CRB1 gene therapy coming of age: mechanistic insight and rAAV assays on mouse & human retinal organoid models

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Chapter 6

GENERAL DISCUSSION

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Introduction: *CRB1*-associated retinal diseases and clinical spectrum

Inherited retinal dystrophies (IRDs) are chronic and disabling disorders of the visual function. The *CRB1* gene is one of more than 279 genes (316 loci & genes) associated with retinal dystrophies (RD) [1]. The RD-*CRB1* patients show an autosomal recessive inheritance pattern. Biallelic genetic mutations of the *CRB1* gene (called *CRB1* variants) can cause retinal pigmentosa (RP), Leber congenital amaurosis (LCA) and occasionally macular dystrophies [2]. *CRB1*-related RDs are one of the more frequently occurring IRDs with 7-17% of all LCA cases and 3-9% of all RP cases [3–7]. Patients with the LCA-form are generally legally blind around birth (*congenital*) and RP-*CRB1* in late adolescence. Currently, no effective therapy exists for RD- or LCA-*CRB1* patients. A large diagnosis overlap exists between LCA-*CRB1* and early RP-*CRB1* [8]. Initial LCA- and RP-*CRB1* symptoms range from night blindness, tunnel vision, and a progressive loss of the peripheral vision in patients, to LCA-only symptoms, such as non-recordable electroretinogram (ERG), nystagmus, and no oculo-digital reflexes [8]. Vision loss in *CRB1*-patients is seen by full-field ERG recordings having lower scotopic a-waves (measuring the photosensitivity of rod photoreceptors), and lower scotopic b-waves (representing a loss of synaptic transmission through the neuroretina). Multifocal ERG recordings demonstrated a loss of ERG signal stemming by large of the rod-rich retinal periphery in early diagnoses and in later life also from a loss of central vision related to the loss of cone photoreceptor function [9].

So far, the *CRB1*-patient retinal morphology has only been studied by non-invasive imaging methods, such as fluorescein angiography, confocal Scanning Laser Ophthalmoscopy (FA cSLO, vascular leakage seen on *en-face* scans) and spectral-domain optical coherence tomography (SD-OCT, overall retinal morphology on *en-face* and OCT b-scans [retinal layer-by-layer visualization]), and similar to many other orphan diseases with limited patient numbers has not been studied on the morphology of cadaveric donor eyes. SD-OCT, cSLO and FA scans on LCA-*CRB1* patients may show a thickened neuroretina with no clearly visible synaptic layers, atrophy of retinal pigment epithelium (RPE), retinal oedema, and microvascular changes and osteoclast depositions (also called *bone-spicules* or *pigments*) close to venules [10,11]. The most common RP-*CRB1* morphological phenotypes are thinned photoreceptor nuclei layers (also called *outer nuclear layer* [ONL]), osteoclast depositions, Coats-like exudative vasculopathies, preserved para-arteriolar retinal pigment epithelia (PPRPE; definition: RPE cells are only preserved in proximity to arterioles), and preserved central fovea's; however, more severe phenotypes such as cystoid macular edema (CME) and macular atrophy can also be seen in late-stage and sporadically in early-stage RP-*CRB1* patients [2,12,13]. An absent ERG response and a thickened retina seen on SD-OCT in the first years of the life of some LCA-*CRB1* patients illustrates that LCA-*CRB1* is a developmental disease potentially affecting the embryonic retina [10,14].

A mix of morphology and retinal function test is the Fundus-controlled perimetry (FCP, also called *microperimetry*), a type of a visual field test. The FCP measures the light sensitivity of a defined retinal area(s) under scotopic (rods), mesopic (rods & cones), and photopic (cones) conditions. FCPs on RP-*CRB1* natural history studies are very promising for the preparation of *CRB1* gene therapy clinical studies because the photosensitivity of retinal areas can be related back on the SD-OCT/cSLO-based spatial markers (e.g. fundus) permitting tracking of specific scotomas (visual field *lesions*) and treated areas over time with a high retest-reliability in patients with no stable fixation [2,15,16]. The main treatment route of gene therapy products for ophthalmic diseases (**Chapter 1**) is delivered by local subretinal injection(s) preserving a limited retina area (called *island*s) that can be tracked by FCPs [17]. Other tests such as the classical Best-corrected visual acuity (BCVA) also showed promise in clinical trial preparations [2]. An rodent-adapted version of the BCVA is the optokinetic head-tracking response (OKT) [18]. It was further developed for measuring under low light and low contrast conditions [19,20]. The previous *proof-of-concept* (POC) *CRB* gene therapy in rodents showed a rescue on ERG and retinal morphology [21,22] (and **Chapter 1**). We also show that OKT contrast sensitivity is a very promising technique for screening *CRB* gene therapy vectors in mice *in vivo* (**Chapter 2**). We also generated retinal explants *ex vivo* (**Chapter 3**) and retinal organoids *in vitro* (**Chapter 4, 5**) which can be both modified for variant *CRB1* (RP-*CRB1* patient) expression. It will be interesting to correlate more patient findings to *CRB1* retinal organoids and other CRB models to generate better predictors (i.e., biomarkers) for monitoring the disease progression in patients and gene therapy trials. For example, the first preliminary experiments have measured retinal function and SD-OCTs on retinal organoids and organ-on-a-chip retina models [23–25].

CRB1 and CRB2 protein location and function in the neuroretina

Epidemiologic studies show a high genotype and phenotype heterogeneity in RD-*CRB1* patient pools [2,6,26]. Nowadays, RD-*CRB1* patients can be efficiently diagnosed by Next Generation Sequencing (NGS). Generally, RD-*CRB1* patients are found by linking a retinal phenotype to genes by whole-exome sequencing of retinal disease genes *only*. However, many (non-retinal disease) modifier genes may be missed by this method [2,12]. Efforts have been undertaken to screen also non-retinal diseases, such as *CRB2* along NGS of retinal patients [27] but the price for NGS and the limited information on retinal modifier genes have been obstacles in the field. RD-*CRB1*-based cell & molecular biology research studies what biological changes drive a RP-*CRB1* phenotype to an LCA-*CRB1* or *vice versa* (by therapies) by studying the *CRB1* gene function and (gene) therapies in different models. Research in the field may find (a) novel biomarkers (**Chapter 2, 5**), (b) develop therapies (**Chapter 1, 2, 3, 4**), (c) find modifier genes (e.g., other LCA/RP genes, apicobasal genes, unknown transcription factors, and other unidentified genes. See also **Chapter 2, 5**), (d) identify complex transcription and proteomic changes in cells affected by the loss of the CRB1 protein

or variant CRB1 protein expression (**Chapter 5**), and (e) predict the stability, folding, and trafficking of variant *CRB1* proteins by experimental methods (**Chapter 5**).

The mouse neuroretina like the human neuroretina contains rods and cones but the mouse retina lacks a cone-rich fovea. Human CRB1 (but not human CRB2) was found in both human PRCs and human MGCs at the subapical region in two-day-old human cadaveric retinas. Human CRB2 was found in the PRC inner segments but not at the subapical region. But in dissected non-human primate retinas and cultured human-derived retinal organoids (**Chapter 4**), CRB2 was found at the subapical region and in the PRC inner segments [28–30]. We propose that CRB2 location was lost at the subapical region in two-day-old cadaver human eyes due to post-mortem degradation processes because fresh non-human primate and cultured human organoids showed CRB2 expression at the subapical region adjacent to the adherens junctions at the outer limiting membrane (**Chapter 4**). In mouse, CRB1 is found exclusively in MGCs-villi at the subapical region and CRB2 in both PRCs and MGCs at the subapical region [31]. We also studied the onset of human CRB1 and CRB2 protein expression in retinal organoids and foetal retina showing that CRB2 precedes CRB1 during retinal development (**Chapter 4**).

The CRB1 protein, belonging to the CRB protein family member (CRB1, CRB2, or CRB3A), is a relative large protein of 160 kDa (1405 aa in mice and 1406 aa in humans; 200-220 kDa when glycosylated) [32]. CRB1 and CRB2 also share a large extracellular domain of three laminin A/globular(LamG)-like and 19 and 14 epidermal growth factor(EGF)-like-domain repeats [33]. Both CRB1, CRB2, and CRB3A proteins share a similar intracellular 37-aa C-terminal tail containing two protein-protein interaction motifs: the FERM domain (**4**.1 protein/**e**zrin/**r**adixin/**m**oesin) and the glutamic acid-arginine-leucine-isoleucine (ERLI)/PDZ (**P**ostsynaptic density/**D**iscs large/**Z**O-1) domain [34–38]. FERM domains are used by many transmembrane proteins as adaptors to the actin cytoskeleton [39]. CRB protein family members (CRB1, CRB2, or CRB3A) can participate in two apical polarity complexes: The core Crumbs (CRUMBS/PALS1/PATJ) and PAR complex (PAR3/ PAR6/aPKC). They are initiated by binding of Protein associated with Lin Seven 1 (PALS1; also called MAGUK p55 [MPP5]) or Partitioning defective-6 homolog (PAR6) to the ERLI/PDZ motif of the Crb protein [38,40,41]. The CRB apical polarity complex containing a CRB protein family member (CRB1, CRB2, or CRB3A) can be in complex with PALS1-MUPP1/MPP3/MPP4, PALS1-PATJ, EPB41L5(Yurt or Moe orthologue; cytoskeletal regulator)/PALS1/PAR6, PALS1/PAR6-LIN7c/PATJ, PAR6-CDC42/aPKC, or PAR6-PAR3(aPKC inhibitor & substrate)-FRMD4A/B-CYTH1 [3,32,42–45]. Yet, it is not clear whether MUPP1 and PATJ are in the same cell types and in the same complex with CRB1-PALS1(MPP5). Also, ablation of PALS1(MPP5) specifically in the neural retina in a Pals1 conditional short hairpin RNA (shRNA) knockdown mouse model using the Chx10Cre transgene did not significantly affect the retinal morphology or the Crumbs complex in photoreceptors/Müller glial cells, whereas

a severe RPE degradation phenotype was observed upon ablation of PALS1(MPP5) in both the RPE and neural retina in a Pals1 shRNA knockdown mouse model using the CrxCre transgene. These experiments indicated that PALS1 is essential at the tight junctions in RPE but not for the CRB apical complex in mouse photoreceptors/Müller glial cells albeit being overabundantly present [46]. Other protein complexes have been associated with the Crumbs complex and CRB binding, such as YMO1 (yurt/mosaic eyes like 1), EHM2, and moesin (FERM domain) proteins on the Crumbs complex; and, 14.3.3, CDC42, HOMER1-3, STXBP4 (Hippo pathway regulator) for the related PAR protein complex (Baz/PAR3-PAR6-aPKC) [36,44,47–50]. Interestingly, CRB's C-terminal phosphorylation by PKCa permits PALS1 binding but inhibits moesin binding indicating that the initiation of the Crumbs complex is less regulated by phosphorylation and more by CRB apical trafficking or ERLI/PDZ motif binding partners [50]. The CRB apical polarity complex in the neuroretina supports the maintenance and recruitment of tight-junction adhesion molecules (cadherins, catenins, zonula occludens proteins) at the adherens junction constituting the outer limiting membrane (OLM), acts as an intermediary for regulating development pathways (e.g. Notch & Hippo pathway), and regulates the recruitment of actomyosin cytoskeletal proteins [38,42,51–53].

The intracellular domain of CRB2 can also interact with the retromer sorting protein VPS35 [54]. The zebrafish CRB2A (a human *CRB2* orthologue) can interact with the recycling endosome protein RAB11A [55]. RAB11A and VPS35 are both essential proteins for efficient turn-over of membrane proteins on early endosomes, such as the cell surface proteins NOTCH1 and CRB1 (**Chapter 5**). We found a reduction in VPS35 and RAB11A in patient retinal organoids compared to their isogenic controls (**Chapter 5**). The long extracellular domain of CRB1 or CRB2 was proposed to form homophilic interaction *in trans* for stabilization at the apical membrane based on *Crb* fruit fly and zebrafish studies [56–58]. For example, the extracellular domain of the zebrafish CRB2A form homophilic interaction *in trans* contributing to the CRB2A apical membrane stabilization (apical protein turn-over) and overall cell adhesion [58]. Recently, also an *in cis* fruit fly Crumbs-Notch interaction on the extracellular domain was demonstrated that suppresses Notch receptor endocytosis and repress the Notch pathway activity [59]. We show that human CRB1-NOTCH1 can also interact on the extracellular domain in retinal organoids with little apical interaction and little apical NOTCH1 found in RP-*CRB1* retinal organoids (**Chapter 5**), potentially in *trans* or *cis* because we found NOTCH1 solely in MGCs and CRB1 in PRCs & MGCs. The results point to CRB1 stabilizing NOTCH1 at the OLM on apical early endosomes determining the apical protein turn-over (see section 3).

In previous studies, genetically modified mice were generated to knockout the *Crb1* gene and conditional knockout the *Crb2* gene in specific retinal cells (PRCs, rod PRC only, Müller glial cells [MGCs], or retinal progenitor cells [RPCs]) to study the function of the *Crb1* and

Crb2 gene [29,47,60–64]. The mouse studies (see also **Chapter 2**) and the RP-*CRB1* retinal organoid studies (**Chapter 4, 5**) show that the loss of CRB1 or CRB2 proteins coincide with the focal loss (*gaps*) of tight-junction adhesion molecules (cadherins, catenins, ZO-1) and subapical proteins (e.g., PALS1, MUPP1, PAR3, PATJ) at the OLM (called *OLM breaks*). The loss of the Crumbs complex in mice [29,47,60–64] (see also **Chapter 2**) and RP-*CRB1* retinal organoids (**Chapter 4, 5**) facilitates loss of cell adhesion between PRCs-PRCs, PRCs-MGCs, and MGCs-MGCs; and dysregulates the apical polarity signalling in the neuroretina (**Chapter 4, 5**).

The endolysosomal system and the apical CRB1 protein trafficking

The endosomal-lysosomal (endolysosomal) system is a complex dynamic intracellular vesicular trafficking and positioning system on the endocytic and secretory pathway. For example, the system regulates the sorting of proteins in correct vesicles, transporting the protein-loaded vesicles to correct microdomains at the cell periphery (along the cell-spanning endoplasmic reticulum [ER]), designation of proteins and vesicles to be sorted for degradation, recycling of proteins, and assisting the autophagic pathway [65,66]. The secretory pathway vesicles are termed secretory vesicles (includes lysosomes), transport vesicles (made on the *trans*-golgi network [TGN]), and exosomes. The endocytic pathway contains endocytic vesicles (clathrin-dependent/independent endocytosis), early endosomes (EE), recycling endosomes, late endosomes (LE), multivesicular bodies (MVB), and lysosomes. The secretory pathway, the endocytic pathway, and the autophagic pathway can converge on almost all vesicles. The endolysosomal system research field is rapidly expanding, even though complicated by the dynamic nature of the system. For example, many classical markers of endosome subpopulation of early/late endosomes and lysosomes are frequently found on all of them making identification of subpopulations difficult, different cells employ different endosome protein(s) (markers) for the regulation of the endolysosomal system, and many studies have been performed on single cells to track individual vesicles *in vitro* but vesicle-trafficking on cell-dense tissues may be quite different because of different apical protein expression profiles. Nonetheless, it is certain that a functional endolysosomal system is central to the health of all cells.

Some of the most studied diseases on (dys-)regulations of the endolysosomal systems are diseases with protein aggregation phenotypes (Parkinson's, Alzheimer's, Huntington's Disease), immune cells fighting pathogens, and monogenetic diseases on receptor-mediated endocytosis. Here, we focus on receptor-mediated endocytosis of intact or variant CRB proteins localized at the apical plasma membrane immediately adjacent to adherens junctions at the outer limiting membrane. At this apical membrane, cell surface CRB transmembrane proteins are internalized by clathrin-mediated endocytosis and packaged in endocytic vesicles maturing to early endosomes from where the vesicles can either be reintegrated back into the apical membrane or become designated for degradation in the lysosome. Recently, it

was indicated that NOTCH and CRB are endocytosed in the same apical vesicles [55]. The membranes of early endosomes (containing CRB/NOTCH) expand forming tubular vesicles containing retromer protein sorting complexes (SNX1/2, SNX5/6, VPS26, VPS29, and VPS35). The early endosomes then briefly peak in levels of PtdIns(3)P to bind to FYVE protein early-endosome-antigen-1 (EEA1), which is required for RAB11A-mediated removal of recycling cargo from endosomes [67,68]. The recycling endosomes then transport back the cargo to the membrane for membrane (re-)integration. It is thought that in an undisturbed endolysosomal system, most CRB/NOTCH is efficiently recycled and only a small fraction (e.g., variant CRB proteins) stays in early endosomes which then mature to late endosomes / lysosomes for protein degradation. New CRB protein is continuously produced in the ER, modified in the Golgi, and loaded on vesicles transported on the TGN moving on plus to minus-strand microtubules by dynein motors to the periphery (anterograde transport). Close to the apical membrane, the vesicles acquire RAB11A and Myosin V (attached on F-actin) guiding the cargo-release and CRB apical membrane integration [69]. The anterograde transport is mediated by a CDC42-dependent positive feedback loop and repressed (preventing overexpression) by Stardust (Sdt)/PALS1 [70,71].

Little is known about the specific regulation of variant CRB1 protein trafficking. Interestingly, the intracellular domain of CRB2 can bind to RAB11A and VPS35 potentially regulating the sorting and cargo-release on early endosomes [54,55]. Also, the loss of retromer induces an increase of lysosome degradation and a reduction of the apical Crumbs complex membrane-occupied area [54]. The intracellular domain of fruit fly Crumbs shows high sequence similarity to the intracellular domain of (human) CRB1 and CRB2 potentially also permitting mediation of sorting and cargo-release on early endosomes by CRB1/CRB2. We found a decrease of variant CRB1, NOTCH1, VPS35, and RAB11A protein expression at the apical membrane as well as an increase of degradative vesicles in *CRB1* patient retinal organoids (**Chapter 5**) providing evidence that variant CRB1 proteins are less efficiently sorted to the apical membrane and potentially dysregulating the CRB1/CRB2-VPS35 and CRB1/CRB2-RAB11A interactions. Interestingly, we also found an increase of two FYVE domain containing proteins EEA1 and WDFY1 on early endosomes in CRB1 patient organoids. The FYVE domains of EEA1 and WDFY1 can bind PtdIns(3)P. Overexpression of *Wdfy1* transcripts was also found in a RNAseq screen of *Crb1Crb2* cKO vs wildtype mice at E15.5 (**Chapter 5**). This suggest that variant CRB1 represses early endosome maturation causing an increase in degradative vesicle accumulation.

It will be interesting to find out (1) how cells upregulate *WDFY1* expression as a cellular response to inefficient sorting of variant CRB1 proteins, and (2) if the integration of more WDFY1 on early endosomes has a biological function such as initiating a less-well studied cell apoptosis pathway. WDFY3 (a WDFY1 family member which is 10x larger) mediates ATG5-guided selective autophagy by binding to the ubiquitin-binding protein p62 [72]. P62,

an autophagosome cargo protein, was also upregulated in *CRB1* patient retinal organoids (**Chapter 5**). Yet, the BEACH binding motif for p62 is not present on WDFY1. It is tempting to think that all WDFY proteins evolved around selective autophagy thus regulating variant CRB1 degradation. Moreover, an increase of WDFY1 protein can induce NF-κB activation and pro-apoptotic pathways in immune cells [73], but conversely, NF-κB activation was not found in neuronal cells expressing increased levels of WDFY1 [74]. These results further underline how the endolysomal system is inherently differently regulated in immune and neuronal cells, and that more research is needed to delineate the effect of WDFY1 on RP-*CRB1* pathology.

Very little is known on how specific variant CRB1 proteins are regulated by the endolysosomal system in retinal cells. For example, when a human *CRB1* mutation was knocked in on a *Crb1*-null mouse ($Crb1^{C249W/-}$), normal amounts of CRB1 protein levels were found at the OLM suggesting that the CRB1 variant trafficking is intact. However, the OLM was more sensitive to light-stress demonstrating a loss of adhesion mediated by variant CRB1 [75]. Also, the natural occurring $Crb1^{rd8}$ mouse – having a single nucleotide deletion on exon-9 corresponding to ~1150th aa of the 1406 aa in humans (the CRB1 variant contains only the extracellular domain) – express variant CRB1 proteins apically but at reduced levels in Müller glial cells [76]. However, the exact variant protein expression may be much lower because the antibody used in these studies to detect the variant CRB1 may also detect CRB2. Finally, fruit fly studies on a *Crb*-null background compared the effect of reintroducing different CRB variants including a variant similar as the $Crb1^{C249W}$ (Crb^{C749W} in fruit fly). Very little variant CRB protein was detected in the rhabdomere (eight photoreceptor cells arranged in a stalk as seen in insects) [77]. Other *Crb* mutants in the LamG3 & EGF-like domain 21/22 on the extracellular domain (Crb^{T1386M}; Crb^{N1486S}; Crb^{C1540Y}) showed a much higher CRB protein expression at the rhabdomere compared to the wildtype control. What is more, these variant CRB1 proteins formed aggregates and mislocalized showing a typical gain-of-function phenotype. We found a reduction of potentially four different variant CRB1 proteins ($CRB1^{M1041T}$, $CRB1^{Y631C}$, $CRB1^{E995*}$, $CRB1^{C948Y}$; expressed in three RP-*CRB1* patients: $CRB1^{M1041T/\ M1041T}$; $CRB1^{Y631C/E995*;}$ $CRB1^{M1041T/C948Y}$) in patient retinal organoids (**Chapter 5**). It will be exciting to study why certain variants become overabundantly expressed at the OLM and others are efficiently removed from the system. For example, $Crb1^{C249W}$ showed protein expression in mice but a very similar Crb^{C749W} did not in fruit fly. Can the difference be explained by differences in the endolysosomal system in species? A study of the $CRB1^{C250W}$ in human retinal organoids may solve the outstanding question. Furthermore, knowing what regulates CRB levels at the OLM will not only broaden our understanding of CRB function but may also contribute developing second generation *CRB* gene supplementation therapies where CRB overexpression can be prevented by including CRB regulators on the vector cassette. Fortunately, overexpression is most likely a lesser problem for gene therapy treated RP-*CRB1* patients because a low dose of CRB may already

be sufficient as indicated by the phenotypic rescue found in the isogenic *CRB* organoids expressing a variant *CRB1* on one allele and a wildtype *CRB1* on the other (**Chapter 5**), the mild phenotype seen in *Crb1*^{KO}*Crb2*^{LowMGC} mice (**Chapter 2**), and the overall tolerability of rAAV high vector doses injected to the mouse eye [21,22] (see also **Chapter 2**).

The total CRB levels at the OLM in photoreceptors and Müller glial cells determines the phenotype

The similar protein location and the mouse (conditional) knockout studies showed that CRB1 and CRB2 proteins have compensatory and overlapping functions in the neuroretina [29,47,60–64]. However, we did not know the contribution of CRB2 protein to photoreceptors and Müller glial cells. A neuroretina consists of roughly ten photoreceptors per Müller glial cell. And one Müller glial cell wraps around ~10 photoreceptor nuclei column in the ONL. Thus, raising the question if a *CRB* gene supplementation therapy needs to target many photoreceptors, many Müller glial cells or a mix of PRCs-MGCs?

First, we generated a new *CRB1*-RP-like mouse model where we knocked out *Crb1* and conditionally knocked out one allele on the *Crb2* gene reducing the levels of Crb2 in MGCs at the OLM (Mouse model: *Crb1*$^{-/-}$*Crb2*$^{Floxed/wildtype}$Pdgfrα-*Cre*$^{Transgene/+}$. Shorted name: *Crb1*^{KO}*Crb2*^{LowMGC}). Early retinal disease phenotypes were seen on morphology, such as misplaced nuclei in the subretinal space (between the OLM and the RPE layer) and the outer plexiform layer (OPL; a synaptic layer between the photoreceptor nuclei and the inner retinal cells) in the inferior quadrants at 1 and 3 months-old animals. This was accompanied by an increase in gliosis (Glial fibrillary acidic protein [GFAP] expression), a reduction in MGC-microvilli length, and a reduction of PRC inner/outer segment length in 3-month-old *Crb1*^{KO}*Crb2*^{LowMGC} retinas. Then we found a correlation between the CRB1, CRB2 and p120-catenin protein expression at the OLM and the number of OLM disruptions within four different *CRB1*-RP-like mouse models (*Crb1*^{KO}; *Crb2*$^{\Delta Rods}$; *Crb1*^{KO}*Crb2*^{LowMGC}; *Crb1*^{KO}*Crb2*$^{\Delta Rods}$) at 3-months-of-age. The study showed that PRCs and MGCs provide around half of the total CRB2 protein to the OLM and that the *Crb1*^{KO}*Crb2*^{LowMGC} neuroretina had a 67% reduction of CRB2 protein at the OLM (**Chapter 2**). Also, the more photoreceptor-based model (*Crb1*^{KO}*Crb2*$^{\Delta Rods}$) mouse had the highest number of OLM breaks compared to the other studied *CRB1*-RP-like models but a surprisingly well-preserved retinal function measured by ERG and retinal thickness for the first 3 months-of-age [61]. When *Crb2* was ablated in Müller glial cells lacking *Crb1* (*Crb1*^{KO}*Crb2*$^{\Delta MGC}$) then no retinal function was detected with a severe retinal phenotype on morphology at 1-month-of-age [29]. These studies demonstrate that *CRB1/CRB2* expression in Müller glial cells is of high importance for gene supplementation therapies and should not be neglected by choosing only a photoreceptor-specific promoter. The current CRB gene supplementation therapy contains a ubiquitous promoter (CMV or CMVmin) that expresses the transgene in Müller glial cells as well as photoreceptor cells [21,78,79] (see also **Chapter 2+3+4**).

Viral vector-based gene supplementation therapy for RP-*CRB1* patients

Finally, we show that the regenerative capacity of the neuroretina/OLM in a *CRB1*-RP-like mouse model can be reversed by supplementing human *CRB1* (h*CRB1*) or *CRB2* (h*CRB2*) cDNA to MGCs by recombinant adeno-associated viral (rAAV) vectors (**Chapter 2**). Gene supplementation therapies by rAAVs are promising techniques because the viral vectors are relatively safe (relative nonintegrative in the genome, replication deficient), many capsids with different cell & tissue-specific infection properties (managed tropism) are available, and the transgene expression can last for many years. Previously, we and others demonstrated that the rAAV6 capsid variant ShH10^{Y445F} can efficiently infect PRCs, MGCs and the epithelium of the ciliary body, and likely stem cells, and immune cells (e.g. dendritic cells, microglial cells). We injected the rAAVs by intravitreal injection targeting at least around 60% of all MGCs [79]. Our vector expresses upon cell infection and integration in the nuclei concatemerized cDNA of h*CRB1* or h*CRB2* under the control of a ubiquitous promoter (a minimal or full length CMV, respectively). We show that rAAV-based gene supplementation of h*CRB1* or h*CRB2* to *Crb1*^{KO}*Crb2*^{LowMGC} MGCs protected the neuroretina on morphology from degenerating under stress measured by retinal thickness, ectopic cells, photoreceptor inner/outer segments, MGC-microvilli length, and on SD-OCT imaging. Interestingly, only h*CRB2* protected the vision measured by ERG and OKT contrast sensitivity. Surprisingly, the h*CRB1* even reduced the ERG dark-adapted a-wave response and the OKT contrast sensitivity measured at 0.031, 0.064, and 0.092 cycles per degree. Further analysis indicated ectopic hCRB1 expression in the ciliary body, an increase in vascular markers at the ciliary body and some infiltrating cells at the inner limiting membrane. We cannot exclude the possibility that the *Crb1* naïve mice upregulated an immune response upon being exposed to the (*de novo*) hCRB1 protein in the eye, indicative that it is rather a species-specific response. More studies are needed to delineate the vision deficit with the therapeutic vector. Nevertheless, the study demonstrates that (a) hCRB1 as well as the hCRB2 protein, which are very similar in their protein domains, can support the regenerative morphological capacity of the neuroretina/OLM in a *CRB1*-RP like mouse model, and that (b) intravitreal injection is a powerful tool because one can supplement much more MGCs with the rescue vector. The vision deficits found in mice exposed to hCRB1 also show that the rAAV-h*CRB1* therapy injections done intravitreally compared to subretinal injections have a higher risk of evoking side-effects and may require additional safety measures limiting (*de novo*) protein exposure to immune cells, preventing ectopic protein expression, and limiting the exposure of potential cis-regulatory toxicity found in some ubiquitous promoters to potentially sensitive cell populations. Several methods can be employed to reduce the risk (reviewed in **Chapter 1**): (a) more infective capsids such as AAV2-GL and AAV2-NN vectors may lower the dose requirement but these capsids tend to be overall less cell-specific, (b) more cell-specific capsids such as the AAV6 variant ShH10^{Y445F} infects more efficiently glial cells than the parent vector but AAV6 also infects immune cells (see also **Chapter 2+3+4**), (c) capsids that

pass more readily the rodent inner limiting membrane after intravitreal injection such as AAV2.7m8, (d) delineate the species-specific tropism in especially human retina models (**Chapter 2+3+4**), and (e) generate retina cell-specific promoters. We have shown that rAAV5 can more efficiently infect human photoreceptors and Müller glial cells in human retinal organoids and human retinal explants compared to rAAV9 (**Chapter 4**) without having the (theoretical) potential drawback of the rAAV6 variant ShH10Y infecting immune cells.

Conclusion

In conclusion, the thesis provides much needed information on rAAV-h*CRB* gene supplementation such as (a) rAAV vector optimization in the ophthalmic field (**Chapter 1**), (b) vector safety related to ectopic transgene expression (**Chapter 2**), (c) the overlapping and compensatory roles of both hCRB1 and hCRB2 protecting the OLM from stress in a challenged RP-*CRB1* mouse model (**Chapter 2**), (d) the development of rAAV-vector screens on the same donor neuroretina in parallel (**Chapter 3**), (e) rAAV5 infecting both human MGCs and PRCs on donor retinas and retinal organoids (**Chapter 4 part 1**), (f) the Crumbs complex changes during retinal development in retinal organoids and foetal retinas alike (**Chapter 4 part 2**), (g) the generation of a human RP-*CRB1* disease model on retinal organoids (**Chapter 4 part 3**), (h) that the human RP-*CRB1* retinal organoids have little variant CRB1 at the OLM which suppresses NOTCH1 expression at the OLM causing a dysregulation in the endolysosomal system (**Chapter 5**). With the previous studies, the new evidence in this thesis, and the ongoing natural history studies, the rAAV-*CRB* gene supplementation project paves the way for clinical studies in near future.

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