Viral gene therapy approaches for CRB1 retinal disease

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Chapter 6

General Discussion

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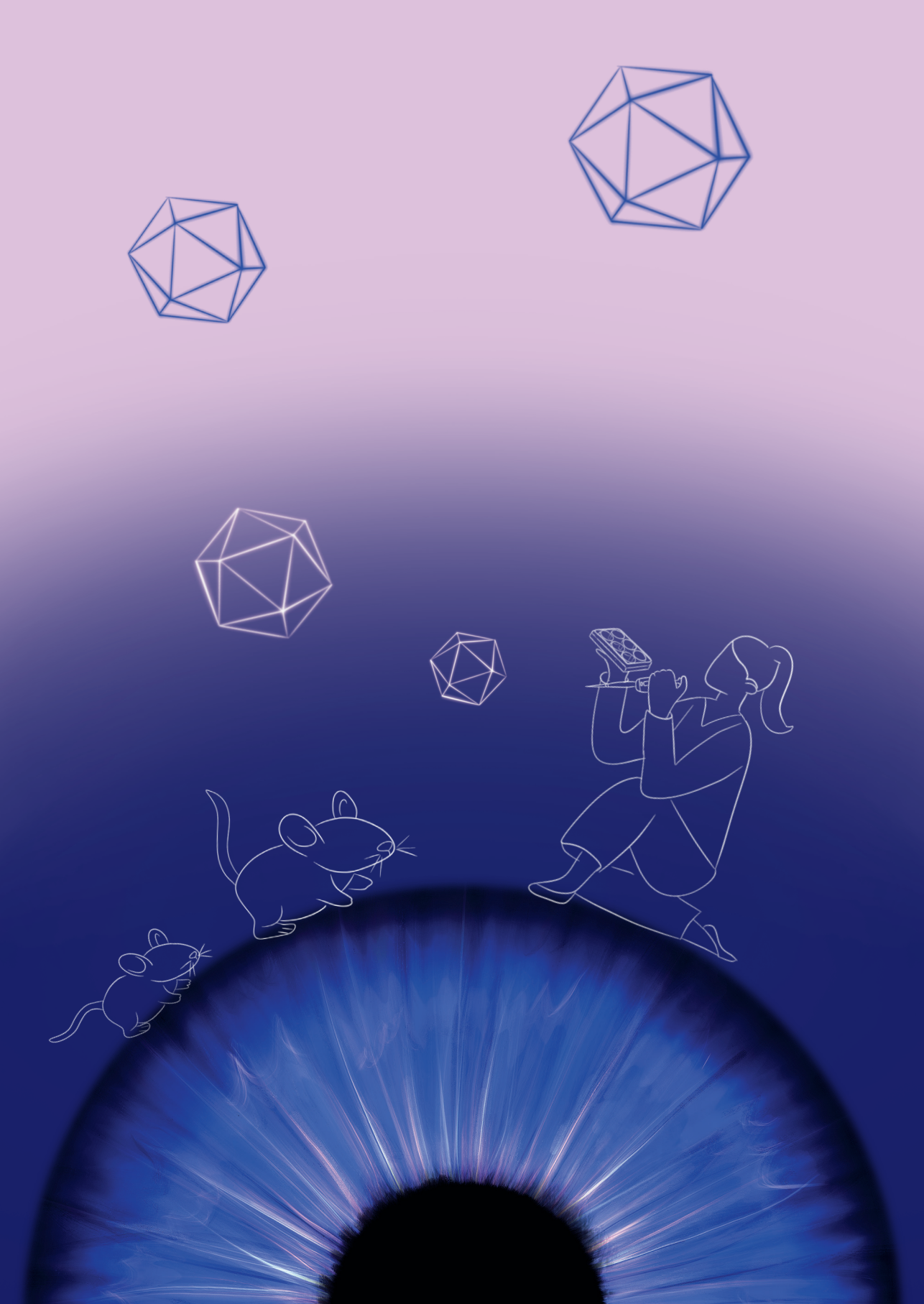

Abstract

Mutations in the *CRB1* gene can cause inherited retinal diseases such as retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA). So far there are no treatment options for patients with a mutation in the *CRB1* gene. In this last chapter of the thesis we will describe (1) CRB1 in retinal disease, (2) CRB protein localization and function, (3) different models to study *CRB1* related retinal dystrophies, (4) AAV-mediated gene augmentation therapy for *CRB1* related retinal dystrophies, and (5) the future perspectives.

CRB1 in retinal disease

Inherited retinal dystrophies affect about 2 million people worldwide and share progressive degeneration of photoreceptors and/or retinal pigment epithelium (RPE) cells. Examples of retinal degeneration include Retinitis Pigmentosa (RP) or Leber congenital amaurosis (LCA), RP affects at least 1 in 3500 to 4000 people and LCA affecting at least 1 in 32000 to 80000 people worldwide [1,2]. RP patients typically experience night blindness followed by progressive visual field loss and complete loss of vision in early or middle-life [3–5], whereas LCA patients experience more severe retinal dystrophy causing serious visual impairment or immediate blindness at birth [6]. Approximately 10% of all LCA cases and 3-9% of all RP cases are caused by mutations in the *Crumbs homologue 1 (CRB1)* gene. There are over 200 different mutations described along the *CRB1* gene resulting in these retinal dystrophies, but so far there is no clear genotype-phenotype correlation [3,7]. The observed clinical variability of disease onset and severity, even within a patient cohort with the same homozygous mutations, supports the hypothesis that the phenotype is potentially modulated by other factors [8]. So far, there are no treatment possibilities for patients with mutations in the *CRB1* gene.

CRB protein localization and function

The human *CRB1* gene is mapped to chromosome 1q31.3 and contains 12 exons consisting of 210 kb of genomic DNA [9]. Canonical CRB1 protein is, like its

Drosophila homologue, a transmembrane protein consisting of a large extracellular domain with multiple epidermal growth factor (EGF) and laminin-globular like domains and a short intracellular domain with a conserved PDZ binding motif [10]. Multiple transcripts of *CRB1* have been described [9,11], including an alternative shorter transcript of *CRB1*, *CRB1-B*, lacking exons 1 to 5 and exon 12 but with substantial overlap encoding the extracellular domain [12]. CRB1 together with CRB2 and CRB3A are members of the Crumbs family. CRB2 displays similar protein structure as CRB1, with a depletion of four epidermal growth factor domains in the extracellular domain, while CRB3 lacks the entire typical extracellular domain but contains the transmembrane domain and the conserved intracellular domain [11,13]. Various research has shown that CRB1 and CRB2 are apical polarity factors, essential for the formation and function of epithelial tissues [14]. The evolutionary conserved CRB complex regulates the apical-basal polarity and maintains cell adhesion [15]. The CRB complex is formed by interaction of CRB with protein associated with Lin Seven 1 (PALS1), also known as membrane-associated guanylate kinase p55 subfamily member 5 (MPP5). The multiple PDZ proteins PATJ and multiple PDZ domain 1, MUPP1, recruit PALS1 to the apical membrane [16,17]. PALS1 can interact with MPP3 or MPP4 at the subapical region in the mouse retina [18–20].

In mammalian tissue, retinal CRB1 and CRB2 are predominantly expressed at the outer limiting membrane (OLM) at the subapical region adjacent to adherence junctions in the inner segments of photoreceptors or at the apical villi of Müller glial cells. Immuno-electron microscopy allows the identification of the subcellular localization of CRB1 and CRB2 in the retina of different species. In mouse [21] and brown Norway rats retina (**Chapter 3**), Crb1 was present at the subapical region above the adherence junctions of solely Müller glial cells (MGC) whereas Crb2 was present at the subapical region above the adherence junctions of MGC as well as photoreceptor cells (Figure 1A) [21,22]. However, in adult non-human primates CRB1 and CRB2 are localized at the subapical region of MGC and photoreceptors (Figure 1B) [21]. In accordance, data of human fetal retina and human-induced pluripotent stem cell-derived retinal organoids show that both CRB1 and CRB2 are located at the subapical region above the adherence junctions of both MGC and photoreceptor cells (Figure 1B)

[23]. Single cell RNA-sequencing of late-stage human-derived retinal organoids confirmed that *CRB1* and *CRB2* transcripts are present in MGC and photoreceptor cells (**Chapter 4**) [24]. Knowledge of this discrepancy in CRB localization is pivotal for future research and understanding of the observed phenotype in the research models.

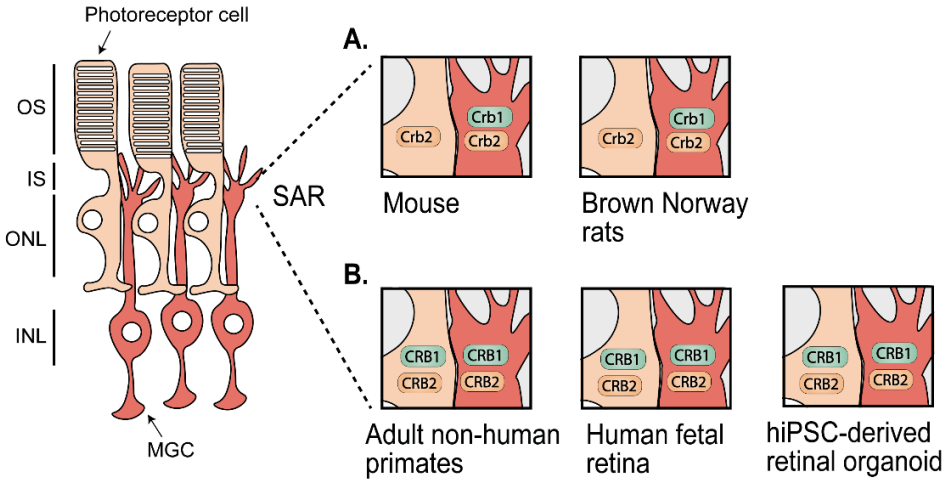

Figure 1. Schematic representation of subcellular localization of CRB1 and CRB2. (A, B) Subcellular localization of CRB1 and CRB2 in (A) mouse and brown Norway rats and (B) hiPSC-derived retinal organoids, human fetal retina, and adult non-human primates at the SAR of MGC (dark orange) or photoreceptor cells (light orange). Note: INL = inner nuclear layer; IS = inner segments; MGC = Müller glial cell; ONL = outer nuclear layer; OS = outer segments; SAR = subapical region.

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Models to study the *CRB1*-related retinal dystrophies

Multiple rodent-derived models are described which mimic parts of the heterogeneous disease phenotype observed in *CRB1* patients. This is visualized by the tremendous number of distinct animal models previously characterized over the years: four LCA and ten RP-like mouse models and one *Crb1* rat model [25–33]. These models show the effect of loss of CRB1 or CRB2 proteins in cell types such as retinal progenitors, photoreceptor progenitors, rod photoreceptors or Müller glial cells.

In **Chapter 2**, two more RP-like mouse models were characterized: $Crb2^{\Delta Rods}$ and $Crb1^{KO}Crb2^{\Delta Rods}$ mice [34]. In these mice *Crb2* was specifically ablated from rod photoreceptor cells ($Crb2^{\Delta Rods}$) or had concomitant loss of *Crb1* from MGC

(*Crb1*^{KO}*Crb2*$^{\Delta Rods}$). While measuring retinal function using electroretinography (ERG), similar ERG responses were observed in one month *Crb2*$^{\Delta Rods}$ and *Crb1*^{KO}*Crb2*$^{\Delta Rods}$ mice compared to age-matched controls. At 3 months of age, a slightly reduced a-wave response, indicating alterations in rod photoreceptor function, was observed in *Crb1*^{KO}*Crb2*$^{\Delta Rods}$ mice whereas the ERG response of *Crb2*$^{\Delta Rods}$ mice was still similar to age-matched controls. The ERG response reduced further over time, and in 9 and 12 months mice both *Crb1*^{KO}*Crb2*$^{\Delta Rods}$ and *Crb2*$^{\Delta Rods}$ mice had a statistically significant reduced a-wave response compared to age-matched controls. Next, visual function was measured using optokinetic head tracking response (OKT) that showed a significant decrease in contrast sensitivity in 3, 7, and 9M *Crb1*^{KO}*Crb2*$^{\Delta Rods}$ mice. Interestingly, no difference in OKT contrast sensitivity was observed in *Crb2*$^{\Delta Rods}$ mice, indicating that the visual function impairment is due to cumulative loss of Crb1 and Crb2. Histological analysis confirmed the degenerative phenotype. Normal retinal lamination was observed in 1 month old *Crb1*^{KO}*Crb2*$^{\Delta Rods}$ and *Crb2*$^{\Delta Rods}$ mice. When the mice age the number of photoreceptor nuclei mainly at the peripheral superior retina were lost. A statistically significant decrease of number of photoreceptor nuclei in a row was observed in *Crb1*^{KO}*Crb2*$^{\Delta Rods}$ at 6M of age mainly in the peripheral superior retina. Moreover, disruptions at the OLM in *Crb1*^{KO}*Crb2*$^{\Delta Rods}$ mice was observed throughout the retina. Transmission electron microscopy (TEM) showed disrupted adherens junctions in *Crb1*^{KO}*Crb2*$^{\Delta Rods}$ and also observed collapsed MGC apical villi in the peripheral retina due to lack of structural support from neighbouring photoreceptor inner segments. Using immunohistochemistry the disturbed radial alignment of MGCs was shown [34].

In comparison to the other *Crb* mouse models, the phenotype of these *Crb1*^{KO}*Crb2*$^{\Delta Rods}$ mice is relatively mild. Mouse models with ablation of *Crb2* from immature photoreceptor cells or from retinal progenitor cells show the morphological phenotype onset from embryonic day 15 (E15) to postnatal day 10 (P10) [26,28,35,36], whereas ablation of *Crb2* from rod photoreceptor cells show at foci first signs of degeneration at 1 month of age. Mice with ablation of *Crb2* from MGC show similarly as mice with loss of Crb1 from MGC a very mild morphological phenotype with no functional consequence measured by ERG [27,28,30]. Concomitant ablation of *Crb1*

and *Crb2* in MGC enhanced the observed phenotype, making some of the RP phenotypes to a more severe LCA-like phenotype [25,28]. An ablation of *Crb1* with reduced levels of *Crb2* in MGC results in a RP-like phenotype [37]. Interestingly, ablation of exclusively *Crb1* or *Crb2* from MGC results in no functional phenotype, whereas ablation of both *Crb1* and *Crb2* from MGC results in a severe LCA-like phenotype [38]. This phenotype is comparable to when *Crb1* is ablated from MGCs and *Crb2* is ablated from immature photoreceptor cells or retinal progenitor cells [25,28]. Suggesting that, either CRB1 or CRB2 in MGC and CRB2 in photoreceptor cells are essential for proper retinal lamination and function in mice.

Not only *Crb1* mutant mouse models have been characterized, in **Chapter 3** the phenotype of a Brown Norway rat (BN/OrlRj) with a spontaneous mutation in the *Crb1* gene is described in more detail. Using immunohistochemistry, the first signs of retinal degeneration were observed at P10 indicated by OLM breaks and protrusions of photoreceptor cell nuclei into the photoreceptor segment layers. Using spectral domain optical coherence tomography (SD-OCT), the retina of control and *Crb1* mutant Brown Norway rats were followed over time. Retinal lamination appeared to be similar in control and *Crb1* mutant rats at P17 but degenerated over time, degeneration was indicated by an increasing number of hyperreflective regions in the INL and ONL of *Crb1* mutant rats. Quantification of the SD-OCT B-scans revealed a statistically significant decrease of laminated retina in *Crb1* mutant rats compared to controls starting at 1 month of age and continuing to degenerate up to at least 3 months of age. This degenerative phenotype was observed using immunohistochemistry in more detail. Displaced SOX9 positive MGCs and loss of rhodopsin-positive and recoverin-positive photoreceptor inner and outer segments were observed in 1 month of age *Crb1* mutant rats. Moreover, measurements by ERG showed a statistically significant decreased retinal function in 1 month of age *Crb1* mutant rats compared to control Brown Norway rats. Visual function as measured by OKT spatial frequency was statistically significant decreased at 3 months of age in *Crb1* mutant rats [22].

Interestingly, the observed phenotype in the *Crb1* mutant rats is more severe than the ones observed in *Crb1* mouse models. These discrepancies could be because of (1) species differences with different genetic backgrounds; (2) different types of

mutations affecting different CRB isoforms, thereby expressing a distinct CRB1^{INDEL} protein in the *Crb1* mutant rats; or (3) the total expression levels of CRB2 might be lower in new-born rats compared to new-born mice. Decreased levels of CRB2 or dysregulation of other CRB-interacting proteins could result in less stabilization of the adherens junction complex at the OLM, resulting in a more severe phenotype. These discrepancies between distinct animal models highlight the importance of using human derived models as well.

In **Chapter 4 and 5** the use of human induced pluripotent cells (hiPSC) derived retinal organoids are described. Differentiating hiPSC into retinal organoids allows access to previously limited or inaccessible human-derived materials. Numerous research groups use or try to improve the differentiation method to generate well laminated retinal organoids [39–43]. RNA sequencing profiling demonstrated that *in vivo* retinogenesis was recapitulated in the retinal organoids in terms of temporal expression of cell differentiation markers, mRNA alternative splicing, and retinal disease genes [44]. In addition, single-cell RNA sequencing (scRNA-seq) revealed that retinal organoids and fetal retina have similar cellular composition at equivalent ages [45]. Moreover, CRB1 and CRB2 expression in retinal organoids also recapitulates those observed in human fetal retina [23]. Therefore, retinal organoids are of great interest for investigating mechanisms of retinal degeneration, developing therapeutic strategies, and many more.

In **Chapter 4**, three *CRB1* RP patient-derived hiPSC were differentiated into retinal organoids up to differentiation day 230 (DD230) [24]. The phenotype was analysed at DD210, where a significantly reduced number of photoreceptor nuclei in a row and a significantly thinner ONL was observed in the *CRB1* RP patient-derived retinal organoids in comparison to the isogenic controls. scRNA-seq data analysis has shown that all the major retinal cell types were equally present in DD230 *CRB1* patient-derived and isogenic control retinal organoids. Interestingly, levels of *CRB1* transcript was similar in *CRB1* patient-derived retinal organoid and the isogenic control, whereas variant patient CRB1 protein was strongly diminished in *CRB1* patient-derived retinal organoids [24]. The data indicate that a variant CRB1 protein is most likely produced, but it potentially has an increased turnover because it does not localize to its expected

location at the OLM. Alternatively, the trafficking machinery or endolysosomal system of CRB1 might be affected in the *CRB1* patient-derived retinal organoids. In *Drosophila* studies it is shown that the Crb trafficking is mediated by transport along microtubules by Rab11- and retromer containing endosomes [46,47]. Here, our gene ontology analysis on *CRB1* patient-derived retinal organoids suggests an aberrant endosomal vesicular system in Müller glial cells and rod photoreceptor cells. The reduced level of variant CRB1 protein in *CRB1* patient-derived retinal organoids is being studied by putative changes in RAB11- and retromer-mediated receptor cycling and the lysosomal compartment [48].

In **Chapter 5**, *CRB1*^{KO} and *CRB1*^{KO}*CRB2*$^{+/-}$ hiPSC were generated from a control hiPSC line and subsequently differentiated into retinal organoids. In the *CRB1*^{KO} hiPSC, a single nucleotide deletion was introduced in *CRB1* exon 2, resulting in a frameshift with premature stop and thus a knockout of the gene of interest. Since concomitant loss of *Crb2* can enhance the phenotype in *Crb1* mutant mice [25,28], *CRB1*^{KO}*CRB2*$^{+/-}$ hiPSC were used as well. Here, mutations were introduced in exon 2 of *CRB1* and in exon 3 of *CRB2*, resulting in a frameshift mutation with a premature termination codon and a homozygous knockout of CRB1 and a heterozygous knockout of CRB2. Three clones of each were used for differentiation into retinal organoids. The *CRB1*^{KO} and *CRB1*^{KO}*CRB2*$^{+/-}$ retinal organoids show a statistically significant decrease in number of photoreceptor nuclei in a row compared to the isogenic control at DD180 and DD210, and a statistically significant increase of number of photoreceptor nuclei above the OLM at DD180 for *CRB1*^{KO} and at DD180 and DD210 for *CRB1*^{KO}*CRB2*$^{+/-}$ retinal organoids. The *CRB1*^{KO} and *CRB1*^{KO}*CRB2*$^{+/-}$ retinal organoids develop an extended phenotype that includes disruptions at the inner retina, indicated by misaligned SOX9 positive MGC and ISLET1-2 positive rod and ON-cone bipolar cells at DD210.

The outer retina phenotype observed in the retinal organoids from **Chapter 4** is similar to the ones observed in *Crb1* RP mutant mouse models [30], an extended phenotype affecting the inner and outer retina was described in **Chapter 5** retinal organoids which was more similar to LCA mouse models [38]. Making the retinal

organoids described in this thesis a suitable model to study RP or LCA treatment possibilities.

AAV-mediated gene augmentation therapy for *CRB1*-related retinal dystrophies

In December 2017, the FDA approved gene augmentation therapy using adeno-associated viral (AAV) vectors for young RP and LCA patients with biallelic mutations in the *RPE65* gene, using AAV2 to deliver by subretinal injection a functional copy of the *RPE65* gene into the RPE (voretigene neparvovec, Luxturna) [49]. Nowadays, there are numerous clinical studies exploring the potential of gene augmentation therapies for retinal dystrophies using AAV vectors [50–52]. AAVs are the leading platform for gene therapy approaches because of their capability to transduce both non-dividing and dividing cells, they show limited integration into the host genome, AAVs show low toxicity and immune response, and AAV capsid variants display distinct cell tropism [50,52]. However, despite these advantages, there were recently a subset of patients observed with progressive atrophy after subretinal AAV treatment. A study by the University Eye Hospital in Tübingen confirmed that subretinal injection of voretigene neparvovec (VN, Luxturna, Novartis, Basel, CH) can potentially lead to RPE atrophy with consequent photoreceptor loss in and outside of the bleb area [53]. These atrophic regions can progress over a period of over 1.5 years. However, stable visual function improvement was observed in all patients in the observation period [53]. Longer follow up of these patients and studying the cause of RPE atrophy in more detail is important to understand and prevent this phenomenon. Further studies will reveal whether the toxicity is due to the overexpression of RPE65, the subretinal injection technique, impurities in the AAV preparation, or cellular response to the naked AAV or contaminating DNA. Studies showed that some but not all naked DNA can induce an innate immune response mediated by Toll-like-receptor 9 (TLR-9). The TLR-9 response can potentially be reduced by reducing the number of non-methylated CpG in the gene therapy vector construct. Alternatively, the TLR-9 response could potentially be reduced by incorporation of specific TTAGGG repeat DNA sequences into the AAV vector [54–57]. One could consider an alternative method of gene therapy application

in the retina such as intravitreal injection. Intravitreal injection is a more commonly performed ocular procedure and enables exposure of the vector to the entire retina. However, the intravitreal injection might transduce more off-target cells [58]. In addition, intravitreal injection of AAV raised sustained cellular inflammation in the vitreous of non-human primate eyes [59]. Therefore, continuing improving research applications and detection techniques is important for the future of gene therapy for patients.

AAV.GFP transduction comparison of distinct animal and human-derived models

Different AAV capsids display distinct cell tropisms, and therefore it is essential to define the AAV tropism in multiple models such as in mouse, rats (**Chapter 3**), and human-derived retinal organoids (**Chapter 4 and 5**).

Intravitreal injection of AAV2/5 and AAV2/9 showed relatively poor transduction efficiency of both photoreceptors and other cells in the inner nuclear layer (INL) in new-born rats [22]. Other researchers also showed a poor transduction of cells in the INL upon intravitreal delivery of all serotypes tested in this study (including AAV2/9) in two-month-old Sprague-Dawley rats [60]. However, an efficient transduction of mainly MGCs was observed after intravitreal injection of AAV6/ShH10^{Y445F} in new-born rats [22]. Efficient transduction of mainly MGCs in the INL after intravitreal injection of ShH10^{Y445F} was also observed in other models [61–63]. For subretinal injection of AAV2/5 and AAV2/9 in new-born rats, the RPE and photoreceptor cells were successfully transduced, whereas subretinal injection of AAV6 variant ShH10^{Y445F} was transducing the RPE, photoreceptor cells, MGCs, and other cell types in the INL [22]. Interestingly, subretinal injection of AAV2/9 or ShH10^{Y445F} in mice were the most powerful capsids to target RPE, MGCs and photoreceptor cells [61]. So subretinal injection of AAV2/9 seem to be different between these mice and rats. However, in adult Sprague-Dawley rats subretinal injection of AAV9 mainly transduced cells in the ONL, but also some (1-5 GFP positive cells per 100µm) cells in the INL [60]. Indicating that not only the species, but also the age of rodents could cause differences in AAV tropism.

Moreover, tropism differences between healthy and degenerated retina has also been described for specific serotypes. A significantly enhanced cellular transduction after intravitreal injection of AAV2 or AAV9 was observed in two-month old diabetic retinopathy mice treated with streptozotocin (STZ) compared to nondiabetic mice [64]. However, short duration STZ treatment (two weeks) did not have a significant effect on the transduction patterns of AAV vectors in the retina. Moreover, in both two weeks and two months STZ treatment diabetic mice, intravitreal injection of AAV5 did not show a statistically significant enhancement compared to nondiabetic control mice [64]. This suggests that certain retinal structural changes are needed to enhance some of the AAV capsids transduction in diabetic retinas. In **Chapter 3** healthy and *Crb1* mutant brown Norway rats showed similar AAV transduction for both subretinal as well as intravitreal injection at P5 or at P8. However, it might be possible that when control and *Crb1* mutant rats are injected at one month of age or older, when the retinal degeneration is further advanced, that AAV transduction of certain serotypes might be different.

Next, AAV tropism of certain serotypes (packed with *GFP*) was defined in human-derived models such as retinal organoids. Transduction of AAV2/5 and ShH10^{Y445F} significantly outperformed AAV2/9 at transducing MGCs of DD220 retinal organoids [23]. Moreover, Achberger *et al*. showed that retinal organoids transduced with self-complementary AAV2/2 (scAAV2/2) have a relatively low eGFP fluorescence intensity expression after 7 days of treatment at DD80 or DD300, whereas the scAAV2/7m8 capsid variant show significantly higher eGFP expression [63]. In **Chapter 4** we show that both single-stranded AAV2/2 and AAV2/5 with the CMV promotor and Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) sequence transduce very efficiently photoreceptor cells and, to a lesser extent, MGCs at DD120 retinal organoids [24]. In **Chapter 5** a similar transduction efficiency and tropism was observed when control retinal organoids were transduced with AAV2/2 and AAV2/5 at DD135 and DD200. In accordance with this, transduction of AAV2/5 with the CAG promotor at DD150 retinal organoids also showed efficient photoreceptor cell transduction [65]. In addition, efficient photoreceptor cell and MGC

transduction was previously shown with transduction at DD220 [23]. Differences in transduction efficiency might be caused by the maturity of the retinal organoids; it might very well be that certain receptors responsible for taking up the AAVs are less accessible at certain differentiation days. In conclusion, we have shown that AAV2/2 and AAV2/5 are capable of infecting Müller glial cells and photoreceptor cells in hiPSC-derived retinal organoids at DD120 (**Chapter 4)**, DD135 (**Chapter 5**), DD200 (**Chapter 5**), and DD220 [23,24].

Another alternative is using human retinal explants which are derived from (cadaveric) human donor eyes. Wiley *et al.* showed a variable tropism of seven AAV serotypes (AAV2/1, AAV2/2, AAV2/4, AAV2/5, AAV2/6, AAV2/8, and AAV2/9) in three human donor eyes [66]. This intra-donor variability was least apparent in AAV2/8, which consistently inefficiently transduced cells in the ONL. Which is interesting, since AAV8 is used in many *in vivo* transductions. Whereas AAV2/1 and AAV2/5 were particularly efficient at solely transducing photoreceptor cells, and AAV2/4 and AAV2/6 efficiently transduced cells in the ONL and the INL [66]. Quinn *et al.* showed that AAV2/5 and ShH10^{Y445F} have a higher potency in transducing photoreceptor cells in the ONL then AAV2/9. No intra-donor variability was observed here; AAV transduction of a serotype was similar in the three independent cadaveric human donor retinal explants investigated [23]. However, they observed that the photoreceptors of cadaveric human retinal explants were only efficiently infected in the presence of intact photoreceptor segments, indicating a role for the segments in the photoreceptor uptake of AAV particles [23]. The intra-donor variability on transduction efficiency of distinct AAV capsids need to be investigated in more detail.

AAV.hCRB treatment of animal and human-derived models

For AAV-*CRB* mediated gene augmentation studies the correct cell type needs to be targeted. For *Crb1* mutant rats mainly MGC with loss of CRB1 should be targeted, for this reason AAV6 variant ShH10^{Y445F} was used for AAV-mediated gene augmentation therapy in **Chapter 3**. In **Chapter 4 and 5**, both photoreceptor cells and MGCs with loss of CRB1 should be targeted in the *CRB1* patient or knockout retinal organoids and therefore AAV2/5.h*CRB* was used.

However, the main disadvantage of AAVs is the limiting package capacity of ~4.7 kb: larger gene expression cassettes than 4.5 kb do not fit in a single stranded AAV vector containing two inverted terminal repeats. Because of this limiting packaging capacity, the development of AAV-mediated gene therapy is challenging. *CRB1* cDNA plus the full length cytomegalovirus (CMV) ubiquitous promotor exceeds the package limit. However, this problem can be circumvented by using a minimal CMV promotor. Intravitreal injection of ShH10^{Y445F} with a CMV minimal promotor showed significant levels of GFP expression in MGCs of *Crb1* knockout mice [61]. Subretinal injection of AAV9 with minimal CMV promotor or hGRK1 promotor mediated substantial levels of Crb1 protein expression at the OLM [61]. Pre-clinical studies have shown that full length or short form of CRB1 were potentially deleterious or causing subretinal toxicity in *Crb1* mutant mouse models [37,58,61]. However, both mouse models have shown that Crb2 can compensate for the loss of Crb1 in *Crb1* mutant mice [37,58]. An improved photoreceptor layer morphology and ERG response was detected in a *Crb1* mutant mouse after AAV9.*CRB2* delivery targeting both photoreceptor and MGC [58]. However, no rescue was observed when either only photoreceptors or only MGCs were targeted with *CRB2* [58], indicating again the importance of targeting the correct cells. Here, both AAV.CMVmin.h*CRB1* and AAV.CMV.h*CRB2* were used for gene augmentation strategies in **Chapters 3, 4, and 5**.

In **Chapter 3**, AAV-mediated gene augmentation therapy using 1 µl of a dose of 1 x 10^{13}gc/mL ShH10^{Y445F}.CMVmin.h*CRB1* (ShH10Y.h*CRB1*) or ShH10^{Y445F}.CMV.h*CRB2* (ShH10Y.h*CRB2*) was explored in new-born *Crb1* mutant rats. Retinal function or visual function of individual *Crb1* mutant rats analysed at 3-months-old were not significantly different between ShH10Y.h*CRB1* and PBS injected, nor when treated with ShH10Y.h*CRB2*. Due to the absence of an enhanced retinal function after AAV treatment, we hypothesized that (1) the intravitreally injected 1 µl of a dose of 1 x 10^{13} gc/mL AAV might not been a high enough dose to spread well through the retina, (2) the injection efficiency and thereby number of AAV particles taken up by the retinal cells might not have been optimal, or (3) the injection at postnatal day 5 (P5) does not allow timely expression of the h*CRB1* or h*CRB2* transgenes to

diminish the severe retinal degeneration. Therefore, intravitreal delivery of ShH10Y.h*CRB1* or ShH10Y.h*CRB2* at P3 was explored as well. No difference was observed in visual function response measured by OKT at 3M. However, a statistically significant decrease in retinal function measured by ERG was observed in rats treated with ShH10Y.h*CRB1*, ShH10Y.h*CRB2*, or PBS in comparison with non-injected eyes. Since the PBS injection already resulted in a statistical significant decrease in ERG response, the surgical technique at P3 might be damaging to the *Crb1* rat retina. In addition, because of the variability between untreated Brown Norway litters, backcrossing these rats into a more defined genetic background might decrease the observed variability. To conclude, no timely rescue of the severe retinal degeneration was observed after AAV-mediated gene augmentation therapy in *Crb1* mutant rats. Future experiments could focus on treatment with immediate expression of transgenes or *in utero* gene therapy approaches.

Previously, proof-of-concept studies of AAV-mediated gene therapy for *CRX*-LCA and *RP2* knockout retinal organoids have been described [65,67]. Here, AAV-mediated gene augmentation therapy was performed on hiPSC-derived *CRB1* RP patient-derived as well as *CRB1*^{KO} LCA retinal organoids (**Chapter 4, 5**). The retinal organoids were transduced with a dose of 3,3 x 10^{10}gc at DD120 and were collected and analyzed at DD210. For the *CRB1* RP patient-derived retinal organoids in **Chapter 4**, AAV.h*CRB1* as well as AAV.h*CRB2* transduction resulted in a significant increase in the number of photoreceptor nuclei in a row as well as the ONL thickness [24]. Moreover, AAV.h*CRB2* treatment resulted in a significantly decreased number of photoreceptor nuclei above the OLM in the *CRB1* patient-derived retinal organoids. In addition, scRNA-sequencing revealed a partial restoration of transcriptional effect on the endosomal system after AAV.h*CRB1* or AAV.h*CRB2* treatment in *CRB1* patient-derived retinal organoids [24]. In **Chapter 5** a statistically significant increase in number of photoreceptor nuclei in a row was observed at DD210 after AAV.h*CRB2* treatment in *CRB1*^{KO} LCA retinal organoids. To our knowledge this is the first time that an improved phenotype after AAV.h*CRB* gene augmentation in *CRB1* RP patient-derived and *CRB1*^{KO} LCA retinal organoids is observed, providing essential

information for future gene therapy possibilities in patients with a mutation in the *CRB1* gene.

Future perspectives

Even though for some studies *in vivo* animal models are far from being completely replaced by human-derived models, it is pivotal for future studies to understand and use the knowledge of differences between rodents and humans. For instance, the subcellular localization of CRB1 and CRB2 are different in human-derived tissue in comparison to rodents [21–23]. In addition, mouse mRNA isoforms revealed that the *Crb1-b* isoform could potentially be associated with photoreceptor death [12], whereas reassessment of the phenotype-genotype correlation of 50 *CRB1* patients has shown that variations in the canonical full length form of CRB1 is mainly causative for retinal degeneration [68]. Therefore, showing that the AAV-mediated gene augmentation therapy was successful in mouse [37,58] as well as in retinal organoids (**Chapter 4 and 5**) [24] provides the essential information needed to continue with future gene therapy applications for patients with mutations in *CRB1*.

Nevertheless, current human-derived models can be improved to better mimic the human situation. First, improving the differentiation efficiency of retinal organoids is of interest. Currently, multiple groups describe a wide variability in differentiation efficiency between distinct iPSC lines [69–72]. In our studies, we also observe a wide variability in differentiation efficiency, even within the same line using the same differentiation protocol (unpublished data). Eliminating the factors causing this variability would be crucial for future studies. Moreover, the use of a bioreactor has shown to be efficient in scaling up the manufacture of retinal organoids in combination with an increased yield of photoreceptor cells bearing cilia and nascent outer-segment like structures [42]. Moreover, one could use a retina on a chip to further mimic the human situation. iPSC can be differentiated into RPE using for example small molecules [73] or using a mix of growth factors [74–76]. If iPSC-RPE and retinal organoids are combined on a chip (RoC), they can interact with each other and thereby better mimic the human situation. These RoC are able to form a physiological interaction of the iPSC-RPE and photoreceptor outer segments of the retinal organoids,

which is essential for the visual cycle in humans [77]. In addition, the microfluidic concept through a bottom channel and constant nutrient supply adds a vascular-like perfusion to the RoC [77]. In addition, a choroid on a chip (CoC) model is available which mimics the tissue vascularization, pigmentation, and immune response in the presence of circulating immune cells [78]. Combination of these RoC with CoC further mimic the human situation in a human-derived model system.

Conclusion

In conclusion, this thesis provides novel information on AAV.h*CRB* gene augmentation therapy in multiple *CRB1* mutant animal and human-derived models. We show the phenotype of (1) a novel mouse model with *CRB2* ablation specifically in rod photoreceptor cells with loss of retinal function (**Chapter 2**), (2) a *Crb1* mutant brown Norway rat with severe and early onset progressive vision loss (**Chapter 3**), (3) *CRB1* RP patient-derived retinal organoids (**Chapter 4**), and (4) $CRB1^{KO}$ and $CRB1^{KO}CRB2^{+/-}$ LCA-like retinal organoids (**Chapter 5**). Next, AAV-mediated gene augmentation strategies were explored in *Crb1* mutant rats (**Chapter 3**) and *CRB1* RP patient-derived and $CRB1^{KO}$ LCA retinal organoids (**Chapter 4 and 5**). Finally, single-cell RNA-sequencing was performed on AAV.h*CRB* treated and untreated *CRB1* RP patient-derived retinal organoids (**Chapter 4**). To our knowledge this is the first time that an improved phenotype after AAV.h*CRB* gene augmentation in *CRB1* RP patient-derived and $CRB1^{KO}$ LCA retinal organoids is observed, providing essential information for future gene therapy possibilities in patients with a mutation in the *CRB1* gene.

Article information

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Abbreviations:

AAV	Adeno-associated viral vectors
CRB1	Crumbs homolog-1
cKO	Conditional knockout
CNV	Copy number variation
CMV	Cytomegalovirus
CoC	Choroid on a chip
DD210	Differentiation day 210
E15	Embryonic day 15
EGF	Epidermal growth factor
ERG	Electroretinography
GFP	Green Fluorescent Protein
hiPSC	Human-induced pluripotent stem cell
INL	Inner nuclear layer
LCA	Leber congenital amaurosis
MGC	Müller glial cell
OKT	Optokinetic head tracking response
OLM	Outer limiting membrane
ONL	Outer nuclear layer
OPL	Outer plexiform layer
P5	Postnatal day 5
PALS1	Protein associated with Lin Seven 1
RoC	Retinal organoids on a chip
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
SAR	Subapical region
scRNA-seq	Single cell RNA-sequencing
SD-OCT	Spectral domain optical coherence tomography
SOX9	SRY-Box Transcription Factor 9
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element

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