Viral gene therapy approaches for CRB1 retinal disease

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Chapter 1

1.1 Research Models and Gene Augmentation Therapy for *CRB1* Retinal Dystrophies

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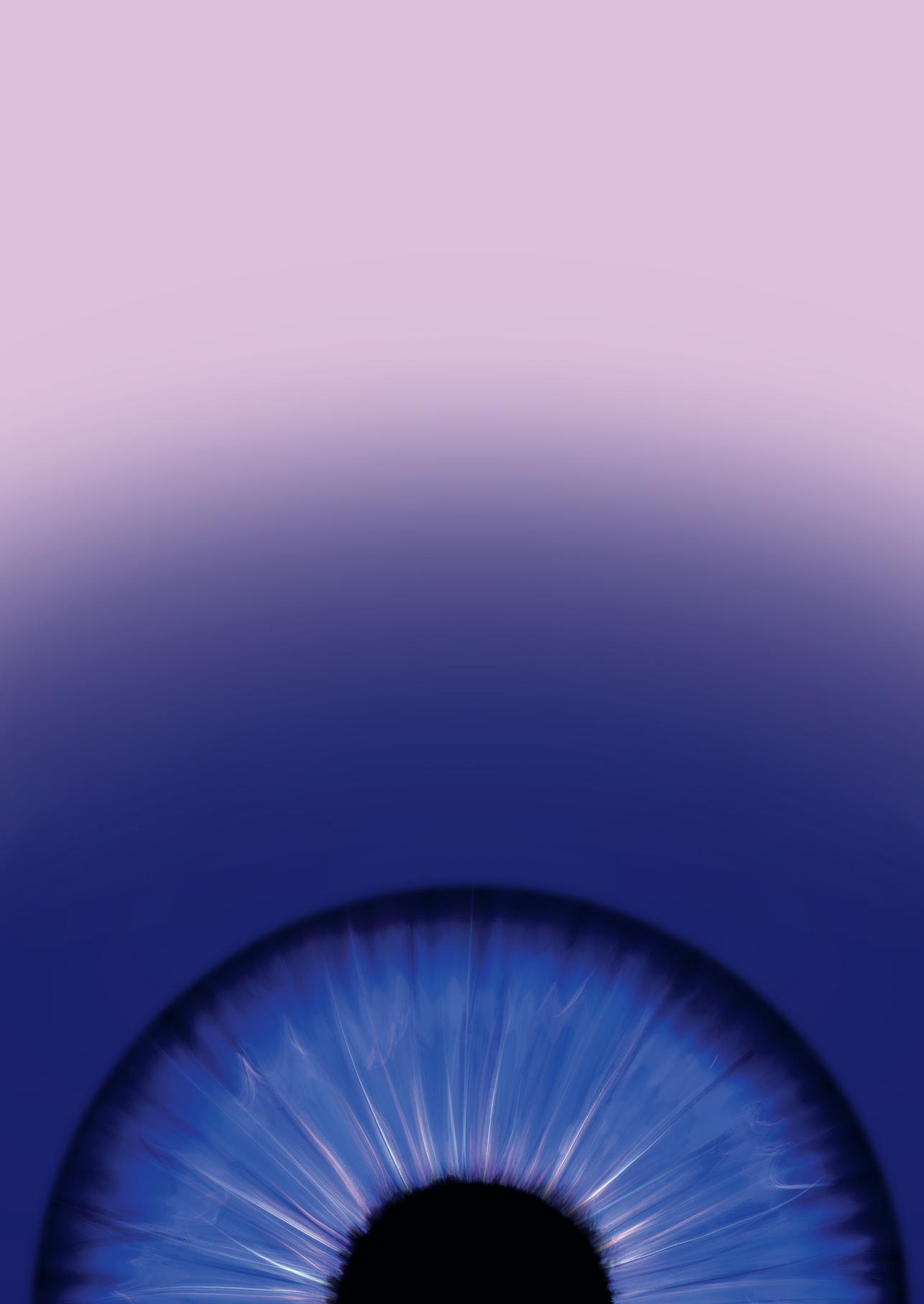
1.2 Aims and Outline of this Thesis



Abstract

Retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA) are inherited degenerative retinal dystrophies with vision loss that ultimately lead to blindness. Several genes have been shown to be involved in early onset retinal dystrophies, including *CRB1* and *RPE65*. Gene therapy recently became available for young RP patients with variations in the *RPE65* gene. Current research programs test adeno-associated viral gene augmentation or editing therapy vectors on various disease models mimicking the disease in patients. These include several animal and emerging human-derived models, such as human induced pluripotent stem cell (hiPSC) derived retinal organoids or hiPSC-derived retinal pigment epithelium, and human donor retinal explants. Variations in the *CRB1* gene are a major cause for early onset autosomal recessive RP with patients suffering from visual impairment before their adolescence and for LCA with newborns experiencing severe visual impairment within the first months of life. These patients cannot benefit yet from an available gene therapy treatment. In this review, we will discuss the recent advances, advantages and disadvantages of different *CRB1* human and animal retinal degeneration models. In addition, we will describe novel therapeutic tools that have been developed, which could potentially be used for retinal gene augmentation therapy for RP patients with variations in the *CRB1* gene.

CRB family members

Crumbs (crb) is a large transmembrane protein initially discovered at the apical membrane of *Drosophila* epithelial cells [1]. Several years later, it was found that mutations in a human homologue of the *Drosophila* melanogaster protein crumbs, denoted as CRB1 (crumbs homologue 1), was involved in retinal dystrophies in humans [2]. The human *CRB1* gene is mapped to chromosome 1q31.3, and contains 12 exons, has 12 identified transcript variants so far, 3 CRB family members, and over 210 kb genomic DNA (Den Hollander et al., 1999); http://grch37.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000134376;r=1:197170592-197447585). Canonical CRB1 is, like its *Drosophila* homologue,

a large transmembrane protein consisting of multiple epidermal growth factor (EGF) and laminin-globular like domains in its extracellular N-terminus (Figure 1A). The intracellular C-terminal domain contains a FERM and a conserved glutamic acid-arginine-leucine-isoleucine (ERLI) PDZ binding motives. An alternative transcript of CRB1, *CRB1-B*, is recently described and suggested to have significant extracellular domain overlap with canonical CRB1 while bearing unique 5' and 3' domains [3]. In mammals, CRB1 is a member of the Crumbs family together with CRB2 and CRB3 (Figure 1A). CRB2 display almost the same protein structure as CRB1, except a depletion of four EGF domains. CRB3A lacks the entire typical extracellular domain but contains the transmembrane domain juxtaposed to the intracellular part with the FERM-binding motif and a the ERLI PDZ sequence. A second protein (isoform CRB3B) arises from the same *CRB3* gene due to alternate splicing of the last exon, resulting in a different C-terminus with a cysteine-leucine-proline-isoleucine (CLPI) amino acid sequence, and thus lacks the PDZ domain [4, 5]. Interestingly, the CRB3B isoform is found in mammals, but not in zebrafish or *Drosophila* [4]. Further details about CRB isoform details can be found in Quinn et al. 2017 [6].

CRB localization in the retina

In mammalian tissue CRB1 and CRB2 are predominantly expressed in the retina, however CRB2 expression is also found in other tissues such as in kidney podocytes, in the subventricular zone of the brain, and in the spinal cord [7-10]. Within the retina, CRB proteins are localized at the subapical region (SAR) above the adherens junctions between photoreceptor and Müller glial cells, multiple photoreceptor cells , or between multiple Müller glial cells (MGC) [11, 12]. In addition, unlike CRB1, CRB2 is also localized in the retinal pigment epithelium (RPE). Defining the subcellular localization is essential to understand the function of CRB proteins (Figure 1B). Mouse studies have shown that full length Crb1 protein is exclusively present in MGC at the SAR while Crb2 and Crb3 are present in both photoreceptor cells and MGC [13]. Serial tangential cryosectioning of the retina followed with western blotting displays Crb1-B transcript expression in outer segments of photoreceptor cells in mice [3]. The first ultrastructural data in postmortem human retina revealed a different localization, where CRB2 is

located in MGC at the SAR and at vesicles in photoreceptor inner segments, whereas CRB1 is located in both MGC and photoreceptor cells at the SAR [14, 15]. CRB3A was found in microvilli of MGC at the SAR and in inner segments of photoreceptor cells [14, 15]. However, recently, it was shown that CRB1 and CRB2 are located in MGC and photoreceptor cells at the SAR in the second trimester of human fetal retina and in human iPSC-derived retinal organoids, whereas in the first trimester only CRB2 was detected [16]. Single-cell RNA sequencing data of human fetal retina and RPE confirms that CRB1 is present in retinal progenitor cells and MGC but not in RPE, which is in accordance with mouse versus primate localization studies [17]. In addition, similar localization for CRB1, CRB2 and CRB3A were detected in rhesus and cynomolgus macaques [18]. CRB3A was also detected in the inner retina and RPE of both rhesus and cynomolgus macaques [18]. These recent data suggest that, in humans, CRB2 might also be present at the SAR membranes in photoreceptor cells rather than only in vesicles of photoreceptor inner segments. The discrepancies in CRB2 pattern at the SAR observed in postmortem human retinas versus monkeys, fetal and retinal organoids could be explained by the age of the donors studied, the quality of the samples (processed within 48h after death), or by technical issues. We could speculate that either CRB2 may have a different location in human aged retinas, CRB2 at the SAR might have not been detected, or CRB2 might have been endocytosed from the photoreceptor cells plasma membrane following donor death. Additional experiments with fresh human retina defining the subcellular localization of CRB1 and CRB2 could potentially resolve these differences. In summary, according to all these evidences, we hypothesize that in primates, including humans, CRB1 and CRB2 are located in both cell types at the SAR. Therefore, both MGC and photoreceptor cells should be targeted to prevent retinal degeneration in RP patients.

Recently, Crb trafficking to the correct apical location in *Drosophila* epithelium has been further investigated (Figure 1C) [19-22]. Crb is correctly localized by Rab11-containing endosomes using motor-driven transport along polarized microtubules and F-actin filaments. Interestingly, upon loss of microtubule minus-end director protein dynein, Rab11 endosomes containing Crb are transported basally rather than apically [19]. Once Crb is successfully addressed to the apical membrane, it is delivered at the

correct localization on the plasma membrane using exocyst-mediated delivery. Mutant clones for sec15 or sec5, subunits of the exocyst, strongly disrupts the apical localization of Crb, confirming the essential requirement of the exocyst in delivery of Crb to the apical membrane [19]. In cultured mammalian cells, the exocyst associates with adherens junctions and PAR3 [23, 24]. Because of the known interaction between CRB, PAR6, and PAR3 in mammals, mentioned below, this trafficking model might be conserved among species. Further investigations are required to test whether the mechanisms of epithelial polarization are conserved in humans.

CRB protein function in mammalian tissues

Various research studies have shown that CRB1 and CRB2 are apical polarity factors, and apical-basal cell polarity is essential for the formation and function of epithelial tissues [25]. More research is required to define the function of the recently described CRB1-B isoform because of its distinct 5' and 3'domain. Below, we will describe the canonical function of CRB in maintaining cell adhesion and morphogenesis, and its role in cell division and development.

Maintaining cell adhesion and morphogenesis

The prototypic ERLI sequence of CRB proteins is important for interaction with key adaptor proteins. The core CRB complex is formed by interaction of CRB and protein associated with Lin Seven 1 (PALS1), also known as membrane-associated guanylate kinase p55 subfamily member 5 (MPP5), where PALS1 binds to the conserved C-terminal PDZ domain of CRB [5, 11, 26]. Ablation of *Mpp5* in mouse retinal pigment epithelium causes early-onset retinal degeneration, whereas ablation of *Mpp5* in the neural retina does not, suggesting an essential role of PALS1 at the tight junctions of RPE but not in the neural retina [27]. The core CRB complex is evolutionary conserved and regulates apical-basal polarity and maintains cell adhesion [28]. PALS1 can interact with MPP3 and MPP4 at the subapical region in the mouse retina (Figure 2A) [29-31]. *Mpp3* conditional knockout (cKO) mice with MPP3 specifically ablated in the retina showed disrupted localization and reduced levels of PALS1, indicating that MPP3 is

essential to maintain levels of PALS1 at the subapical region near the outer limiting membrane [31].

Additionally, binding of PALS1 and CRB can lead to the recruitment of PATJ or multiple PDZ domain protein 1 (MUPP1) to the apical membrane (Figure 2B) [26]. PATJ connects and stabilizes apical and lateral components of tight junctions in human intestinal cells [32]. In some cells, both MUPP1 and PATJ complexes co-exist, and MUPP1 regulates the cellular levels of the PALS1/PATJ polarity complex [33]. Co-immunoprecipitation studies showed that MUPP1 interacts in the retina with CRB1 and PALS1, but not or less with PATJ [11]. PATJ preferentially binds with PALS1 and partitioning defective-6 homolog (PAR6) (Figure 2B) [33, 34]. PAR6 is a key adaptor protein that interact with the ERLI PDZ domain of CRB in mammalian cells [35, 36]. PAR6 leads to recruitment of PAR3, atypical protein kinase C (αPKC) and cell division control 42 (CDC42), known as the PAR complex [36-42]. This PAR6-CDC42 complex is required for the apical-basal polarity and cell adhesion [42].

Alternatively, the importance of a CRB-PALS1-EPB4.1L5 complex in mammals has been described [43-45]. Co-expression and co-localization studies suggested that in several epithelial derived tissues Epb4.1l5 interact with at least one Crumbs homologue and with PALS1 (Figure 2C). In addition, in the adult retina, Epb4.1l5 showed substantial overlap at the OLM with CRB1 and PALS1 [43]. Overexpression of Epb4.1l5 in polarized MDCK cells affects tightness of cell junctions and results in disorganization of the tight junction markers ZO-1 and PATJ [43]. However another group discovered, unlike in the zebrafish and *Drosophila* orthologues, that mouse Crumbs proteins are localized normally in absence of Epb4.1l5 [46]. Additional research should be performed to define its precise molecular mechanism in mammalian cells.

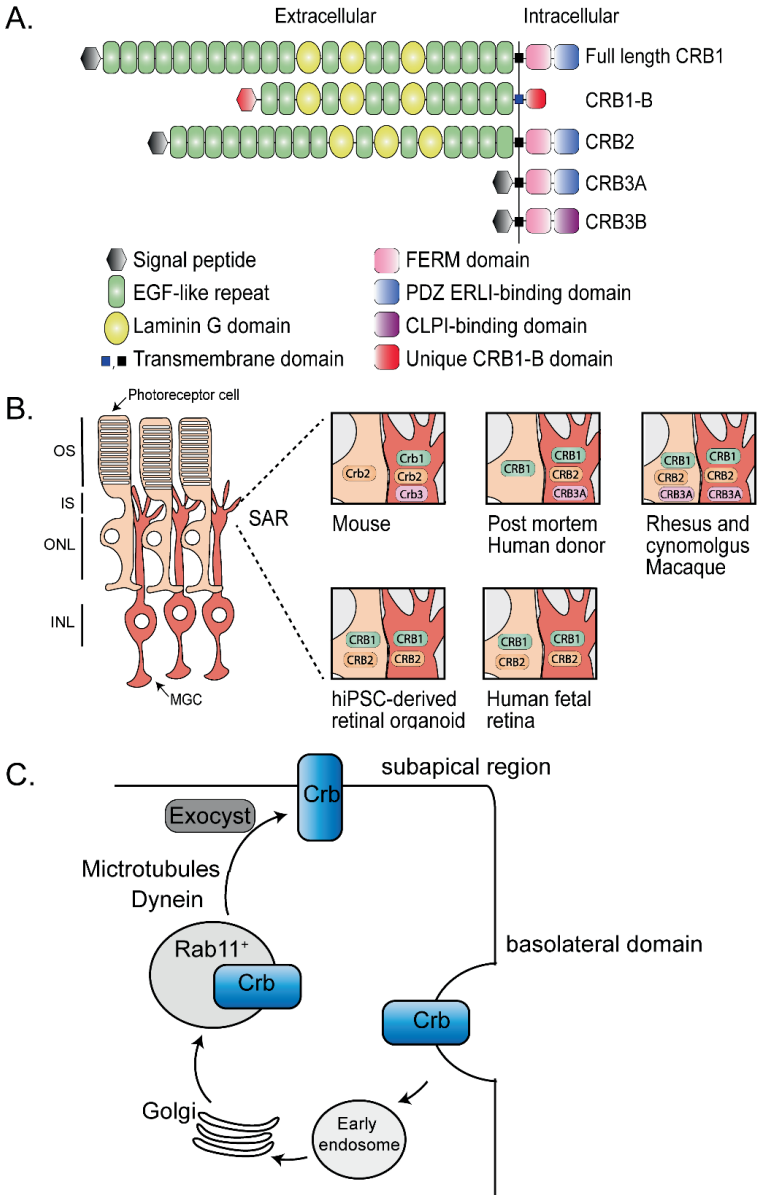


Figure 1. Schematic representation of CRB subcellular localization and proposed trafficking mechanism (A) Schematic overview of full length CRB1 (CRB1-A), CRB1-B, CRB2, CRB3A and CRB3B proteins. (B) Subcellular localization of CRB1, CRB2, and CRB3A in the retina of mouse, post mortem human donor, macaque, hiPSC, and human fetal retina. (C) Trafficking mechanism in *Drosophila* suggested to be conserved among species, adapted from

(Aguilar-Aragon et al., 2020). Note: INL = inner nuclear layer; IS = inner segments; MGC = Müller glial cell; ONL = outer nuclear layer; OS = outer segments; SAR = subapical region.

The alternative isoform CRB3B contains a distinct carboxy terminal motif namely the CLPI motif, suggesting different binding partners in epithelial cells. CRB3 is widely expressed in epithelial cells. A *Crb3* KO mouse demonstrates extensive defects in epithelial morphogenesis, the mice die shortly after birth with cystic kidneys and lung proteinaceous debris throughout the lungs [47]. Interestingly, these defects are also seen in Ezrin knockout mice, which is in line with the detected interaction between CRB3B and Ezrin in mice and mammalian cells (Figure 2D) [47, 48]. This indicates that CRB3B is also crucial for epithelial morphogenesis and plays a role in linking the apical membrane to the underlying cytoskeleton [47-49]. Therefore, the roles of the two CRB3 variants in the mouse lungs remains to be determined.

D*rosophila* Crb has also been found to inhibit the positive-feedback loop of phosphoinositide 3-kinase (PI-3K) and Rac1, thereby repressing the activation of Rac1 as well as PI-3K and maintaining proper apical domain and epithelial tissue integrity (Figure 2E) [50]. This process could potentially be conserved in different species, however, more research in mammalian cells is required to support this hypothesis.

CRB function in cell proliferation

In Müller glial cells of *Drosophila* and *Xenopus,* YAP plays an important role in damaged retina [51-53]. YAP is a core member of the Hippo pathway, which regulates several biological processes including cell proliferation, and survival [54]. The intracellular domain of *Drosophila* Crb interacts with Expanded and thereby regulates the activity of Hippo pathway kinases [54, 55]. In mammalian lung epithelial and breast cancer cells, CRB3 expression also correlates with the Hippo pathway [56, 57]. More specifically, CRB3 affects the Hippo pathway by interacting with Kibra and/or FRMD6 (FRMD6 is the homologue of *Drosophila* Expanded; Figure 2 F). With low CRB3 expression levels, the Hippo-pathway is inactivated, YAP is not phosphorylated and can move to the nucleus where YAP target genes are expressed leading to increased cell proliferation and decreased apoptosis [56]. Also in *Crb1*^{rd8} versus wild-type mouse retina several Hippo signaling related genes were differentially expressed [51]. In

addition, CRB1 and CRB2 deletion also lead to YAP signaling dysregulation in developing murine retinas [58]. These data suggest an essential role of the Hippo pathway in the control of cell proliferation.

CRB1 and CRB2 in retinal diseases

Mutations in the *CRB1* gene are associated with a wide spectrum of retinal dystrophies, such as retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA). RP is a clinically and genetically heterogeneous disease affecting more than 1.5 million people worldwide, where patients typically experience night blindness followed by progressive visual field loss ultimately leading to complete loss of vision in early or middle-life [59, 60]. The age at symptom onset for RP patients ranged from 0 to 47 years, with a median onset of 4 years [59]. Approximately 3 to 9% of non-syndromic cases of autosomal recessive RP are caused by a mutation in the *CRB1* gene [61-63]. LCA is a more severe retinal dystrophy, causing serious visual impairment or blindness in newborns [64]. Mutations in the *CRB1* gene account for approximately 7 to 17% of all LCA cases [61-64]. There are more than 200 different mutations described along the *CRB1* gene resulting in retinal dystrophies without a clear genotype-phenotype correlation with RP or LCA, *CRB1* patients may display unique clinical features such as pigmented paravenous chorioretinal atrophy, macular atrophy alone, retinal degeneration associated with Coats-like exudative vasculopathy, para-arteriolar preservation of the retinal pigment epithelium, or nanophthalmia [61, 62, 65]. The clinical variability of disease onset and severity, even within a patient cohort with the same homozygous mutations, supports the hypothesis that the phenotype of patients with *CRB1* mutations is modulated by other factors [66]. So far, no treatment options exist for patients with mutations in the *CRB1* gene. To study *CRB1*-related retinal dystrophies for treatment options, several models have been used. Below, *CRB1*-related human- and animal-derived retinal models are described.

Until recently, no RP patients were described with mutations in the *CRB2* gene. However, Chen *et al* discovered, using whole exome sequencing (WES), a homozygous *CRB2* p.R1249G mutation in a consanguineous Chinese family presenting RP. This mutation disturbs the stability of CRB2 protein and thereby induces RPE degeneration,

impairs RPE phagocytosis, and accelerates RPE apoptosis [67]. However, only a limited number of patients with this mutation are described, identification of *CRB2* mutations in more RP patients is warranted to better support its pathogenicity.

Human-derived retinal models

The use of human-induced pluripotent stem cell (hiPSC) models for research is an emerging strategy to explore patient phenotypes *in vitro.* These techniques allow access to previously limited or inaccessible material and have been explored in many ophthalmic laboratories worldwide. A commonly used method is the differentiation of hiPSC into retinal organoids. Since the first one, numerous groups have adapted or created their own method to more efficiently generate well laminated retinal organoids [68-72]. Nevertheless, a wide variability in differentiation efficiency across hiPSC lines is often reported [73-76]. Chichagova *et al.* have shown that the ability of three different hiPSC lines to differentiate into retinal organoids in response to IGF1 or BMP4 activation was line- and method-dependent [75]. Hallam *et al.* differentiated five hiPSC lines with a variability in efficiency, but by 5 months of differentiation all the retinal organoids were able to generate light responses and contained a well-formed ONL with photoreceptor cells containing inner segments, cilia, and outer-like segments [77]. Attempts have been made to decrease this wide variability, Luo *et al* described that the use of a Wnt signalling pathway antagonist, Dickkopf-related protein 1 (DKK-1), efficiently generated retinal organoids in all six hiPSC lines [72]. Bulk RNA-sequencing profiling of retinal organoids demonstrated that the retinal differentiation *in vitro* recapitulated the *in vivo* retinogenesis in temporal expression of cell differentiation markers, retinal disease genes, and mRNA alternative splicing [78]. These results make the retinal organoids, despite their high variability, of great interest in a wide range of applications including drug discovery, investigating the mechanism of retinal degeneration, developing cell-based therapeutic strategies and many more.

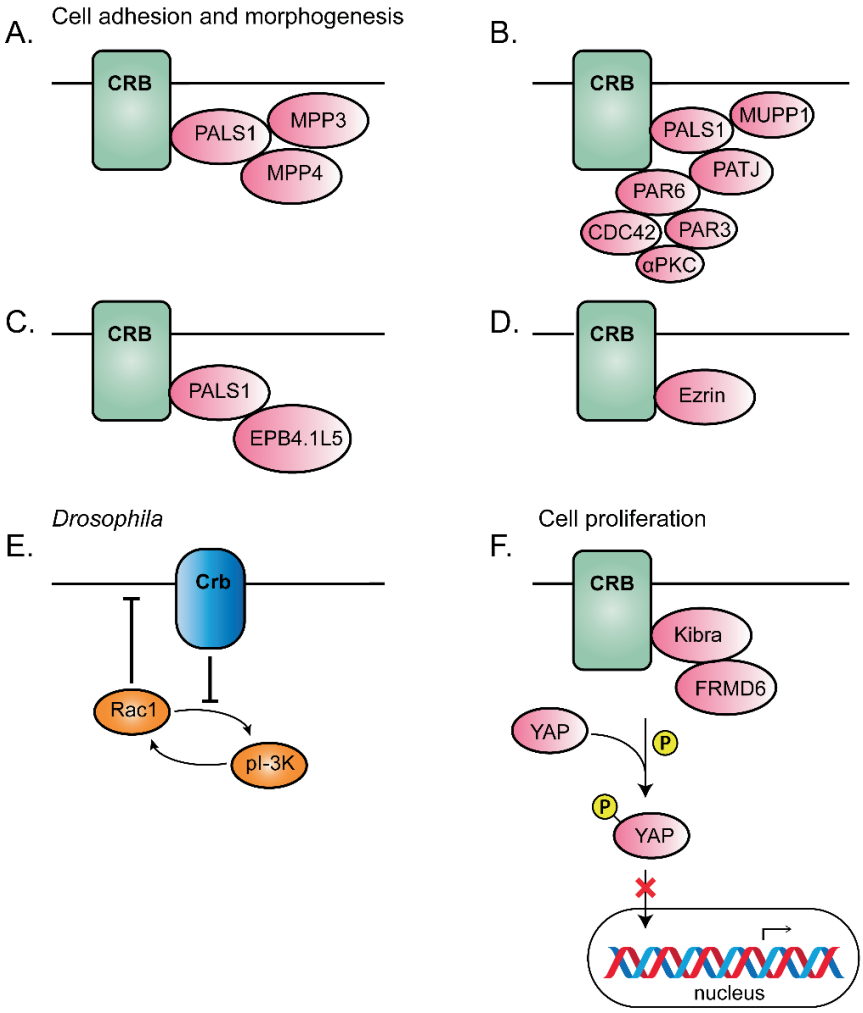


Figure 2. Schematic representation of CRB interaction partners. (A-D) Proposed interaction partners and formed CRB complexes in mammals involved in cell adhesion and morphogenesis. (E) Proposed interaction partner in Drosophila suggested to be conserved among species. (F) Proposed interaction partners involved in cell proliferation.

Defining the localization and onset of expression of the CRB complex members has been achieved in healthy hiPSC-derived retinal organoids. Several members of the CRB complex, CRB2, PALS1, PATJ, and MUPP1, were detected at the outer limiting membrane as early as differentiation day 28 (DD28), typical and clear puncta-like

staining patterns for CRB1 were found only after DD120. All CRB complex members together with adherens junction markers, p120-catenin and N-cadherin, were still detectable in DD180 retinal organoids [16]. The onset of CRB1 and CRB2 protein expression recapitulates those observed in the human fetal retina, with a clear onset of CRB2 expression before CRB1 expression [16].

Three *CRB1* patient hiPSC lines containing a homozygous missense mutation (c.3122T>C), or heterozygous missense mutations (c.2983G>T and c.1892A>G, or c.2843G>A and c.3122T>C) were successfully differentiated into retinal organoids and analyzed at differentiation day 180. Here, all three retinal layers were developed: retinal ganglion cell layer marked by Tuj1 positive dendrites, neuroblast layer marked by SOX9 positive retinal progenitor cells, and an outer nuclear layer marked by recoverin positive photoreceptor cells. However, frequently, there were ectopic recoverin positive cells found above the outer limiting membrane and all missense *CRB1* organoid lines developed small but frequent disruptions of localization of CRB complex members at the OLM that were not found in control lines [16]. Data from these *CRB1* patient hiPSC retinal organoids suggest a retinal degeneration phenotype similar to that previously found in mice lacking CRB1, mice expressing the C249W CRB1 variant, or mouse retina lacking CRB2 [11, 79, 80]. Another study shortly describes the successful differentiation of hiPSC carrying a compound heterozygous mutation in the *CRB1* gene (c.1892A>G and c.2548G>A) to retinal organoids. All three germ layers and expressed markers of retinal progenitor cells, including N-cadherin, rhodopsin, and PAX6, after 35 days of differentiation were present, but no phenotype was described in this paper [81]. To our knowledge, only these two papers have reported the generation of *CRB1* patient hiPSC-derived retinal organoids. The reproducible phenotype observed in these three *CRB1* patient lines [16] provides a good model for assessing potential gene therapy approaches.

Another frequently used method in the ophthalmic field is the differentiation of hiPSC into retinal pigment epithelium (RPE) monolayers. Efficient protocols for differentiating hiPSC into RPE monolayers using a mixture of growth factors have been established [82-84]. However, attempts are currently made to use nonbiological

products, such as small molecules, that would limit the risks of infection or immune rejection when transplanted. Maruotti *et al.* developed a protocol which uses chemotin (CTM) in combination with a previously known neural inducer nicotinamide (NIC) to efficiently differentiate hiPSC into RPE monolayers [85]. In three independent hiPSC lines, RPE differentiation was efficient, and key RPE markers such as microphthalmia-associated transcription factor (MITF), PMEL17, and tight junction protein zonula occludens 1 (ZO-1) were strongly expressed. When left longer in culture to mature, bestrophin 1 (BEST1) and retinal pigment epithelium-specific protein of 65kDa (RPE65) were also strongly expressed [85]. Using a slightly adapted protocol, Smith et al. differentiated six more hiPSC lines into hiPSC-RPE monolayers, all six also expressed the key RPE markers ZO-1, BEST-1, and MITF. Another study revealed by RNA sequencing data that hiPSC-RPE grouped with fetal RPE samples, indicating that their gene expression was highly correlated and similar [86]. In addition, Zahabi et al. provided proof-of-concept that multiple retinal-disease specific hiPSC lines, including two RP lines, can be differentiated into RPE monolayers [84]. Altogether, this data illustrates the potential of hiPSC-RPE as a model system for retinal diseases with mutations in the RPE. Related to CRB, recent studies have shown that CRB2 but not CRB1 is expressed in the human RPE during the differentiation into retinal organoids [16]. In addition, there are RP patients described with specific *CRB2* variations expressed in RPE cells [67]. Therefore, the method of generating hiPSC-RPE could be used to explore treatment possibilities for patients with specific variations in *CRB2*.

CRB1-related animal retinal degeneration models

Numerous research groups focus on animal models to gain, understand, and develop gene therapy strategies that potentially can be used to treat retinal degeneration of RP and LCA patients. Over the years there are multiple animal models developed mimicking the CRB1-related phenotype in patients. These models vary from mild to more severe, early- to late-onset, and Müller glial cell or photoreceptor specific phenotypes. Double retinal knock-outs of *CRB1* and *CRB2,* have helped to understand the contribution of the two CRB proteins to the retinal disease etiology, and explain the relatively mild phenotype observed in *Crb1* variant mouse models [11, 79, 87, 88].

	Crb1$^{KO/C249W}$	*Crb2* $^{\Delta MGC}$	*Crb1*$^{del-B}$	*Crb1*^{rd8}	*Crb1*^{KO}	*Crb1*^{null}	*Crb2*$^{\Delta Rods}$	*Crb1*^{KO} *Crb2*$^{\Delta Rods}$	*Crb1*^{KO} *Crb2*$^{low-imPRC}$	*Crb1*^{KO} *Crb2*$^{low-RPC}$	*Crb2*$^{\Delta RPC}$	*Crb2*$^{\Delta imPRC}$
Severity	+	+	-	++	++	++	++	+++	+++	+++	++++	++++
CRB1-A ablation	MGC	-	-	Natural occurring mutation	MGC	MGC	-	MGC	MGC	MGC	-	-
CRB1-B ablation	-	-	PRC & MGC	Natural occurring mutation	-	PRC & MGC	-	-	-	-	-	-
CRB2 ablation	-	MGC	-	-	-	-	Rod PRC	Rod PRC	50% in immature PRC	50% in RPC	RPC	Immature PRC
Morphologic phenotype onset*	8M	1M	NA	1M	P14	3M	3M	1M	P10	P10	E18.5	E15.5
ERG differences*	No	No	NA	No	No	NA	Yes (9M)	Yes (3M)	Yes (3M)	Yes (3M)	Yes (1M)	Yes (1M)
Affected areas	NA	Mainly periphery	NA	Inferior nasal quadrant	Inferior temporal quadrant	Inferior retina	Superior retina	Superior retina	Inferior retina	Throughout retina	Throughout retina	Throughout retina
OLM disruptions	Yes, sporadic	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
PRC nuclei protrusions	Yes, sporadic	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Table 1: Schematic overview RP mouse models. MGC, Müller glial cells; PRC, photoreceptor cell; RPC, retinal progenitor cell. * The phenotype onset and ERG differences can be marginal on the indicated ages, the severity of the phenotype increases over time.

LCA-like mouse models

Four mouse models showing a *CRB1* LCA-like phenotype have been reported: $Crb1^{KO}Crb2^{\Delta RPC}$ where both Crb1 and Crb2 are ablated in retinal progenitor cells [58], secondly the $Crb1^{KO}Crb2^{\Delta imPRC}$ where Crb1 is ablated in MGC and Crb2 is ablated in immature photoreceptor cells with remaining Crb2 levels in MGC and progenitor cells [89], thirdly the $Crb1^{KO/WT}Crb2^{\Delta RPC}$ mouse model with reduced levels of Crb1 in MGC and ablation of Crb2 in retinal progenitor cells [58], and finally $Crb1^{KO}Crb2^{\Delta MGC}$ in which both Crb1 and Crb2 are ablated in MGC [18]. All four models exhibit vision loss indicated by a reduced ERG response. In addition, retinal degeneration was observed by outer limiting membrane disruptions, abnormal retinal lamination, intermingling of nuclei of the ONL and INL, and ectopic localization of retinal cells. These mouse models show an early onset phenotype with distinct severity indicated by the order mentioned above. In short, in the most severe mouse model, $Crb1^{KO}Crb2^{\Delta RPC}$, the phenotype onset was found as early as embryonal day 13 (E13) which was observed throughout the retina [58]. Retinal degeneration in $Crb1^{KO}Crb2^{\Delta imPRC}$ was detected at E15 in the whole retina, but in adult mice the superior retina was more affected than the inferior retina [89]. Also in $Crb1^{KO/WT}Crb2^{\Delta RPC}$ mice retinal degeneration was detected at E15, but was mostly affecting the peripheral retina [58]. Finally, the $Crb1^{KO}Crb2^{\Delta MGC}$ mice showed the first signs of degeneration at E17, where mostly the peripheral retina was affected. More subtle differences between these models are described and summarized before [18]. These data show that all four *Crb1* mouse models mimic the LCA phenotype in patients and could therefore be used for future therapy development.

RP-like mouse models

Twelve *CRB1* RP-like mouse models have been described so far, including (1) $Crb1^{KO/C249W}$ with a missense variation in the *Crb1* gene [79], (2) $Crb2^{\Delta MGC}$ where only Crb2 is ablated in MGC [90], (3) the $Crb1^{rd8}$ mice with a naturally occurring nonsense mutation in the *Crb1* gene [87], (4) the $Crb1^{KO}$ where the full length Crb1 protein is ablated from retinal radial glial progenitor cells, Müller glial cells, and the rest of the

body [11], (5) the *Crb1*$^{del\text{-}B}$ were *Crb1-B* is abolished from photoreceptor and Müller glial cells [3], (6) the *Crb1*^{null} where a deletion of alternate exon 5a up to intron 7 disrupts all *Crb1* isoforms [3],(7) *Crb2*$^{\Delta rods}$ where Crb2 is ablated only in developed rod photoreceptor cells [91], (8) *Crb1*^{KO}*Crb2*$^{\Delta rods}$ where Crb1 is ablated in MGC and Crb2 in rod photoreceptor cells [91], (9) *Crb1*^{KO}*Crb2*$^{low\text{-}imPRC}$ with absence of Crb1 and reduced levels of Crb2 in immature photoreceptors [89], (10) *Crb1*^{KO}*Crb2*$^{\Delta low\text{-}RPC}$ with absence of Crb1 and reduced levels of Crb2 in retinal progenitor cells [14], (11) *Crb2*$^{\Delta RPC}$ where Crb2 is ablated in retinal progenitor cells [80], and finally, (12) *Crb2*$^{\Delta imPRC}$ with Crb2 ablation in immature photoreceptor cells [90]. The different Cre mouse models show variations in mosaicism (mutant adjacent to wildtype cells), but eleven out of twelve *Crb* models show disruptions at the outer limiting membrane with rows or single photoreceptor nuclei protruding into the subretinal space or ingressing into the outer plexiform layer leading to a degenerative retinal phenotype (table 1).

The *Crb1*^{rd8} mice have a naturally occurring single base deletion in exon 9 of the *Crb1* gene causing a frame shift and premature stop codon, thereby truncating the transmembrane and cytoplasmic domain of CRB1. This results in a photoreceptor degeneration mainly observed in the inferior nasal quadrant of the eye, caused by retinal folds and pseudorosettes [87]. In the *Crb1*^{KO} mouse model the retinal lamination is predominantly maintained, and degeneration is found in the inferior temporal quadrant of the retina. Degeneration is indicated by single or groups of photoreceptor cells protruding into the subretinal space, rosette formation, and neovascularization. In 18M-old mice there was no loss of overall retinal function measured by electroretinography, suggesting that a major part of the retina was not affected by loss of Crb1 [11, 88]. Light exposure experiments reveal that light exposure doesn't initiate but rather enhances the retinal degeneration [88]. The mild phenotype observed in these *Crb1* mouse models suggest that Crb2 protein may compensate the effect of CRB1 deletion.

In the *Crb1*$^{del\text{-}B}$ the alternative *Crb1-B* isoform is abolished from photoreceptor cells, while the *Crb1*^{null} disrupts all potential *Crb1* isoforms [3]. The *Crb1*$^{del\text{-}B}$ mouse do not show significant disruptions in the OLM, while the disruptions in the *Crb1*^{null} mice were comparable with *Crb1*^{rd8} [3]. Although not mentioned by Ray *et al.*, also the *Crb1*^{KO}

mice shows significant OLM disruptions strongly depending on genetic background as well as exposure to light [11, 88]. As the IrCaptureSeq suggested that *Crb1-B* is the most abundant transcript in mouse and human retina, a cross-breeding of $Crb1^{null}$ with $Crb1^{del\text{-}B}$ mice was performed to define the relevance of Crb1-B in the retina. This heterozygous $Crb1^{null/del\text{-}B}$ showed similar OLM disruptions with the homozygous $Crb1^{null}$ mouse [3]. Similar OLM disruptions were thus found in $Crb1^{rd8}$, $Crb1^{null}$, $Crb1^{null/del\text{-}B}$, and $Crb1^{KO}$ mice. It is essential to perform retinal function measurements on the mouse models affecting *Crb1-B* to understand its function and to compare it with previously described *Crb1* mouse models. In addition, the most severe retinal phenotype so far derives from *Crb1* mice with concomitant loss of Crb2.Both $Crb2^{\Delta RPC}$ and $Crb2^{\Delta imPRC}$ show an early onset retinal degeneration at embryonic day 18.5 (E18.5) and E15.5 respectively, the difference is caused by the distinct expression pattern and timing of the Cre recombinase and morphological phenotypes result in differences in the scotopic and photopic ERG conditions already at 1M of age [80, 90]. Interestingly, in contrast to $Crb2^{\Delta RPC}$ mice, the lamination of Müller glial, ganglion and amacrine cells were also misplaced in $Crb2^{\Delta imPRC}$ mice. These lamination defects were also observed in $Crb1^{KO/WT}Crb2^{\Delta RPC}$ mice [58].

Another mouse model, $Crb2^{\Delta rods}$, show a mild and late onset phenotype limited to the superior retina [91]. In some 3M and all 6M old $Crb2^{\Delta rods}$ mouse, disruptions of the outer limiting membrane, a thinned photoreceptor layer at the peripheral superior retina, and photoreceptor cells protruding into the subretinal space were observed. A reduction in ERG scotopic a-wave was observed in 9M old mice, while no difference in optokinetic head tracking response (OKT) spatial frequency or contrast sensitivity was observed. Interestingly, while most photoreceptor cells were affected, all the remaining photoreceptor cells showed mature inner- and outer-segments. Remaining rods were functional and the cones showed normal morphology. This phenotype is enhanced by a concomitant loss of Crb1 in Müller glial cells [91]. These mice, $Crb1^{KO}Crb2^{\Delta rods}$, show a similar but enhanced phenotype in comparison to $Crb2^{\Delta rods}$ mice. Here, a reduction in ERG scotopic a-wave was observed at 3M and became more apparent at 6M onwards. In addition, a significant decrease in OKT contrast sensitivity was found from 3M of age onwards [91]. The difference in phenotype onset between all these

mouse models might be explained by the onset of Cre expression during retinogenesis and by cell-type specific ablation; ablation of Crb2 from a later time point resulted in a milder phenotype. Interestingly, when Crb2 is ablated in MGCs, $Crb2^{\Delta MGC}$, a very mild morphological phenotype with no functional consequences measured using ERG was observed [90], but ablation of both CRB1 and CRB2 from Müller glial cells caused a severe LCA phenotype [18]. This data suggests that CRB2 has a redundant function in Müller glial cells, while in photoreceptor cells it is essential for proper retinal lamination and function.

Interestingly, the $Crb1^{KO}$ phenotype is located at the inferior temporal quadrant whereas the $Crb2^{\Delta rods}$ phenotype is mainly observed at the peripheral and central superior retina. These differences might be related to higher levels of CRB2 in the inferior retina while CRB1 is expressed at higher levels in the superior retina [14]. In addition, there might be modifying factors present which are enriched in either the superior or inferior retina causing the different phenotypes.

In addition to these mouse models, a rat with a spontaneous mutation in *Crb1* exon 6 was discovered mimicking human macular telangiectasia type 2 [92]. The autosomal recessive indel mutation causes an early-onset phenotype with a strongly reduced ERG response in three-week old rats. These rats showed a focal loss of retinal lamination, OLM disruptions, and photoreceptor cells, MGC and RPE alterations [92]. Differences between this and the *Crb1* mouse models could result from different types of mutations or from different genetic setups displayed by these different animal species

Gene augmentation strategies for *CRB1* retinal dystrophies

There is an emerging interest in gene augmentation strategies for retinal dystrophies. Recently, gene therapy became available for young RP and LCA patients with biallelic mutations in the *RPE65* gene**.** Voretigene neparvovec-rzyl, or its commercial name: LUXTURNA™, uses the adeno-associated viral vector serotype 2 (AAV2) to deliver by subretinal injection a functional copy of the *RPE65* gene into the RPE cells. *RPE65* transgene expression results in the production and correct localization of RPE65 protein in RPE cells, thereby compensating for the loss of the protein and restoring the visual

cycle in these patients [93]. Nowadays, there are numerous clinical studies ongoing which explore the use of AAV as a therapeutic vector for retinal diseases such as retinitis pigmentosa (RP), wet age-related macular dystrophy (AMD), Leber congenital amaurosis (LCA) and many more [94]. However, so far, no treatment options are available for RP and LCA patients with mutations in the *CRB1* gene. Below, we will describe novel therapeutic tools which could be promising for *CRB1* retinal gene augmentation therapy in RP patients.

Currently, AAVs are the leading platform for gene delivery in the treatment of retinal dystrophies. AAVs are mainly investigated because of their low toxicity, their capability to transduce both dividing and non-dividing cells, they do not integrate into the host genome, and AAV capsid variants display distinct cell tropisms. A complete overview of basic AAV biology, AAV vectorology, and current therapeutic strategies and clinical progress was recently reviewed by Wang *et al* [94]. The major disadvantage of AAVs is their limited package capacity, bigger gene expression cassettes than 4.5 kb are not able to fit within a single AAV. Because of this, the development of AAV-mediated *CRB1* gene therapy is also challenging. Full-length cytomegalovirus (CMV) ubiquitous promoter and *CRB1* cDNA exceeds the AAV package limitation. However, using an engineered minimal CMV promoter and codon optimized *CRB1* cDNA allowed sufficient expression levels of full-length CRB1 protein in Müller glial cells in mice [95]. Pre-clinical studies in mouse using this AAV.CMVmin.*hCRB1* have shown that expression of *CRB1* was deleterious in *CRB1* mouse models but not in wild-type mice [15]. In addition, there are more potential *CRB1* transcript variants which could be targeted [6]. Interestingly, alternative strategies using the codon-optimized structural and functional family member *CRB2* to rescue *CRB1*-related retinopathies restored retinal function and structure in *Crb1* mouse models [15]. In that study, improved photoreceptor layer morphology and ERG response was detected after *CRB2* delivery. The combination of AAV9 with a full-length CMV promoter was used to target both photoreceptor and Müller glial cells, whereas no rescue was observed when either photoreceptor or Müller glial cells were targeted with *CRB2* [15]. In addition, several groups explored the possibility to overcome the limiting AAV package capacity by dual or triple-AAV approaches [96-98] or by increasing the package capacity [99]. Yet, the

studies performed so far have shown that the dual AAV approaches have lower expression levels compared to a single AAV vector [96, 97].

Several research groups focus on AAV-mediated gene delivery to define the tropism in hiPSC-derived retinal organoids and/or RPE. Out of four different AAV capsids packaged with the CAG promoter and GFP (AAV2, AAV2-7m8, AAV8, AAV9), Garita-Hernandez *et al* showed with AAV2-7m8 infection at day 44 the most efficient transduction of the hiPSC-derived retinal and RPE organoids. Limited transduction of AAV9 was found [100]. Another study showed, using AAV-CMV-*GFP* constructs, a more efficient retinal organoid transduction at DD220 especially in MGC with AAV5 or Shh10Y445F capsids in comparison with AAV9 [16]. Recently, Lane *et al* demonstrate efficient photoreceptor cell transduction in DD150 retinal organoids using an AAV5 packaged with the CAG protomer [101]. Moreover, AAV5-mediated gene augmentation with human *RP2* was able to rescue the degeneration found in *RP2* KO retinal organoids by preventing ONL thinning and restoring rhodopsin expression [101].Therefore, testing CRB1- or CRB2-expressing vectors in retinal organoids will aid in the discovery of *CRB* gene therapy treatment for *CRB1*-related retinal dystrophies in patients. In addition, there are different non-viral mediated approaches for gene therapy. An example is the use of nanoparticles. Three representative nanoparticles, namely metal-based, polymer-based, and lipid-based nanoparticles, were recently reviewed describing their characteristics and recent application in ocular therapy [102]. Shortly, the most extensive characterized nanoparticle is the polymer-based CK30-PEG nanoparticle, which contains plasmid DNA compacted with polyethylene glycol-substituted lysine 30-mers [103, 104].Theoretically, these approaches have an unlimited gene packaging capacity, which display a big advantage for large genes such as *CRB1*. These nanoparticles are also able to infect RPE and photoreceptor cells, and can drive gene expression on a comparable scale and longevity than AAVs in mice [104, 105]. CK30-PEG nanoparticles have a tolerable safety profile and is nontoxic in mouse and non-human primate eyes [106, 107]. To our knowledge, there is no successful clinical study described using these nanoparticles so far. More research is required to define the clinical relevance of these nanoparticles in retinal gene therapy. Further pre-clinical research and clinical trials will aid in the discovery of new gene

therapy approaches for *Crb1*-related retinal dystrophies in patients. A retrospective *CRB1* natural history study (NHS) has been published [59], and their prospective NHS with strategies for evaluation of the efficacy of novel clinical therapeutic interventions in the treatment of *CRB1*-retinal dystrophies will be reported in the end of 2020. Recently, a French biotech company called HORAMA signed an exclusive license agreement with Leiden University Medical center targeting *CRB1* gene mutations to treat inherited retinal dystrophies. Based on current timelines, HORAMA expects initiating a Phase I/II clinical study with the drug candidate in 2023 [108].

Conclusion

In this review we have discussed (1) CRB protein function in mammalian cells, (2) recent advances and potential tools for *CRB1* human and animal retinal degeneration models, and (3) described therapeutic tools which potentially could be used for retinal gene augmentation therapy for RP and LCA patients with mutations in the *CRB1* gene.

1.2 Aims and outline of this thesis

The general aim of the research described in this thesis was describing multiple model systems for *CRB1* related retinal dystrophies, where **Chapter 2** describes the use of a mouse model, **Chapter 3** describes a rat model, and **Chapter 4 and 5** describe human-derived models. In addition, AAV-mediated *CRB* gene augmentation therapy on rats and retinal organoids was explored in **Chapter 3, 4, and 5**.

Chapter 1 serves as an introduction for this thesis, where I review (1) CRB1 and CRB2 function and localization in the retina, (2) the current status of human-derived retinal models, (3) the different generated *Crb1*-related animal retinal degeneration models, and finally (4) describe the current gene augmentation strategies and possibilities for *CRB1* retinal dystrophies.

In **Chapter 2** we describe the effect of CRB2 in rod photoreceptor cells and whether ablation of CRB2 in rods enhance the observed phenotype in $Crb1^{KO}$ mouse models. For this, we assessed the morphological, retinal, and visual functional consequences of loss of CRB2 from rods with or without concomitant loss of CRB1.

Chapter 3 describes the phenotype of *Crb1* mutant rats in more detail using electroretinography (ERG), spectral domain optical coherence tomography (SD-OCT), and morphological analysis. The subcellular localization of CRB1 and CRB2 in *Crb1* mutant and control rats was explored. In addition, the tropism of three distinct viral vectors administrated in two different injection ways were analysed in *Crb1* mutant and control rats. Finally, AAV-mediated gene augmentation therapy was explored in new-born *Crb1* mutant rats.

Chapter 2 and **Chapter 3** discussed our findings on *Crb1*^{KO} mouse and *Crb1* mutant rat models, but in **Chapter 4** and **Chapter 5** we switch the focus to using human induced pluripotent stem cells (hiPSC) to differentiate into *CRB1* human derived models (retinal organoids).

Chapter 4 describes the use of hiPSCs from three different *CRB1* RP patients and isogenic controls. These *CRB1* patient derived retinal organoids were analysed for a morphological phenotype at differentiation day 210 (DD210). In addition, single cell RNA-sequencing data analysis was performed and shows disruptions in the endosomal system in Müller glial cells and rods of *CRB1*-patient derived retinal organoids. Then, AAV-mediated gene augmentation therapy was explored in these retinal organoids. AAV.h*CRB1* or AAV.h*CRB2* treatment partially improved the phenotype of *CRB1* patient derived retinal organoids, providing essential information for future gene therapy approaches for patients with mutations in the *CRB1* gene.

In **Chapter 5** retinal organoids derived from *CRB1*^{KO} and *CRB1*^{KO}*CRB2*$^{+/-}$ iPSC show a significantly decreased number of photoreceptor nuclei in a row and a significantly increased number of photoreceptor nuclei protruding above the OLM at DD180 and DD210. AAV-mediated gene augmentation therapy showed that AAV.h*CRB2* could partially restore the phenotype observed in *CRB1*^{KO} retinal organoids.

Chapter 6 consist of the general discussion and provides future perspectives of the above mentioned chapters in this thesis, and **Chapter 7** includes the summary, the "Nederlandse samenvatting" and abbreviations, and a list of publications, curriculum vitae, and acknowledgements of the author of this thesis.

Article information

Conflict of Interest: The authors declare that the research was conducted without any commercial or financial relationship that could be construed as a potential conflict of interest. The LUMC is the holder of patent number PCT/NL2014/050549, which describes the potential clinical use of *CRB2*; JW and LPP are listed as co-inventors of this patent, and JW is an employee of the LUMC.

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Abbreviations:

αPKC	Atypical protein kinase C
AAV	Adeno-associated viral vectors
AMD	Age-related macular dystrophy
BEST1	Bestrophin 1
cKO	Conditional knockout
CLPI	Cysteine-leucine-proline-isoleucine
CMV	Cytomegalovirus
CRB1	Crumbs homologue 1
CTM	Chemotin
DD	Differentiation day
DKK-1	Dickkopf-related protein 1
E13	Embryonal day 13
EGF	Epidermal growth factor
ERG	Electroretinography

ERLI	Glutamic acid-arginine-leucine-isoleucine
hiPSC	Human-induced pluripotent stem cell
LCA	Leber Congenital Amaurosis
MGC	Müller glial cells
MITF	Microphthalmia-associated transcription factor
MPP5	Guanylate kinase P55 subfamily member 5
MUPP1	Multiple PDZ domain protein 1
NIC	Nicotinamide
OKT	Optokinetic head tracking response
OLM	Outer limiting membrane
PALS1	Protein associated with Lin Seven 1
PAR6	Partitioning defective-6 homolog
PI-3K	Phosphoinositide 3-kinase
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
RPE65	RPE-specific protein of 65kDa
SAR	Subapical region
WES	Whole exome sequencing
YAP	Yes-associated protein 1
ZO1	Zonula occludens-1

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