The Retinal Crumbs Complex: from animal models and retinal organoids to therapy

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Chapter 8

8.1 Retinogenesis of the Human Fetal Retina: An Apical polarity perspective

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8.2 Summary and discussion

ABSTRACT

The Crumbs complex has prominent roles in the control of apical cell polarity, in the coupling of cell density sensing to downstream cell signalling pathways, and in regulating junctional structures and cell adhesion. The Crumbs complex acts as conductor orchestrating multiple downstream signalling pathways in epithelial and neuronal tissue development. These pathways lead to the regulation of cell size, cell fate, cell self-renewal, proliferation, differentiation, migration, mitosis and apoptosis. In retinogenesis, these are all pivotal processes with important roles for the Crumbs complex to maintain proper spatiotemporal cell processes. Loss of Crumbs function in the retina results in loss of the stratified appearance resulting in retinal degeneration and loss of visual function. In this review, we begin by discussing the physiology of vision. We continue by outlining the processes of retinogenesis and how well this is recapitulated between the human fetal retina and human stem cell-derived retinal organoids. Additionally, we discuss the functionality of *in utero* and preterm human fetal retina and the current level of functionality as detected in human stem cell-derived organoids. Finally, we discuss the roles of apical-basal cell polarity in retinogenesis with a focus on Leber congenital amaurosis which leads to blindness shortly after birth.

Keywords
Apical Polarity, Crumbs Complex, Fetal Retina, PAR complex, Retinal Organoids, Retinogenesis.

The Physiology of Vision

Vision is perhaps the most dominant sense in daily life and both non-correctable unilateral and bilateral vision loss severely impact the quality of life (1). Vision begins with the processing of light, which is electromagnetic radiation that travels as waves. Light waves, as with all waves, can be characterised by their wavelength (distance between wave peaks), frequency (number of wavelengths within a time period) and amplitude (the height of each peak or depth of each trough). Visible light is a narrow group of wavelengths between approximately 400 nm and 760 nm which we interpret as a spectrum of different colours (2). Light can be reflected (bounce of a surface), absorbed (transfer of energy to a surface) or refracted (bending of light between two mediums).

When light first enters the eye, it is refracted by the cornea through the pupil, which size is controlled by the iris. The iris, the coloured part of the eye, controls the amount of light entering the eye while the lens focuses the light through the vitreous humour and on to the proximal surface of the retina. The adult retina consists of one glial cell type, the Müller glial cells, and six major types of neurons, the rod and cone photoreceptors, bipolar cells, amacrine cells, horizontal cells, ganglion cells. Their cell bodies are distributed across three nuclear layers, the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL). Two synaptic layers, the outer plexiform layer (OPL) and inner plexiform layer (IPL), contain the axonal and dendritic processes of the cells (3). Whereas there is one type of rod photoreceptor, there are various subtypes of cone photoreceptor, bipolar, amacrine, horizontal and ganglion cells that differ in their functional roles and morphology (4). Besides Müller glial cells there are two other glial cell types that serve to maintain retinal homeostasis, the astrocytes and resident microglia (5). Light must be channelled through the retina and absorbed by its three light responsive cells: the rod and cone photoreceptors and the intrinsically-photosensitive retinal ganglion cells (ipRGCs). The mammalian retina contains various opsin proteins involved in the photoreception synchronisation of circadian rhythms (photoentrainment). These are the cone opsins (M/LWS, red/green opsin; SWS1, blue opsin) responsible for high visual acuity, resolution and colour vision (photopic vision), and rod opsin (RH1, Rhodopsin) responsible for dim light vision (scotopic vision) and ipRGCs opsin (OPN4, Melanopsin) responsible for synchronisation of the circadian rhythms and ambient light perception (6–9). The cones are less sensitive to light and rods are more sensitive to light and are also used together under intermediated light conditions (mesopic vision) (10). Most forms of inherited retinal dystrophy negatively affect the function of photoreceptors, resulting in progressive loss of rod and/or cone photoreceptors. Müller glial cells mediate the channelling of light through the retina towards the photoreceptors (11, 12). Müller glial cells can channel different wavelengths of light to specific subsets of photoreceptors to optimise day vision (13). The visual pigments of the photoreceptors contain an opsin protein covalently linked to the chromophore 11-*cis*-retinal. Upon the absorption of a photon 11-*cis*-

retinal becomes isomerised to all-*trans*-retinal, this leads to an activated opsin intermediate (metarhodopsin II, rods; Meta-II, cones). This active intermediate leads to triggering of a transduction cascade resulting in hyperpolarisation of the photoreceptors, due to the closure of cGMP-gated channels, and a reduction in glutamate release (14). This electrophysiological signal is then further propagated to the inner retina and can be propagated through many different pathways to the ganglion cells. Prototypically these signals can be direct, from photoreceptor (PRC) to bipolar cells to ganglion cells. However, it can also be indirect with lateral modulation of the electrophysiological signals being made by horizontal cell processes in the OPL or by amacrine cell processes in the IPL (10, 15, 16). Thus creating radially aligned "functional units" of photoreceptors, bipolar cells, amacrine cells, horizontal cells and ganglion cells. The fovea contains a specialised pathway, termed the midget pathway, which helps account for its ability to provide high visual acuity (17–19).

The visual system, however, is not solely comprised of the eye but also the topographically mapped ganglion cell axonal projections connecting the retina to the superior colliculus (SC) and lateral geniculate nucleus (LGN) in the brain (20). The ganglion cell axonal projections exit the left and right eye as bundles, the optic nerves, and they extend to below the hypothalamus to the optic chiasm. The optic chiasm is the crossover point for the nasal axons of each eye which combine with the opposing eyes temporal axons. The two optic tracts extend from the optic chiasm to the SC and the LGN, with the optic radiations further extending from the LGN to the primary visual cortex (21). The SC, LGN and pulvinar nuclei are all involved in the process of relaying and refining visual information to the primary visual cortex (22, 23). Interestingly, despite the severe retinal dysfunction of Leber congenital amaurosis-2 (LCA2) patients, recovery of both retinal function but also reorganisation and maturation of synaptic connectivity in the visual pathway is found upon administration of a gene therapy treatment (24). Such recovery highlights the relative plasticity of the human visual system.

Retinogenesis

The retina, part of the central nervous system, offers an extremely accessible and relatively immune-privileged model system for investigating the mechanisms of neural development and vision (25). A high conservation of the genes involved in retinal development exists across species allowing us to gain an in-depth fundamental knowledge of these mechanisms. Retinal development is both a pre- and postnatal process. The development of the retina begins when the anterior neural plate subdivides into a number of domains, with the medial region specifying as the eye field. The formation of the eye field is coordinated by expression of the eye field transcription factors (EFTFs), shortly after gastrulation. There are a number of EFTFs in mammals including Pax6, Rax, Lhx2, Six3 and Six6. The eye field consists of all the progenitors which go on to form all the neural-derived cell types and

structures of the eye (26–30). The progenitors of the eye field begin to specialise very early in development, hence the large number of bilateral diseases of eye morphogenesis (28). From the eye field, bilateral optic sulci form and evaginate from the diencephalon at human fetal embryonic day (E)22 forming optic vesicles at E24. The optic vesicles extend towards the surface ectoderm remaining connected to the forebrain through the optic stalk, which eventually develops into the optic nerve. The hyaloid artery, running from the optic stalk and into the retinal neuroepithelium through the optic fissure, provides the basis for the vascularisation of the retina and developing eye. As the optic vesicles invaginate forming the two-layered optic cups by E32, the surface ectoderm thickens forming the lens placode and further develops into lens vesicle, sitting behind the surface ectoderm. The anterior rim of the optic cup will become the iris and ciliary body, while the posterior will become the pigmented and neural retina. The outer layer of the posterior optic cup remains as a single cuboidal layer becoming the retinal pigment epithelium (RPE). The single inner layer of the posterior optic cup proliferates and differentiates, beginning in the 7th fetal week (Fwk), developing into the multilayered neural retina (28, 31).

The processes of the newborn progenitors of the inner optic cup, the retinal neuroepithelium, extend and attach both apically through adherens junctions (AJs) at the outer limiting membrane (OLM), and basally through integrin and proteoglycan based focal adhesions at the inner limiting membrane (ILM) (32, 33). Retinal progenitors undergo interkinetic nuclear migration in which their nuclei move in an apical-basal manner in phase with the cell cycle, this occurs in mainly a stochastic manner but becomes briefly directed at cell division (33–35). Progenitors initially undergo symmetric cell division leading to an increase in the pool of progenitors and thus thickening of the neuroepithelium. After that the progenitors go through asymmetric divisions, and produce one daughter cell to maintain the stem cell pool and one terminally differentiated postmitotic cell. Later in development depletion of the retinal progenitor pool occurs through symmetric divisions leading to two postmitotic terminally differentiated daughter cells (34, 36). Cell intrinsic and extrinsic factors govern cell fate choice and thus tissue architecture and function. The retinal cells governed by these factors progress from multipotent retinal progenitors to competent postmitotic precursors, which undergo further specification before becoming the final differentiated adult cell type (4, 37–39).

The birth of the seven major cell types of retina occur from the early multipotent retinal progenitor cells and happens in an orderly and overlapping manner (39). The genesis of the major cell types group into an early phase and a late phase. The early phase consists of the birth of the first ganglion cells, cone photoreceptors, horizontal cells and amacrine cells. The overlapping late phase consists of the birth of the first rod photoreceptors, Müller glial cells and bipolar cells (39). Recently, both Aldiri et al (2017) and Hoshino et al (2017) described similar retinal time courses for the developing human retina based on RNA-Seq analysis (40, 41). The newborn postmitotic cell types must become positioned correctly within the retina; this occurs through migration of cells along the radial axis (apical-basal) of the retina

or by tangential migration of cells perpendicular to the radial axis of the retina.

Interestingly, only the early born cell types (ganglion cells, cone photoreceptors, horizontal cells and amacrine cells) exhibit tangential migration (42, 43). There are a number of modes of radial migration for newborn neurons including: Glial cell-guided, the migration of neurons along radial glial progenitors; Somal translocation, the movement of nuclei across inherited apical or basal processes; Multipolar migratory mode, nuclei movement due to multiple cell processes with no retention of apical or basal attachment to facilitate nuclei movement; No translocation, inefficient migration due to retention of the apical or basal process and slow release of opposing process. These various modes of migration are cell type-specific (44–47). Tangential dispersion is driven by a mix of diffusible signals and/or contact-mediated interactions that drive a local spacing rule to keep a minimum distance between neighbouring cells of the same cell type (48).

Retinal mosaic is the term used for the distribution of a neuronal cell type orthogonal to the apical-basal axis in a particular retinal layer. There is a highly ordered mosaic architecture in the mammalian retina leading to the non-random distribution of its cell bodies and dendritic process. This mosaic patterning is essential for retinal functionality, tying information together in a regularly patterned/ordered way from radially aligned "functional units" such that complete sampling and coverage of an image is achieved. Development of mosaics may be due to a combination of tangential dispersion (for early born cell types), programmed cell death and lateral inhibition (45, 48, 49). Interestingly, mosaic patterning can apply to a group of cells that have yet to reach their final developmental position, suggesting a pre-orchestrated cell intrinsic process (45).

Thus, retinogenesis is a precise orchestration of spatiotemporal processes such as symmetric and asymmetric cell division, cell fate choice (determination, competence, specification and differentiation), cell migration (interkinetic nuclear migration, radial migration and tangential migration) and maturation (integration and specialisation of retinal spatiotemporal processes to provide adult functionality). The developing retinal neuroepithelium has a large amount of plasticity to accommodate these spatiotemporal process while maintaining its tissue integrity and architecture.

Morphological and Molecular Recapitulation of the Human Fetal Retina

Studying retinal development in animal models is useful to understand the underlying cellular processes, but some of these processes are remarkably different in the human retina. There are molecular and morphological similarities between mammalian retina, but subtle differences highlight caution when applying information from other species to the human retina and its diseases, even between non-human primate and human retina (50–53). Additionally, limited access to human fetal and adult cadaveric donor retina limited the studies in human retina at a tissue or single cell type level in regards to pathobiology, signalling dynamics and

physiology. Retinal organoids derived from human embryonic stem cells or induced pluripotent stem cells mimic the three-dimensional laminated structure of the retina allowing us to study basic eye development, disease modelling, drug potency assays, gene augmentation and cell therapeutic strategies (54–59).

Multiple recent studies have directly compared human fetal retina with human stem cell-derived retinal organoids, analysing their morphology or transcriptome or both (60–63). Additionally, a large body of research exists regarding the morphology and transcriptome of either human fetal retina (8, 40, 71–74, 41, 64–70) or human stem cell-derived retinal organoids (54, 55, 83–85, 75–82). Together, these studies provide a unique insight into human retinogenesis and allow us to have a reference point between *in vivo* human retinal development and *in vitro* models of it. Do human stem cell-derived retinal organoids sufficiently recapture the cell type diversity including all sub-types, morphological cues, synaptic wiring and light sensitivity of the human fetal retina and subsequent adult retina?

Human retinal organoids have been found to recapitulate the main temporal and spatial cues of the *in vivo* human retina producing all the retinal layers and containing the 7 major cell types (55, 58, 86). The spatiotemporal birth and maturation of retinal cell types differ depending on their location within the retina. In the human fetal retina, photoreceptors are born and remain located in the apical retina. However, in human stem cell-derived retinal organoids photoreceptors reside mostly apically but are also seen in more basal positions particularly in early developmental stages, suggesting incomplete positional cues. Interestingly, Kaewkhaw et al (2015) were able to track basally located photoreceptors from DD42 to DD44 with live cell imaging, finding that they translocated their soma apically (79). The mammalian retina matures in a centroperipheral manner with the peripheral retina organising its adult like lamination later than the central retina (65). This delayed peripheral maturation is also detected in gene expression profiles comparing these regions. Cell type expression patterns, analysed by immunohistochemistry, can be delayed by at least 50 days in the peripheral compared to more central retina. Differences are also found between nasal and temporal retina (41). Additionally, sustained differences in gene expression profiles of peripheral and macula of the human adult retina is found, but this has been attributed to the anatomical differences between these two regions (87). Mammals are known to contain a peripherally located ciliary marginal zone (CMZ), a stem cell-like niche able to contribute postmitotic cells to the retina in addition to radial glial progenitor cells (88, 89). However, little data is available on the CMZ of the human fetal retina, but studies on human and mouse stem cell-derived retinal organoids have highlighted its presence (75, 76).

The human retina contains a central region termed the macula in which the 1.5 mm fovea centralis resides. The fovea, required for high visual acuity and colour vision, in particular, was found to develop and maturate much earlier than the rest of the retina (41, 90). The developing fovea, already free of mitotic cells at Fwk8.4, initially consists of a single row of cone photoreceptors. The rapidly maturating foveal inner retina already consists of the main components of the midget pathway as well

as of presynaptic markers present in the OPL and IPL by Fwk13.7 (41). However, a small depression called the foveal pit develops from Fwk25 which leads to a gradual receding of all retinal layers except for the outer nuclear layer which begins to thicken. Foveal development continues in early childhood with elongation of cone outer segments and is considered adult-like in early- to mid-adolescence (70, 73, 74, 91, 92). Newborns still have underdeveloped outer segments at the cone-rich fovea after birth. Therefore, suggesting that human newborns rely on extrafoveal vision initially (51). Immature cones are present from Fwk8 and immature rods from Fwk10. Cone S opsin expresses from Fwk12 while cone L and M opsin and rhodopsin express at Fwk 15 (8, 65, 69, 72, 74). Rod photoreceptor maturation defined as the development of both inner and outer segments, occurs quicker in the mid-peripheral human retina than the parafoveal region (71). Full maturation of the human retina does not occur until early adolescence. The average adult human retina contains 4.6 million cones, decreasing sharply in density outward from the fovea. However, the average number of rods in the adult human retina, 92 million, far exceeds the number of cones. The density of rods is highest around the optic nerve and decrease in density towards the peripheral retina and are absent from the central fovea (66).

Retinal organoids do not yet form a separate specialised fovea. However, they do form all of the main photoreceptor subtypes including photoreceptors with Rhodopsin, L/M-opsin and S-opsin (59, 93, 94). Retinal organoid photoreceptors form both inner and outer segments and contain many of the prototypic morphological structures including: mitochondria, basal body, centriole, connecting cilium and outer segment discs. Additionally, adjacent structures such as the adherens junctions of the OLM and the microvilli of radial glial progenitors and subsequently Müller glial cells are also found (55, 80, 81, 83, 84, 86, 94). The quality of these structures greatly varied between studies and in particularly outer segments had very few disk stacks, or they were disorganised. Recently, Eldred et al (2018) found that the DD200 retinal organoids can recreate the temporal generation of cones having a similar distribution, gene expression profile and morphology as adult human retina. They also found a recreation of the temporal switch which specifies specification of S cones to the generation of L/M cones through thyroid hormone signalling. Going one step further they also found that the two thyroid hormone states, T3 and T4, were modulated by different deiodinase enzymes in early or late organoids to specify the change from S to L/M cone subtypes and found that temporal gene expression of these enzymes correlated with data from Hoshino et al (2017) on the human developing retina (41, 59). Interestingly, preterm human infants that have low T3/T4 are more likely to have defects in colour vision (95). Perhaps further building on this work by local modulation of thyroid signalling in retinal organoids may be able to specify a more cone-rich, fovea-like region, making them a step closer to the *in vivo* human retina. Such modulation of thyroid signalling in retinal organoids might be achieved through optogenetically controlled protein expression.

Light Responsiveness and Synaptic Transmission of the Fetal Retina and Stem Cell-derived Retinal Organoids

Light responsiveness and synaptic transmission are critical milestones for a functioning retina. As previously mentioned, although not considered fully mature until post birth, the fetal retina does have both rods and cones with developing outer segments as early as Fwk15. The eyelids open by approximately Fwk 25, a milestone that suggests the fine tuning of the retina to visual stimulation can begin from this point (96). *In utero* visual stimulation is considered very limited. However, fetal responses to visual stimulus as measured by heart rate, physical movement and brain activity have been reported. Transabdominal illumination has been reported to increase fetal heart rate at Fwk36 (Smyth et al, 1965: Fwk not reported, Kiuchi et al, 2000: Fwk 36-40) and fetal movement at Fwk26 (97, 98). Fetal heart rate increase has also been shown during amnioscopy in which a cold light source was exposed to the amnion and fetus for 30 seconds at Fwk38 (99). An increase in fetal heart rate in response to light with increasing gestational age as analysed by actocardiogram is seen from Fwk18 to 41 (100). However, fetuses have only been reported to reliably respond to light stimulation from Fwk37; this is likely due to differences in abdominal and uterine wall thicknesses, light sources used, the distance of light source and its focus on the fetus eyes (100–102). Magnetoencephalography (MEG) studies have recorded visually evoked brain activity from as early as Fwk28 (103–106). Functional magnetic resonance imaging (fMRI) has also been successfully used to measure fetal response to transabdominal illumination, finding activity in the frontal lobes not the visual cortex from Fwk36 (107).

Two main techniques have been utilised to measure preterm neonatal vision, the electroretinogram (ERG) for retinal activity and visual evoked potentials (VEP) for brain activity. VEP studies suggest that extrauterine age accelerates the development of the fetal visual system once a maturational threshold has been reached, post Fwk25 (108–111). Interestingly, this coincides with the approximate age of eyelid opening. Similarly, ERG studies on preterm neonates, from as early as Fwk31, suggest that improvements in retinal activity (decrease in a-wave and b-wave latency, increase in amplitude) are correlated with postconception age and extrauterine age (111–113). Because the eyelids are yet closed before Fwk25, preterm ERG studies cannot be used to assess if *in vivo* retinal activity also has a maturational threshold. Other features of preterm ERG include decreasing rod threshold with increasing postconception age, adult-like b-wave sensitivity is reached at six months after normal birth (114, 115). Rod functional maturation occurs peripherally and then parafoveally (116). Preterm birth, however, has been consistently linked with reduced rod and cone function when compared to usual term infants (117–119). This suggests that exposure of the retina to light too early is harmful, this may also apply to cultured stem cell-derived retinal organoids.

As previously mentioned, human retinal organoids develop photoreceptor outer segments of different quality between Wk16-28 (55, 59, 78, 80, 81, 83, 84, 86, 94,

120). Retinal and brain organoids have been shown to respond to light (55, 83, 121). Zhong et al (2014) found that 2 out of 13 rod cells from Wk25-27 hiPSC-derived retinal organoids, which had putative outer segments, responded to light as measured by perforated-patch recordings in the voltage-clamp mode. The sensitivity of these cells was less than found in adult non-human primate photoreceptors, and they were unable to elicit multiple responses, likely due to a depletion in components required for phototransduction (55). Hallam et al (2018) used a 4096 channel multielectrode array (MEA) on which they flattened longitudinally opened Wk21.4 (DD150) hiPSC-derived retinal organoids ganglion side down and found changing spike activity from pulses of white light. They also used puffs of cGMP, which depolarises photoreceptors leading to an unstimulated condition (Dark Current), to show that the light response they found were driven by phototransduction in photoreceptors and not the potential activity of ipRGCs (83). Quadrato et al (2017) found that eight month hiPSC-derived brain organoids exhibited spontaneously-active neuronal networks, using high-density silicon microelectrodes. Additionally, they found subpopulations of neurons which were responsive to 530 nm light in 4 out of 10 organoids. However, they were unable to attribute the responses directly to the photosensitive cells or the downstream neuronal networks (121).

Human stem cell-derived retinal organoids can develop some synaptic maturity. Electron microscopy data show the presence of electron-dense ribbons surrounded by synaptic vesicles and IHC markers such as RIBEYE (as detected by CtBP2) and Bassoon confirm the presence of ribbon synapse, along with other synapse markers PSD-95, vGlut1, PNA, Synaptophysin and Syntaxin 3, in human stem cell-derived retinal organoids. These markers roughly aligned at the OPL of retinal organoids. However, this varied between protocols and degeneration of the inner retina in ageing organoids may also play a factor (61, 78, 81, 83, 86, 93). Interestingly, Dorgau et al (2018) showed that blocking of Laminin γ3, an extracellular matrix protein, in late-stage retinal organoids led to the disruption of ribbon synapse marker Bassoon. This may be due to the significant disruption of Müller glial cell end feet at the inner limiting membrane (61). Wahlin et al (2017) showed that both excitatory (L-aspartate, glutamate) and inhibitory (GABA, glycine) neurotransmitters of the retina were present in Wk43 (DD300) hiPSC-derived retinal organoids. L-aspartate was found in the ONL, glutamate and GABA throughout the retina and glycine in the INL (81). Hallam et al (2018) used puffs of GABA to highlight the emerging functional neural networks in Wk21 (DD150) human stem cell-derived retinal organoids (83). Wahlin et al (2017) used whole-cell patch clamp recordings to elicit membrane capacitance changes as an index of voltage-dependent synaptic vesicle release in retinal organoid photoreceptors (81). Similarly, Deng et al (2018) were able to show the electrophysiological response from whole-cell patch clamp recorded rod photoreceptors from hiPSC-derived retinal organoids (93).

Together, these data highlights the potential for producing light responsive human stem cell-derived retinal organoids with maturating synapses that are capable of transferring information from the photoreceptors to the inner retina.

At the least, retinal organoids have “functional units” of photoreceptors, bipolar cells, and ganglion cells. Work needs to be carried out to assess the quality of these neural networks and how well all cell types contribute. More emphasis needs to be put on comparing the early *in vivo* and *in vitro* retinal response to light and how that ties in with the maturational stage. Unsurprisingly, retinal organoids represent an immature/incomplete development stage, and thus functional responses reflect this. Improved culturing methods and better control of the microenvironment might help in further maturation of the retinal organoids.

Improved Retinal Organoid Modelling

Despite ongoing issues with batch to batch variations, many current differentiation protocols lead to the generation of well laminated retinal organoids that contain all the primary retinal cell types but have putative photoreceptor segments or in long-term culture have degeneration of the inner retina. This may be due to the lack of correct microenvironmental cues and structural support. Degeneration of the inner retina is likely due to the lack of access by medium components, particularly as the retina thickens during development. Furthermore, misregulation of ECM components in human stem cell-derived retinal organoids may significantly affect their correct lamination (61). Many teams are focusing on new methodologies to improve the quality of retinal organoids by tweaking protocols, using bioreactors or microfluidic systems, (78, 80, 81, 84). The use of bioreactor setups, instead of static culture setups, may help solve a number of these problems as they allow for improved aeration and distribution of nutrients as well as allow for scaling up of organoid production. Bioreactor setups report stem cell-derived retinal organoids with better lamination and enhanced differentiation, an increased yield of photoreceptors with outer segment structures, improved cone formation and a better recapitulation of the spatiotemporal development of *in vivo* retina (78). While the initial stages of differentiation require hypoxic conditions, improved oxygen diffusion at later stages is essential for greater cell proliferation and ganglion cell survival. In the absence of vascularised stem cell-derived retinal organoids, bioreactors help facilitate this mechanism.

Many of the differentiation procedures used to derive human retinal organoids lead to the concomitant production of RPE. However, it produces RPE that is consistently not directly adjacent to the photoreceptor segments when using a 2D to 3D differentiation protocol. It does allow however medium that is conditioned by both the retinal organoids and RPE, which may provide some essential diffusible factors for both structures. Production of full-length photoreceptor segments by Wahlin et al (2017) suggested that contact of RPE is non-essential for their development/maturation (81). However, correctly located RPE may provide essential structural support as well as help facilitate a number of the physiological roles of photoreceptor segments such as phototransduction. Microfluidic systems for retinal organoids may help promote improved cell to cell interaction and additionally provide tighter

control of the microenvironment (122). One of the future uses of human stem cell-derived retinal organoid technology is likely to be as a source of transplantable tissue and in particularly photoreceptor cells to treat retinal dystrophies (86, 123–125). Integration and functionality of transplanted photoreceptors into host retina has been shown to be much more limited than initially thought, with material transfer of cytoplasmic components including fluorescent reporter proteins being found to predominate (126–128). Transplanted photoreceptors may not facilitate their physiological roles fully due to lack of interaction with host RPE. One way to enhance the photosensitivity of transplanted photoreceptors from human stem cell-derived retinal organoids is to use optogenetically transformed photoreceptors (129). Material transfer is found from conjugates formed from transplanted NTPDase2-positive CellTracker Green labelled Müller Glial Cells (130). Material transfer represents a novel route to develop cell-based therapeutics which may be able to transfer "healthy components" to diseased retinal cells (131).

The Apical CRB and PAR Complexes

Apical-basal polarity is pivotal for the formation and functionality of epithelial tissues being governed by conserved canonical factors that define the apical domains (Crumbs-homologue (CRB), Protein Associated with Lin Seven 1 (PALS1), partitioning defective-6 (PAR6), atypical protein kinase C (aPKC), junctional such as PAR3 and basolateral such as Scribbled (SCRIB), Discs large (DLG), Lethal giant larvae (LGL). However, recent work in the fruit fly *Drosophila* mid gut indicates that there are alternative apical polarising factors other than the canonical epithelia polarity factors and that this may also extend to some types of vertebrate epithelia (132). Additionally, there also exists planar cell polarity (PCP) in tissue epithelia, which is orthogonal to the apical-basal axis (133). The retinal sub-apically localised CRB and PAR complexes are pivotal in maintaining the spatiotemporal processes of retinogenesis. The CRB complex has a prominent role in the control of apical-basal polarity acting as a sensor for cell density and upon polarisation leading to regulation of Adherens Junctions (AJs) to promote maintenance of cell adhesion (134, 135). Disruption of the CRB complex leads to loss of polarity and can lead to subsequent loss of adhesion, ectopic localisation of progenitors and postmitotic cells due to disrupted apically anchored process and coordinated cell migration, increase in cycling progenitor cells and late-born cell types, increase in early retinal apoptosis and disruption of lamination. A long-term consequence of loss of retinal apical polarity is mild to severe retinal degeneration with a concurrent loss of retinal function in line with morphological deficit (136–141). The complex acts in the role of conductor coordinating multiple downstream signalling pathways which have essential roles in development, such as the Notch and Hippo pathways (134, 142, 143). Thus leading to the regulation of cell size, cell fate determination, cell self-renewal, proliferation, differentiation, mitosis and apoptosis. However, how these intertwined cellular responses are mediated collectively by the core complex

remains ambiguous.

The mammalian retinal CRB complex comprises of at least one of the three CRB family members, CRB1, CRB2 and CRB3 (two isoforms CRB3A and CRB3B), in addition to PALS1 (also called MPP5), PATJ, MUPP1, MPP3 and MPP4. Both CRB1 and CRB2 have a large extracellular domain with epidermal growth factor (EGF) and laminin-globular domains. CRB1, CRB2, and CRB3A have a single transmembrane domain juxtaposing a short intracellular C-terminus of 37 amino acids which contains a FERM-binding motif (4.1, ezrin, radixin, moesin) and PSD-95/Discs-large/ZO-1 (PDZ)-binding motif ERLI (Glu-Arg-Leu-Ile) (57, 135, 143, 144). Alternatively, the non-prototypic CRB3B isoform, which has a role in ciliogenesis and cell division, contains a C-terminal PDZ-binding motif CLPI (Cys- Leu-Pro-Ile) that does not interact with the PAR complex (145, 146). The FERM motif can bind to proteins such as EPB4.1L5 which plays a role in epithelial-to-mesenchymal transition during gastrulation in mice and is a negative regulator of photoreceptor size in *Drosophila* and zebrafish (147–151). EPB4.1L5 oligomerization is essential for its binding to CRB and is mediated through its FERM and FERM adjacent (FA) domains (152). EPB4.1L5 controls the actomyosin cytoskeleton at both apical junctions and basal focal adhesions (147, 153). EPB4.1L5 is predominantly located basolaterally in early development, repressing CRB, but is recruited by CRB apically at later stages of differentiation. In the adult mammalian retina EPB4.1L5 has been found to localize to the OLM (150, 151). PAR complex member aPKC can bind and phosphorylate the FA domain of EPB4.1L5 leading to the dismantling of the EPB4.1L5 oligomer. This phosphorylation by aPKC prevents the premature apical localization of EPB4.1L5, in turn EPB4.1L5 restrains aPKC signalling thus antagonizing each other, leading to tightly controlled segregation of apical/basal membrane domains (152, 154). The 4 amino acid PDZ-binding motif ERLI of CRB allows for interaction with adaptor proteins such as PALS1 and PAR6 (155, 156). Binding of PALS1 to the C-terminal PDZ domain of CRB leads to recruitment of PATJ or MUPP1 through binding of the PALS1 N-terminal L27 domain to the L27 domain of PATJ or MUPP1 (157, 158). Additionally, PALS1 can recruit MPP3 and MPP4 to the apical complex (159, 160).

Binding of PAR6 to the C-terminal PDZ domain of CRB leads to the recruitment of the other PAR complex members PAR3, aPKC and cell division control 42 (CDC42). PAR6 can interact with PAR3 through their PDZ domains, with aPKC through their N-terminal Phox and Bem1 (PB1) domains, and with CDC42 through their semi-CDC42- and Rac-interactive binding (CRIB) domains (161–164). The activity of aPKC is suppressed by PAR6 binding, but this suppression is partially relieved when GTP bound CDC42 interacts with the complex (165, 166). However, the activity of aPKC has also been shown to be promoted by PAR6 (167). PAR3 is both an inhibitor of aPKC activity but also its substrate (164). At adherens junctions, PAR3 can also bind to the scaffolding proteins FERM domain containing 4A (FRMD4A) and FRMD4B (also known as GRSP-1) leading to the recruitment of cytohesin-1 (CYTH)1 and causing subsequent activation of ARF6, this complex being essential for epithelial polarity (168). FRMD4B and CYTH3 (also known as GRP1) also exist in a complex

(169). Recently, in the mouse retina a variant of FRMD4B, *Frmd4b*^{Tvrm222}, led to the suppression of OLM fragmentation and photoreceptor dysplasia in *Nr2e3*^{rd7} and *Nrl*$^{-/-}$ mice. Whole exome sequencing revealed the *Frmd4b*^{Tvrm222} variant had a substitution of serine residue 938 by proline (S938P). Transfection of COS7 cells with either wild-type FRMD4B or S938P and the addition of insulin, an agonist of the phosphoinositide 3-kinase (PI3K)-AKT pathway, revealed that the FRMD4B variant does not translocate to the plasma membrane as occurs with wildtype FRMD4B. The *Frmd4b*^{Tvrm222} mice showed reduced AKT phosphorylation and an increase in cell junction proteins, activated AKT leads to loss of apical-basal polarity. Therefore, the interactions of the FRMD4B variant and cytohesin-3 may modulate both the PAR3 activated ARF6 pathway and/or PI3K-AKT pathway to prevent retinal dysplasia in *Nr2e3*^{rd7} and *Nrl*$^{-/-}$ mice (170). CDC42 in addition to its role in regulating aPKC has recently been shown to regulate another kinase to the apical domain, p21-activated kinase-1 (PAK1) (166, 171). Loss of PAK1 or aPKC leads to a moderate polarity phenotype however when both are inactive a dramatic loss of epithelial polarity was detected. Both aPKC and PAK1 act redundantly downstream of CDC42, PAK1 expression is found throughout the mouse retina (171, 172). The CRB and PAR complexes may interact additionally through direct interaction of the amino terminus of PALS1 and the PDZ domain of PAR6, the interaction being regulated by GTP bound CDC42 (173). PAR6, PAR3 and aPKC are located at the OLM in embryonic mouse retina while CDC42 is located throughout the retina (174–176).

The Localisation of the mammalian Retinal CRB complex

The developing mammalian retina expresses both CRB1 and CRB2. In mouse retina, both CRB1 and CRB2 are expressed at the subapical region adjacent to adherens junctions of progenitor cells at embryonic day 12.5, equivalent to 1st trimester human fetal retinal development (40, 41, 138, 150, 177). However, in 1st trimester human fetal retina, while CRB2 labelling is found at the subapical region adjacent to adherens junctions in putative photoreceptor inner segments and the apical villi of radial glial progenitor cells, CRB1 is only sporadically found. Subsequently, in 2nd trimester human fetal retina CRB1 and CRB2 labelling could be clearly detected at the subapical region adjacent to adherens junctions between putative photoreceptor inner segments and in the apical villi of radial glial progenitor cells/ Müller glial cells. The onset of CRB1 and CRB2 expression in the human fetal retina was found to be recapitulated in early versus late differentiated induced-pluripotent stem cell (iPSC)-derived retinal organoids ((178); unpublished data PQ and JW) .

In the adult mammalian retina CRB1, CRB2 and CRB3 proteins localize at the subapical region in the Müller glial cells of mice, non-human primates and humans. However, while CRB3 is present at the subapical region of photoreceptors in all three species, the expression patterns of CRB1 and CRB2 at the subapical region of photoreceptors differs between the three species. CRB2 is present at the subapical region of mouse photoreceptors, whereas CRB1 is not. In non-human primates both

CRB1 and CRB2 are present in photoreceptors. In cadaveric human retinas collected 2-days after death CRB1 is present at the subapical region whereas CRB2 is present in the photoreceptor inner segments but at some distance from the subapical region (141, 179–181). Interestingly, all 3 CRB proteins in the adult human retina are detected in the photoreceptor inner segments at a distance from the outer limiting membrane (182). Additionally, CRB3 is also detected in the inner retina of mice, non-human primates and humans (179, 181, 183). All of the ultrastructural immuno-electron microscopy studies using anti-CRB on adult human cadaveric retina were carried out using two-days-old tissue samples, and might for that reason differ with the results obtained when using freshly collected non-human-primate retinas. In other studies, a negative correlation between protein abundance and post-mortem time has been found in the human retina (184, 185).

CRB1 and Leber Congenital Amaurosis

Leber congenital amaurosis (LCA) is an early-onset disease leading to blindness from near birth with *CRB1* mutations accounting for 7-17% of cases and affecting approximately 10,000 patients worldwide (143, 186–188). Currently, there is no treatment available for *CRB1*-LCA patients, but *proof-of-concept* gene supplementation studies have shown functional rescue in *CRB1* retinitis pigmentosa mouse models which sets a ground work for *proof-of-concept* in *CRB1*-LCA-like mouse models (182). *CRB1* gene mutations cause LCA8 with patients having severely attenuated or non-recordable ERG, abnormal pupillary reflex and nystagmus whiles their retinas have abnormal layering with reports of the retina being thickened, thinned or unchanged (189–198). Human stem cell-derived retinal organoids which mimic retinal disease are an excellent tool for understanding disease mechanisms as well as a platform for testing therapeutic strategies. Recently, Zhang et al (2018) reported that retinal organoids could be derived from induced pluripotent stem cells made from the dermal fibroblasts of a human retinitis pigmentosa patient with two missense mutations in the *CRB1* gene (NM_201253.2): c.1892A > G (p.Tyr631Cys) and c.2548G > A (p.Gly850Ser) (199). Other teams, including our own, are investigating pathobiology and disease mechanism of stem cell-derived retinal organoids from *CRB1* patients. However, these studies are in their early stages. Many rodent models of CRB retinal degeneration exist and have provided us with a deep insight into the pathobiology and mechanisms underlying CRB disease (57, 143). New *CRB1*-LCA-like models have further built on our previous data (181, 200). One of the main working hypothesis that we draw from our four *CRB1*-LCA-like models is that CRB2 protein levels may be lower or that a less functional variant is of CRB2 is expressed in *CRB1* LCA patients compared to less severe *CRB1* retinal dystrophies (181, 200, 201). Clinical reports of *CRB1* LCA patients have shown that degeneration can affect all quadrants of the retina while other reports show restriction to the inferior retina (194, 197, 202, 203). This is highly suggestive of the presence of a modifying factor, variants of *CRB2* have been associated with retinal

aberrations (204). Additionally, CRB2 is present in the fetal human retina in the first-trimester, whereas CRB1 expression starts from the second trimester ((178); unpublished data PQ and JW). In the mouse, both CRB1 and CRB2 are found in the early embryonic retina (138, 150, 177). Therefore, missense variants of *CRB2* in humans are likely to have small but important effects on retinal dystrophies. A number of transcript variants have been reported for CRB1, and new novel isoforms of CRB1 are reported in the mouse and human retina and may also lead to the phenotypic variability in CRB1 patients (57, 205).

The four *CRB1*-LCA-like models had both alleles of *Crb2* disrupted in either retinal progenitor cells (*ΔRPC*), immature photoreceptors (*ΔimPRC*), or Müller glial cells (ΔMG) on genetic backgrounds with either reduced levels of ($Crb1^{KO/WT}Crb2^{\Delta RPC}$) or complete knockout of *Crb1* ($Crb1^{KO}Crb2^{\Delta RPC}$, $Crb1^{KO}Crb2^{\Delta imPRC}$, $Crb1^{KO}Crb2^{\Delta MG}$). All of these models had abnormally layered and transiently thickened retina, disruptions of the outer limiting membrane and ectopic localisation of mitotic progenitors, cycling cells, and immature photoreceptors. The thickened retinas observed were in part due to the ectopic birth or displacement of early progenitors which we found increased in the $Crb1^{KO}Crb2^{\Delta RPC}$ and $Crb1^{KO}Crb2^{\Delta imPRC}$ mouse retinas, but not in the $Crb1^{KO}Crb2^{\Delta MG}$. This led to adult retinas in which we detected ectopic cells in the ganglion cell layer either sporadically ($Crb1^{KO}Crb2^{\Delta RPC}$), at the peripheral retina ($Crb1^{KO}Crb2^{\Delta MG}$), or within most of the retina ($Crb1^{KO}Crb2^{\Delta imPRC}$) or throughout the retina ($Crb1^{KO}Crb2^{\Delta RPC}$). We hypothesize that *CRB1* LCA patients which exhibit a thickened retina and abnormal layering do so due to similar mechanisms as found in our *CRB1*-LCA-like mouse models, displacement or the ectopic birth of progenitor cells, cycling cells and immature photoreceptor cells (181, 194, 200, 201). Additionally, the reported thinned or unchanged retinal thickness of *CRB1* LCA patients is likely due to measurements made when significant retinal degeneration had already occurred. In our *CRB1*-LCA-like mouse models we detected transient changes in retinal thickness (197, 198).

While in the $Crb1^{KO/WT}Crb2^{\Delta RPC}$ and $Crb1^{KO}Crb2^{\Delta RPC}$ mouse models no differences in retinal degenerations were reported between superior/inferior or central/peripheral retina, the $Crb1^{KO}Crb2^{\Delta imPRC}$ and $Crb1^{KO}Crb2^{\Delta MG}$ retina showed these phenotypes, respectively (181, 200, 201). Interestingly, a new *Crb1Crb2* double knockout mouse model which disrupted both alleles of *Crb2* in rods (Δrods) on a genetic background lacking *Crb1* ($Crb1^{KO}Crb2^{\Delta rods}$) does not have an LCA-like but RP-like phenotype. The $Crb1^{KO}Crb2^{\Delta rods}$ retinas have a phenotype that mainly affects the peripheral and central superior retina (unpublished data Henrique Alves and JW, 2018). These differences may be attributed to opposing gradients of CRB1 and CRB2 at the subapical region between the superior and inferior retina as well as the contribution of CRB1 and CRB2 to either photoreceptors (CRB2) or Müller glial cells (CRB1 and CRB2) (141, 179, 180). Another common feature of the *CRB1*-LCA-like mouse models is the early formation of retinal rosettes (181, 200, 201).

Rosette formation has been extensively described in many retinal conditions including retinitis pigmentosa, diabetic retinopathy and retinoblastoma (206–208).

The formation of rosettes has been attributed to the disruption of the OLM both chemically and genetically (137, 140, 141, 174, 209–213). A defining hallmark of CRB mouse models is the formation of photoreceptor rosettes which may be concurrent with the loss of polarity in a CRB dependent manner. The more severe CRB models have a low level of total CRB, less stable AJs and thus an earlier phenotype onset and rosette formation. The less severe CRB models have a higher level of total CRB, more stable AJs and thus a later phenotype onset and rosette formation (57). Rosette formation is preceded by aberrant localisation of retinal cells into the subretinal space at foci where loss of adhesion is found. In the developing retina, this is usually seen as "volcanic-like" cell eruptions, while in the mature retina this is seen as a loss of complete rows of photoreceptors (138, 139, 141, 179, 181, 200, 201). The *Crb1*^{KO}*Crb2*$^{\Delta MG}$ retina has a peripheral to central degenerative phenotype. At P1 in the *Crb1*^{KO}*Crb2*$^{\Delta MG}$ retina rosettes can be detected peripherally while only protrusions into the subretinal space are found in the central retina. By P14 the peripheral rosettes are gone due to advancement of the phenotype, but rosettes can now be found in the central retina (181). Differences in the number of aberrant cells in early- versus late-disrupted OLM phenotypes likely arise from both the extent of OLM disruption as well as the development stage of the retina. Earlier cells are less mature/competent and are still undergoing division and migration changes. Rosette formation is likely independent of any increased cell proliferation seen in early-onset RP-like and LCA-like CRB models as rosettes are also present in later onset CRB, CRB-related and non-CRB mouse models that do not exhibit changes in proliferation (138, 139, 141, 181, 200, 201, 211, 212, 214).

Although the mechanism by which rosettes are formed in retinal disease is not fully alluded to, it has been related to changes in the extracellular matrix, adhesion molecules and the cytoskeleton, all of which can be affected by an imbalance between apical and basal polarity domains (215–218). Loss of CRB in mutant mouse models may not be uniform due to opposing gradients of CRB1 and CRB2 in the superior versus inferior retina as well as well as the non-uniform localisation of CRB between photoreceptors (CRB2) and Müller glial cells (CRB1 and CRB2), leading to imbalance of CRB levels at the OLM in the mutant models (141, 179, 180). Differences in adjacent apical levels of CRB are well studied as this is part of the process for tube invagination, similar mechanisms in opposing CRB levels between adjacent cells may lead to rosette formation, which are in fact invaginations of the apical retinal surface (219–221).

Concluding remarks

Human stem cell-derived retinal organoids faithfully recapture in part many of the facets of the human fetal retina, including retinal cell type diversity, morphological cues, synaptic wiring and light sensitivity. Improved retinal organoid culturing methods using bioreactors or microfluidic organ-on-a-chip technology, which also allows for tight control of the physiological microenvironment, bring us a further

step towards an *in vivo* like retina (78, 222, 223). Furthermore, retinal organoid models derived from patients will be able to be used in conjunction with two-photon imaging and light sheet microscopy as well as tissue clearing methods such as DISCO (47, 80, 224, 225). This will allow unparalleled analysis of live and fixed cellular events including spatiotemporal process such as proliferation, differentiation and migration. Additionally, new methods such as ferrofluid droplets as mechanical actuators allow analysis of the mechanics of 3D developing tissues. This tool in combination with optogenetics or calcium imaging would provide insight into how neuronal and mechanical responses may influence each other in retinal development and disease (226, 227). Together, these are all valuable tools to further evaluate the mechanisms by which the misregulation of the apical CRB and PAR complexes affects retinogenesis leading to the severe retinal degeneration seen in LCA patients.

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8.2 SUMMARY AND DISCUSSION

Approximately 250 genes are linked to hereditary retinal disorders such as retinitis pigmentosa (RP) and Leber's congenital amaurosis (LCA). The Crumbs complex is crucial for cell polarity and epithelial tissue function, playing an essential role during retinogenesis. Disruption of the Crumbs (CRB) complex will interrupt the precise orchestration of spatiotemporal process during retinal development, such as cell fate choice, division, migration and differentiation. In turn this can cause retinal degeneration leading to impairment of retinal function and thus vision. Mutations in the *Crumbs homologue-1* (*CRB1*) gene account for around 80,000 persons with RP and LCA worldwide. The *CRB1* gene therefore is an essential target to develop therapies for the treatment of RP and LCA. However, this requires an understanding of the pathobiology and signalling mechanisms that underlie these diseases. Animal models which mimic *CRB1*-RP-like and *CRB1*-LCA-like phenotypes help us to understand the function of the CRB complex in retinal development. Additionally, they are also pre-clinical tools to study retinal degeneration and test therapeutic strategies. Intriguingly, there is no clear genotype phenotype correlation for *CRB1* mutations suggesting that there are other modifying factors. We hypothesised that conditional disruption of *Crb2* in either retinal progenitor cells (RPC), immature photoreceptor cells (imPRC) or Müller Glial cells (MG) on a genetic background lacking *Crb1* may substantially modify its phenotype from *CRB1*-RP-like to *CRB1*-LCA-like. The $Crb1^{KO/WT}Crb2^{\Delta RPC}$, $Crb1^{KO}Crb2^{\Delta RPC}$ (**Chapter 2**), $Crb1^{KO}Crb2^{\Delta imPRC}$ (**Chapter 3**) and $Crb1^{KO}Crb2^{\Delta MG}$ (**Chapter 4**) *CRB1*-LCA-like mouse models all have transiently thickened and abnormally layered retina with disruptions at the outer limiting membrane. Additionally, they have ectopic localization of mitotic progenitors, cycling cells, and immature photoreceptors. These *CRB1*-LCA-like models proof the modulating effect CRB2 has above a CRB1 phenotype and better allude to the pathobiology of *CRB1* LCA patients which have a thicker inner retina. In these four animal models the thickened retina we find is in part due to the displacement or ectopic birth of retinal progenitor and cycling cells. This may also be the case in *CRB1* LCA patients.

Previously, we have analysed $Crb1^{KO}$ mice which lack the CRB1 protein in retinal progenitor cells and develop a slow-progressing RP-like phenotype restricted to foci in the inferior retina. We have also analysed $Crb2^{\Delta RPC}$ mice which lack CRB2 protein in retinal progenitor cells and develop an early-onset RP-like phenotype leading to retinal laminations disruptions throughout the retina. Whiles $Crb1^{KO}$ mice have no functional deficit as measured by electroretinography, $Crb2^{\Delta RPC}$ have moderate attenuation of retinal function. In **Chapter 2** we studied mouse models in which both alleles of *Crb2* are disrupted in retinal progenitor cells, on genetic backgrounds with either reduced levels of ($Crb1^{KO/WT}Crb2^{\Delta RPC}$) or lacking ($Crb1^{KO}Crb2^{\Delta RPC}$) *Crb1*. We hypothesised that there was a potential overlap of function for CRB1 and CRB2 proteins in retinal progenitors. The $Crb1^{KO/WT}Crb2^{\Delta RPC}$ retina had a severe morphological phenotype however maintained distinctive retinal lamination and

did not exhibit a thickened ganglion cell layer. The *Crb1^{KO}Crb2^{ΔRPC}* retina failed to form an outer plexiform with significant mixing of the outer nuclear layer (ONL) and inner nuclear layer (INL) and ectopic cells in the ganglion cell layer. Both the *Crb1$^{KO/WT}$Crb2^{ΔRPC}* and *Crb1^{KO}Crb2^{ΔRPC}* retina had severe attenuation of retinal function mimicking a *CRB1*-LCA-like phenotype. Analysis of all retinal cell types in the *Crb1^{KO}Crb2^{ΔRP}* retina found an increase in the number of late-born cell types such as bipolar cells, rod photoreceptors and Müller glial cells but not early-born cell types. This coincided with an increase in retinal progenitors and late-born precursor cells due to aberrant regulation of the cell cycle. There was dysregulation of a) the Notch siginalling pathway which regulates cell proliferation, fate, differentiation, and apoptosis and b) the Hippo signalling pathway which regulates cell proliferation, stem cell self-renewal and apoptosis in *Crb1^{KO}Crb2^{ΔRP}* retina. This highlights a crucial role for CRB1 and CRB2 in regulating these pathways. The study data shows that CRB2 is a modulating factor of CRB1 phenotype in the retinal progenitor cells.

In addition to the *Crb1^{KO}* and *Crb2^{ΔRPC}* mouse models previously described we have also specifically ablated *Crb2* in immature mouse photoreceptors. Similarly to the *Crb2^{ΔRPC}* mouse model the *Crb2^{ΔimPRC}* mouse retinas represent an early-onset *CRB*- RP-like phenotype leading to lamination defects and loss of retinal function. In **Chapter 3** to investigate the physiological roles of CRB2 in immature photoreceptors and adjacent retinal progenitor cells we studied disruption of a single allele (*Crb1^{KO}Crb2$^{Low-imPRC}$*) or both alleles (*Crb1^{KO}Crb2^{ΔimPRC}*) of *Crb2* in immature photoreceptors, on a genetic background lacking *Crb1*. The *Crb1^{KO}Crb2$^{Low-imPRC}$* mouse model mimicked a severe *CRB1*-RP-like phenotype showing a phenotype above a *Crb1^{KO}* but still mostly restricted to the inferior retina. These retinas had a progressive loss of visual function with the severest foci of degeneration in the inferior retina showing loss of photoreceptors and extensive neovascularisation. In contrast, additional disruption of *Crb2* in the *Crb1^{KO}Crb2^{ΔimPRC}* mouse retina lead to a severer phenotype in the superior than inferior retina with extensive ectopic cells in the ganglion cell layer. Similar to the *Crb1^{KO}Crb2^{ΔRPC}* mouse retina the *Crb1^{KO}Crb2^{ΔimPRC}* mouse retina did not form an outer plexiform layer and had extensive mixing of the nuclei of the ONL and INL. Despite having an increase in mitotic cells the *Crb1^{KO}Crb2^{ΔimPRC}* retina unlike the *Crb1^{KO}Crb2^{ΔRPC}* retina did not have an increase in late-born photoreceptor cells. This may be due to the concomitant increase found in programmed cell death at P1. The data presented in chapter 3 highlights the need for physiological levels of CRB proteins in adjacent photoreceptors and Müller glial cells. It also shows that CRB2 is required to determine the superior-inferior symmetry of the developing retina and that it is a modulating factor of CRB1, in this case specifically in photoreceptors. Ablation of either CRB1 or CRB2 proteins from Müller glial cells leads to mild retinal degeneration with no functional deficit. This suggested an overlap of function of CRB1 and CRB2 proteins in Müller glial cells. In **Chapter 4** to prove this point we disrupted both alleles of *Crb2* in Müller glial cells, on a genetic background lacking *Crb1* and studied the morphological and functional outcome in the *Crb1^{KO}Crb2^{ΔMG}* mouse retinas. The remaining CRB2 protein in the adjacent photoreceptors was

not sufficient to maintain retinal structure and function. The *Crb1*^{KO}*Crb2*$^{\Delta MG}$ retinas exhibited an early onset peripheral to central retinal degeneration with disruptions at the outer limiting membrane and complete loss of retinal function between 1- and 3-months of age. Early displacement of mitotic progenitors, cycling cells and immature photoreceptors was detected. However, unlike the *Crb1*^{KO}*Crb2*$^{\Delta RPC}$ and *Crb1*^{KO}*Crb2*$^{\Delta imPRC}$ mouse retina, no increase in mitotic progenitors was found. Ectopic adult photoreceptor cells could be detected at the peripheral retina, leading to a transiently thickened ganglion cell layer at 1-month of age. This data confirms the hypothesised overlap of function for CRB1 and CRB2 proteins in mouse Müller glial cells. The study also adds further weight to the data showing CRB2 as a modulating factor of CRB1, in this case specifically in Müller glial cells.

Future safety and biodistribution studies for *CRB*-mediated gene therapy vectors will be undertaken in non-human primates. Therefore it is essential to understand the localisation of CRB1 and CRB2 in these retinas to assess the outcome of such experiments. In **Chapter 4** using immuno-EM, CRB1 and CRB2 were detected at the subapical region in photoreceptor inner segments and Müller glial cells apical villi. This data suggests the compensatory mechanism found in mouse Müller glial cells may also be present in non-human primate Müller glial cells and perhaps also photoreceptors.

In **Chapter 5 and 6** we explored gene and cell therapy-based strategies for *CRB* retinal disease. Specifically in **Chapter 5**, we looked at the feasibility of a *CRB*-based gene therapy approach in mouse models mimicking a *CRB1*-RP-like phenotype. To this aim we codon optimized both *CRB1* and *CRB2* cDNA and used either ShH10Y-CMV-*CRB(n)* (intravitreal injection) to target Müller glial cells, AAV9-GRK1-*CRB(n)* (subretinal injection) to target photoreceptors and AAV9-CMV-*CRB(n)* (subretinal injection) to target both photoreceptors and Müller glial cells. For CRB1 expression we had to use a minimal CMV promoter due to AAV packaging limitations and for CRB2 expression we could use the full length CMV promoter. When targeting either photoreceptors or Müller glial cells separately we did not find a rescue as measured by electroretinography and morphological analysis despite finding expression of the proteins. However, when targeting both cell types together, CRB2 but not CRB1 lead to both a morphological and functional rescue in the *CRB1*-RP-like models. Expression of CRB1 in the eye resulted in poorer morphology of not only the retina but also the ciliary body and iris leading to reduced retinal function. This coincided with an increase in infiltration of CD11B and CD3 immune cells in the retina and ciliary body. This study highlighted, using immuno-EM, expression of CRB protein family members additionally away from the subapical region within structures of the photoreceptor inner segment in adult human retina. In summary, the proof-of-concept *CRB* gene therapy studies in mouse models showed that the *CRB1*-RP-like phenotype can be rescued using AAV-*CRB2* gene therapy vectors, and this technology may be further developed for use in the ophthalmic clinic.

Müller glial cells are pivotal in providing homeostatic, metabolic, structural and functional support to retinal neurons. Therefore they are potential ideal to use in cell

transplantation studies to treat retinal degeneration. In **Chapter 6.1** we developed a Fluorescence-Activated Cell Sorting (FACS) technique to enrich for immature and mature mouse retinal Müller Glial cells using the cell surface marker NTPDase2. Subsequently, in **Chapter 6.2** we described transplantation experiments in which we either used NTPDase2-positive sorted cells labelled with cell tracker green (CTG) or NTPDase2-positive sorted cells from transgenic tdTomato mouse retinas. We found using both methods clumps of transplanted cells which stayed in the subretinal space and did not integrate into the retina fully. Additionally, cell tracker green conjugates formed from NTPDase2-positive cells and were preferentially taken up by choline acetyltransferase Starburst amacrine cells.

Although the CRB Complex of developing retina in mice has been well studied this was not the case in human retina. Therefore in **Chapter 7** we studied the onset of tissue expression of CRB complex proteins in the developing human retina by immunohistochemical analysis of human fetal retina and human induced Pluripotent Stem Cell (hiPSC)-derived retinal organoids. Specifically, using immuno-EM, we found occasional and limited labelling for CRB1 protein in photoreceptor inner segments and apical villi of radial glial progenitor cells in 1st trimester fetal retina. During the 2nd trimester however when that retina maturates forming all adult cell types we found CRB1 labelling at the subapical region adjacent to adherens junctions between photoreceptor inner segments and in the apical villi of radial glial progenitor cells/ Müller glial cells. In contrast we found pronounced CRB2 labelling at the plasma membrane and at the subapical region adjacent to adherens junctions between photoreceptor inner segments and in the apical villi of radial glial progenitor cells/ Müller glial cells from the 1st trimester onwards. We found that this tissue expression pattern recapitulated in young versus old hiPSC-derived retinal organoids. Together this data suggest CRB2 is the predominant CRB member in very early retinal development and CRB1 becomes involved during the maturation of the retina. Additionally, with immuno-EM we found CRB2 labelling above tight junctions and in the apical microvilli of human fetal retinal pigment epithelium and hiPSC-derived retinal pigment epithelium.

Human iPSC-derived retinal organoids now offer us alternatives to animal models for testing transgene expression and biological activity. Thus, in **Chapter 7**, we used both hiPSC-derived retinal organoids and human donor retinal explants to test the specificity of AAV9, AAV5 and ShH10Y445F serotypes in combination with the ubiquitous cytomegalovirus (CMV) promoter to target photoreceptor cells and Müller glial cells. We found that AAV5 and ShH10Y445F serotypes are more potent at transducing Müller glial cells than AAV9 in hiPSC-derived retinal organoids and that AAV5 outperformed AAV9 in the transduction of photoreceptor cells in human cadaveric retina. Taken together, we identified AAV serotype 5 to be a good candidate for delivery of a CRB-mediated gene therapy. Additionally, our study found that a lack of photoreceptor segments in human cadaveric retinal explants significantly changed the tropism patterns of AAV serotypes compared to human cadaveric retinal explants with photoreceptor segments. Taken together with the

AAV serotype testing on hiPSC-derived retinal organoids this suggests that the bioavailability of AAV vectors change to target less abundant/preferred receptors in conditions were there is less well formed photoreceptor segments. From a therapeutic perspective this implies an important role for photoreceptor segments in the uptake of various serotypes of AAV and this should be considered during the timing and administering of AAV mediated therapeutics.

In conclusion, by investigating the roles of CRB proteins in different cell types of the mouse retina we were able to better understand the likely pathobiology of *CRB1*-RP and *CRB1*-LCA patients (**Chapter 2, 3 and 4**). We developed gene and cell therapy-based tools as therapeutic strategies for alleviation of these diseases (**Chapter 5 and 6**). By using human fetal retina and hiPSC-derived retinal organoids, we were able to understand the possible roles of CRB proteins in the developing human retina (**Chapter 7**). Finally, the insights derived from 1) the localisation of CRB proteins in adult non-human primates (**Chapter 4**), 2) the proof-of-concept *CRB* gene therapy on *CRB1*-RP-like mouse models (**Chapter 5**), 3) AAV serotype testing on retinal organoids and adult donor human retina (**Chapter 7**) sets a platform to launch CRB-mediated therapy into preclinical trials.

Chapter 9

9.1 Appendix I.
Production of iPS-Derived Human Retinal Organoids for Use in Transgene Expression Assays

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Methods Mol. Biol., 2018, 1715, 261–273.

9.2 Appendix II.
Nederlandse Samenvatting
Abbreviations
List of Publications
Curriculum Vitae
Acknowledgements