal dystrophies associated with CRB1 variants.

Here, we study the cellular localization of CRB1 and CRB2 proteins in human retina and test if the Crb2 gene may influence the severity of CRB1-related retinal dystrophies in mice. We found that in contrast to mice, in human retina CRB1 protein was expressed at the subapical region in photoreceptors and Müller glia cells and CRB2 only in Müller glia cells. Genetic ablation of one allele of Crb2 induced a mild phenotype in heterozygous Crb1 mutant mice and lead to an earlier and more severe phenotype limited to the entire inferior retina in homozygous Crb1 knockout mice.

RESULTS
CRB1 and CRB2 expression and localization in human retinas

We showed previously that retinas lacking CRB1 develop a mild RP but no LCA phenotype, whereas retinas lacking CRB2 develop a severe RP phenotype, and retinas lacking CRB2 with decreased levels of CRB1 develop a severe LCA phenotype. We previously performed immunoelectron microscopy on mouse retinas and determined the localization of CRB1 and CRB2 proteins in Müller glia cells and photoreceptors (10). Since human retinas lacking CRB1 function develop either a severe RP or LCA phenotype, we decided to extend our immunoelectron microscopy studies to human retinas. Immunoelectron microscopy on human donor retinas revealed strong CRB1 staining at the subapical region above the adherens junctions in the microvilli of Müller glia cells and in the inner segments of photoreceptor cells and in the Henle’s fiber structure of the fovea in Müller glia cells surrounding the axons of the photoreceptors (Fig. 1A and C; Supplementary Material, Fig. S1A). The antibody raised against the extracellular epitope of CRB1 showed a strong staining in the vicinity of the membranes of the two cell types (Fig. 1C). CRB2 staining was obvious in the microvilli of Müller glia cells, but barely detectable in photoreceptor cells (Fig. 1B). A similar pattern was obtained at the subapical region using a different CRB2 antibody (unpublished data). CRB3A staining was found in the microvilli of Müller glia cells and in the inner segments of photoreceptor cells (Fig. 1D).

Several transcript variants have been reported for the human CRB1 gene in GenBank but only the transcripts for full-length CRB1 (transcript variant-1) and the variant CRB1 lacking exons encoding the C-terminal cytosolic and transmembrane domains (transcript variant-4) have been described in the literature (4,21). Using quantitative PCR, we confirmed that the full-length and the variant-4 CRB1 were expressed in the human retina and in similar levels in the macula and the peripheral retinas and were not detectable in retinal pigment epithelium (RPE)/choroid tissues (Fig. 2A and B; Supplementary Material, Table S1). In addition, we found that the transcript variant-2, which has an in-frame deletion of exons 3 and 4 leading to the deletion of the EGF domains 4–6, was expressed at similar levels in the macula and peripheral retina but was not detectable in RPE/choroid tissues (Fig. 2A and B; Supplementary Material, Table S1). Using different primer sets (Supplementary Material, Table S1), CRB2 transcripts were found at similar levels in the macula and peripheral retina but were not detectable in RPE/choroid tissues (Fig. 2A). Using primers recognizing both CRB3A and CRB3B variants, the transcripts were found at low but similar levels in the macula and peripheral retina and were not detectable in adult RPE/choroid tissues (Fig. 2A). Using specific primers, CRB3A transcript was detected, but CRB3B transcript was not amplified from any of these tissues (unpublished data). Neural retina or RPE-specific or MPP5 transcripts were amplified in the expected tissues (Fig. 2C; Supplementary Material, Table S1).

Immunohistochemistry of human retinas showed strong and similar intensity at the subapical region of the macula and in the peripheral retina for CRB1, CRB2 and CRB3 (Fig. 2D–F). CRB1 antibody raised against the extracellular domain (amino acids 26–135), which recognized all the CRB1 protein variants, and CRB1 antibody against the intracellular domain that recognized only the full-length CRB1 protein, showed expression at the subapical region and in the cone inner segment of the macula (Supplementary Material, Fig. S2A and E). CRB1 and CRB2 co-localized at the subapical region, but CRB1 co-localized partially with glutamine synthetase (a marker for Müller glia cells), whereas CRB2 co-localized completely (Supplementary Material, Table S2B–D). CRB3 localized at the SAR but also at the synaptic outer plexiform layer (Supplementary Material, Fig. S2F).

Depletion of one Crb2 gene allele in Crb1 heterozygous retinas leads to a mild retinal phenotype

In order to test our hypothesis that Crb2 may act as a contributing factor of Crb1-related retinal dystrophies in mice, we crossed conditional Crb2F/+ Chx10CreEG+/– mice with Crb1+/– and Crb1–/– mice to generate Crb1+/–/Crb2F/+ Chx10CreEG+/– (Crb1+/–/Crb2F/+ cKO) and Crb1–/–/Crb2F/+ Chx10CreEG+/– (Crb1Crb2F/+ cKO). The mice were bred with Chx10Cre
transgenic mice, which express Cre recombinase fused to EGFP throughout the developing retina starting at E11.5 (22).

The single mutant heterozygote Crb1+/− or Crb2Flox+/− cKO mice do not show any alteration in retinal activity or morphology in aged or light exposed animals (11,14,17). In vivo functional and structural analysis were performed on 1- to 12-month-old double mutant Crb1+/−/Crb2F+/− cKO and Crb1Crb2F+/+ cKO mice, using electroretinography (ERG), spectral domain optical coherence tomography (SD-OCT) and scanning laser ophthalmoscopy (SLO). As shown previously, the retinal activity is not affected in Crb1+/−/Crb2F+/− cKO up to 12 months of age (Fig. 3) (19).

In vivo imaging analysis revealed no change in Crb1+/−/Crb2F+/− cKO retinas in fundus appearance and in retinal layer morphology in 1- to 12-month-old animals (Supplementary Material, Fig. S3) (19). Histological sections showed mild alteration with some photoreceptor ingresses in the outer plexiform layer from 3 months of age (black arrows; Fig. 4A–D). Retinas of mice exposed to constant white light of 3000 lux during 72 h displayed increased phenotype accompanied with many ingresses of photoreceptors into the outer plexiform layer and sporadically protrusions of photoreceptors into the subretinal space compared with Crb1+/− exposed to light (black arrows; Fig. 4E–G). The outer nuclear layer thickness in the Crb1+/−/Crb2F+/− cKO exposed to light was significantly decreased to similar thinning observed in 12-month-old retinas in contrast to cycled light Crb1+/− exposed to constant light retinas at 3 months (light exposed 50.6 ± 4.6 μm versus non-exposed 62.7 ± 2.3 μm) and 6 months (light exposed 49.3 ± 1.9 μm versus non-exposed 65.5 ± 0.9 μm or Crb1+/− exposed 59.8 ± 0.9 μm; Fig. 4H).

In vivo imaging analysis revealed no change in Crb1+/−/Crb2F+/− cKO retinas in fundus appearance and in retinal layer morphology in 1- to 12-month-old animals (Supplementary Material, Fig. S3) (19). Histological sections showed mild alteration with some photoreceptor ingresses in the outer plexiform layer from 3 months of age (black arrows; Fig. 4A–D). Retinas of mice exposed to constant white light of 3000 lux during 72 h displayed increased phenotype accompanied with many ingresses of photoreceptors into the outer plexiform layer and sporadically protrusions of photoreceptors into the subretinal space compared with Crb1+/− exposed to light (black arrows; Fig. 4E–G). The outer nuclear layer thickness in the Crb1+/−/Crb2F+/− cKO exposed to light was significantly decreased to similar thinning observed in 12-month-old retinas in contrast to cycled light Crb1+/− exposed to constant light retinas at 3 months (light exposed 50.6 ± 4.6 μm versus non-exposed 62.7 ± 2.3 μm) and 6 months (light exposed 49.3 ± 1.9 μm versus non-exposed 65.5 ± 0.9 μm or Crb1+/− exposed 59.8 ± 0.9 μm; Fig. 4H).

No alterations were found in localization of Crb1, Crb2, Crb complex members MUPP1 or PALS1/MPP5 or in adherens junction markers P120-catenin or Nectin1 (Supplementary Material, Fig. S4). However, from 6 months onward, stress and gliosis occurred in these retinas as shown by the increased expression of glial fibrillary acidic protein (GFAP) in Müller glial cells up to the outer nuclear layer compared with control (Fig. 4I and J).

Figure 1. Ultrastructural localization of CRB1, CRB2 and CRB3A at the subapical region of human retina. (A) Immunoelectron microscopy against the intracellular domain of CRB1 revealed that CRB1 is located apically to the adherens junctions in microvilli of Müller glial cells and in the inner segments of photoreceptor cells. (B) CRB2 is expressed in microvilli of Müller glial cells just above the adherens junctions and barely in photoreceptor cells. (C) Immunoelectron microscopy against the extracellular domain of CRB1 revealed that CRB1 is found membrane-associated and inside the microvilli of Müller glial cells and the inner segments of photoreceptor cells. (D) CRB3A is located in the microvilli of Müller glial cells and in the inner segment of the photoreceptor cells at the SAR. IS, inner segment; MGC, Müller glial cells; μ, microvilli of MGC; PRC, photoreceptor cells. Scale bar: 1 μm.
Figure 2. Expression of CRB1 variants, CRB2 and CRB3 in human retinas. (A) Quantitative PCR in five postmortem human donor eyes showed that the variant-4 isoform (ex11'), the isoform deleted of exons 3/4 (ex2/5) and the full-length isoform of CRB1 were detected in the retina. CRB1, CRB2 and CRB3 transcripts were expressed at similar level in the periphery and in the macula of the retina and were not detectable in RPE/choroid tissues. (B) Schematic representations of the full length (tv1), deleted exons 3 and 4 (tv2) and truncated (tv4) isoforms of CRB1 transcripts and their corresponding primers. (C) Quantitative PCR in the same five postmortem human donor eyes showed that RPE and retina-specific genes were amplified in RPE/choroid and macula/periphery tissues, respectively, and MPP5 and RLBP1 were amplified in all the tissues. Immunohistochemistry of CRB1 (D, extracellular epitope), CRB2 (E) and CRB3 (F) showed similar levels of expression at the SAR of the human macula and the peripheral retina. CRB1 was also found in the soma of cones (arrowheads in D) and CRB3 in the synaptic terminals in the outer plexiform layer and in endothelial cells (arrowhead in F; see also Supplementary Material, Fig. S2F). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; SAR, subapical region; tv, transcript variant. Scale bar: 50 μm.
Depletion of one Crb2 gene allele in Crb1 KO retinas leads to severe degeneration in the inferior but not superior retina

Three-month-old Crb1 KO retinas showed sporadic alterations of the photoreceptor layer in the inferior temporal quadrant progressing to degeneration in aged mice, but loss of retinal activity is not detectable by ERG (11,19). Whereas at 1 months the amplitudes of electroretinograms were not different from Crb1+/− Crb2+/− cKO retinas, Crb1Crb2+/− cKO retinas showed reductions at 3 and 6 months and a pronounced reduction.

Figure 3. Retinal function is affected in Crb1Crb2F/+ cKO. Single-flash ERG age series (1, 3, 6 and 12 months) in Crb1Crb2F/+ cKO (red) and Crb1+/− Crb2+/− cKO (black) mice. (A) Quantitative evaluation (box-and-whisker plot) of scotopic (left column) and photopic (right column) b-wave amplitude data. Boxes indicate the 25 and 75% quantile range, whiskers indicate the 5 and 95% quantiles and the asterisks indicate the median of the data. In Crb1Crb2F/+ cKO mice, the scotopic and photopic b-waves abnormally declined with increasing age up to 12 months. (B) Scotopic (top) and photopic (bottom) single-flash ERG responses from representative animals at the age of 12 months. The a- and b-wave are indicated by open arrows. The reduced scotopic ERG a-wave indicated photoreceptor degeneration (filled arrow).
at 12 months (Fig. 3B) indicated that the retinal activity alteration is primarily due to loss of photoreceptor cells.

In vivo imaging analysis in Crb1Crb2Δ/Δ cKO mice revealed many spots and sporadic patchy areas in the photoreceptor layer mainly in the inferior retina at 1 months in contrast to control retinas (Fig. 5A–D; Supplementary Material, Fig. S3) followed by a rapid degeneration of this layer in the two inferior retinal quadrants at 3 months of age (Fig. 5E–H). In 6- and 12-month-old retinas, the degeneration seemed to affect the entire inferior retina and extended above the optic nerve in the superior retina in contrast to control (Fig. 5I–P, Supplementary Material, Figs S3 and S5D) (16). The first disruptions of the outer limiting membrane associated with photoreceptor cell protrusions in the inferior retina were observed at postnatal Day 10 (P10) in histological sections (Fig. 6A). At P14, this extended to a larger area where many photoreceptor cells were displaced close to the RPE layer and folded into the inner nuclear layer (Fig. 6B). At 1 months the outer plexiform layer was disrupted, the photoreceptor segments were reduced and their soma located close to the RPE (Fig. 6C and G–J). The photoreceptor cells from the inferior retina were rapidly degenerating from 1 to 3 months where only one row of photoreceptors remained (Fig. 6D and K). In 6- and 12-month-old inferior retinas, photoreceptor cells disappeared and the inner and ganglion cell layers and RPE/choroid were affected (Fig. 6E, F, K). The superior retina from 6 months onward displayed a mild phenotype similar to Crb1Δ/Δ–Crb2Δ/Δ cKO (Figs 4C–D and 6E–F).

Already at P14, stress and gliosis (measured by increased levels of GFAP) was present especially in the inferior retina accompanied with perturbed localization of the apical Crb complex members, PALS1/MPP5 and MUPP1, and rod (rhodopsin positive) and cone [cone arrestin (CAR)-positive] photoreceptors in the lumen of the outer nuclear folding (Supplementary Material, Fig. S5A–C). Many more apoptotic cells (cleaved caspase 3, c-caspase 3) were found in the inferior compared with the superior retina, and these rhodopsin-positive cells were identified as being mainly rods (Fig. 7A and B). At 3 months, the stress and gliosis increased and all Müller glia cells strongly expressed GFAP, especially around the remaining row of photoreceptors (Fig. 7C). In contrast to the superior retina where rhodopsin located exclusively in the outer segments at 3 months, in the inferior retina rhodopsin was found in the soma of the remaining rods (Fig. 7D). Whereas the Chx10CreGFP-positive bipolar cells were not different in the inferior compared with the superior retina, the number of rods and cones was decreased by a factor of 15 and 2, respectively (Fig. 7E).

**Differences between superior and inferior retinas in mice**

The main difference between the superior and the inferior murine retina is that M-cones mainly localized in the superior retina, whereas S-cones mainly localized in the inferior retina (Supplementary Material, Fig. S6A) (23). The thyroid hormone gradient established a gradient of M-opsin by inhibiting S-opsin expression through the Thrβ2 receptor (24). Thrβ2Δ/Δ retinas have only S-cones throughout the retina (25).

In order to understand why Crb1Δ/Δ and Crb1Crb2Δ/Δ cKO retinas are affected in the inferior temporal retinal quadrant and the whole inferior retina, respectively (Figs 5–7) (11), we crossed Crb1Δ/Δ and Thrβ2Δ/Δ mice hypothesizing that the
**DISCUSSION**

In this study, we showed that CRB1 and CRB2 in human retinas have an opposite pattern of expression in Müller glia and photoreceptor cells compared with mouse retinas, and that Crb2 influences the severity of the murine Crb1-linked retinal dystrophies.

At the subapical region, in human retinas, CRB1 seems to be expressed in Müller glia and photoreceptor cells whereas CRB2 only in Müller glia cells. In mouse retinas, an opposite expression pattern was detected. Many genes involved in retinal dystrophies have been reported to show differences between human and mice and in temporal and spatial expression patterns inside the retina. This reversed pattern of CRB1/CRB2 cellular expression might be a good explanation why mice lacking Crb1 protein show a mild phenotype in contrast to humans and why Crb2 cKO retinas exhibit the characteristics of human RP associated with CRB1 variants. Mouse Crb1 was first identified by RT–PCR and rapid amplification of cDNA ends and thereafter, Crb2 and Crb3 were found in database searches using the highly conserved cytoplasmic domain. We may also hypothesize that in the retina, the expression patterns of CRB1 and CRB2 proteins are reversed and that, functionally, they have switched their identities.

Surprisingly, no clear genotype–phenotype correlation has been established for RP and LCA due to mutations in the CRB1 gene. It has been identified that some LCA patients also carry mutations in other LCA genes that may influence the severity of the disease.
variants also carries a mutation in the Aryl-hydrocarbon-interacting protein-like 1 LCA gene, which may explain why this patient developed LCA and not RP phenotype (31,32). In addition, around a quarter of the general population carries a null variant in a gene responsible for retinal dystrophy (33). In the present study, we showed that the mouse Crb2 gene influenced the severity of Crb1-related retinal dystrophy. Whereas Crb1−/− retinas show a mild patchy disorganized phenotype...
Figure 7. Severe loss of photoreceptors in Crb1Crb2<sup>F</sup> inferior retinas. (A) Many c-caspase 3-positive cells were found in 1-month-old inferior retinas that colocalized with rhodopsin, a marker for rod photoreceptor cells. (B) The number of c-caspase 3-positive apoptotic cells at P14, 1 month and 3 months from 20 sections of 3 Crb1Crb2<sup>F</sup> cKO animals showed an increase in the number of apoptotic cells in the inferior retinas in contrast to the superior retinas. (C) A strong increase in GFAP intensity, a marker for retinal stress, limited to the entire inferior retina of 3-month-old Crb1Crb2<sup>F</sup> cKO retinas whereas the superior retina showed only few GFAP fibers in the INL. (D) Cone and rod photoreceptors (cone arrestin and rhodopsin, respectively) were severely reduced in 3-month-old Crb1Crb2<sup>F</sup> cKO inferior retinas in contrast to the superior retinas. (E) Whereas the number of Chx10<sup>CreGFP</sup>-positive bipolar cells was unchanged at 3 months, the number of rods and cones was reduced by 15 times and 2 times, respectively. GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; INL, inner nuclear layer; IS, inner segment; ONL, outer nuclear layer; OS, outer segment. Data are presented as mean ± SEM. **P < 0.01; ***P < 0.001. Scale bar: 50 μm (A, C and D).
in the inferior temporal quadrant (11), and Crb1<sup>+</sup>/Crb2<sup>−−</sup> retinas in the inferior nasal quadrant (16), the Crb1Crb2<sup>+/−</sup> cKO retinas show severe degeneration of the whole inferior retina at 3 months of age. Whereas Crb1<sup>+/−</sup> and Crb2<sup>+/−</sup> cKO retinas appear normal, Crb1<sup>+/−</sup>/Crb2<sup>−−</sup> cKO retinas show a mild phenotype throughout the retina, which was increased with exposure to constant light for 72 h. This study suggests that the CRB2 gene might be a good candidate modifier gene for the severity of the CRB1-retinal dystrophies also in humans. The human CRB2 gene itself has not been linked to retinal dystrophies, but revealed 11 single allele variants (20). Due to expression in vital organs (20), complete loss of CRB2 function in mice leads to embryonic lethality (34) and explains why no double allele mutations in the CRB2 gene have been identified. However, in a patient carrying double allele variants with unknown functional effect in the CRB1 gene, an additional mutation in the CRB2 gene may influence the severity of the retinal dystrophy from RP to LCA. Similarly, we hypothesize that other members of the CRB complex such as PALS1/MPP5, MPP4, MPP3 and MUPP1 (35–39) might be candidate modifier genes for the severity of the CRB1-retinal dystrophies. Therefore, further genetic investigations on these genes in LCA and RP patients carrying CRB1 variants may reveal the genotype–phenotype correlation.

Crb1<sup>+/−</sup> mice show a mild sporadic phenotype in one quadrant of the inferior retina leading ultimately to photoreceptor degeneration in 12-month-old mice, but without detectable loss of the retinal activity measured by ERG (14). Crb1Crb2<sup>+/−</sup> cKO retinas show already at P10 disorganization of the outer limiting membrane leading to severe degeneration of the photoreceptor cells in the whole inferior retina at 3 months with impairment of the retinal activity. Studying Crb1 and Crb2 protein levels, we found Crb1 (expressed in Müller glia cells) (10) substantially lower in the inferior retina, whereas Crb2 (expressed in photoreceptors and Müller glia cells) (10) higher in the inferior. In Crb1<sup>−−</sup> and Crb1<sup>+/−</sup>/Crb2<sup>−−</sup> mutant retinas, levels of Crb2 mRNA and Crb2 and Crb3 proteins were found unchanged in comparison to wild-type retinas (11,15). Levels of Crb1 and Crb3 mRNA and Crb1 protein in Crb2 cKO retinas were also not different than control retinas (17,40). It seems that compensation by other members of the family does not occur between Crb1 and Crb2 proteins. Therefore, we hypothesize that the decrease of Crb1 and Crb2 proteins in the inferior retinas is critical and might explain why the inferior retinas are affected in Crb1<sup>−−</sup> and Crb1Crb2<sup>+/−</sup> cKO retinas. These mice are good mouse models of RP phenotype due to CRB1 variants and are of interest to test and develop CRB1 gene therapy vectors.

**MATERIALS AND METHODS**

**Animals**

Animal care and use of mice was in accordance with protocols approved by the Animal Care and Use Committee of the Royal Netherlands Academy of Arts and Sciences. All mice used were maintained on a 50% C57BL/6J Ola Hsd and 50% 129/Ola genetic background. Mice did not have rds8 or pde6b mutations. Animals were maintained on a 12 h dark/dim light cycle and supplied with food and water ad libitum. Crb1<sup>−−</sup> mice (11), Crb2<sup>+/−</sup>/Chx10Cre<sup>+/−</sup> clone P1E9 (Crb2<sup>+/−</sup> cKO) generated previously (17) were crossed to generate Crb1<sup>+/−</sup>/Crb2<sup>+/−</sup>/Chx10Cre<sup>+/−</sup> (Crb1<sup>+/−</sup>/Crb2<sup>+/−</sup> cKO) and Crb1<sup>−−</sup>/Crb2<sup>+/−</sup>/Chx10Cre<sup>+/−</sup> (Crb1Crb2<sup>+/−</sup> cKO). Crb1Crb2<sup>+/−</sup> cKO retinas were compared with littermate Crb1<sup>+/−</sup>/Crb2<sup>+/−</sup> and Crb1<sup>+/−</sup>/Crb2<sup>+/−</sup> cKO retinas. Chx10CreGFP<sup>+/−</sup> mice (Jackson Laboratory) express the CRE recombinase in the retina from E11.5 onward (21). Chromosomal DNA isolation and genotyping were performed as previously described (17).

For light exposure experiments, after a 12-h dark period, mice were continuously exposed for 72 h to diffuse white fluorescent light 3000 lux (TL-D-18W/33tubes, Philips; 350–700 nm) without pupillary dilation (14). Immediately after these 72 h of light exposure, the animals were killed.

In **vivo analysis**

SLO, SD-OCT and ERG measurements were performed at 1, 3, 6 and 12 month(s). The groups consisted of in 4–6 animals of each genotype. Electroretinograms were recorded binocularly as described previously (41). Mice were anesthetized using ketamine (66.7 mg/kg body weight) and xylazine (11.7 mg/kg body weight). The pupils were dilated and single-flash responses were obtained under scotopic (dark-adapted overnight) and photopic (light adapted with a background illumination of 30 cd m<sup>−2</sup>) starting 10 min before recording) conditions. Single white-flash stimuli ranged from ~4 to 1.5 log cd s/m<sup>2</sup> under scotopic and from ~2 to 1.5 log cd s/m<sup>2</sup> under photopic conditions. Ten responses were averaged with inter-stimulus intervals of 5 s (for ~4 to ~0.5 log cd s/m<sup>2</sup>) or 17 s (for ~0 to 1.5 log cd s/m<sup>2</sup>). Retinal morphology of the anesthetized animals was visualized via SLO imaging with an HRA 1 (Heidelberg Engineering, Heidelberg, Germany) (42). Briefly, the HRA 1 system features two lasers (488/514 nm) in the short (visible) wavelength range and two (795/830 nm and 785/815 nm) in the long (infrared) wavelength range. For fundus visualization, the 514 nm (red-free) laser was used. The 488 and 795 nm lasers are used for fluorescence (FLA) and indocyanine green angiography, respectively, whereas autofluorescent images were obtained with the 488 nm laser (AF) with a barrier filter at 500 nm. In the same session, SD-OCT imaging was performed with a commercially available Spectralis HRA+OCT device from Heidelberg Engineering. This equipment features a broadband superluminescent diode at λ = 870 nm as low coherent light source (43). Each 2D B-scan recorded with the equipment set to 30° field of view consists of 1536 A-scans acquired at a speed of 40 000 scans/s. Optical depth resolution is ∼7 μm with digital resolution reaching 3.5 μm. Imaging was performed using the proprietary software package Eye Explorer (version 3.2.1.0, Heidelberg Engineering) and for the work-up of the images, we used CorelX3 (Corel Corporation, Ottawa, ON, Canada).

**Real-time quantitative PCR**

This study was performed in agreement with the declaration of Helsinki on the use of human material for research. Postmortem human donor eyes (from 40 to 85-year-old donors) were acquired from the Euro Cornea Bank and were processed within 48 h after death. Retinas were dissected from the eyecup, and the vitreous was removed. RNA was isolated from retina (periphery and macula) and RPE/choroid tissues from 5 postmortem human donor eyes using TRIZOL reagent (Gibco Life Technologies) according to the manufacturer manual, and
after the final precipitation dissolved in RNase-free water. After genomic DNA degradation with RNase-free DNase I (New England Biolabs), 2 μg of total RNA was reverse transcribed into first-strand cDNA with Superscript III Plus RNase H-Reverse Transcriptase (Invitrogen) and 50 ng random hexamer primers during 50 min at 50°C in a total volume of 20 μl. To the resulting cDNA sample, 14 μl of 10 mm Tris, 1 mm EDTA was added. From all samples, a 1/20 dilution was made and used for qPCR analysis. Primer pairs (Supplementary Material, Table S1) were designed with a melting temperature of 60–62°C, giving rise to an amplicon of 80–110 bp. Real-time qPCR was based on the real-time monitoring of SYBR Green I dye fluorescence on an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The PCR conditions were as follows: 12.5 μl SYBR Green PCR 2× mastermix (Applied Biosystems), 20 pmol of primers and 2 μl of the diluted cDNA (ca. 6 ng total RNA input). An initial step of 50°C for 2 min was used for AmpliTaq incubation followed by 15 min at 95°C to inactivate AmpliTaq and to activate the AmpliTaq. Cycling conditions were as follows: melting step at 95°C for 1 min, annealing at 58°C for 1 min and elongation at 72°C, for 40 cycles. At the end of the PCR run, a dissociation curve was determined by ramping the temperature of the sample from 60 to 95°C while continuously collecting fluorescence data. Non-template controls were included for each primer pair to check for any significant levels of contaminants. Values were normalized by the mean of the four reference genes: glyceraldehyde 3-phosphate dehydrogenase, elongation factor 1α, peptidylprolyl isomerase A or cyclophilin A and ribosomal protein large P0.

Immunohistochemical analysis
Mouse eyes were enucleated and eyes orientated. Subsequently, the mouse eyes and human retinas were fixed during 20–30 min in 4% paraformaldehyde in PBS, cryo-protected with 5 and 30% sucrose in PBS, embedded in Tissue-Tek OCT Compound (Sakura, Finetek) and used for cryosectioning. Cryosections (7–10 μm) were rehydrated in PBS and blocked for 1 h using 10% goat serum, 0.4% Triton X-100 and 1% bovine serum albumin (BSA) in PBS. The antibodies used are listed in Supplementary Material, Table S2. The primary antibodies were diluted in 0.3% goat serum, 0.4% Triton X-100 and 1% BSA in PBS and incubated overnight at 4°C or 2 h at room temperature in a moist chamber. Secondary antibodies were diluted in 1% BSA in PBS and incubated for 1 h at room temperature in a moist chamber. Nuclei were counterstained and mounted in Vectashield hardset mounting medium containing DAPI (Vector Laboratories). Images were generated on a Leica SP5 confocal laser scanning microscope and analyzed with Adobe Photoshop CS4. Representative inferior and superior sections of the whole retina from three different animals were stained with c-caspase 3 (a marker for apoptosis) or cone arrestin and rhodopsin (markers for cones and rods, respectively). The positive cells were manually counted and corrected by the length of each section (measured using ImageJ software fiji-win32).

Electron microscopy
One-month-old Crb1Crb2+/+ cKO mice were perfused with 4% paraformaldehyde, 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After the retinas were dissected free, they were postfixed in 1% osmium tetroxide. Tissues were thoroughly rinsed and stained with 2% uranyl acetate in 70% ethanol. Samples were then dehydrated in a graded series of ethanol and embedded in epon 812 (Polysciences). Ultrathin sections of the superior and inferior retinas were examined with a Zeiss 912 electron microscope. Immunelectron microscopic analysis was performed as previously described (44). Briefly, 40 μm thick sections were incubated with the appropriate first antibody for 48 h (Supplementary Material, Table S2), then incubated with rabbit peroxidase antiperoxidase 2 h, then developed in a 2,2-diaminobenzidine solution containing 0.03% H2O2 for 4 min and then the gold substitute silver peroxidase method applied. Sections were embedded in epoxy resin, ultrathin sections made and examined with an electron microscope (FEI Tecnai 12; Fei Company, Eindhoven, The Netherlands).

Statistical analysis
Normality of the distribution was tested by Kolmogorov–Smirnov test. Statistical significance was calculated by using t-test of 3–5 independent retinas (20 sections)/genotype/age. Values were expressed as mean ± SEM. Values of *P < 0.05, **P < 0.01 and ***P < 0.001 were considered to be statistically significant. Calculations and graphs were generated using GraphPad Prism 5.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS
The authors thank Penny Hartsuiker and Feline Spiering for technical assistance and Jacoline Ten Brink for technical advices. The authors also thank Henrique Alves and Jeroen Dudok for advice on the manuscript. The authors thank Dr Douglas Forrest for provision of Thrβ2−/− mice.

Conflict of Interest statement. None declared.

FUNDING
This work was supported by Rotterdamse Vereniging Blindenbelangen, Landelijke St. voor Blinden en Slechtiendien, St. Blindenhulp, St. Oogfonds Nederland, St. Retina Nederland, Netherlands Institute for Neuroscience, Foundation Fighting Blindness (TA-GT-0811-0540-NIN and TA-GT-0313-0607-NIN) and The Netherlands Organisation for Health Research and Development (ZonMw grant 43200004 to J.W.), European Union (HEALTH-F2-2008-200234 to A.L.B. M.W.S., J.W.),
The Deutsche Forschungsgemeinschaft (DFG, grants Se837/5-2, Se837/6-1, Se837/6-2, Se837/7-1 to M.W.S.), and the German Ministry of Education and Research (BMBF, grant 0314106 to M.W.S.). The French National Research Agency (ANR n°BLAN07-2-186738 to A.L.B.).

REFERENCES


REFERENCES


REFERENCES


