Crumbs and MAGUK proteins, from interaction to function
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Chapter 1

General introduction

The role of Crumbs and associated PDZ proteins in epithelial polarity and retinal dystrophies
Chapter 1

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>adRP</td>
<td>autosomal dominant retinitis pigmentosa</td>
</tr>
<tr>
<td>AF-6</td>
<td>afadin 6</td>
</tr>
<tr>
<td>AIP1</td>
<td>aryl hydrocarbon receptor-interacting protein-like 1</td>
</tr>
<tr>
<td>AJ</td>
<td>adherens junctions</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical protein kinase C</td>
</tr>
<tr>
<td>ARM</td>
<td>armadillo</td>
</tr>
<tr>
<td>arRP</td>
<td>autosomal recessive retinitis pigmentosa</td>
</tr>
<tr>
<td>Baz</td>
<td>bazooka</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>C1</td>
<td>protein kinase C conserved region</td>
</tr>
<tr>
<td>CA</td>
<td>cadherin repeat</td>
</tr>
<tr>
<td>Caco2</td>
<td>human colon adenocarcinoma line 2</td>
</tr>
<tr>
<td>CAMKII</td>
<td>calcium/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CAR</td>
<td>coxsackievirus and adenovirus receptor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>Crb (CRB)</td>
<td>crumbs</td>
</tr>
<tr>
<td>CRX</td>
<td>cone-rod homeobox</td>
</tr>
<tr>
<td>Dlg</td>
<td>Discs Large</td>
</tr>
<tr>
<td>Dm</td>
<td><em>Drosophila melanogaster</em></td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ERGs</td>
<td>electroretinograms</td>
</tr>
<tr>
<td>FERM</td>
<td>4.1/ezrin/radixin/moesin</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factors</td>
</tr>
<tr>
<td>FHA</td>
<td>forkhead associated</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell line-derived neurotrophic factors</td>
</tr>
<tr>
<td>GUCY2D</td>
<td>guanylate cyclase 2D</td>
</tr>
<tr>
<td>GuK</td>
<td>guanylate kinase</td>
</tr>
<tr>
<td>has</td>
<td>heart and soul</td>
</tr>
<tr>
<td>hASH1</td>
<td>human protein absent small or homeotic</td>
</tr>
<tr>
<td>HOOK</td>
<td>variable hinge (flexible region)</td>
</tr>
<tr>
<td>ICAMs</td>
<td>intercellular adhesion molecules</td>
</tr>
<tr>
<td>IG</td>
<td>immunoglobulin like</td>
</tr>
<tr>
<td>IGV-type</td>
<td>immunoglobulin V-type</td>
</tr>
<tr>
<td>INL</td>
<td>inner nuclear layer</td>
</tr>
<tr>
<td>JAM</td>
<td>junctional adhesion molecule</td>
</tr>
<tr>
<td>JEAP</td>
<td>junction enriched and associated protein</td>
</tr>
<tr>
<td>L27</td>
<td>domain found in Lin2 and Lin7 proteins</td>
</tr>
<tr>
<td>LCA</td>
<td>Leber congenital amaurosis</td>
</tr>
<tr>
<td>Lgl</td>
<td>lethal giant larvae</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine rich repeats</td>
</tr>
<tr>
<td>MAGI</td>
<td>MAGUK inverted</td>
</tr>
<tr>
<td>MAGUK</td>
<td>membrane associated guanylate kinase</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>mDlg</td>
<td>mammalian Dlg1</td>
</tr>
</tbody>
</table>
mLgl mammalian Lgl
Mpp membrane palmitoylated protein
MUPP1 multiple PDZ domain protein 1
N-CAM neural cell adhesion molecule
NMDA N-methyl-D-aspartate
nNOS neural nitric oxide synthase
OLM outer limiting membrane
ONL outer nuclear layer
P0 protein 0
Pals1 protein associated with Lin7 (Mpp5)
PAR partitioning defective
PATJ protein associated with tight junctions
PB1 Bem1 protein
Pd pupal development
PDZ Postsynaptic density 95/Discs large/Zonula occludens
PPCRA pigmented paravenous chorioretinal atrophy
PPRPE para-arteriolar preservation of the retinal pigment epithelium
prph peripherin
RA Ras association
rAAV recombinant AAV
Rd (RD) retinal degeneration
Rho Ras homology
RP retinitis pigmentosa
RPE retinal pigment epithelium
RPE65 retinal pigment epithelium-specific 65 kDa protein
RPGRIP1 retinitis pigmentosa GTPase regulator-interacting protein 1
sAJ spot AJ
SAP synapse associated protein
SAR subapical region
Scrib (scrib) scribble
Sdt (sdt) stardust
siRNA small interfering RNA
SJ septate junction
SJs septate junctions
Sorb sorbin homologous
Src Src homology 3
SynN syntaxin N-terminal
TJ tight junction
TJs tight junctions
TM transmembrane
SNARE soluble N-ethylmaleimide-sensitive factor attachment protein receptors
VIN vinculin binding region
WD40 Trp-Asp 40 repeats
XLRP X-linked retinitis pigmentosa
ZA zonula adherens
ZO zonula occludens
1. **Adherens junctions, tight junctions and control of cell polarity**

   The adherens and tight junctions are composed of two main classes of proteins:

   1: intracellular attachment proteins, which form a plaque on the cytoplasmic face of the plasma membrane and connect the junctional complex to the actin cytoskeleton.

   2: transmembrane linker proteins, whose cytoplasmic domains bind to one or more intracellular attachment proteins, while their extracellular domains interact with the extracellular matrix or with the extracellular domains of transmembrane linker proteins on another cell.

   Still other proteins are involved in vesicular trafficking to the junctions or in cell signaling.

   **Adherens junctions** mechanically attach cells and their cytoskeletons to their neighbors or to the extracellular matrix.

   In vertebrates there are two types of actin filament attachment sites:

   A. **Cell-cell adherens junctions** (e.g. adhesion belts).

      In epithelial sheets they often form a continuous adhesion belt (zonula adherens) around the apex of each epithelial cell. The adhesion belts in neighboring cells are directly opposed, and the interacting plasma membranes are joined together by transmembrane proteins. Within each cell a contractile bundle of actin filaments lies adjacent to the adhesion belt, localized parallel to the plasma membrane, to which it is bound through a set of intracellular attachment proteins. The actin bundles in adjacent cells are thus connected into a broad transcellular network.

   B. **Cell-matrix adherens junctions** (e.g. focal contacts).

      These junctions enable cells to get a hold on the extracellular matrix by connecting their actin filaments to the matrix at specialized regions of the plasma membrane called focal contacts, where bundles of actin filaments terminate.

   **The tight junctions** play two distinct roles in selective barrier function: controlling transcellular and paracellular transport. Transcellular transport depends on two sets of membrane-associated carrier proteins: one is limited to the apical (or basolateral) surface of the epithelial cell and transports selected molecules into the cell while the other, which is confined to the basolateral (or apical) surface, allows the same molecules to leave the cell by facilitated diffusion or ATP-dependent transport into the extracellular fluid on the opposite side. In order to maintain this polarized transport, the apical (or basal) set of proteins must not migrate out of the apical (or basal) surface. In addition, the spaces between epithelial cells must be sealed, to prevent the transported molecules from diffusing back through the intercellular space (the paracellular route). The tight junctions are believed to block both types of diffusion.

   Tight and adherens junctions play an important role in setting up polarity in many cell types, as mutations in proteins localized to these structures cause loss of membrane asymmetry. The establishment and maintenance of cell polarity within various tissues are crucial for the correct development of both invertebrates and vertebrates. Cell adhesion proteins mediate the establishment of various cell-cell junctions that are organized asymmetrically along the lateral cell membrane.
Fig. 1 Comparison of the organization of cell junctions in *Drosophila* vs. vertebrates. AJ, adherens junction (small pale dots); SAR, subapical region (large pale dots); SJ; septate junction (black dots); TJ, tight junction (large pale dots); ZA, zonula adherens (small pale dots).

The adherens junctions (AJ) are located just basal to the TJ. In *Drosophila*, the zonula adherens (ZA), the invertebrate counterpart to the AJ, represents the most apical site of cell adhesion. The septate junctions (SJ) exist below AJ (Fig. 1). Similar structures in different species tend to be made up of similar proteins. Interestingly, the surface of cell types lacking TJs, such as neurons, and the *Caenorhabditis elegans* zygote, *Drosophila* neuroblasts or migrating cells, can also be highly polarized in the absence of tight junctions and adhesion belts. Genetic studies in *Drosophila* have recently shown that membrane asymmetry can be generated in the absence of SJs. Studies of cell-cell junctions and of cell-cell adhesion were once quite different endeavors, stemming from two distinct experimental approaches, junctions through
electron microscopic description, and adhesion through functional tests and biochemistry. Only a decade ago have these two approaches begun to come together in an integrated view of the molecular basis of cell junctions and adhesion.

1.1 Adherens junction proteins

A major component of the adherens junctions are the cadherins. The cadherin superfamily comprises glycoproteins responsible for calcium-dependent, homotypic cell interactions. All members of the superfamily are single-pass transmembrane proteins and have a variable number of extracellular 110 amino acid (cadherin) domains. Classical cadherins contain five cadherin domains and are distinguished from other members of the superfamily by the presence of a conserved cytoplasmic tail that associates with cytoplasmic proteins, the catenins. Homotypic interactions between extracellular domains of cadherins are necessary but not enough for cell-cell adhesion. They must be stabilized through cytoplasmic adapter proteins that link the integral membrane proteins to the actin cytocortex. Linkage of the cadherin cytoplasmic domain to three cytosolic proteins, named α-catenin, β-catenin, and plakoglobin is required (Fig. 5 and 6). For example E-cadherin binds directly to β-catenin and plakoglobin, while α-catenin binds to β-catenin. Plakoglobin (previously also called γ-catenin) is highly homologous to β-catenin and sometimes substitutes for β-catenin in the cadherin-catenin complex. α-catenin binds to actin. Nectins comprise a sub-family of the immunoglobulin superfamily. They promote cell adhesion in a calcium-independent manner. Nectin-2 is a component of the adherens junctions. It is linked to the microfilaments of the cytoskeleton via the intracellular protein, l-afadin which interacts with α-catenin and ponsin (Fig. 5 and 6).

Some of the best described AJ proteins are:

**Transmembrane proteins**
- Cadherins
- Selectins
- Integrins

**Ca²⁺-dependent Cell-Cell Adhesion Molecules**
- Cadherins
- Selectins
- Integrins

**Ca²⁺-independent Cell-Cell Adhesion Molecules**
- Immunoglobulin Superfamily of Proteins
- Neural cell adhesion molecule (N-CAM)
- Intercellular adhesion molecules (ICAMs)
- Nectins

**Cytoplasmic Plaque AJ proteins**
- Afadin
- Ponsin
- Vezatin
- α-Catenin
- β-Catenin
- Vinculin
- p120

Further details on adherens junction proteins can be found in dedicated reviews.
1.2. **Tight junction proteins**

Specialized transmembrane proteins such as claudins, occludin, and junctional adhesion molecule (JAM) colocalize at the apical-most tip of the lateral membrane and constitute the backbone of TJ strands. Plaque proteins like zonula occludens (ZO)-1, ZO-2, and ZO-3 tether the claudins and occludin to the TJ cytocortex (Fig. 5). Many more proteins are involved in the build up of TJ, but this chapter is not aiming at covering them all since they have been described in several reviews.

Table 1 summarizes the ones to be mentioned in this chapter.

### Transmembrane proteins of the TJ

**Tetraspan proteins**
- Occludin
- Claudins
- TJ proteins that belong to the immunoglobulin superfamily
- JAM (Junctional Adhesion Molecule)
- CAR (Coxsackievirus and Adenovirus Receptor)
- P0 (Protein 0)

**Plaque proteins of the TJ**
- PDZ (Postsynaptic density 95/Discs large/Zonula occludens)-containing proteins.
  - PAR (Partitioning defective) proteins of the TJ
  - MUPP1 (Multiple PDZ domain Protein 1)
  - PATJ (Protein Associated with Tight Junctions)
  - AF-6/Afadin
- The MAGUK (Membrane Associated Guanylate Kinase) proteins of the TJ
  - ZO-1 (zonula occludens-1)
  - ZO-2
  - ZO-3
  - Pals1 (Protein Associated with Lin7) (Mpp5) (Membrane palmitoylated protein 5)
- MAGI, the (MAGUK Inverted) proteins of the TJ
  - MAGI-1
  - MAGI-2.
  - MAGI-3.

**TJ proteins lacking PDZ domains**
- Cingulin
- Symplekin
- Barmotin
- Rab proteins
  - Rab13
  - Rab3B
- Pilt
- JEAP (Junction Enriched and Associated Protein),
- hASH1 (Absent Small or Homeotic).

### Heterotrimeric G proteins

Some of the homologue proteins have different names in the different species. Table 1 summarizes the ones to be mentioned in this chapter.
Table 1. Names of SAR/TJ and ZA/AJ proteins in *Drosophila* and vertebrates.

<table>
<thead>
<tr>
<th><strong>Drosophila</strong> SAR/TJ</th>
<th><strong>Vertebrate</strong> SAR/TJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bazooka</td>
<td>PAR3/ASIP</td>
</tr>
<tr>
<td>DmPar6</td>
<td>PAR6</td>
</tr>
<tr>
<td><em>DaPKC</em> (atypical protein kinase C)</td>
<td>aPKC</td>
</tr>
<tr>
<td>Stardust</td>
<td>PALS1/MPP5</td>
</tr>
<tr>
<td><em>DPATJ</em> (formerly called Discs lost)</td>
<td>hINADL/PATJ</td>
</tr>
<tr>
<td><strong>Drosophila</strong> ZA/AJ</td>
<td><strong>Vertebrate</strong> ZA/AJ</td>
</tr>
<tr>
<td>Shotgun</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>Armadillo (Arm)</td>
<td>β-catenin</td>
</tr>
</tbody>
</table>

The photoreceptor cells possess epithelial and neuronal characteristics. They are differentiated from neuroepithelium and have axon-dendrite polarity, and linear arrangement of AJs.\(^41\) The subapical region located above the AJ contains many of the TJ proteins, but does not play a real barrier function (Fig. 2).\(^42,43,44\)

---

**Fig. 2** Organization of Tight junctions / Subapical region and Adherens Junctions in epithelial and photoreceptor/Müller glia cells.

1.3. **PDZ and MAGUK proteins** concentrate at cell contacts like tight junctions, adherens junctions and synapses.\(^45,46,47,48,49,50,37\) They participate in various protein
complexes as we shall see later in this review, and will be briefly introduced in this section. MAGUK proteins are a subclass of PDZ domain containing proteins. The domain composition of MAGUKs usually involves one or more PDZ domains, followed by Src homology 3 (SH3) and C-terminal Guanylate kinase (GuK) domains. Some of them have additional domains, which classifies them into several subfamilies\(^{51,52}\): resembling DLG1, LIN2, ZO-1, p55 (Fig. 3).

**Fig. 3 Domain organization of MAGUK subfamilies.**

PDZ, Postsynaptic density 95/Discs large/Zonula occludens domain; SH3, Src homology 3 domain; GuK, Guanylate kinase domain; L27, domain found in Lin2 and Lin7 proteins; HOOK, variable hinge (flexible region); CAMKII, Calcium/calmodulin-dependent protein kinase II domain.

PDZ domains normally bind to specific C-termini of target proteins, but also can heterodimerize, and can bind internal sequences.\(^ {53,54,55,56,57,58} \)

SH3 domains are protein-protein interaction modules that are seen often in proteins with divergent function.\(^ {59} \) They typically bind proline rich motifs.\(^ {60,61,62,63} \)

GuK domains share homology with *Saccharomyces cerevisiae* guanylate kinase enzyme (Guk1) that catalyzes the phosphorylation of GMP to GDP using ATP as a donor.\(^ {64} \) MAGUK GuK domains however are catalytically inactive.\(^ {65} \)

GuK domains in MAGUKs can bind their SH3 domains, preferentially in intramolecular fashion.\(^ {66,67,68,69,70} \) This interaction is non-canonical for the SH3 domains, as they do not bind proline-rich sequences in GuK domains. In postsynaptic density protein 95 (PSD95) the HOOK (variable hinge or flexible region) insert is part of the SH3 domain and replaces a conserved helix involved in proline recognition.\(^ {71} \) This model of interaction is another way of building supramolecular complexes.

L27 domains can heteromultimerize. Individual domains are unfolded, but upon binding they form rigid structures comprising a tetramer made of two heterodimers.\(^ {72} \) Thus the rest of the MAGUKs’ domains are free for additional targets building large protein complexes.
1.3.1. Regulation of MAGUK complexes:  
1.3.1.1. Palmitoylation is a posttranslational modification adding a fatty acid, which alters protein-membrane and protein-protein interactions. For example it allows the clustering of PSD95 with ion channels.\(^{73}\)

1.3.1.2. Alternative splicing is a common mechanism for variant mRNA transcripts influencing protein composition and thus localization, interactions, and function. DLG1 and PSD95 are examples of functional difference of splice variants. N-terminal variations encoding proline-rich regions of DLG1 influence its interaction with SH3 domains of tyrosine kinases, and facilitate DLG1 multimerization.\(^{74,75,58}\) Alternative splicing of the PSD95 N-terminus replaces the region for palmitoylation in the L27 domain, which mediates binding to CASK.\(^{76}\)

1.3.1.3. Phosphorylation dynamically regulates MAGUKs. Activity dependent changes in synaptic structure and composition are regulated by CamKII-dependent phosphorylation of *Dm*-DLG.\(^{77}\) Phosphorylation of PSD95 blocks channel clustering.\(^{78}\)

1.3.1.4. Nuclear translocation of the GuK domain of CASK enables interaction with nuclear CINAP and Tbr-1 proteins, and regulates the expression of the N-methyl-D-aspartate (NMDA) receptor 2B.\(^{79}\)

1.3.2. Scaffolding roles of MAGUKS at TJ and synapses:  
1.3.2.1. Epithelial cell polarity. MPP5 binds the PDZ domain of PAR6, a member of the PAR3/PAR6/aPKC complex that is tethered to the TJ via binding of PAR3 to JAM.\(^{80,81}\) MPP5 binds the C-terminal ERLI-motif in transmembrane protein Crumbs (CRB)1-3. More on these proteins will be presented later in this chapter.

1.3.2.2. Anchoring TJ adhesion proteins to the cytoskeleton. ZO-1,-2,-3 directly bind to transmembrane proteins such as occludin, claudin and JAM\(^{82,83,84}\) and in addition to actin filaments.\(^{85,36,86}\)

1.3.2.3. Synaptogenesis. The central nervous system (CNS) expresses the greatest diversity of MAGUKs in the body.\(^{87}\) PSD95 is a major component of the post-synaptic densities and associates with receptors as NMDA, adhesion molecules like neuronal Nitric Oxide Synthase (nNOS)\(^{45,48,47,46}\). Overexpression of PSD95 enlarges synapse size and accelerates its development.\(^{88}\)

1.3.2.4. Clustering of receptors. PSD93, and PSD95 are known to bind many channels, and receptors like K+ channel Kv1.4, NMDA.\(^{46,45}\)

1.3.2.5. Organizing signaling complexes. PSD95 binds both to the NMDA receptor and nNOS, thus coupling the Ca++ influx with activation of nitric oxide signaling molecule producing nNOS\(^{55}\).

1.3.2.6. Altering synaptic plasticity. The change in synaptic strength is achieved by adjusting the density of AMPA receptors, while the plasticity is ensured by Ca++ flow through NMDA receptors.\(^{89,90}\) PSD95 binds NMDA directly\(^{53,91}\) and AMPA receptors indirectly via Stargazin, and thus regulates their density at membranes.\(^{92}\)

2 Hierarchy in cell polarity  
*Drosophila* represents an excellent model for genetic studies of developmental processes, many of which have been later confirmed in vertebrates. As much of the pioneer work on cell polarity has been performed in *Drosophila*, this is where we will start reviewing the current knowledge on this topic.
Three polarity protein complexes, each consisting of one or more PDZ proteins in *Drosophila*, have been intensively studied in recent years.\(^3\) The first complex consists of the transmembrane protein Crumbs (Crb), along with its associated cytoplasmic proteins, Stardust (Sdt) and DPATJ formerly called Discs Lost (Dlt). The second complex consists of Bazooka (Baz), *Dm*Par6, and DaPKC. Scribble (Scrib), Discs Large (Dlg), and Lethal Giant Larvae (Lgl) are components of the third complex. The first two complexes are localized at the SAR, apical to the AJ, while the third one is at the lateral membrane.\(^2\)

In *Drosophila* epidermis the ZA is the most apical region of cell adhesion. In contrast, the TJ represents the most apical cell contact in vertebrate epithelial cells. The mammalian homologues of Crb/Sdt/DPATJ and Baz/*Dm*Par6/DaPKC complexes, Crb/Mpp5/PATJ and Par3/Par6/aPKC, respectively, reside at the TJ. Mammalian Dlg1 (mDlg) and mammalian Lgl (mLgl) localize at the lateral membrane underneath the TJ. Of the above mentioned nine proteins, six contain PDZ domains, strengthening the concept that PDZ proteins can assemble protein scaffolds which have essential functions.

### 2.1 Crb protein complex

#### 2.1.1 Crb complex members in *Drosophila*

The Crb/Sdt/DPATJ complex localizes at the SAR, which resides just above the most apical side of cell contact, the ZA\(^2\), and is important for the maintenance of cell polarity in some embryonic epithelia.\(^2,101,102\) *Drosophila* embryos lacking expression of Crb, Sdt, or DPATJ exhibited apicobasal polarity defects.\(^103,104,105\) Furthermore, in the absence of Crb or Sdt, a continuous ZA did not form from so-called spot AJs (sAJs).\(^106,107\) Sdt mediates the indirect interaction between Crb and DPATJ.\(^108\) In the absence of Sdt, Crb and DPATJ are mislocalized, indicating that the integral complex is stably retained at the subapical region.\(^109,110\) Overexpression of Crb in the embryo can lead to apical surface increase, disorganization of the ZA, and multilayering of epithelia; nevertheless, the overall polarity was retained.\(^111,112\) Thus, Crb is an important apical surface determinant,\(^113,2,102\) a notion supported by its ability to organize the apical spectrin/actin cytoskeleton through *Dm*oesin, which is a member of the band 4.1 superfamily of actin-associated proteins.\(^114,115\) There are two known domains within the 37 amino acid cytoplasmic tail of Crb: a consensus motif predicted to bind proteins of the band 4.1/ezrin/radixin/moesin (FERM) superfamily and a PDZ domain binding sequence (ERLI) at the extreme C- terminus.\(^113,101\) Crb binds to *Dm*oesin via the FERM binding motif and to the PDZ domain of Sdt via the C-terminal ERLI sequence.\(^109,110,114\)

#### 2.1.2 Crb family members and complex in vertebrates

While only one Crb gene exists in *Drosophila*, three CRB genes (CRB1, CRB2, and CRB3) are present in vertebrates.\(^116,3,117\) CRB1 is expressed in the retina and in the brain.\(^118\) CRB2 is expressed in the retina, retinal pigment epithelium (RPE)/choroid, brain, kidney, lung, placenta, and heart.\(^117\) CRB3 is expressed in lung, kidney, retina, colon, muscles, testis and MDCK epithelial cell line.\(^98,42,119\)

All three members of the CRB family are expressed in the mammalian retina.\(^42\) While CRB1 and CRB2 proteins comprise numerous extracellular epidermal growth factor (EGF)-like repeats and laminin A G-like domains, CRB3 consists of only 120
amino acids. All CRB members have 37 amino acid-long cytoplasmic tails, which are highly conserved in sequence (Fig. 4).\(^{117,101}\)

**A**

![Diagram showing CRB family members and their domains](image)

CRB1 1406 aa  
CRB2 1285 aa  
CRB3 120 aa  

**B**

<table>
<thead>
<tr>
<th>CRB1</th>
<th>1370</th>
<th>SNKRATQGTYSPSREKESVRVEMNLMPPPAMERLI</th>
<th>1406</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRB2</td>
<td>1249</td>
<td>RKREQEGTYPSQDEVACREMOSVLFVPEERLI</td>
<td>1285</td>
</tr>
<tr>
<td>CRB3</td>
<td>84</td>
<td>REKREQGTYPSQDEQVVARPPTPNLKPEERLI</td>
<td>120</td>
</tr>
</tbody>
</table>

**Fig. 4** Schematic representation of human CRB family members and their C-termini. (A) The three CRB family members. (B) Comparison of the 37 amino acid C-termini of the CRB family members. Amino acids conserved between all three Crumbs family members are underlined, while those shared by two members are in bold.

The C-terminal ERLI motifs of CRB1 and CRB3 bind the PDZ domain of MPP5, the mammalian Sdt homologue.\(^{98,108}\) MPP5 consists of multiple protein-protein interaction domains besides its PDZ domain (Fig. 3).\(^{120,108}\) The N-terminal L27 (L27N) domain of MPP5 associates with the N-terminal L27 domain of PATJ. MPP5 and the multiple PDZ domain protein 1 (MUPP1) interact in a similar manner.\(^{108}\) A L27-L27 type of interaction could mediate the direct binding between Sdt and DPATJ.\(^{108}\) Association of MPP5 and PATJ is required for the localization of MPP5 to TJs as MPP5 lacking its L27N domain is mislocalized apically.\(^{108}\) However, this interaction is not responsible for the targeting of PATJ itself.\(^{96}\)

Mutations have been identified in the human Crumbs homolog-1 (CRBI) gene in individuals with various forms of Retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA) (see below).\(^{121,122,123}\)

### 2.2 The Par3 protein complex

#### 2.2.1 Par3 complex members in Drosophila

In epithelia, the Baz (DmPar3)/DmPar6/DaPKC complex colocalizes with the Crb/Sdt/DPATJ complex at the SAR, and has been shown to be essential during *Drosophila* establishment of epithelial polarity and morphogenesis. The localization of
DmPar6 and DaPKC to the subapical region was dependent on that of Baz. Baz was mistargeted in the absence of DmPar6 or DaPKC, indicating that only a complex containing all three components was stably associated with the membrane at the subapical region. Drosophila embryos lacking Baz, DmPar6, or DaPKC showed disrupted apico-basal polarity of epithelia, mislocalized Crumbs protein, defects in ZA formation and epithelia apoptosis.

2.2.2 Par3 complex members in vertebrates

Par3/Par6/aPKC has been shown to be critical for TJ formation and epithelial polarization. Par3 and Par6 bind to the kinase domain and N-terminus of aPKC, respectively. Par-3, which itself is a substrate of aPKC, could inhibit the kinase activity, while Par-6 could promote it. Ser827 of Par3 is a phosphorylation target for aPKC and lies within the aPKC binding site, and its modification destabilized the Par3/aPKC complex. This dynamic interaction is important for TJ formation, as the Par3 S827A mutant acted as a dominant negative protein disrupting TJ assembly. The function of this complex is not limited to epithelia both in invertebrates and vertebrates. Par3/Par6/aPKC plays also a role in establishing polarity in astrocytes and hippocampal neurons.

The role of this protein complex in polarity might involve signaling events, as the Par3/Par6/aPKC complex binds to the activated form of two members of small GTPases: Cdc42 and Rac1.

2.3 Scribble protein complex

2.3.1 Scribble complex members in Drosophila

The third polarity complex includes Scrib, Dlg, and Lgl. They colocalize in epithelia, show strong genetic interactions, and act together in regulation of cell polarity. Mutations in the genes dlg and lgl affected epithelial polarity and growth control in imaginal disc epithelia. Loss of Scrib, Dlg, or Lgl led to apical surface increase and mistargeting of ZA proteins towards more lateral positions of the membrane in embryonic epithelia, similarly to the Crumbs overexpression phenotype. dlg and scrib encode PDZ domain proteins; while lgl encodes a protein with WD-40 repeats (Fig. 6).

In lgl or dlg mutant embryos, Scrib is mislocalized, while in dlg or scrib mutant epithelia, Lgl was mistargeted. These findings indicate that the three proteins probably form a complex. However, biochemical data to confirm this hypothesis is not available.

2.3.2 Scribble complex members in vertebrates

Homologues of Scrib, Dlg, and Lgl exist in mammals: Scrib, mDlg (synapse associated protein 97 or SAP97; DLG1), and mLgl, respectively. It has been demonstrated that mDlg binds mLin-2/CASK, Dlg2, and Dlg3 thus influencing the targeting of mDlg/SAP97 to the lateral membrane. As in invertebrates it is not known if mLgl associates with Scrib or SAP97.

mLgl was repositioned from the cytoplasm to the lateral membrane after Madin-Darby Canine Kidney (MDCK) cells formed cell-cell contacts and expressed a polarized phenotype. mLgl can be phosphorylated, and this seems to exclude it from the apical domain. Due to its association with Syntaxin 4, which is a part of the basolateral
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exocytotic apparatus, mLgl is considered to play a role in apico-basolateral polarity by regulating exocytosis.100

The evolutionarily preservation of these proteins in vertebrate epithelia suggest that they function cooperatively during the establishment of basolateral membrane domain during apicobasal polarization.

mLgl binds mPar-6 and forms a complex with aPKC. Furthermore, the phosphorylation of mLgl is required for polarization and TJ formation in different cell types.141,142

2.4. Molecular and functional interactions of the partners

ZA defects in crb or sdt null mutants were observed at a later stage of gastrulation, than the onset of ZA defects in baz null flies.7,107 This is in agreement with the notion that Crb is required to maintain Baz at the subapical region but is dispensable for the initial localization of Baz during early gastrulation.7 The early ZA defects observed in baz sdt double-mutant embryos were reminiscent of those seen in baz null flies, suggesting that the Baz complex functions upstream of the Crb complex.107 Together, these results indicated that the Baz/DmPar6/DaPKC and Crb/Sdt/DPATJ complexes function in a coordinated fashion during ZA formation. While the Baz/DmPar6/DaPKC complex is involved in polarization in numerous cell types, the function of the Crb/Sdt/DPATJ complex could be restricted to epithelial cells. This was manifested in sdt mutant embryos, where the polarity and asymmetrical cell division of neuroblasts were unaltered.110

The opposite but common temporal characteristics of the Crb/Sdt/DPATJ and Scrib/Dlg/Lgl mutant phenotypes indicate that the activity of these complexes is balanced precisely, determining apical and basolateral membrane domains and ZA position.7,143

Mutations in dlg or scrib resulted in an enhancement of the Crb overexpression phenotype and suppress the crb null phenotype.143 In embryos with mutations in the dlg and crb group genes, the phenotype was identical to the one of dlg group null mutants. This is an evidence that dlg group mutations are epistatic to sdt and crb mutations7,143. It has been hypothesized that the Baz protein complex could be substituted for the Crb complex in sdt dlg and crb scrib double mutants.143 The double mutant analysis demonstrated that expansion of the apical membrane can occur in complete absence of Crb.7,143 Obviously, there is an additional activity that can facilitate the establishment of the apical membrane domain. The logical candidates for this activity are the baz group genes. Indeed, the phenotypes of dlg-baz group double-mutants were highly similar to baz single mutants,7,143 demonstrating that the baz group genes are epistatic to the dlg group.

In fact, a hierarchical model to explain this has been proposed.7 During early epithelial polarization spot AJ material assembles and apical membrane identity is established by Baz/DmPar6/DaPKC. This complex localizes Crb/Sdt/DPATJ to the apical membrane. At the same time, Scrib/Dlg/Lgl is localized to the lateral membrane and balances the apicalizing effects of the Crb complex. In a crb mutant, Dlg group activity is too high and overwhelms the activity required for formation of the apical membrane, presumably the Baz group. As Crb is necessary to maintain Baz localization, it is expected that Crb/Sdt/DPATJ and Baz/DmPar6/DaPKC function cooperatively in establishing an apical membrane domain. Scrib, Dlg, and Lgl proteins are independently targeted to the basolateral domain, and counteract the activity of the apical complexes. Spot AJs fuse at
the apical side of cells, forming a continuous, belt-like ZA, eventually leading to the proper establishment and maintenance of apicobasal polarity.

The genetic interactions between apical and basolateral protein complexes, and between members of the two apical complexes are strongly supported by recent biochemical data. Specifically, the components of the Baz complex, namely PAR-6, and of the Crb complex, namely MPP5, physically interact in cultured cells.\textsuperscript{144} CRB3 binds Par6.\textsuperscript{119} In addition, mLgl was able to physically interact with mPar-6 and formed a complex with aPKC, and the phosphorylation of mLgl was required for polarization and TJ formation in different cell types.\textsuperscript{145,141,142} And last but not least, the intracellular domain of Crumbs was a substrate for aPKC in vitro, while Patj, which also binds aPKC, affected the phosphorylation negatively.\textsuperscript{146}

Expression of exogenous CRB3 in cells that normally do not form TJs, induced formation of functional TJs, and TJ markers were recruited to these structures.\textsuperscript{147} Both the C-terminal PDZ binding motif and FERM binding motif are required for this function. The FERM binding motif is found in many adaptor proteins that link transmembrane proteins to the actin cytoskeleton.\textsuperscript{148} MPP5 also contains a FERM binding motif, called HOOK domain.\textsuperscript{120}

Interestingly overexpression of CRB3 in cells that form TJs and express endogenous CRB3, led to delay in the formation of TJs, without affecting the AJ or the overall polarity of the cells.\textsuperscript{95,119} However, the tightness of the junctions was decreased though.\textsuperscript{119} The phenotype is dependent on the FERM binding motif and the last four amino acids of the cytoplasmic tail of the protein, which binds MPP5 and Par6.\textsuperscript{119,95} Thus, CRB3 plays via C-terminal interactions a role in apical domain morphogenesis and TJ regulation. The CRB3-MPP5-PATJ complex localized to the TJ in mammalian epithelia.\textsuperscript{116,98} Overexpression of CRB3 resulted in expansion of the apical membrane in agreement with certain \textit{Drosophila} data.\textsuperscript{112} Both Par6 and CRB3 complexes have been detected in mammalian TJs.\textsuperscript{130,128,95}

\textit{MPP5} silencing in MDCK cells resulted in loss of expression of MPP5, a protein that binds CRB3 and PATJ.\textsuperscript{149} The association of CRB3 with the Par3-Par6-aPKC complex decreased, although the expression of all these proteins was unchanged.\textsuperscript{149} This is not in line with results in \textit{Drosophila}, where mutations in \textit{stardust} lead to loss of proper Crumbs localization.\textsuperscript{110,150} The \textit{MPP5} silenced cells exhibited delayed formation of TJs and decreased trans-epithelial resistance.\textsuperscript{149} These effects might be due to decreased amounts of aPKC in the TJ and proper downstream signaling events. The Par3-Par6-aPKC complex has been demonstrated to regulate the assembly of TJs, and the kinase activity of aPKC is essential for this role.\textsuperscript{144,149,151,129}

Upon \textit{PATJ} silencing in Caco2 (a human colon adenocarcinoma line) or MDCK cells, MPP5, along with other TJ proteins like ZO-3 and occludin were no longer associated with the TJ, and CRB3 accumulated in early endosomes.\textsuperscript{152} The formation of TJs was delayed.\textsuperscript{153} Interestingly, the overall polarity was not affected, and the levels of MPP5 and CRB3 proteins were not changed.\textsuperscript{152,153} Thus, PATJ stabilized the CRB3 complex, including the PATJ binding partners ZO-3 and occludin.\textsuperscript{96} Therefore, the mammalian Crb complex represents an evolutionarily conserved complex that plays an essential role during epithelial polarization.

Cells expressing a dominant negative form of PATJ did not target MPP5, ZO-1 and aPKC properly to the TJ, and affected TJ genesis.\textsuperscript{144} The TJ localization of MPP5 has
been previously shown to be dependent on PATJ. Because CRB3 can bind Par6, one possibility is that CRB3 recruits Par6 directly to the developing TJ, or if CRB3 is needed to recruit MPP5 to the TJ, then MPP5 can promote the association of CRB3 with the PAR6 complex.

It seems that there is sufficient redundancy among proteins, protecting the TJ from loss of their function. For example lack of occludin, cingulin or ZO-1 did not affect polarity or the barrier function of the TJ.

Expression of a dominant-negative form of aPKC led to mislocalization of PAR3, and delayed the formation of TJs and affected the barrier properties. Mutant Par6 that no longer interacted with aPKC led to delay of TJ formation.

The failure of aPKC to associate with TJs may result in perturbation of polarity signals. For example Lgl binds Par6 and is phosphorylated by aPKC.

In vertebrate epithelia the establishment of polarity begins when two cells form first cell contacts via E-cadherin and nectin. Nectin recruits JAM to these initial adhesive contacts. ZO-1 can directly interact with claudins and JAM, and Par3 directly binds JAM. In the following stage, Par3-complex proteins like Par6 and aPKC are recruited. CRB1 binds MPP5 and PATJ, which in turn interacts with ZO-3 and claudin-1, while MPP5 binds Par6. The CRB1/ MPP5/PATJ complex can be linked to claudin-1 and JAM via MUPP1, which binds to MPP5. Taken together, these results suggest a contributing role in mammalian epithelial cells for claudins in the anchorage of the two scaffolds, the Par3-Par6–aPKC and the Crb1–MPP5–PATJ complex at the TJ. Eventually the premature AJs form belt-like continuous AJs and TJs around the apical side of cells (Fig. 5).
Fig. 5 Apicobasal polarity complexes in epithelial cells.
Fig. 6 Domain organization of junctional and polarity proteins
RA, Ras association domain; FHA, Forkhead associated domain; SynN, Syntaxin N-terminal domain; t-SNARE, Helical region found in soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs); Sorb, Sorbin homologous domain; Rho,
Ras homology domain; ARM, Armadillo/β-catenin-like domain; LRR, Leucine rich repeats; CA, cadherin repeat; VIN, Vinculin binding region; IGv-type, Immunoglobulin V-type; IG, Immunoglobulin like domain; WD40, WD (Trp-Asp) 40 repeats; C1, Protein kinase C conserved region 1; PB1, Bem1 protein domain; TM, transmembrane domain; PDZ, Postsynaptic density 95/Discs large/Zonula occludens domain; SH3, Src homology 3 domain; GuK, Guanylate kinase domain; L27, domain found in Lin2 and Lin7 proteins; HOOK, variable hinge (flexible region). CRB proteins are depicted in Fig. 4.

3 Crumbs related photoreceptor and retinal degeneration
3.1 Photoreceptor degeneration in Drosophila

3.1.1 Structure of the Drosophila eye

The photo-transduction apparatus of vertebrate and Drosophila photoreceptors is confined in morphologically distinct apical domain specializations called the photoreceptor outer segment and rhabdomere, respectively. Both of these structures arise from the extensive growth of the photoreceptor apical domain, resulting in stacks of membrane packed with photopigments. During pupal development (pd), apical membranes in fly photoreceptors differentiate into two regions: the rhabdomere at the apical apex and stalk membranes connecting the rhabdomere and ZA. The Drosophila eye is composed of about 800 ommatidia, cylindrical, barrel-like structures, with eight photoreceptor cells in the center and additional support cells, such as the primary, secondary and tertiary pigment cells and the cone cells (Fig. 7). All cells of the adult eye develop from a single layered epithelium of the imaginal disc. During the pupal stage the apical surface of the photoreceptor cells are shifted by 90° and come to lie laterally (Fig. 7). AJs anchor photoreceptors to the surface and floor of the retina (Fig. 7a–c). At about 37% pupal development the apical membrane undergoes remarkable expansion and highly pleated microvilli are formed. The microvilli give rise to the rhabdomere. The part of the apical membrane between the ZA and the rhabdomere differentiates into the stalk membrane.

Fig. 7. Developmental stages of Drosophila photoreceptors
A. Third-instar eye disc. Apical domain of photoreceptors (orange) is separated from the baso-lateral membrane by the AJ (green).
B. 37% pupal development. Apical domains turn and face each other.
C. 67% pupal development. The rhabdomere (blue) and the stalk membrane form.
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D. Schematic representation of a tangential section of an adult ommatidium. Rhabdomeres in blue; AJ in green; stalk membrane in orange; bristle in beige; secondary pigment cells in purple; tertiary pigment cells in grey.

3.1.2 Localization of the Crumbs complex in the Drosophila eye

The transmembrane protein Crb, and membrane-associated Stardust (Sdt) and DPATJ, co-localize at the apical side of undifferentiated cells and differentiating photoreceptors throughout eye development.\(^{113,166,150}\) When morphologically distinct rhabdomere and stalk domains are formed, Crb, Sdt and DPATJ become restricted to the stalk membrane, and are excluded from the rhabdomere.\(^{166,115}\) The developmental expression patterns of Sdt, Crb, and DPATJ suggest that they form a complex in developing photoreceptors as in embryonic epithelia.\(^{166}\)

3.1.3 Crumbs mutant photoreceptor phenotype

Crb has an essential role in rhabdomere shape, ZA integrity, and stalk membrane formation, independently of its role in determining apicobasal polarity in the embryo.\(^{113,115}\) A dynamic requirement for Crb in ZA formation was observed during distal-to-proximal expansion of photoreceptor apical membrane.\(^{164}\) Rhabdomeres of Crb mutants extended only 50\% of their normal distal-to-proximal length, and were restricted to the distal portion of the retina.\(^{113,115}\) The affected rhabdomeres were shorter and thicker and often in contact with neighboring rhabdomeres.\(^{113,115}\) In addition, the stalk membrane was reduced in length.\(^{165}\) \(crb-/\)- photoreceptors extended down to different levels in depth of the retina.\(^{113}\) In addition, some secondary and tertiary pigment cells died in \(crb\) mutant clones and the eyes also showed irregularities in the arrangement of ommatidia.\(^{115}\) \(crb-/\)-cells in third-instar larval eye discs showed normal epithelial morphology and ZA integrity.\(^{115}\) Upon initial expansion at 50\% pupal development, ZA were strongly fragmented and adherens junction material was more scattered into the lateral membrane. Less-severe defects were observed at 70\% pupal development, indicating a significant recovery from initial defects in constant absence of Crb.\(^{115}\)

Crb is also required for the survival of photoreceptor cells when exposed to light. When flies carrying \(crb-/\)- eyes were kept in constant light for 7 days, the retina showed extensive degeneration. Signs of degeneration included the dissolution of the highly pleated rhabdomere structure, rounding up of nuclei, and condensation of nucleolus, nucleoplasm, and cytoplasm indicative of apoptotic cell death.\(^{165}\) One way to explain programmed cell death upon light exposure is by accumulation of unusually stable metarhodopsin/arrestin complexes through an unknown mechanism.\(^{165}\) This is supported by the observation that photoreceptor cells in \(crb-/\)- mosaic flies, raised on vitamin A-deficient medium, showed morphogenetic defects similar to those of \(crb-/\)- mutants raised on normal medium and kept in the dark.\(^{165}\)

3.1.4 Stardust mutant photoreceptor phenotype

Mutations in the interaction partner of Crb, Sdt\(^{110,109}\), also causes an eye phenotype. In early \(sdt-/\)- photoreceptors, the overall phenotype was comparable to that of \(crb-/\)-,\(^{166,115,113,150}\), although during early pupal development ZA formation was only mildly affected. The shape of rhabdomeres in \(sdt-/\)- photoreceptors was irregular, frequently expanded, split, or in contact with neighboring rhabdomeres. They were
missing in the proximal region, but were relatively well organized in the distal area. 
Unlike in crb/- mutants, however, in sdt/- the microvilli basal membrane seemed to be 
severely disrupted by vesicle-like membrane structures. The stalk membranes were 
reduced like in crb/- photoreceptors. At late pupal stages the ZA defects in sdt/- photoreceptors were no longer detectable. The recovery appeared to be more complete 
than in crb photoreceptors.

3.1.5 **DPATJ mutant photoreceptor phenotype**

In *Drosophila* eyes, throughout their development, DPATJ colocalizes with Crb 
and Sdt. Both Crb and Sdt are required for the stabilization of DPATJ.

One mutation in *DPATJ* resulted in the production of a truncated protein 
comprising the L27 and the first PDZ domains. Before the formation of the stalk 
membrane, Crb and Sdt could be found at the apical membrane in *DPATJ* mutant 
photoreceptors, and rhabdomeres were normal. Interestingly, the truncated DPATJ was 
targeted to the apical membrane, similar as in the wild type. The phenotype was 
considerably changed at 70% pd when the staining of Crb, Sdt and DPATJ itself was 
ascent from the stalk membrane, where it should normally be located at this stage of 
development. *DPATJ* eyes of adult flies were somewhat disorganized, and Crb and Sdt 
could not be detected. Some rhabdomeres were disorganized, but the overall polarity was 
ot affected. The stalk membrane was reduced by approximately 40%, which is less than 
the decrease observed in *crb/-* flies. In contrast, to the *crb* and *sdt* mutant 
phenotypes, there were no defects on photoreceptor elongation. These observations 
suggest that at late pupal development, DPATJ is required for the stabilization of 
Crb/Sdt/DPATJ complex at the stalk membrane, and the mutant form can do that only 
until about 70% pd. Like Crb, DPATJ is required to protect photoreceptors from 
degeneration in the light. Upon constant light exposure for seven days, *DPATJ* mutant 
photoreceptors showed clear signs of degeneration.

It has been shown that Crb is needed for the stabilization of Sdt and DPATJ at the 
stalk membrane, and Sdt is required for the proper targeting of Crb and DPATJ. It 
appears that DPATJ is necessary for the localization of Sdt and Crb, indicating that each 
member of the complex is needed to stabilize the other two. All three proteins are also 
required for proper stalk membrane formation.

3.1.6 **Bazooka, *DmPar6* and *DaPKC* mutant photoreceptor phenotype**

Components of the second apicobasal polarity complex, Baz, *DmPar6*, and *DaPKC* also give eye phenotypes. In wild type photoreceptors at 38–43% pd, Baz was 
colocalized with Arm at the ZA but did not overlap with the apical localization of DPATJ 
and Crb. At 70% pd, Baz staining disappeared from the ZA and became concentrated in 
the area of the developing rhabdomere. *DaPKC* did not overlap with Baz in early pupal 
stages, but was localized to the apical region and stalk membrane until 55% pd. At later 
stages, *DaPKC* also began to concentrate into the rhabdomere similarly to Baz. Thus, a 
protein complex of Baz and *DaPKC* did not form during early pupal photoreceptor 
development.

Baz is essential in establishing apical-basal polarity in pupal photoreceptors. In 
baz-/ clones within mosaic ommatidia at either 37% or 47% pd the Arm staining was
severely disrupted\textsuperscript{166,150}, indicating a complete failure of ZA formation, which is the hallmark of apical–basal polarity in photoreceptors. Developing rhabdomeres became fragmented, randomly positioned, or lost. Although in third instar larval eye discs DPATJ staining was not altered, at later stages the apical localization of DPATJ was lost or randomized.\textsuperscript{166,150} The size of \textit{baz} clones in eye discs was much smaller, and \textit{baz} clones were never found in late pupal retinas.\textsuperscript{166}

Most clones of \textit{par6} and \textit{DaPKC} mutations in mosaic eyes were very small, having only a few cells, suggesting that Par6 and DaPKC are important for proliferation and/or survival of retinal cells. In \textit{par6-/-} photoreceptors, DPATJ and Arm (\(\beta\)-catenin) were strongly reduced and/or mislocalized. DPATJ and Arm were also mislocalized in \textit{DaPKC-/-} photoreceptors. DPATJ failed to localize at the membrane, resulting in a diffused distribution pattern. These results showed that Par6 complex proteins are essential for proper localization of the Crb complex and AJ components. Importantly, the Crb complex was lost or unable to localize to the membrane in the absence of the Par6 complex. On the contrary, the Par6 complex remained in the membrane although in the absence of the Crb complex it was mistargeted.\textsuperscript{150} This phenotypic disparity suggests that the Par6 complex may play a pivotal role for membrane localization of the Crb complex, and Crb complex proteins could be vital for maintaining the Par6 complex.

3.2. Retinal degeneration in the mouse and other species

3.2.1. Structure of the mammalian retina

Loss of vision is a major health care problem: approximately one in three elderly persons has some form of vision-reducing eye disease by the age of 65.\textsuperscript{167} The eye is the organ, which gives the sense of sight. The eye has three main layers. These layers are positioned flat against one another and make up the eyeball. The outer layer is called sclera. The slight swell in the sclera at the front of the eye is a transparent tissue called cornea. The middle layer is the choroid. The front of the choroid contains eye muscles and the iris. The inner layer is the retina, situated in the back of the eyeball. The retina consists of two layers: the sensory retina, containing nerve cells that process visual information; and the RPE, which lies apically to the sensory retina. Between the two layers lies the sub-retinal space.
The RPE is a monolayer of columnar epithelium with various functions: production of extracellular matrix, adhesion of the neuro-sensory retina, absorption of light, reduction of light scatter, transport and storage of metabolites, provider of selective barrier between the choroid and neuro-sensory retina, and phagocytosis of rod and cone outer segments.

The neuro-sensory retina is thin and transparent. Light stimuli are transformed into neuronal impulses in the retina. These are then integrated to some extent before being transferred to the brain via the ganglion cell axons.

Apart from the neural cells the retina consists of several other cell types including glial cells, vascular endothelium, microglia, and pericytes. The three main neuronal cell types are photoreceptors, bipolar cells and ganglion cells. Their activity is modulated by horizontal, and amacrine cells. Retinal cells are arranged in a highly organized manner in layers.

Photoreceptors are of two types: rods and cones. The human retina contains around 120 million rods and about 6 million cones. Rods are responsible to sense contrast, brightness and motion while cones provide fine and spatial resolution as well as color vision.

There are three types of pigments in the cones that enable the eye to see colors: cyanolabe, chlorabe, and erythrolabe absorbing blue, green, and red light, respectively. These pigments allow us to perceive more than 200 colors. The pigment of the rods is called rhodopsin. The visual pigments are responsible for absorption of light and initiation of the neuro-electrical impulse.
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Each photoreceptor is a long narrow cell with an inner and outer segment connected by a cilium which transfers metabolites between the inner and outer segments. These segments are separated from the cell body by the outer limiting membrane (OLM). This structure contains adherens junctions between Müller cells and photoreceptors (Fig. 2). Photoreceptor cell bodies lie in the outer nuclear layer (ONL) (Fig. 8). The photoreceptor axons pass into the outer plexiform layer to form synaptic terminals (cone pedicle or rod spherule) with bipolar and horizontal cells.

The central difference between rods and cones is in the outer segment, containing the visual pigment. In rods the visual pigment is found on numerous flat internalized discs. The discs are produced at the bottom of the outer segment and travel to the tips enclosed by the microvilli of the RPE. Daily, outer segments are shed off and phagocytosed by the RPE. In cones the pigment is found on invaginations of the membrane, in free communication with the interphotoreceptor space.

Bipolar cells transmit signals from photoreceptors to ganglion and amacrine cells, between which they are located. Their cell bodies form the inner nuclear layer (INL) (Fig. 8). Their dendrites form synapses with photoreceptors and horizontal cells, while the axons form synapses with ganglion and amacrine cells.

Ganglion cells are the last link in the retinal component of the visual pathway. Their axons form the nerve fiber layer of the retina and contact cells in the lateral geniculate nucleus of the thalamus. The axons form the optic nerve (Fig. 8).

Horizontal cells have an integrative role in retinal processing.

Amacrine cells are positioned in the inner nuclear layer and their dendritic processes ramify and terminate mostly in the inner plexiform layer. These cells play a role in modulation of signals reaching ganglion cells.

Retinal glia includes the neuroglia and the microglia.

Astrocytes form a scaffold between vessels and neurons perpendicular to the Müller cells, isolating the receptive surfaces of neurons.

Müller cells are the major supporting glial cells of the retina, analogous to oligodendrocytes. They have a radial orientation and extend through the depths of the retina from the inner limiting membrane to the outer limiting membrane. They surround blood vessels and neuronal cell bodies forming “tunnels”. The cytoplasm of Müller cells contains rich endoplasmic reticulum and microtubules underlining their role in protein synthesis, transport and secretion.

Microglial cells in the eye are represented by a specialized sub-population of the mononuclear phagocyte system of the central nervous system. Following injury to the retina, microglia behave as phagocytes.

3.2.2 Localization of the Crb family complex in retina

Crb1 is colocalized with Mpp5 at the SAR in human and mouse retina, adjacent to adherens junctions at the OLM. In the mouse retina aPKC and the multiple PDZ proteins Patj and Mupp1 are detected at the same structure. Interestingly, Crb2 and Crb3 are also present at the SAR. Crb1 was shown to be associated with Mpp5 and Mupp1 in the retina, similar to what was demonstrated in vitro for epithelial cells. The SAR is just above the zonula adherence, and thus corresponds to the SAR in Drosophila epithelia, and the lowest part of the Drosophila photoreceptor stalk
membrane. Crumbs was detected at the SAR of fly epithelia, and in the stalk membrane of fly photoreceptors.²,¹¹⁵,¹¹³

3.2.3. Clinical features of Leber congenital amaurosis (LCA) and Retinitis pigmentosa (RP)

Leber congenital amaurosis was named after the German ophthalmologist Theodor von Leber, who in 1869 described the disease. LCA is a severe childhood retinal dystrophy, characterized by a non-detectable scotopic and photopic electroretinogram (ERG), nystagmus, sluggish pupillary reaction, and pigmentary retinopathy soon after birth.¹⁷⁰ LCA, considered the most severe form of retinitis pigmentosa, is a genetically heterogeneous disorder. Although a severe and early-onset disease, LCA presents with variable expression, which can be explained by genetic differences. Distinguishing LCA from early-onset RP is complicated because these disorders have a range of phenotypes often caused by mutations in the same gene(s). LCA often resembles an ocular phenotype observed in several systemic disorders including Refsum disease, Senior-Loken syndrome, and Joubert syndrome.¹⁷⁰ LCA-associated proteins show great variability in their cellular localization and function. Mutations in these proteins affect variable cellular processes from transcription to photo-transduction and vitamin A metabolism.¹⁷⁰

Retinitis pigmentosa is a name for a group of hereditary progressive retinal disorders with a combined prevalence of about 1 in 3000 in the population.¹⁷¹ This group is phenotypically and genetically heterogeneous. RP is characterized by bony spicule pigmentation in mid-peripheral retina, signifying a degenerative process of the retina initially affecting the rod photoreceptors and the retinal pigment epithelium. Apoptosis has been proposed as a mechanism for photoreceptor cell death in RP.¹⁷²,¹⁷³ RP patients experience early night blindness, loss of peripheral vision and ultimately of central vision and frequently myopia. This condition is often associated with perivascular pigmentation, decrease of the retinal arterioles, and pale appearance of the optic discs. Central vision is usually spared until later stages of the disorder.¹⁷⁴ In addition, RP can be a secondary condition in origin and may be linked with more than 30 syndromes including Usher syndrome, Bardet–Biedl syndrome, and Refsum disease.¹⁷⁵

3.2.4. Causes of hereditary LCA and RP

Diseases of the retina include a continuum of photoreceptor phenotypes mapped to >150 loci on the human genome (RetNet™; www.sph.uth.tmc.edu/Retnet/home.htm). About half of the underlying genes have been identified (RetNet™). RP is inherited in various modes: 20–25% of the cases are autosomal recessive (arRP), 15–20% are autosomal dominant (adRP), 10–15% are X-linked (XLRP), and the remaining 40–55% are genetically unclassified, and are called simplex RP (SRP).¹⁷¹,¹⁷⁴ At present, 20 arRP, 14 adRP, and five XLRP loci have been mapped and 32 genes from these loci were cloned (RetNet™). The majority of RP forms are monogenic, but digenic–diallelic and digenic–triallelic inheritances have also been described.¹⁷⁶,¹⁷⁷ LCA can result from mutations in at least nine loci (RetNet™). Six LCA disease genes have been identified. The disease genes are inherited mainly in autosomal recessive and seldom in autosomal dominant manner. Collectively, variants in aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1)¹⁷⁸, Crumbs homolog-1 (CRB1)¹²⁵, cone-rod
homeobox (CRX)\textsuperscript{179}, guanylate cyclase 2D (GUCY2D) \textsuperscript{180}, retinal pigment epithelium-specific 65 kDa protein (RPE65)\textsuperscript{181,182}, and retinitis pigmentosa GTPase regulator-interacting protein 1 (RPGRIP1)\textsuperscript{183} account for less than half of all LCA patients. The other loci concerned with LCA are LCA3 on 4q24\textsuperscript{184}, LCA5 on 6q11-q16\textsuperscript{185,186}, and LCA9 on 1p36.\textsuperscript{187}

3.2.5. Mutation and phenotype analysis of \textit{CRB1} in LCA and RP

To isolate candidate genes for chorioretinal diseases, human cDNA libraries enriched for retina- and (RPE)/choroid-specific cDNAs have been constructed through a suppression subtractive hybridization method.\textsuperscript{188,189} 35\% of the analyzed cDNAs were expressed predominately in the retina or RPE/choroid. One third of them (11) co-localized with loci involved in retinal disorders.\textsuperscript{188} One of these cDNA fragments mapped in a previously described region for autosomal recessive retinitis pigmentosa (RP12).\textsuperscript{190,191,192} Northern blot analysis detected transcripts in neural retina; RT-PCR detected expression additionally in adult and fetal brain.\textsuperscript{121,118} Overlapping cDNA fragments encoded an extracellular protein with 19 EGF-like domains, and 3 laminin-A G-like domains. The protein had a length of 1,376-amino acids, was 35\% identical to the protein 'Crumbs' (Crb) of \textit{Drosophila melanogaster}, and was named \textit{CRB1}, for Crumbs homolog-1. The first full-length \textit{CRB1} gene described was divided into 11 exons. Later on, an alternative 3’ splice form was described, giving rise to a 1406 amino acid protein with a trans-membrane region comprising 22 amino acids and a cytoplasmic tail of 37 amino acids. The intracellular region was 72\% similar to that of \textit{Drosophila} Crumbs. Two intracellular sub-domains critical for the function in the fly were highly conserved.\textsuperscript{118}

\textbf{\textit{CRB1} mutations cause RP with PPRPE:} In the studies of Den Hollander et al. (1999) patients suffering from RP with para-arteriolar preservation of the RPE (PPRPE) were identified with homozygous, compound heterozygous, or mutations on only one of the \textit{CRB1} alleles (probably due to unsuccessful screening for a mutation in the second allele)\textsuperscript{121,123} Another study confirmed the involvement of \textit{CRB1} mutations in RP with PPRPE in two unrelated Pakistani families with homozygous mutations.\textsuperscript{193} The specific RPE changes observed in RP12 patients suggested that \textit{CRB1} mutations cause a novel mechanism of photoreceptor degeneration. It has been suggested that PPRPE is due to the release of a permeable factor or oxygen from retinal arterioles which preserves RPE and retinal function in the proximity of the arteriole.\textsuperscript{194} Because of the early onset of symptoms in patients who have RP with PPRPE and the observation that mutations in \textit{RPE65} and \textit{CRX} can lead to both LCA and RP\textsuperscript{181,195,196,197} \textit{CRB1} was considered to be a good candidate gene for LCA as well.\textsuperscript{122}

\textbf{\textit{CRB1} mutations cause LCA:} Homozygous mutations were detected in patients with LCA from the Netherlands, Germany, and the United States. Among the patients with LCA, nine distinct sequence changes in the coding region or splice sites of \textit{CRB1} were found. Two of the changes had been identified previously\textsuperscript{121} in patients who had RP with PPRPE, but seven of them had not been identified.

More individuals with LCA were screened in another study\textsuperscript{198} having mutations in the \textit{CRB1} gene. In that particular group of LCA patients, coding sequence variations were observed in the \textit{CRB1} gene more frequently than in any of the other five known LCA-associated genes.\textsuperscript{198} Among the mutations described were small 1-7 bp insertions and deletions in the coding sequence of \textit{CRB1}, six substitutions of cystein with another amino
acid or stop codon, other amino acids mutations, >100 bp poly A insertion, and even synonymous codon changes. Later on a genome-wide screen for homozygosity in a large consanguineous family originating from Palestine was performed.\textsuperscript{199} The affected family members were found to be homozygous for a 10-bp deletion in the 12\textsuperscript{th} exon of the \textit{CRB1} gene.\textsuperscript{118} This sequence change led to the deletion of four amino acids at positions 1374-1377, a frame shift and truncation of the cytoplasmic end of the protein. One additional study reported \textit{CRB1} homozygous mutations in Pakistani patients with LCA.\textsuperscript{193}

Onset of human genetic retinal disease usually results in retinal thinning due to progressive photoreceptor degeneration. However, Jacobson et al (2003) described two new mutations in \textit{CRB1} leading to LCA, and characterized the retinal organization in vivo of LCA patients with \textit{CRB1}, \textit{RPE65}, or \textit{GUCY2D} mutations by optical coherence tomography.\textsuperscript{200} It was found that, unlike other inherited retinal degenerations, the \textit{CRB1} mutant retinas were 1.5 times the normal thickness in cross section and lacked the distinct layers of normal adult retina. This LCA phenotype was specific for \textit{CRB1} mutations, as patients with mutations in other genes had normal or reduced retinal thickness. \textbf{\textit{CRB1} mutations cause severe autosomal recessive and juvenile RP:} The phenotype, and sometimes the genotype, of juvenile RP shows partial overlap with LCA.\textsuperscript{196} Juvenile RP patients normally have central vision during the first decade of life. Mutations in \textit{CRB1} have been reported to cause this form of RP; some of them have been reported previously to be associated with the LCA phenotype, whereas other mutations appear to be unique for juvenile RP.\textsuperscript{201}

\textit{CRB1} mutations were initially described in patients with RP12.\textsuperscript{121} In later studies, it appeared that mutations in the \textit{CRB1} gene were also detected in patients with severe autosomal recessive RP without preservation of the para-arteriolar retinal pigment epithelium (PPRPE), further suggesting that \textit{CRB1} mutations give rise to multiple phenotypes.\textsuperscript{202} Thirty-seven patients from two Pakistani families with severe autosomal recessive retinitis pigmentosa without PPRPE were screened for mutations in \textit{CRB1}.\textsuperscript{202} Two novel \textit{CRB1} mutations were discovered. In one of the families a homozygous non-conservative mutation of a cystein was found. In the second family, a homozygous 10 base-pair deletion resulting in a downstream stop codon was described. The stop codon probably leads to an early-truncated protein and is deleterious to the protein function. This hypothesis is supported by the early onset of the disease in this family starting from age five on. \textbf{\textit{CRB1} mutations cause RP with Coats-like exudative vasculopathy:} \textit{CRB1} mutations were detected also in patients suffering from RP with Coats-like exudative vasculopathy\textsuperscript{122,123}, a complication of RP that may result in a partial or full retinal detachment. Because most patients had the condition in one eye and not all siblings with RP had the complication, \textit{CRB1} mutations were considered a risk factor for Coats-like exudative vasculopathy, although its progress may require other genetic and/or epigenetic factors. Some of the mutations were unique for this condition.\textsuperscript{122,123} \textbf{\textit{CRB1} mutations cause Pigmented Paravenous Chorioretinal Atrophy:} Further proving that mutations in \textit{CRB1} can result in various phenotypes, it has been described that a novel dominant Val162Met mutation within the fourth EGF-like domain is associated with Pigmented paravenous chorioretinal atrophy (PPCRA).\textsuperscript{203} Of the seven family members tested, the variation was found to co-segregate with six affected individuals and was not found in unaffected family members. PPCRA was dominantly
inherited in this family in accordance with previous studies on PPCRA\textsuperscript{204,205}, but exhibited variable expressivity. PPCRA is an unusual retinal degeneration characterized by accumulation of pigmentation along retinal veins. Patients are often asymptomatic, and the diagnosis is based on a typical fundus appearance, during routine ophthalmic inspection. PPCRA differs from RP in the lack of night blindness, minimal ERG abnormalities and its typical fundus appearance.\textsuperscript{206} In silico homology modeling was performed on the mutated protein domain. The Val162Met variation is adjacent to the fourth highly conserved cysteine and is replaced with a larger non-aliphatic methionine amino acid. Val162 is not conserved, but is involved in hydrogen bonding within the major β-sheet.\textsuperscript{203} Contrary to the reported thick retinas in cases of LCA with \textit{CRB1} mutations, patients with PPCRA phenotype had normal lamination and retinal thickness, suggesting that different mutations in \textit{CRB1} cause various retinal phenotypes.\textsuperscript{200,203}

Among mutations reported in \textit{CRB1}, the amino acid substitution Cys948Tyr is the most common, having been associated with LCA, RP, RP with Coats’-like exudative vasculopathy and RP with PPRPE.\textsuperscript{122,121,198} Affected individuals homozygous for the Cys948Tyr mutation experience a severe LCA phenotype.\textsuperscript{122} The main structural feature of all EGF-like domains is a central two-stranded β-sheet that is located in a region containing disulfide bonds.\textsuperscript{203} The 19 EGF domains comprise about 50\% of CRB1 but contain approximately 70\% of the non-truncating mutations, half of which substitute one of the six cysteines forming disulfide bonds, thus stabilizing the fold of the domain. Some \textit{CRB1} mutations introduce cysteine amino acids that could incorrectly form disulfide bonds.\textsuperscript{198}

\textit{CRB1} mutations are not a frequent cause for classic RP, but are often detected in RP with PPRPE, Coats-like exudative vasculopathy and early onset RP without PPRPE. The frequency of LCA caused by \textit{CRB1} mutations varies dramatically (0-13\%) between populations.\textsuperscript{122,198,123} Different mutations have been identified in the \textit{CRB1} gene, including amino acid changes, frame shifts, non-sense changes, splice site mutations, in-frame deletions, and large insertions.\textsuperscript{123} A concentration of mutations is found in exons 7 and 9, coding for the third laminin A G-like domain. More than half of the amino acids substitutions are in the laminin A G-like domains, while 40\% of the mutations are in the EGF domains. The majority of the mutations in EGF domains affect highly conserved cysteines, used in protein folding.\textsuperscript{207} Numerous proteins crucial for neuronal development, for instance reelin, agrin, tenascin and members of the notch family, contain multiple EGF-like domains.\textsuperscript{208}

Most nonsense and frame shift mutations are predicted to result in a truncated protein and lack the transmembrane domain and intracellular domain. Alternatively, these changes could lead to unstable transcripts destined for degradation.\textsuperscript{206} LCA patients have null mutations more frequently than RP patients (37\% vs. 19\%), but still the percentage of LCA patients with two null mutations is not significantly higher than for RP.\textsuperscript{123} Finally, the existence of RP patients with null mutations on both alleles indicates that loss of function of \textit{CRB1} is not the only cause for LCA, and additional genetic and epigenetic factors might have a role in determining the severity of the phenotype. A wide range of visual acuity has been reported even among LCA patients with mutations in \textit{CRB1}.\textsuperscript{210}

Because \textit{CRB2} is expressed in the eye and has a very similar structure to \textit{CRB1}, it has been screened for mutations in patients with RP and LCA. Mutations in the \textit{CRB2}
gene are not associated with recessive RP or LCA, although pathogenicity was not completely excluded.\cite{117}

3.2.6 Crb1 retinal phenotype in mice

There are two murine models for Crb1 mutation. Crb1 cDNA from retinal degeneration (rd) 8 mice shows a base pair deletion at nucleotide 3481, leading to a frameshift and a premature stop codon after amino acid 1207. The predicted mutant truncated and secreted protein, if generated, would retain most of the EGF-like and laminin A G-like domains, would contain 47 novel amino acids following amino acid 1160, and lack the transmembrane and intracellular domains of the CRB1 protein.\cite{211}

Rd8 retinas had large spots concentrated in the inferior nasal quadrant of the fundus. These spots corresponded to regions with retinal folds (pseudorosettes) that involve the photoreceptors and often distort the inner nuclear layer. The retina was thin in affected areas. The degeneration in rd8 mice was focal in nature. In older mice the outer nuclear layer could be as thin as one single row of nuclei in affected areas, with nearly normal retina present around. The photoreceptor inner segments were suggested to be about 25% shorter than normal, resembling the findings in Drosophila, where lack of Crb leads to shortened stalk membranes.\cite{115} Stalk membranes correspond to the mammalian inner segments of photoreceptors. Unfortunately, a statistical basis for the observed shortened inner segments has not been described. Photoreceptor outer segments of rd8 mice were also suggested to be shortened. A breakdown of individual photoreceptor lamellae was observed. With age advancing, only a few outer segment fragments remained in the affected regions.\cite{211} Although the inner retina was generally normal, Müller cell processes were unusually prominent. Müller cell apical processes were not observed immediately beneath the regions that did not contain adherens junctions. Rd8 mice showed loss of structural integrity at the retinal outer limiting membrane. Fragmented staining of OLM markers was probably due to loss of adherens junctions. Adherens junction complexes were considerable distances apart and focal expansion of the extracellular space was observed in regions where the ZA was absent. The retinal spotting was strongly dependent on the genetic background, suggesting that genetic modifiers exist.\cite{211}

The second murine model was generated by inactivation of the Crb1 gene by targeted deletion of the Crb1 promoter and the first exon, encoding the amino terminus of the corresponding protein. The resultant animal lacks expression of the gene and the protein, thus representing a Crb1 mouse model that lacks Crb1 protein.\cite{42} Crb1\textsuperscript{−/−} mice show loss of cell adhesion between photoreceptors and Müller glia cells, suggesting a role for Crb1, localized at the subapical region adjacent to adherens junctions, in maintenance of cell adhesion between photoreceptors and Müller glia cells. Retinal abnormalities were detected at 3 months of age in Crb1\textsuperscript{−/−} mice maintained at dimmed light. The mice developed small regions of retinal degeneration. The OLM was ruptured by the protrusion of single or groups of photoreceptors into the subretinal space, and some ingressed into the OPL and inner nuclear layer. Double photoreceptor layers or half rosettes with developed photoreceptor inner segment membranes and an OLM were present immediately underneath the photoreceptor layer. The photoreceptor layer transformed into two layers without a major loss of photoreceptor cells. Except for localized regions of retinal degeneration, the subcellular localizations of β-catenin, ZO-1,
Patj, Mupp1, Mpp5, aPKC, Crb2 and Crb3 at the OLM were normal, suggesting that the polarity was not lost in unaffected regions.

In more severely affected retinas, loss of photoreceptors, cells from the INL, and degeneration of RPE was detected.\(^{42}\) Loss of overall retinal function could not be detected by electoretinography, most likely because less than 25% of the retina was disorganized or degenerated.

Crb1 also has light protective function similar to the \textit{Drosophila} homolog Crumbs.\(^ {165}\) \textit{Crb1}\(^{-/-}\) mice exposed to 3000 lux for 3 days showed a significant increase in number of disorganized areas in mutant mice compared to mutant retinas exposed to a normal dimmed light/dark procedure. Retinal degeneration started at several foci at the inferior temporal quadrant of the \textit{Crb1}\(^{-/-}\) retina, the area most exposed to light in the murine retina, indicating that light is an environmental factor that enhances the onset of retinal degeneration in \textit{Crb1}\(^{-/-}\) retinas. Light damage to the retina was detected early in Müller cells that respond by increase of intermediate filament proteins in regions of detectable retinal degeneration.\(^ {212}\) Fundus photography revealed macroscopically visible retinal spots corresponding to rosette structures and double photoreceptor layers in the sections.

It has been suggested that mammalian CRB1 could have the same function as \textit{Drosophila} Crumbs.\(^ {165,115}\) However, results up to date indicate partial overlap. One should take into account that \textit{Drosophila} has one Crumbs protein, whereas mammals have three Crb proteins, Crb1-3, with potentially redundant or overlapping functions.

A variant short form of Crb1, Crb1s, has been reported in mice. Unlike Crb1 it is expressed ubiquitously.\(^ {213}\) The protein is truncated after the 12\textsuperscript{th} EGF domain. The study described a putative role of Crb1s in skin development. Crb1s was localized at the outer membrane of basal cells of epidermis during embryogenesis. Upon birth Crb1s was detected in the upper layers of the epidermis where tight junctions were mainly localized.\(^ {213}\) Crb1s was secreted into medium upon differentiation of cultured keratinocytes, whereas the protein that remained intracellular was targeted to cell-cell contacts.\(^ {213}\) The physiological role of this Crb isoform is not clear. The overall normal phenotype of our \textit{Crb1}\(^{-/-}\) mice suggests that Crb1s is not essential for survival, although it is widely expressed.

### 3.2.7 aPKC retinal phenotype in mice

aPKC is a member of the apical PAR3/PAR6/aPKC protein complex. Mice with conditional knock-out of aPKC in post-mitotic differentiating neurons failed to develop proper retinal lamination. The disorganization did not affect the cell fate determination, but the photoreceptors were randomly distributed in the retina, and did not form outer segments and inner segments, although the components for these structures were expressed. Normal synaptic terminals were missing as well, although ribbons were present. The expression of proteins localizing at the AJ or at the SAR like β-catenin or Par3 and Par6, respectively, was normal but AJs between photoreceptors and progenitor cells were not formed. AJs were detected between progenitors themselves. These cells were scattered around the retina instead of concentrated at the apical edge. Probably the progenitors did not establish proper adhesion with the differentiated photoreceptors, and also the differentiation occurred at different locations in the retina, leading to the formation of rosettes. These findings indicate that aPKC is needed for the proper
polarization of photoreceptors and lamination of the entire retina.\(^{214}\) This severe phenotype signifies the central role of αPKC in establishing proper polarization on cellular and tissue level.

### 3.2.8 aPKC and Pals1/Mpp5 phenotype in zebrafish

Other vertebrate models further support the notion of importance of proteins involved in complexes establishing apical-basal polarity for the development of proper retinal organization.

*Heart and soul* (has) encodes an aPKC homologue in zebra fish. *Heart and soul* mutants displayed defects within the eye and the neural tube. During early stages of organogenesis, when epithelial phenotypes first manifested, *heart and soul* appeared to regulate the clustering and maintenance of apical adherens junctions. Loss of aPKC\(\lambda\) activity led to defects in spindle orientation during progenitor cell division in retina. Despite the low level of aPKC protein, retinal apical adherens junctions formed in *heart and soul* mutants, but these structures were not maintained. At 72 hours post fertilization, mutant retinas only had round, and disorganized, instead of columnar cells at the ventricular surface. Apical adherens junctions were also progressively lost from the ventricular surface of the neural tube during the same stages. *Heart and soul* mutants displayed an increased number of ectopic cell divisions, suggesting that these ectopically dividing cells may lack all apicobasal polarity.\(^{215}\) In WT animals >90% of total mitotic spindles were oriented parallel to the ventricular surface. During divisions, aPKC localized to the apical side of progenitor cells and appeared to segregate symmetrically into both daughter cells. Only a small fraction of divisions had spindles that oriented at an angle perpendicular to the ventricular surface. In contrast, *heart and soul* mutants showed an increased number of cell divisions with abnormal spindle orientations\(^{215}\), suggesting that aPKC is required for mitotic spindle orientation during retinal progenitor cell divisions in vertebrates. Localization of the PAR3/PAR6/aPKC complex to the tight junction is required for proper apicobasal polarity in mammalian epithelial cells.\(^{130,94}\) In *Drosophila*, Baz (Par3) directs the apical clustering of spot adherens junctions to form the zonula adherens during gastrulation\(^{107}\), which suggests that this particular function of the Baz/Par6/aPKC complex may be conserved between flies and vertebrates. One model for the control of spindle orientation during this process postulates that a single polarity cue aligns the mitotic spindle parallel to the ventricular surface. During asymmetric division, this polarity cue is ignored and spindle orientation is randomized, resulting in a subset of spindles with perpendicular orientation.\(^{216}\) *Heart and soul* mutants may lack the polarity cue required to align the spindle parallel to the ventricular surface. Work from *Drosophila* has suggested that apical adherens junctions anchor the mitotic spindle within the plane of an epithelium.\(^{217}\)

The *nagie oko* cDNA sequence encodes a protein, which is a homolog of mouse Mpp5, human MPP5 and *Drosophila* Stardust. Mutations in *nagie oko* severely disrupted all retinal layers. Individual cell classes could not be distinguished on the basis of their positions or morphology, as they occupy highly abnormal positions. Centrosomes, actin bundles associated with adherens junctions, and M-phase nuclei were found ectopically in the internal region of the retinal neuroepithelium. These data indicate that *nagie oko* is essential for maintaining the correct polarity of the retinal neuroepithelial sheet. The overall polarity of brain neuroepithelium was not affected. One of the two characterized
alleles contains a point mutation that results in a substitution of isoleucine by asparagine at position 329 within the PDZ domain. In most PDZ domains this position is occupied by hydrophobic amino acids: isoleucine, leucine or valine. The second allele resulted in a mutant polypeptide lacking most of its GUK domain. As the GUK domain is conserved in all MAGUK proteins and is presumed to act in inter- and intra–protein interactions, this truncation most likely results in a severe loss of function. The wild type Nagie oko protein was detected apical to the retinal OLM cell junctions, suggesting that it influences cell adhesion within the photoreceptor cell layer. The truncated protein was absent at the apical domain.

It is noteworthy that homologues of nagieoko, and heart and soul in Drosophila, stardust and DaPKC, play a role in the polarity of fruit fly embryonic epithelia. The conservation of both structure and function in this group of genes indicates that embryonic insect epithelia and the neuroepithelium of the vertebrate eye share similar developmental mechanisms.

4. Prospects for gene therapy

There are two main strategies in gene therapy:
1. Correction of the gene defect, which requires delivery of genes to those cells in which the abnormal gene functions. In most recessive conditions, where the mutations are leading to loss of function, introduction of a wild type copy of the gene is needed. Treatment of disorders with gain-of function caused by dominantly inherited genes requires a more complex approach. In such cases, introduction of small interfering RNA (siRNA) or ribozyme, to prevent the production of mutant protein is necessary. Introduction of a normal copy of the gene might be required as well.
2. Introduction of genes encoding growth factors to either counter or diminish the consequences of the mutated gene(s) without correcting the mutation(s). This method can be used for dominant and recessive diseases. Often loss of vision is due to photoreceptor death rather than their abnormal functioning and gene therapy cannot restore the vision. The aim in these cases is to slow photoreceptor death by introducing genes coding for trophic or survival factors.

Progress in genetic engineering has led to a rapidly growing number of transgenic animals, mostly mice, carrying constructs that lead to disruption or overexpression of genes resulting in retinal degenerations. These animal models are used for the study of molecular mechanisms leading to retinal degeneration. These animal models often have a similar phenotype as human patients and therefore constitute a powerful tool for investigation of treatment by gene therapy.

The development of gene therapy will critically depend on safe and efficient delivery of genes to target cells. Different viral vectors have been used for ocular gene transfer in animal models, but most effective for adult photoreceptors are those based on Adeno-Associated Virus (AAV). Adeno-associated virus type 2 (AAV-2) is a human parvovirus that is used as a successful gene transfer vector in a variety of rodent and large animal models. Recombinant AAV-2 (rAAV-2) vectors used for gene therapy were derived from the wild-type virus by deleting the entire viral coding region and replacing them by a reporter or therapeutic transgene. More recently, seven other rAAV serotypes (AAV-1, -3, -4, -5, -6, -7 and 8) have been isolated and cloned. In the retina, following subretinal delivery, AAV-2 vectors transduced RPE and photoreceptor
Intravitreal injection of rAAV-2 led to efficient ganglion cell transduction. Sub-retinal injection of a complete rAAV-5 vector in mouse showed faster expression kinetics and resulted in higher photoreceptor transduction efficiency compared with rAAV-2. Both rAAV-2 and rAAV-5 transduced RPE cells and photoreceptors.

An important issue for safe and successful ocular gene therapy trials using rAAV is an accurate evaluation of vector distribution after ocular viral injection. Some studies have looked at the distribution of rAAV following various routes of administration. There were cases of scatter of virus to the systemic circulation. Administration of rAAV-2/2-lacZ in the vitreous of guinea pigs led to β-galactosidase expression in fibers, glial cells and blood vessels of the optic nerve. One can overcome these problems to some extent by using retina cell-specific promoters driving the expression.

The restoration of vision in dogs lacking functional RPE65 and the morphological and functional rescue of photoreceptors in peripherin-2 (prph2) mouse and rat models provided evidence to support rAAV-mediated gene therapy replacement strategies.

rAAV-mediated gene transfer using neurotrophic factors, such as ciliary neurotrophic factor (CNTF), fibroblast growth factors (FGF) and glial cell line-derived neurotrophic factors (GDNF), showed increased photoreceptor survival in different rodent models of retinal degeneration. Despite the lack of significantly increased electroretinograms (ERGs), the treated rats displayed a greater number of surviving photoreceptors following sub-retinal injection of these vectors, suggesting the ability of these factors to protect photoreceptors from apoptosis.

A key factor for successful gene therapy is the ability to regulate expression. Many proteins have more narrow therapeutic windows, and overproduction could result in toxicity. Several regulation systems, including rapamycin, mifepristone, ecdysone, and tetracycline, have been developed to control transgene expression in vivo. A smart alternative to these systems, which are dependent on administration of exogenous pharmacological agents, is to couple expression to changes in the local tissue environment, which are indicative of need of intervention. For example: use a hypoxia-responsive element (HRE) allowing low expression in normoxic conditions, and high activated expression upon lowering of the oxygen concentration.

Last but not least one should bear in mind that in some cases a novel transgene can cause an immune response. An example for that are studies testing rpe65 gene replacement therapy in a dog model of LCA. The rpe65 gene encodes a 65-kDa-specific protein essential for synthesis of the rhodopsin ligand 11-cisretinal. The fact that there are close similarities between the clinical characteristics of the diseases resulting from RPE65 gene mutations in humans and in the dog make the RPE65/- dog a valuable model for the evaluation of gene therapy. Two studies testing gene replacement therapy using AAV-2/2-mediated deliveries of a canine rpe65 gene have now been reported. They both demonstrated restoration of dark- and light adapted ERG responses and improved psychophysical outcomes in the dogs. Although these results are promising, Narfstrom et al (2003) have reported that uveitis developed in 75% of the rAAV-2/2-rpe65-treated eyes, which could be due to a contaminant in the vector stock or may result from an immune response to the RPE65 protein.
When considering disease with early onset it is particularly encouraging to mention studies reporting that it is possible to restore visual function to the rpe65\(^{-/-}\) mouse following in utero administration of a rAAV vector carrying the rpe65 gene.\(^{254}\) However, many more steps, ranging from safety studies to discussion of ethical implications, must be taken before gene therapy for human retinal diseases can be envisaged. Demonstrating ‘proof of principle’ and safety in a number of animal models and species allows approaching clinical trials with increased confidence.

The Crb1 gene is of a size on the margin allowed to be introduced into modified AAV vectors. Its expression pattern suggests that it is expressed by photoreceptors and/or Müller cells. This indicated that subretinal or intravitreal injections could be utilized. In addition, photoreceptors and Müller cells express a number of unique genes, and thus can be targeted with specific promoters. The LCA and early onset RP phenotype associated with CRB1 mutations suggests that treatment should occur as early as possible to slow down the onset of retinal degeneration. The eye is an easily accessible organ for gene therapy without jeopardizing the general health. Furthermore, it has been shown that rAAV can be administered to the eye without immune response.\(^{255,256}\) Relatively small amounts of virus would be necessary to target retinal cells, therefore lowering the risk of spreading virus outside the target region.
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Chapter 1

Aim

The aim of this thesis was to generate a mouse model for disease(s) caused by mutations in the *CRB1* gene. We planned to investigate the pathogenesis of the anticipated mutation-induced retinal disorder, the time of onset of retinal degeneration, and to examine the histological characteristics in the diseased retina that could point towards specific pathological mechanisms involved. We were also interested in finding interaction partners for the CRB1 protein that could provide us with clues about the cellular pathways that these proteins participate in. At the start of this project only one human *CRB* gene was described, and mutations were reported to be associated with several types of retinal disorders.\textsuperscript{1,2,3,4,5} Available studies on the function of the *Crb* gene had been performed using flies, which are an excellent genetic model but are evolutionary quite remote from mammals. In addition, the expression pattern of Crb in flies differs from CRB1 in mammals.\textsuperscript{6,7,8,9,10,12,13,14} For that reason we started the project by generating a *Crb1* knock out mouse (described in chapter 2). Based on the available literature information and the results obtained early in the study\textsuperscript{15,16} we decided to concentrate on the putative associations between CRB1, MPP3, MPP4, MPP5, and other MAGUK proteins, described in chapters 3 and 4. Finally, our interest in one of the putative CRB1 interacting proteins, MPP4, led to the generation of a *Mpp4* knock-out mouse model, outlined in chapter 5, which gave additional power to our genetic and functional studies. The results obtained will pave the way for future intervention strategies, for which our mammalian model organism is indispensable.

References

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Chapter 2

Crumbs homologue 1 is required for maintenance of photoreceptor cell polarization and adhesion during light exposure

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### Abbreviations List

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AJ</td>
<td>Adherens Junction</td>
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<tr>
<td>aPKC</td>
<td>atypical Protein Kinase C</td>
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<td>CRB1 and Crb1</td>
<td>human and mouse Crumbs homologue 1</td>
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<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
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<td>INL</td>
<td>Inner Nuclear Layer</td>
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<tr>
<td>LCA</td>
<td>Leber Congenital Amaurosis</td>
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<td>MGC</td>
<td>Müller Glia Cell</td>
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<tr>
<td>Mpp4</td>
<td>Membrane Palmitoylated Protein 4</td>
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<tr>
<td>Mupp1</td>
<td>Multiple PDZ domain Protein 1</td>
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<tr>
<td>OLM</td>
<td>Outer Limiting Membrane</td>
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<tr>
<td>ONL</td>
<td>Outer Nuclear Layer</td>
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<tr>
<td>OPL</td>
<td>Outer Plexiform Layer</td>
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<tr>
<td>Pals1</td>
<td>Protein Associated with Lin-7</td>
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<tr>
<td>Patj</td>
<td>Pals1-Associated Tight Junction protein</td>
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<tr>
<td>PDZ</td>
<td>PSD95/Dlg/ZO-1</td>
</tr>
<tr>
<td>PRC</td>
<td>Photoreceptor Cell</td>
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<tr>
<td>RPE</td>
<td>Retinal Pigment Epithelium</td>
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<tr>
<td>SAR</td>
<td>Sub Apical Region</td>
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<td>Sdt</td>
<td>Stardust</td>
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Crumbs homologue 1 is required for maintenance of photoreceptor cell polarization and adhesion during light exposure

Summary
Loss of Crumbs homologue 1 (CRB1) function causes either the eye disease Leber congenital amaurosis or progressive retinitis pigmentosa, depending on the amount of residual CRB1 activity and the genetic background. Crb1 localizes specifically to the sub-apical region adjacent to the adherens junction complex at the outer limiting membrane in the retina. We show that it is associated here with multiple PDZ protein 1 (Mupp1), protein associated with Lin-7 (Pals1 or Mpp5) and Mpp4. We have produced Crb1-/ mice completely lacking any functional Crb1. Although the retinas are initially normal, by 3-9 months the Crb1-/- retinas develop localized lesions where the integrity of the outer limiting membrane is lost and giant half rosettes are formed. After delamination of the photoreceptor layer, neuronal cell death occurs in the inner and outer nuclear layers of the retina. On moderate exposure to light for 3 days at 3 months of age, the number of severe focal retinal lesions significantly increases in the Crb1-/- retina. However, Crb2, Crb3 and Crb1 interacting proteins remain localized to the sub-apical region and therefore are not sufficient to maintain cell adhesion during light exposure in Crb1-/- retinas. Thus we propose that during light exposure Crb1 is essential to maintain, but not assemble, adherens junctions between photoreceptors and Müller glia cells and prevents retinal disorganization and dystrophy. Hence, light may be an influential factor in the development of the corresponding human diseases.

Introduction
Mutations causing amino acid substitutions in the human CRB1 gene, the human homologue of Drosophila Crumbs, can lead to Leber congenital amaurosis (LCA), classic- and retinitis pigmentosa type 12, and retinitis pigmentosa with Coats-like exudative vasculopathy, whereas all known null mutations lead to LCA. LCA is a group of inherited severe retinal diseases and characterized by loss of vision within one year after birth. It accounts for at least 5% of all retinal dystrophies and is one of the main causes of blindness in children. Mutations in six genes were identified that together account for approximately 50% of all LCA patients. CRB1 accounts for 9-13.5% of these cases. The CRB1 protein contains 19 epidermal growth factor-like domains, 3 laminin A globular-like domains, a transmembrane domain and a 37-amino acid cytoplasmic tail with an C-terminal ERLI motif.

In the retina, photoreceptor cells (PRCs) transform the incoming light to a signal which is processed subsequently by other neurons in the retina and brain. The PRCs are packed together, with processes of Müller glia cells (MGCs) for structural and metabolic support, in the retinal outer nuclear layer (ONL). The establishment and maintenance of apical-basal polarization and cell adhesion is crucial for the PRCs. At the apical site of the ONL, an adhesion belt named the outer limiting membrane (OLM) contains specialized adherens junctions (AJs), which are present between the PRCs and MGCs. The AJs consist of multi-protein complexes and are linked to the cell skeleton for cell-shape.

Crumbs, a transmembrane protein, is associated with the formation of adherens junctions between cells in Drosophila. It localizes at a specialized region apical to the AJs, the subapical region (SAR), and is an essential component of the intracellular scaffold for the assembly of the protein complex at the AJ. Crumbs is important in regulating the length of the stalk-membrane in PRCs and in preventing light-induced
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PRC degeneration.\textsuperscript{11,12,8} Also, Crumbs interacting proteins are essential for the correct positioning and the integrity of the AJ for epithelial cell polarity and during PRC morphogenesis. Stardust (Sdt) binds to the C-terminal ERLI motif of Crumbs and deletion of this motif results in complete abolishment of interaction between Sdt and Crumbs. Sdt loss of function results in e.g. multi-layered epithelia and tissue disintegration.\textsuperscript{9,6,13} Another Crumbs interacting partner in \textit{Drosophila} is Pals1 Associated Tight Junction protein (dPATJ; formerly known as Discs Lost), which does not play an essential role in epithelial cell polarity or viability.\textsuperscript{10,14,15} Upon overexpression of \textit{CRB1} in polarized epithelial cells, CRB1 associates with tight junctions.\textsuperscript{16} In the tight junctions it co-localizes with Protein associated with Lin-7 (PALS1 or MPP5), which is the homologue of Sdt, and with Pals1 Associated Tight Junction protein (PATJ).\textsuperscript{17,14}

Although \textit{Drosophila} Crumbs is required for polarity and adhesion in embryonic epithelia and for stalk membrane morphogenesis in PRCs,\textsuperscript{11,12,10,18,8} the role of mammalian Crb1 in retinal dystrophies is still unclear.\textsuperscript{2,19} A clue came from a natural Crb1 mutant mouse, retinal degeneration 8 (\textit{rd8}), which produces a secreted truncated Crb1 protein (Crb1-rd8) of 1207 amino acids that lacks the single transmembrane and the intracellular domain. The \textit{rd8} mouse developed irregularities at the outer limiting membrane and loss of PRCs.\textsuperscript{20}

We have been able, by inactivating both alleles of the \textit{Crb1} gene to produce a complete null to examine the physiological role of Crb1 in the mammalian retina. Moreover, the localization of other components of the scaffolding complex was examined in vivo. We found that Crb1 has a central role in the scaffolding complex and is required for maintaining a single, organized layer of PRCs during light exposure. In its absence the adhesion between PRCs and MGCs is temporarily lost resulting in dramatic structural and functional changes.

Results

The localization of proteins in the outer limiting membrane

The photoreceptor layer in the retina contains a structure called the OLM that contains AJ, a region of cell-cell adhesion between PRCs and MGCs. We examined the localization of mouse Crb1 with mammalian homologues of Crumbs interacting proteins and other AJ and tight junction proteins in wild-type retinas. At the subapical region (SAR), adjacent to the AJ, we detected a number of proteins: Crb1 (Fig. 1A), F-actin (Fig. 1B,C), membrane-associated guanylate kinase (MAGUK) proteins\textsuperscript{9,13} membrane palmitoylated protein 4 (Mpp4)\textsuperscript{21} (Fig. 1C) and Pals1\textsuperscript{22,23} (Fig. 1D), the PDZ-motif containing proteins Multiple PDZ Protein 1 (Mupp1)\textsuperscript{24} (Fig. 1E) and Patj\textsuperscript{14,22} (Fig. 1F) as well as atypical protein kinase C (aPKC) (Fig. 1G). The Crb1 homologues Crb2 (Fig. 1H) and Crb3\textsuperscript{25} (Fig. 1I), co-localized with Crb1 to the SAR. The AJ contained $\beta$-catenin (Fig. 1A), N-cadherin, p120,\textsuperscript{26} ZO-1 and ZO-2\textsuperscript{27} (data not shown).

Patj was also detected in the retinal pigment epithelium (RPE) (data not shown). Mpp4 localized strongly in the synaptic terminal of the photoreceptors in the outer plexiform layer (data not shown and Fig. 5F). CD44 localized in the apical villi of the MGCs, but did not co-localize with Crb1 in the SAR (Fig. 1J). Cdc42 was detected in a similar localization pattern as F-actin, in the inner segments and outer plexiform layer (OPL) (data not shown). Next to the Crumbs complex, there are other complexes involved in
establishing epithelial polarity, such as the Par3-Par6-aPKC-Cdc42 complex. Components of this complex are known to interact with Crb family members and interacting proteins, e.g. Par6 has been shown to interact directly with Crb3 and Pals1. Of this complex, we detected aPKC and Cdc42 in the PRCs (Fig. 1G,M). This is the first study in mouse that defines the localization of several proteins at the OLM, such as Patj, Mupp1, Crb2, Crb3, Mpp4, aPKC and Cdc42. The localization of the homologues of these proteins e.g. Crumbs in the SAR and Armadillo in the ZA in Drosophila, show remarkable similarities with our data in mouse (for review see ).

In the human retina, CRB1 and MUPP1 were detected at the SAR. The AJ was stained with a β-catenin antibody, while CRB1 and MUPP1 were located apical to this region (Fig. 2K-L), similar as in the mouse retina.

**Binding partners of Crb1**

To extend the co-localization studies further, we investigated which proteins associated together in the same complex. Immunoprecipitation of Mupp1 from retinal lysates demonstrated that endogenous Mupp1 interacted in PRCs in a protein-complex with Crb1, Pals1 and Mpp4 (Fig. 2). The antibody against Mupp1 efficiently co-precipitated Crb1, in agreement with co-localization of Crb1 with Mupp1 in the retina. Pals1 was efficiently co-precipitated as well. The Mpp4 protein was efficiently co-precipitated with Mupp1 from the outer limiting membrane given that most of the Mpp4 is localized at the outer plexiform layer (data not shown). The membrane-associated protein Patj was not detected in the Mupp1 precipitate (Fig. 2). In Crb1−/− retinas before the onset of retinal degeneration, the complex between Mupp1, Pals1 and Mpp4 formed in absence of Crb1, indicating that Crb1 is not essential for this interaction (Fig. 2, first column). Pals1 was detected as a doublet. Both Pals1 proteins are present in the Mupp1 immunoprecipitate, with preference for the high molecular weight form. We conclude that in PRCs, Mupp1 is in complex with Pals1, Mpp4 and Crb1 and localized at the SAR.

**Generation of Crb1−/− mice.**

We inactivated the murine Crb1 gene by deleting a 2.9 kb genomic DNA fragment in murine embryonic stem cells (Fig. 3A,B; see Methods). The deletion contained the upstream promoter of the gene and the first exon encoding the amino-terminus of the Crb1 protein. The homozygous mice were healthy and fertile under normal conditions in the animal facilities. Antibodies directed against the carboxyl-terminus of Crb1 did not detect Crb1 in retinal lysates of Crb1−/− mice (Fig. 3C). Using three different antibodies against Crb1, including that was described we observed localization of Crb1 specifically at the SAR in the inner segments of rods and cones of wild-type retinas (Fig. 3A), but not in the outer segments of the cones as previously described (Figs. 1A, 3D,E). In Crb1−/− mice, Crb1 protein was not detected (Fig. 3F,G) indicating that the targeted allele is a true null.

**Retinal morphology in Crb1−/− mice.**

In Crb1−/− mice, maintained in a 12 hours light (100 lux) – 12 hours dark cycle, no abnormalities were detected in 2 or 3 weeks, or 2 months old retinas (n=5 in each group). However by 3 months, Crb1−/− mice had developed small, but significant, regions of
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retinal degeneration that were never observed in wild-type controls (Fig. 4A-C). The OLM was ruptured by the protrusion of single or groups of PRC bodies into the interphotoreceptor space. Moreover, ingressed PRC bodies were also detected in the OPL and inner nuclear layer (INL) (Fig. 4A,B). A striking feature of the Crb1−/− phenotype was the presence of double PRC layers or half rosettes (Fig. 4C). The rosettes resided immediately underneath the PRC layer and developed PRC inner segment membranes and an OLM. The number of PRCs in the upper and lower layers was approximately half the amount compared to the region immediately adjacent to the affected area, suggesting that the single PRC layer transformed into two layers without major loss of the PRCs. At this stage, except for the localized regions of retinal degeneration described in the next section (Fig. 5), the subcellular localizations of N-cadherin, β-catenin, p120, ZO-1, ZO-2, Patj, Muppl, F-actin, Mpp4, Pals1, aPKC, Cdc42, Crb2 and Crb3 were similar to the wild-type (data not shown, Fig. 1). The gross ultra-structure of the AJ and the apical processes of MGCs, which extend for a short distance beyond the AJ, was normal on electron microscopy (Fig. 6). The outer segment membranes of wild-type and mutant mice were flat and well organized, and closely aligned with each other and the plasma membrane. Our results indicate that mouse Crb1 is not essential for the assembly of the SAR and AJ during PRC layer development but rather to maintain the correct position and integrity of the SAR and AJ. Since retinal degeneration occurred in the presence and normal subcellular localization of both Crb2 and Crb3 in Crb1−/− mice, it appeared that the presence of Crb2 and Crb3 is not sufficient to rescue the retinal degeneration phenotype. Further indications for loss of PRC to MGC adhesion at foci in the retina were obtained by the analysis of Crb1−/− mice of 6 months of age. Large ectopic layers of PRCs formed a funnel-shaped layer, abutting the ganglion cell layer and inner limiting membrane (Fig. 4D). The ectopic layers of PRCs resembled giant half rosettes with outgrown inner segments and OLM. In the more severely affected Crb1−/− mutant retinas, ghost-like structures were detected, that may represent remnants of PRC rosettes that underwent cell death (Fig. 4E). In these areas, we detected loss of PRCs as well as cells from the INL, and degeneration of RPE, without significant loss of thickness of the retina (Fig. 4D). These results may explain in part the results by Jacobson et al., who observed thick retinas in LCA patients with CRB1 mutation, compared to thin retinas for e.g. RPE65 mutations.29

Light exposure accelerates retinal degeneration in Crb1−/− mice

Mutations in Drosophila crumbs result in improper PRC morphogenesis and progressive light-induced PRC degeneration.12 Since light could be one of the environmental factors causing the degenerations at foci in the Crb1−/− retinas, we exposed 3 months old pigmented wild-type and Crb1−/− mice to continuous moderate white fluorescent light (3000 lux) for 3 days.30 In a representative experiment there was a significant increase in the number of degenerative areas in moderate light-exposed Crb1−/− retinas, compared to retinas exposed to cycled light (12 hours dark / 12 hours 100 lux) (P< 0.02, n= 4) (Fig. 5A). The number of degenerative regions counted in the Crb1−/− retinas exposed to 3000 lux for 72 h is probably an underestimate of the true number of foci affected, as we observed that several small foci appeared to fuse into larger abnormal regions as can be seen in Fig. 5B when compared to Fig. 4. Retinal degeneration started at several foci at the inferior temporal quadrant of the Crb1−/− retina, the area most
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exposed to light in the murine retina. In the moderate light exposed $Crb1^{-/-}$ mice, retinal degeneration started as described for the cycled light 3–9 months $Crb1^{-/-}$ mice (Fig. 5B). However, 3 days after moderate light exposure, PRCs apical to the rosettes lacked inner and outer segments (Fig. 5C). None of the moderate light-exposed wild-type retinas showed degenerative areas ($n=10$). Overall our results support the hypothesis that light is an environmental factor that enhances the onset of retinal degeneration in $Crb1^{-/-}$ retinas. Light damage to the retina is detected early in MGCs, which respond by altering their protein levels and/or subcellular localization, e.g. by the upregulation and redistribution of intermediate filament proteins. We therefore used glial fibrillary acidic protein (GFAP) to stain intermediate filaments in MGCs. In $Crb1^{-/-}$ mice, ectopic GFAP staining correlated well with regions of detectable retinal degeneration. Strongly stained bundles of newly formed GFAP-positive intermediate filaments were detected through the INL and ONL. Moreover, MGCs extended further through the OLM into the interphotoreceptor space (Fig. 5D-E). In the degenerated areas, staining with markers for the OLM indicated disruptions at the SAR or AJ (Fig. 5F-J). Staining with markers for the OPL indicated losses of neuronal synapses at local regions (Fig. 5F-G). Remarkably, only a small number of TUNEL-positive cells were detected specifically at these sites of retinal degeneration in moderate light-exposed $Crb1^{-/-}$ mice (Fig. 5K-L), suggesting a delayed initiation of cell death after loss of PRC-MGC adhesion.

Electroretinography and fundus photography

The retinal function was examined by electroretinography. No loss of overall retinal function was detected in either the 3, 6 (data not shown), and 9 month old $Crb1^{-/-}$ mice exposed to cycled light, nor in 3-months old $Crb1^{-/-}$ mice exposed for 72 hours to 3000 lux of light (Fig. 7). This is not surprising as the focal retinal degeneration observed is very localized, whilst the remainder of the retina appears grossly normal. In the 72 hours 3000 lux exposed $Crb1^{-/-}$ mice, fundus photography revealed macroscopically visible retinal spots (Fig. 5M) that correlated with rosette structures and double PRC layers in the sections. In mice with rosette or pseudo-rosette formation but without double PRC layers, similar spots were detected. No spots were observed on SLO in 3, 6 or 9 months old $Crb1^{-/-}$ retinas exposed to cycled light, most likely because the areas of retinal degeneration are very small.

Discussion

In this study, we present the generation and characterization of $Crb1^{-/-}$ knock out mice. We demonstrate that complete disruption of $Crb1$ results in light-inducible retinal degeneration. The onset of the disease is due to transient loss of cell adhesion between PRCs and MGCs. When PRCs re-adhere, rosette structures and double PRC layers are formed. It has been demonstrated that MGCs rescue nearby PRCs from apoptosis and prolong their survival by the expression of growth factors. These MGC-derived growth factors might be essential in the transient rescue of PRCs in the ectopic rosette structures and double PRC layers in $Crb1^{-/-}$ retinas. Hence, Crb1 is not essential for the initial assembly of the AJ between PRCs and MGCs, but rather for the maintenance of the AJs during light exposure.

In most polarized vertebrate epithelial cells, tight junctions are located apical to the AJ. In the retina, the OLM harbours an AJ and a distinct region apical to the AJ, the
SAR. We provide novel data on Crb1 co-localization with Mpp4, Cdc42, F-Actin, Patj, Mupp1, aPKC, Crb2 and Crb3 in the SAR. Localization of Pals1 in the SAR is confirmed.\textsuperscript{20} Cell-cell contact in the OLM is not tight, since large molecules such as colloidal thorium and peroxidase\textsuperscript{34,35} readily diffuse from the vitreous through the OLM, into the interphotoreceptor space. In accordance, claudins 1-5 were not detected at the SAR.

The complex of Crb1, Pals1 and Mpp4 with Mupp1 has not been demonstrated before in vivo. In cultured epithelial cells it was shown by Roh et al.\textsuperscript{16,22} that PALS1 binds to CRB1 and that PALS1 binds to the L27-like domain in PATJ and MUPP1.\textsuperscript{16,22} MPP4, a family member of Pals1, also interacts with CRB1 when overproduced in 293 human embryonic kidney cells (A.K. and J.W., unpublished results). It is therefore likely that separate complexes of Crb1-Pals1-Mupp1 and Crb1-Mpp4-Mupp1 exist, or since MAGUK proteins may form heterodimers or homodimers,\textsuperscript{36} that complexes of Crb1-Pals1/Mpp4-Mupp1 are present in the PRCs. Conditions used for Patj immunoprecipitation were not optimal for the demonstration of a Patj-Pals1-Mpp4-Crb1 complex (data not shown). However, the interaction between Patj and Pals1 has been shown by others with overexpression in epithelial cell culture.\textsuperscript{14,22} Since Patj co-localizes with Crb1 and Mupp1 at the SAR, it is therefore conceivable that Crb1-Pals1/Mpp4-Patj complexes also exist.

The many different mutations in the human \textit{CRB1} gene cause variable eye phenotypes e.g. LCA, retinitis pigmentosa type 12, classic retinitis pigmentosa, and retinitis pigmentosa with Coats-like exudative vasculopathy. Full ablation of CRB1 function is likely to result in LCA,\textsuperscript{1} whereas other amino acid substitutions in the extracellular domain of CRB1 are likely to result in different forms of retinitis pigmentosa. The same may be true for truncated CRB1 proteins lacking the transmembrane and cytosolic domains.\textsuperscript{1} Mehalow et al.\textsuperscript{20} recently identified a mutation in the \textit{Crb1} gene to be responsible for the rd8 retinal degeneration phenotype. The \textit{rd8} mutation is most likely not a null allele, but encodes an aberrant secreted truncated Crb1-rd8 protein of 1207 amino acids, of which 47 novel amino acids. This aberrant protein lacks 4 of the 19 epidermal growth-factor domains and one of the three laminin A G-like domains.\textsuperscript{20} The production of Crb1-rd8 in combination with the lack of the native Crb1 protein causes retinal folds (pseudorosettes), very different from the double photoreceptor layers (giant half-rosettes) observed in the \textit{Crb1}\textsuperscript{−/−} mice. In old \textit{rd8} mice the photoreceptor layer declines to a single row of PRCs. In contrast, in foci of \textit{Crb1}\textsuperscript{−/−} retinas, complete loss of PRCs and INL, as well as degeneration of RPE occurs after an initial transformation of single to double photoreceptor layers. The difference in phenotype could be due to genetic background or to residual Crb1 function in the Crb1-rd8 protein.\textsuperscript{20} Also, in the \textit{rd8} mice many irregularly shaped spots are detectable as early as 3 weeks of age, whereas in the \textit{Crb1}\textsuperscript{−/−} mice these spots are sporadic at 3 months of age. An interesting difference is the localization of the spots, which localized to the inferior nasal quadrant of the fundus in \textit{rd8} mice, but to the inferior temporal quadrant in \textit{Crb1}\textsuperscript{−/−} retina correlating to the area most exposed to light. Furthermore, some similarities in phenotype are detected such as discontinuous staining of β-catenin, N-cadherin or Pals1 at affected regions.

We propose that the \textit{Crb1}\textsuperscript{−/−} mouse is a model for those LCA patients completely lacking functional CRB1. Our data suggest that exposure to daylight accelerates retinal degeneration. Our results indicate that Crb1 is not essential for the assembly of the SAR.
Crumbs homologue 1 is required for maintenance of photoreceptor cell polarization and adhesion during light exposure and AJ during PRC layer development but rather to maintain the correct position and integrity of the SAR and AJ. The carboxyl-termini of murine Crb1 and Crb3 interact in vitro with the PDZ domain of Pals1. Redundant functions of Crb1, Crb2 and Crb3 in photoreceptors are not known but their co-localization at the SAR, the high similarity of their C-termini, and interaction with Pals1 suggest a possible overlap or competition in function. Therefore, Crb3 could be involved, by interacting with Pals1-Patj/Mupp1, in the assembly of the SAR and AJ. Also the function and possible redundancy of Crb2, which is highly similar to Crb1, remains to be investigated.

It has been suggested that the mammalian CRB1 could have the same function as Drosophila Crumbs. Our results show that, although there is some overlap, there are differences between mammalian CRB1 and Drosophila Crumbs. In Drosophila only one Crumbs protein is present, whereas in the mouse there may be redundant overlapping functions for Crb1, Crb2 and Crb3, the latter two with yet unknown physiological function(s). Crumbs regulates the length of the stalk membrane in the PRCs of Drosophila, a structure hypothesized to share function with inner segments of PRCs. However, the length of inner segments of mutant and wild-type mice did not differ (data not shown). Therefore, our data suggest that Crb1 does not regulate the length of the inner segments of PRCs, or that other proteins (e.g. Crb2 or Crb3) exert similar functions. Different from the PRCs in the fruit fly, loss of cell polarization and adhesion in Crb1−/− mice is transient as shown by newly formed layers of PRCs with an OLM. These data suggest possible overlap of functions for the Crb1 family members, and a central role for Crb1 as a component of the molecular scaffold that controls maintenance of the PRC AJ during exposure to light. These findings provide insight into PRC layer polarization and retinal degeneration, and they reveal that Crb1 protects PRCs against physiological stress induced by light. Since LCA develops early in life in humans, by the time the pathology of the retinas is examined, the retina has completely degenerated. One study, using optical coherence tomography in vivo on LCA patients until 50 years of age, indicated a change in morphology. However, the onset of the pathology and delamination of the PRC layer have not been described in LCA patients. Therefore, the Crb1−/− mouse model could facilitate in obtaining more knowledge on the onset of LCA. Moreover, understanding the role of light in the onset of LCA, in patients with CRB1 mutations, will gain insight towards prevention of retinal dystrophy.

Materials and Methods
Generation of Crb1−/− mice.

Gene targeting was performed as described before. In short, a characterized 194 bp mouse Crb1 cDNA fragment encompassing 5′-noncoding region and exon 1 encoding the translation start site and transmembrane signal peptide of Crb1 was used to screen an EMBL3 genomic 129/Ola DNA phage library. A targeting vector was constructed by assembling a 5.6 kb BamHI 5′ Crb1 genomic fragment, a hygromycin resistance gene driven by the mouse phosphoglycerate kinase promoter, and a 4.9 kb BglII 3′ fragment of the Crb1 gene. Correct targeting deleted 2.9 kb of Crb1 sequence containing the upstream promoter region, exon 1 encoding the start of the Crb1 protein, and part of intron 1. Two ES clones with normal karyotype were injected into C57BL/6 mouse blastocysts. The homozygous as well as the wild-type mouse stocks were maintained as a cross of C57Bl/6 and 129/Ola (50%/50%), and kept at a 12 hours dark / 12 hours dimmed light
cycle (100 lux). For multiplex-PCR genotype analysis, we detected the wild-type Crb1 allele using a sense primer JW1 (5’-CTGGGAGGGGTGGGACAG-3’) and an antisense primer JW105 (5’-AAAACCTGGACCAGAAGCGC-3’). These primer pairs amplify a 351-bp fragment from wild-type Crb1. The mutant allele was amplified using antisense primer JW105 and a sense primer JW123 (5’-GCTGTGTAGAAGTACTCGCCG-3’). All animals were treated according to guidelines established at the institutions in which the experiments were performed.

**Morphological, immunohistochemical analysis and confocal microscopy.**

Affinity purified rabbit polyclonal antibodies raised against the C-terminal 36 amino acids peptide of human CRB1 (AK7) and the C-terminal 19 aa peptide of mouse Crb1 (AK2), antibodies raised against the C-terminal WNLMPPPAMERLI amino acids (EP13), the extracellular GPAWEGPRCEIRAD amino acids (EP14) and cytoplasmic CARLEMDSVLKVPPEE amino acids domains of human Crb2, and internal 15 amino acids peptides (KCVEADETFESEEL) conserved between human MPP4 and mouse Mpp4 (AK4), were generated and purified following standard procedures. For immunostaining, the following antibodies were used: rabbit pAb’s anti-Crb1 (AK2, AK7, 1:250; 851, Dr. Tepass, 1:50), anti-Crb2 (EP13, EP14, 1:5000, Dr. P. Rashbass) (A1, 1:200, Dr. A. Le Bivic), anti-Crb3 (1:500, Dr. Margolis), anti-Mpp4 (AK4, 1:1000), anti-Mupp1 (1:500, Dr. Le Bivic), anti-Patj (1:100, Dr. Le Bivic or Dr. Margolis), anti-Pals1 (1:500, Dr. Margolis), anti-GFAP (1:250), anti-ZO-1 (1:500, Zymed, San Francisco, CA), mouse mAb’s anti-PKCα (cross-reacts with PKCδ, 1:250), anti-p120 (1:1000), anti-moesin (1:500), anti-Dig (1:500), anti-N-cadherin (1:250), anti-β-catenin (1:500), anti-Cdc42GAP (1:500) (Transduction Laboratories, Alphen aan de Rijn, The Netherlands), anti-pan-cadherin (CH-19, 1:500; Sigma), rat mAb anti-CD44 (1:200, Dr. van der Neut) and goat pAb’s anti-Par6 (N18, 1:100), anti-Par6 (T20; 1:100), anti-ZO-2 and anti-ZO-3 (1:500, SantaCruz). Secondary antibodies were conjugated with Cy3 (1:600) or Alexa 488 (1:500, Molecular Probes, Leiden, The Netherlands). Cone segments and pedicles were stained with peanut agglutinin-fluorescein (1:100, Vectorlabs, Burlingame, CA) and F-actin was stained by rhodamine-phalloidin (1:500, Molecular Probes) during incubation with the primary antibody.

Snap-frozen human eyes were obtained from the Cornea Bank Amsterdam, with a post mortum period of 8-24 hours. Cryosections (7 µm) were rehydrated in PB and blocked for 1 hour using 10% goat or donkey serum, 0.4% Triton X-100 and 0.1% BSA in PB. Murine eye-cups were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline, pH 7.4 for 30 minutes. After rinsing in PBS, eyecups were immersed in 5% sucrose in PBS, followed by 30% sucrose in PBS for 3 h. Tissue was blotted dry, embedded in TissueTek (Sakura Finetek, Zoeterwoude, The Netherlands) and frozen on dry-ice. Cryosections (7 µm) were rehydrated in PB and blocked for 1 hour using 10% goat or donkey serum, 0.4% Triton X-100 and 0.1% BSA in PB. Tissues for anti-Pals1 staining were permeabilized in 10% goat or donkey serum, 1% SDS and 0.1% BSA in PBS for 1 hour. Primary antibodies (see above) were diluted in 0.3% goat or donkey serum, 0.4% Triton X-100 and 0.1% BSA in PB and incubated for 16 h. Secondary antibodies were diluted in 0.1% goat or donkey serum in PB and incubated for 1 h at RT. Sections were then washed in PB, mounted using vectashield (Vectorlabs, Burlingame, CA). Sections
Crumbs homologue 1 is required for maintenance of photoreceptor cell polarization and adhesion during light exposure

were imaged on a Zeiss 501 confocal laser scanning microscope. Confocal images were processed with the Zeiss LSM image browser v3.2 and figures were assembled in Adobe Photoshop v7.0.

**PCR analysis of Rpe65 genotype at codon 450.**

Codon 450 is CTG encoding a leucine in light sensitive vs. ATG encoding a methionine in light insensitive mouse strains. We carried out PCR using primers and genomic DNA from mouse-tails, and determined their DNA sequences using an ABI prism 310 sequencer (ABI, Applera, Nieuwekerk a/d IJssel, The Netherlands). Details are available on request. A correlation between codon 450 polymorphism in Rpe65 and light-sensitivity in Crb1−/− or wild-type retinas was excluded.

**Light exposure and analysis of retinal damage.**

Prior to the start of the light exposure, all mice were maintained in a 12 hours dark / 12 hours dimmed light (100 lux) cycle. After a 12 hours dark period mice were continuously exposed for 72 hours to 3000 lux of diffuse white fluorescent light (TLD-18W/33 tubes, Philips; 350-700 nm) without pupillary dilation. A correlation between codon 450 polymorphism in Rpe65 and light-sensitivity in Crb1−/− or wild-type retinas was excluded. Immediately after light exposure the eyes were orientated and thereafter enucleated, fixed either for cryosectioning or for 3 μm sectioning in Technovit 7100 (Kultzer, Wehrheim, Germany). In the latter, the whole retina was examined for protrusions and ingressions. Statistical analysis was performed using the Mann-Whitney test. Significance was accepted if P < 0.05. For apoptosis analysis, the rhodamine in situ cell death detection kit (Roche, Mijdrecht, The Netherlands), a TdT-mediated dUTP nick-end labeling (TUNEL) assay, was used on 7 μm cryosections, according to the manufacturers suggested protocol and included 1:5 dilution of the labelling mix. As positive control, sections treated with DNase were used according to manufacturers protocol. Images were obtained with a Leica DMRD microscope and DC350F digital camera (Leica, Rijswijk, The Netherlands).

**Immunoprecipitation experiments.**

Retinas were isolated from 3 months old wild-type and Crb1−/− mice and homogenized in lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, 50 mM Tris-HCl, pH 7.4 containing a complete protease inhibitors cocktail). Crude lysates were cleared by centrifugation (10,000g for 15 minutes at 4°C) and were used for immunoprecipitation with AK2. Membrane fractions were prepared by homogenization of retinas in extraction buffer (10 mM NaCl, 3 mM MgCl2, 1 mM DTT, 1 mM PMSF, 1mM Na3VO4, 10 mM HEPES, pH 7.9 supplemented with a complete protease inhibitors cocktail) and subsequent differential centrifugation. Membrane fractions were resuspended in lysis buffer and cleared by centrifugation, the supernatants were used for immunoprecipitation with monoclonal anti-Mupp1 (Transduction Laboratories, Alphen aan de Rijn, The Netherlands). As a control, mouse IgG’s (Sigma) were used to perform immunoprecipitation. The precipitated proteins were washed 4 times with lysis buffer, eluted with sample buffer and analyzed by western blotting.
Electroretinography and scanning-laser ophthalmoscopy.

ERGs and SLOs were obtained according to previously reported procedures.39 Electroretinography. Briefly, following dark adaptation over night, mice were anesthetized with ketamine (66.7 mg/kg) and xylazine (11.7 mg/kg), and the pupils were dilated. The ERG equipment consisted of a Ganzfeld bowl, a DC amplifier, and a PC-based control and recording unit (Multiliner Vision; Jaeger-Toennies, Hoechberg, Germany). Band-pass filter cut-off frequencies were 0.1 and 3000 Hz. Single flash recordings were obtained both under dark-adapted (scotopic) and subsequently light-adapted (photopic) conditions. Light adaptation was achieved with a background illumination of 30 cd/m² starting 10 minutes before photopic recordings. Single flash stimulus intensities were increased from 10-4 cd*s/m² to 25 cd*s/m², divided into ten steps of 0.5 and 1 log cd*s/m². Ten responses were averaged with an inter-stimulus interval (ISI) of either 5s or 17 s (for 1, 3, 10, 25 cd*s/m²).

Fundus imaging was performed with a HRA scanning-laser ophthalmoscope (SLO; Heidelberg Instruments, Heidelberg, Germany) that provides two Argon wavelengths (blue, 488 nm, and green, 514.5 nm) and an infrared diode laser (690 nm) for fundus visualization. The confocal diaphragm of the SLO allows imaging of different planes of the posterior pole, ranging from the surface of the retina down to the retinal pigment epithelium (RPE) and the choroid. Different planes can be viewed by varying the focus by about +/- 20 diopters.
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Figures

Figure 1. Confocal images of 3 months old wild-type mice and human retinas. These images are high power insets of the OLM. (A) Crb1 is confined to the SAR, while β-catenin localized more basally at the AJ. (B) Crb1 localized at the basal part of the F-actin localization in the PRC inner segments. Mpp4 (C), Pals1 (D), Mupp1 (E) and Patj (F) localized at the SAR, compared to the location of β-catenin at the AJ. (G) aPKC co-localized with Crb1 to the SAR. (H-I) Crb2 and Crb3 localized to the SAR. A similar staining was detected in Crb1-/- retina. (J) CD44 localized in the MGC apical villi, but did not co-localize with Crb1 in the SAR. Par6, moesin or ZO-3 were not detected in the OLM (data not shown). Claudin-1 to –5 and occludin were not detected in the retina, but claudin-2 and occludin were detected in the retinal pigment epithelium (RPE). (K) CRB1 localized at the SAR of the human retina, apical to β-catenin in the AJ. (L) MUPP1 localized at the SAR of the human retina. (M) Schematic diagram of the localization for the different proteins at the SAR or AJ. Bars represent 2.5 μm.
Figure 2. Mupp1 immunoprecipitation (IP) co-precipitates Crb1, Mpp4 and Pals1. Crb1 was co-immunoprecipitated from retinal lysates of wild-type but not Crb1−/− mice. Patj was not found in the precipitated protein complex but was present only in the total retinal lysate (L). Anti-Pals1 detected a Pals1 doublet. In the control (Pre), mouse IgG’s were used for immunoprecipitation.
Crumbs homologue 1 is required for maintenance of photoreceptor cell polarization and adhesion during light exposure

Figure 3. Generation of Crb1−/− mice and confocal images of mouse retinas. (A) Crb1 disrupted by insertion of the targeting vector. E1, exon 1; E2, Exon 2; pA, polyadenylation signal; PGK, phosphoglycerate kinase promoter; B, BamHI; RV, EcoRV; Bg, BglII. (B-C) Deletion of the exon encoding the N-terminal signal peptide prevents the production of Crb1 protein with C-terminal transmembrane and intracellular domains. (B) EcoRV Southern blot analysis using a 750 bp BglII-AccI fragment probe in the 3' flanking region. (C) Immunoprecipitation of Crb1, with AK7, from lysate of wild-type but not of the Crb1−/− retina. As positive control 293/CRB1 cell lysates were used. Crb1 was stained using AK2. Asterisks represent cross-reacting bands with AK2 in 293 cells. (D-G) Localization of Crb1 (red) in the OLM and staining of cone segments and pedicles by peanut agglutinin (green) in the retinas of wild-type (D) and Crb1−/− (F) mice. Detail of the localization of Crb1 at the SAR for the wild-type (E) and Crb1−/− retina (G). Bars represent 30 μm.
Figure 4. Retinal phenotype of Crb1<sup>−/−</sup> mice exposed to cycled light (12 hours dark – 12 hours 100 lux). (A-C) Different stages of degeneration in 3 months old Crb1<sup>−/−</sup> retinas. (A) Start of degeneration in which some PRCs protrude through the OLM and ingress into the OPL. (B) More PRCs are involved in larger ingression areas and PRCs ingressed onto the INL. (C) Ingressed PRCs re-aggregate into half rosette structures, and form new inner segments and an OLM (arrow). (D-E) Morphology of 6 months old Crb1<sup>−/−</sup> retinas. (D) Formation of a giant half rosette of PRCs with outgrown inner-segments. Note the presence of cells from the INL, possibly MGC nuclei, closely localized to the edges of and inside this structure (arrowheads). (E) Complete degeneration of the ONL, presence of ghost structure (arrow) and ingression of RPE into the retina (arrowheads). Bar represents 100 µm.
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Figure 5. Retinal phenotype of 3 months Crb1^-/- old mice exposed to 72 hours 3000 lux. (A) Representative experiment indicating number of protrusions, ingressions and total amount in cycled light (12 hours dark/12 hours 100 lux) vs. 72 hours 3000 lux exposed wild-type and Crb1^-/- retinas. Error bars represent SEM. Asterisks indicate statistical difference (P<0.02) between the cycled and 72 hours 3000 lux exposed groups. (B) Numerous ingressions areas through the OPL (arrow) and protrusions through a distorted OLM (arrowhead) in the Crb1^-/- retina. (C) OLM present in rosette (arrow), no segments are present on the disorganized PRCs (arrowhead). (D-J) Fluorescence microscopy images of degenerated areas. Nuclei are stained with Hoechst (blue). Note that in unaffected areas, adjacent to the ingestion areas, localization of the proteins is normal. In both cycled and 72 hours 3000 lux of light exposed wild-type mice GFAP localized near the inner limiting membrane, at the MGC end-feet and in horizontally radiating MGC rootlets in the OPL (data not shown). (E) Detail of (D), strong staining of GFAP in the ONL and through OLM. (F) Areas of protrusions (arrowheads) and ingressions (arrows) where Mpp4 is lost at the OLM and OPL as well as mislocalized into the ONL (asterisk). (G) β-Catenin mislocalized throughout the ONL in ingestion areas (arrows). (H) ZO-2 localization perturbed in a protrusion and ingestion area. (I-J) Mislocalized and loss of Patj or Mupp1 in affected areas (arrowheads). (K) Apoptotic cells are rarely present in WT retinas. (L) Slightly increased apoptosis in Crb1^-/- mice around ingestion areas. Bars represent 50 µm. (M) SLO image (514.5 nm) of a 3 months old 72 hours 3000 lux exposed Crb1^-/- mouse fundus. The multiple dots (arrowhead) indicate areas of rosette formation in the inferior temporal quadrant of the retina.
Figure 6. Electronmicroscopic image of the region containing the outer limiting membrane in a 3 months old $Crb1^{-/-}$ retina.
Arrow indicates an electron dense adherens junction, which are similar as the adherens junction in the wild-type mouse. M= apical villi of a Müller glia cell, IS= inner segment of the photoreceptor cell, PRC= photoreceptor cell body. Bar represents 0.5 $\mu$m.

Figure 7. Electrophysiology of 9 months old $Crb1^{-/-}$ mice.
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(A) Scotopic b-wave amplitude vs. log Intensity (VlogI) function. Boxes indicate the 25% to 75% quantile range, whiskers the 5% and 95% quantiles, and the asterisk the median of the Crb1−/− data. The normal range is delimited by solid lines indicating the 5% and 95% quantile of the control group. There is no sign of impaired retinal function in Crb1−/− mice.

(B) Typical scotopic intensity series in a wild-type (right column) and a Crb1−/− mutant mouse (left column). Log light intensities (from top to bottom) were -4, -3, -2, -1.5, -1, -0.5, 0, 0.5, 1, 1.5 log cd*s/m². No differences in amplitude or waveform are visible. (C-D) Electrophysiological results in 3 months old light exposed Crb1−/− mice. Also under these conditions, there is no sign of impaired retinal function in Crb1−/− mice. No differences in amplitude or waveform are visible. Calibration marks: Vertical 200 μV/div., horizontal 40 ms/div.

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Chapter 2

References


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MPP5 recruits MPP4 to the CRB1 complex in photoreceptors

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\(^\dagger\) Contributed equally to this work and should therefore be considered equivalent authors.

### Abbreviations List

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AJ</td>
<td>adherens junctions</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>Cask</td>
<td>calcium-calmodulin-dependent serine kinase</td>
</tr>
<tr>
<td>CRB1 and Crb1</td>
<td>human and mouse Crumbs homologue 1</td>
</tr>
<tr>
<td>FERM</td>
<td>4.1/ezrin/radixin/moesin</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>GuK</td>
<td>guanylate kinase</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HOOK</td>
<td>variable hinge (flexible region)</td>
</tr>
<tr>
<td>INL</td>
<td>inner nuclear layer</td>
</tr>
<tr>
<td>IS</td>
<td>inner segments;</td>
</tr>
<tr>
<td>L27</td>
<td>domain found in Lin2 and Lin7 proteins</td>
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<tr>
<td>LCA</td>
<td>Leber congenital amaurosis</td>
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<td>MAGUK</td>
<td>membrane associated guanylate kinase protein;</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
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<td>MPP</td>
<td>membrane-associated palmitoylated protein;</td>
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<tr>
<td>MPP4</td>
<td>membrane palmitoylated protein 4</td>
</tr>
<tr>
<td>MUPP1</td>
<td>multiple PDZ domain protein 1</td>
</tr>
<tr>
<td>OLM</td>
<td>outer limiting membrane</td>
</tr>
<tr>
<td>ONL</td>
<td>outer nuclear layer</td>
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<tr>
<td>ONPG</td>
<td>ortho-nitrophenyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>OPL</td>
<td>outer plexiform layer</td>
</tr>
<tr>
<td>OS</td>
<td>outer segments;</td>
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<tr>
<td>Pals1</td>
<td>protein associated with Lin-7</td>
</tr>
<tr>
<td>PATJ</td>
<td>protein associated with tight junctions</td>
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<tr>
<td>PDB</td>
<td>protein data base</td>
</tr>
<tr>
<td>PDZ</td>
<td>postsynaptic density 95 / discs large / zonula occludens 1;</td>
</tr>
<tr>
<td>PPRPE</td>
<td>para-arteriolar preservation of the retinal pigment epithelium</td>
</tr>
<tr>
<td>Psd95</td>
<td>post synaptic density protein 95</td>
</tr>
<tr>
<td>Rf</td>
<td>reading frame</td>
</tr>
<tr>
<td>RP</td>
<td>retinitis pigmentosa</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigment epithelium</td>
</tr>
<tr>
<td>SAR</td>
<td>subapical region</td>
</tr>
<tr>
<td>SH3</td>
<td>Src-homology-3</td>
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<tr>
<td>ZO</td>
<td>zonula occludens</td>
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Abstract

**Purpose.** Mutations in the human *Crumbs homologue 1 (CRB1)* gene are a frequent cause of Leber congenital amaurosis (LCA) and various forms of retinitis pigmentosa. CRB1 is thought to organize an intracellular protein scaffold in the retina that is involved in photoreceptor polarity. This study was focused on the identification, subcellular localization and binding characteristics of a novel member of the protein scaffold connected to CRB1.

**Methods.** To dissect the protein scaffold connected to CRB1, the yeast two-hybrid approach was used to screen for interacting proteins. Glutathione S-transferase (GST) pull-down analysis and immunoprecipitations were used to verify protein-protein interactions. The subcellular localization of the proteins was visualized by immunohistochemistry and confocal microscopy on human retinas, and immunoelectron microscopy on mouse retinas.

**Results.** A novel member of the scaffold connected to CRB1, called membrane palmitoylated protein (MPP) subfamily member 4 (MPP4), a membrane associated guanylate kinase (MAGUK) protein, was identified. MPP4 was found to exist in a complex with CRB1 through direct interaction with the MPP subfamily member MPP5 (PALS1). 3D homology modeling provided evidence for a mechanism that regulates the recruitment of both homo- and heterodimers of MPP4 and -5 proteins to the complex. Localization studies in the retina show that CRB1, MPP5 and MPP4 co-localize at the outer limiting membrane (OLM).

**Conclusion.** These data imply that MPP4 and -5 have a role in photoreceptor polarity and, by association with CRB1, pinpoint the cognate genes as functional candidate genes for inherited retinopathies.

Introduction

The polarized organization of photoreceptor cells is a fundamental feature of the developing retina. Polarity can be either a dynamic event where proteins are shuttled across the cell or a more static process in which proteins are clustered in complexes and retained at particular subcellular locations.\(^1,2\) Detailed studies on the factors that play a role in this site-specific localization could provide knowledge about the general pathways that establish and maintain retinal polarity. It can also help us to understand the pathological pathways in the retina that are triggered by mutations in genes that encode components of such complexes.

Several members of the membrane associated guanylate kinase (MAGUK) protein family are involved in cell polarity through their role in large multiprotein complexes at tight junctions.\(^3,4\) This protein family is characterized by a specific set of protein-binding domains, consisting of one or more PDZ (postsynaptic density 95 / discs large / zonula occludens 1) domains, a SH3 (Src homology 3) domain and a region with homology to a guanylate kinase (GuK) domain.\(^5,6\) Some members such as PALS1
(assigned the name MPP5 by the HUGO Gene Nomenclature Committee; gene.ucl.ac.uk/nomenclature; hosted by University College London. London, UK), ZO1 and PSD-95, have been shown to be localized at sites of cell-cell contact (e.g., synapses and epithelial tight junctions). It is currently assumed that MAGUK proteins serve as scaffolds by recruitment of other MAGUKs, eventually linking them to the cell cytoskeleton or to the carboxyl-terminus of transmembrane proteins.

The MAGUK protein Stardust is the *Drosophila* homologue of MPP5, one of the seven mammalian membrane palmitoylated protein (MPP) subfamily members. *Stardust* mutants exhibit severe disruption in apicobasal polarity of embryonic epithelia. In addition, mutants of the zebra fish homologue *Nagle oko* display severe defects in the organization of the retinal cell layers, and loss of Stardust gives rise to an eye phenotype in *Drosophila* characterized by a shortened stalk membrane and altered rhabdome morphogenesis resembling the Crumbs mutant phenotype.

Crumbs is an apically localized transmembrane protein involved in organizing the apical plasma membrane subdomains. Stardust was found to co-localize with Crumbs and interact physically with the C-terminal ERL1 motif of Crumbs via its PDZ domain. This interaction was recently also identified for their mammalian homologues MPP5 and CRB1 as well as for MPP5 and CRB3. Stardust and Crumbs are both necessary to ensure stability, localization and function in controlling the apicobasal polarity of epithelial cells. The Crumbs-Stardust protein complex also recruits the *Drosophila* protein associated with tight junctions (DPATJ; formerly known as Discs lost). This cytoplasmic multi-PDZ domain protein interacts indirectly, via Stardust, with the cytoplasmic tail of Crumbs. These three proteins co-localize in *Drosophila* photoreceptors during and after eye development. The mammalian CRB1-MPP5-PATJ complex localizes to tight junctions where it may control cell polarity. In the mouse retina, the CRB1-MPP5-PATJ proteins co-localize at the apical region adjacent to adherens junctions of photoreceptors. The *Drosophila* Crumbs protein and the human homologue CRB1, are 35% similar in amino acid sequence and contain the same conserved protein motifs. Mutations have been identified in the *CRB1* gene in individuals with Leber congenital amaurosis (LCA), retinitis pigmentosa (RP) type 12, with preservation of para-arteriolar retinal pigment epithelium (PPRPE), RP with Coats-like exudative vasculopathy and early onset RP without PPRPE. CRB1 has been found to maintain adherens junctions between photoreceptor cells and Müller glia cells, thus preventing delamination of the photoreceptor layer and death of retinal neurons.

In this article we identified a second MPP subfamily member, MPP4, existing in a complex with CRB1 through direct interaction with MPP5. The presence of MPP4 and -5 in this CRB1 protein complex implicates these proteins in photoreceptor polarity, and putatively in inherited retinal dystrophies.

**Results**

**Screening for Interaction Partners of CRB1**

The conserved putative PDZ and FERM protein-binding regions in the intracellular domain of CRB1 suggest target epitopes for different interactions in this relatively small
domain. Therefore, we used this domain as a bait to screen bovine and human yeast two-hybrid retina cDNA libraries. We identified only MPP5 as an interacting protein from the bovine randomly primed library (13 clones out of $1.4 \times 10^7$ cotransformants analyzed). A highly saturated screen of the human oligo-dT-primed retina cDNA library ($1.0 \times 10^7$ cotransformants analyzed) did not reveal any interactors.

**Identification of a Novel Interactor with MPP5**

Although the MAGUK proteins in general and the MPP family of proteins in particular contain different conserved putative protein–protein interaction domains that allow the buildup of a scaffold, only a few of these domains in MPP5 have known ligands (Fig. 1). We used the conserved epitopes of human MPP5, for which no partners have yet been identified, as baits in yeast two-hybrid screens of a human oligo-dT-primed retina cDNA library. We did not identify interactors for the coiled-coil domain (amino acid [aa] 1-122) nor for the GuK domain (aa 470-675; data not shown). However, a bait construct containing the SH3-HOOK region of human MPP5 was found to interact with MPP4. Four different clones containing the C terminus of MPP4 were identified, with the GuK domain flanked by strands E and F (Fig. 1), starting at aa 319 (#228), aa 365 (#73), aa 368 (#221) and aa 390 (#78) (Fig. 2A). The binding affinities of these different clones for MPP5 were measured semiquantitatively in a liquid ortho-nitrophenyl-β-D-galactopyranoside (ONPG) assay, revealing that the peptide stretch of MPP4 containing the regions E, GuK, and F is essential for the interaction. However, when more amino acids were present at the N terminus, the binding affinity increased.

**Association of MPP4 and -5 In Vitro and In Vivo**

The interaction between MPP4 and -5 was confirmed in a GST pull-down assay (Fig. 2B). GST-MPP5\textsuperscript{SH3-HK} fused to glutathione-Sepharose efficiently pulled down His-MBP-MPP4\textsuperscript{365-637} (Fig. 2B).

Furthermore, to test for a physical interaction between MPP4 and -5 in the presence or absence of CRB1, we used 293HEK cells overexpressing MPP4, and/or MPP5, and/or CRB1 in immunoprecipitation experiments (Figs. 2C, 2D). Anti-FLAG antibody coimmunoprecipitated MPP5 from cells overproducing 3xFLAG-MPP4 and MPP5, as well as from cells overproducing 3xFLAG-MPP4, MPP5, and CRB1 (Fig. 2C). The 80-kDa recombinant and the endogenously expressed 70-kDa MPP5 coimmunoprecipitated with MPP4 (Fig. 2C). In a reciprocal experiment, anti-MPP5 antibody SN47 coimmunoprecipitated MPP4 from cells overproducing 3xFLAG-MPP4 and MPP5, as well as from cells overproducing 3xFLAG-MPP4, MPP5, and CRB1 (Fig. 2D). Coimmunoprecipitation of MPP4 or CRB1 with immunoprecipitated endogenous 70- and 80-kDa MPP5 was below detection levels (Fig. 2D, and data not shown). Recombinant MPP5 was efficiently immunoprecipitated by SN47 from cell lysates overproducing MPP5, whereas endogenous MPP5 was not (data not shown). By Western blot analysis we could not detect endogenous expression of CRB1 (data not shown). These results show that MPP4 interacts with MPP5 in the absence as well as presence of CRB1. To test for the presence of a protein complex containing MPP4 and CRB1, we used 293HEK cells overexpressing 3xFLAG-MPP4 and/or myc-CRB1. Anti-MPP4 antibody (AK4)
immunoprecipitated MPP4 from 3xFLAG-MPP4-overproducing cell lines (Fig. 2E), and coimmunoprecipitated CRB1 from cells overproducing 3xFLAG-MPP4 and myc-CRB1 (Fig. 2F), though at a much lower level.

In a reciprocal experiment, anti-myc antibody immunoprecipitated CRB1 from myc-CRB1 overproducing cell lines (data not shown), and coimmunoprecipitated MPP4 from cells overproducing 3xFLAG-MPP4 and myc-CRB1 at similar low levels (Fig. 2G). This result, as well as the anti-Flag immunoprecipitation of MPP4, indicates the presence of MPP4 and CRB1 in the same complex, though probably through endogenously expressed MPP5. The calculated size of CRB1 was 154 kDa, whereas CRB1 polyclonal antibodies recognized a protein >220 kDa on Western blot from 293HEK/CRB1 and MDCKII/CRB1 cells. The PROSITE program predicted 23 putative N-glycosylation sites (Swiss Institute of Bioinformatics). Therefore, we tested to see whether CRB1 is glycosylated in MDCKII/CRB1 cells. Incubation with tunicamycin shifted the molecular weight toward the expected 154 kDa (Fig. 2H), indicating extensive N-glycosylation of CRB1. The endogenous Crb1 from mouse retina appears to be more than 220 kDa, whereas the calculated molecular weight is 153 kDa. This suggests that N-glycosylation also occurs in vivo in mouse retina.

**Computer-Based Molecular Dynamics and In Vivo Interaction of Homo- and Heterodimers of MPP4 and -5**

The interacting domains of MPP4 and -5 were analyzed by using a panel of deletion variants of both proteins in the yeast two-hybrid system (Fig. 3A, above dotted line). A fragment of MPP5 containing the SH3-HOOK region, flanked by strands A and D, specifically interacts with a fragment of MPP4 containing the GuK domain, flanked by strands E and F. When either of these strands was absent, the interaction was fully disrupted (data not shown). Furthermore, no interaction was found between the SH3-HOOK region of MPP5 and the full-size SH3-GuK module of MPP4, or between the full-length MPP5 and -4 proteins (data not shown). These results are analogous to the intramolecular interactions reported for the MAGUK protein PSD-95. In PSD-95, the interaction between the SH3 and GuK domains can either occur within a single peptide chain or between separate peptide chains. It has been proposed before that the interactions observed in PSD-95 are a conserved feature among MAGUK proteins. A similar mechanism could thus be expected for MPP4/MPP5 based on our experimental results and the high percentage of sequence identity to PSD-95, especially at the domain interface. To analyze this possibility, we built 3D homology models of the SH3-GuK domains of MPP4 and -5 (Fig. 3B, left), based on the crystal structures of PSD-95. All four permutations of domain interactions were analyzed: MPP4$^{SH3}$-MPP4$^{GuK}$, MPP4$^{SH3}$-MPP5$^{GuK}$, MPP5$^{SH3}$-MPP4$^{GuK}$, and MPP5$^{SH3}$-MPP5$^{GuK}$. In all cases, the domain interfaces were stabilized by salt bridges and hydrophobic interactions, most prominently by a conserved tyrosine (Y413 in MPP4 and Y466 in MPP5) that forms the core of the SH3 domain, but belongs to the E-strand just before the GuK domain (Fig. 3B, below dotted line). The domain binding energies of the four models are listed in Table 2.

The binding energies of the SH3-GuK modules of MPP4 and -5 are predicted according to the NOVA energy function (in kilocalories per mole). Results were obtained
by subtracting the NOVA energy of the SH3/GuK complex from the energies of separated SH3 and GuK modules; higher energies thus indicate better binding. The highest binding energy (147 kcal/mol) is predicted for the SH3/GuK self-interaction in MPP4. This matches our experimental finding in the yeast two-hybrid system that a full-length MPP4 construct did not bind to MPP5 (data not shown), but obviously prefers the self-interaction. The second strongest binding energy was obtained for MPP5^{SH3}\_MPP4^{GuK}. This model contains several salt bridges that are either missing or less pronounced in the remaining two models (Fig. 3B, right). Indeed, the MPP5^{SH3}\_MPP4^{GuK} interaction was the one we first discovered experimentally in the yeast two-hybrid screening (Fig. 3A). To validate the model further, we analyzed the binding affinities of the SH3 and GuK domains in MPP4 and -5 (Fig. 3A, right). We were able to confirm in the sensitive liquid ONPG assay that the MPP4 domains interact more strongly than the ones in MPP5.

**Expression of CRB1, MPP5, and MPP4**

Expression analysis by semiquantitative RT-PCR on a panel of RNAs from several human tissues showed that CRB1 is predominantly expressed in the retina. An increase in the number of PCR cycles identified a lower level of expression in brain, testis, and fetal eye (Fig. 4). MPP5 is more ubiquitously expressed and is also present in the retina. MPP4 is highly expressed in the retina. An increase in the number of PCR cycles identified a lower level of expression in brain, testis, ARPE cell line and fetal eye.

Low levels of MPP4 RNA were also detected in human retinal pigment epithelium, but MPP4 protein was below detection levels in human and mouse RPE.

**Immunolocalizations of CRB1, MPP4, and MPP5 in Human Retina**

Immunohistochemistry and confocal laser scanning microscopy were used to determine the subcellular protein localization of CRB1, MPP4, and MPP5. Anti-CRB1 antibodies AK2 and -5 detected CRB1 at the outer limiting membrane (OLM) of human retina. Using monoclonal antibodies against human β-catenin as a marker for the adherens junction, localization of CRB1 apical to the adherens junction was detected (Figs. 5A-D). Anti-MPP5 antibodies (SN47) detected the protein at the OLM, also apical to the AJ (Figs. 5E-H), where it colocalized with CRB1 (Figs. 5I-L). MPP4 was detected at the OLM and in the outer plexiform layer (OPL) of human retina with AK4 (and AK8) antibodies. At the OLM, MPP4 was detected apical to the adherens junction. However, the intensity of the signal at the OLM was much lower than the MPP4 staining intensity in the OPL (Figs. 5M-Q).

**Ultrastructural Localization of Mouse Mpp4 in Retina**

Mpp4 was detected with immunoelectron microscopy at the OLM and in the cone pedicles and rod spherules. At the OLM, Mpp4 was present apically to and at the adherens junction contacts (Figs. 6A, 6E). Staining was also detected at membranes of the Golgi area (Figs. 6A, asterisk, 6D) and other small and large vesicles in the inner segments of photoreceptors. In rod photoreceptors, there was a strong association of the
signal with the lateral plasma membranes of the spherules (Figs. 6B, 6F). In the cones, the Mpp4 signal was concentrated at the basal side, which comprises the contacts with horizontal and bipolar cells and also at the lateral side of the pedicle plasma membrane (Figs. 6C asterisk, 6G). In both types of photoreceptors, Mpp4 staining was also associated with vesicles proximal to the presynaptic membrane.

The ultrastructural study showed that retinal Mpp4 is restricted to the photoreceptors. We did not detect any protein in the Müller (Fig. 6E), bipolar, horizontal, or other neuronal retinal cell types. This finding correlates with in situ hybridization analysis on mouse retina showing distribution of mRNA coding for Mpp4 protein in the photoreceptor inner segments and outer nuclear layer (ONL).46,47 We conclude that Mpp4 is localized in rod and cone photoreceptors at the plasma membrane and at membranes of intracellular vesicles around the subapical region (SAR) and adherens junctions, and OPL.

Discussion

MPP4 as a Member of the CRB1 Protein Scaffold

Recent findings have emphasized the central role of CRB1 and its intracellular interactor MPP5/PALS1 in the regulation of epithelial polarity. However, the larger part of both CRB1, containing a putative FERM-binding motif, and MPP5, containing the additional MAGUK modules, have not yet been described to have a role in this process. The putative protein–protein-interacting capacity of these domains in any tissue was still to be shown. This motivated us to search for interactors with these specific domains in the retina by using a yeast two-hybrid approach. In this study, our results showed that the MAGUK protein MPP4 was recruited to the MPP5/CRB1 complex through direct binding of the SH3-GuK modules of both MPP family members. These three proteins form a multiprotein complex at the OLM of the retina. Using antibodies directed against the multiple PDZ protein Mupp1, we recently showed coimmunoprecipitation of endogenous Mpp4, Mpp5, and Crb1 from mouse retinal lysates of wild-type mice and coimmunoprecipitation of MPP4 and -5 from lysates of Crb1 knockout mice.24 We now have identified MPP4 as a binding partner for the SH3-HOOK region of MPP5.

Mechanism of Interaction and Implications for Regulation

The interaction between MPP5 and -4 involves the C-terminal end of MPP4. Alternative interaction through L27 domain dimerization was excluded. In a yeast two-hybrid experiment, no L27 domain binding was found between MPP4 and -5, whereas the L27 domains of PATJ and MPP5 did show interaction (data not shown).

Detailed analysis revealed that the E and F strands flanking the GuK domain of MPP4 are essential for binding. This is in full agreement with the folding according to the crystal structure of PSD-95.39 45 In PSD-95, the SH3-HOOK region interacts both in cis and trans with the E-GuK-F region in vitro. The intramolecular interaction initially prohibits intermolecular interactions by preventing a mechanism called 3D domain swapping. Domain swapping allows proteins to assemble dimers or higher order oligomers by
exchanging complementary substructures. The need of a cofactor or modulator has been postulated to switch the preference from intra- toward intermolecular interactions in vivo, thus enabling specific regulation of MAGUK multimerization at the cytoplasmic membrane.

The 3D homology modeling of MPP5 and -4 pointed to a PSD-95-like interaction mechanism that also matched the results of our yeast two-hybrid binding assays. GST pull-down analysis and immunoprecipitation experiments both confirmed the interactions biochemically and provided in vivo evidence for the proposed regulatory mechanism of heterodimerization. The full-length proteins that were the targets for immunoprecipitation did coprecipitate the full complex of CRB1, MPP4, and MPP5 from HEK293 cells, indicating that in these cells the regulatory factor is present (Figs. 2C 2D). Good candidates for regulation of the dimerization of MPP5 are members of the 4.1 protein family, as the variable hinge or HOOK region of MPP5 contains a conserved 4.1 binding motif. MPP4 does not contain this particular conserved motif in the same region, but does have a predicted -helical stretch. We propose that regulation of dimerization of these MAGUK proteins is one of the factors that governs a dynamic variation of proteins that are present at this polarity-associated protein scaffold.

SH3 domains of tyrosine kinases are usually involved in protein–protein interactions by binding to proline-rich sequences, and have been described to couple substrates to enzymes, thereby regulating enzymatic activities. However, based on the 3D homology models presented herein and the interactions identified in this study, the SH3 module of MPP4 and -5 seems to be functionally different from the conventional ones. The SH3 domain of MPP5 interacts with the GuK domain of MPP4 as well as with its own GuK domain.

**Distribution of Protein Expression in the Human Retina**

Whereas MPP5 is expressed ubiquitously, both CRB1 and MPP4 genes are expressed more selectively in eye and brain. MPP4 RNA is also present in liver, spleen, heart, and testis, but at much lower levels. Previous experiments showed that Crb1 and Mpp4 RNA is expressed in the ONL and inner photoreceptor segments of the retina. Crb1 RNA was also detected at low levels in the inner nuclear layer.

The protein complex CRB1-MPP5-MPP4 localizes subapically to the adherens junction at the OLM of the retina. The localization of Mpp4/MPP4, Mpp5/MPP5, and Crb1/CRB1 appears to be conserved between mice and humans. Our results on MPP4 localization partially overlap with the positioning described for Mpp4 in mouse retina. Differences in genetic background or detection level may explain why Mpp4 was not detected in cones or OLM in previous studies and may explain the localization of MPP4 in the connecting cilia of bovine and porcine.

We observed that Mpp4 is detected at intracellular vesicles and at the plasma membrane. Mpp4 is also located at the presynaptic membrane and proximal vesicles. The localization of MPP4 at more than one functionally different cell structure suggests participation in
different protein complexes. Some MAGUK proteins target and anchor glutamate receptors to the synaptic terminals.\textsuperscript{57} It has been proposed that the complex involving the MAGUK protein CASK acts as a nucleation site for the assembly of proteins involved in synaptic vesicle exocytosis and synaptic junctions.\textsuperscript{58,59} It is tempting to speculate on possible functions of MPP4 in vesicle targeting or fusion complexes at the photoreceptor synapses and the region apical to the adherens junction.

**Implications for Inherited Retinal Degenerations**

Altogether, these facts provide strong evidence for the involvement of CRB1, MPP5, and MPP4 in a common pathway that determines the polarity of photoreceptors in the retina. Based on the recruitment of both MPP4 and -5 to the CRB1 protein scaffold, the disruption of retinal lamination observed with loss of mouse Crb1\textsuperscript{24} and the zebra fish MPP5 homologue *Nagie oko*\textsuperscript{14} and the high expression of MPP4 in the retina, we propose that *MPP5* and *MPP4* are functional candidate genes for inherited retinal degenerations.

The *MPP4* gene has been screened for mutations in RP with 300 patients, and it was mapped in a locus for autosomal recessive retinitis pigmentosa (RP26 locus) at 2q31-33, but no mutations were identified. Recently, mutations in the neighboring *CERKL* gene were found to cause RP26.\textsuperscript{60} However, based on the more severe phenotype that is often observed in patients with mutations in *CRB1*, *MPP4* remains a candidate gene for similar eye disorders. Mutational changes in members of the complex that are closely linked could lead to a similar disrupting effect of the protein complex in the retina. Mutation analysis in selected patient panels could reveal the involvement of either *MPP4* or *MPP5* in inherited retina disorders.

**Materials and Methods**

**DNA constructs**

Human retinal cDNA (Marathon Ready; BD-Clontech, Palo Alto, CA) or mouse B6D2 retinal cDNA synthesized with a cDNA amplification kit (Marathon; BD-Clontech), was used to amplify the full-length cDNAs for human *CRB1*, *MPP5*, *MPP4*, and mouse *Mpp4* with a cDNA PCR kit (Advantage; Clontech). For human *CRB1* the following primers were used: 5'-GGGATCCAAATACCACCATGGGACTTTAAAGAACATTAC-3' (sense) and 5'-GATCCTCGAGACTCTCCTTCTCCATGGCAGG-3' (antisense). Italic sequences denote start and stop codons of the gene. Two consecutive Myc tags were inserted at amino acid (aa) position 1331 with the following primers: 5'-GCGAACAAAAATACCATCTTCTCAGAAGAGATCTG-3' (sense), and 5'-GCAGATCCTCTTCTGAGATGAGTTTTGTC-3' (antisense). The human *MPP5* PCR was performed with primers 5'-GATCCCGGGCCATCTCAGAAGAGGATCTG-3' (sense), and 5'-GCGAGATCCTCTTCTGAGAAGATCTGTTTC-3' (antisense). The human *MPP5* was synthesized with primers 5'-GATCCCGGGCCATCAGAAGAGGATCTG-3' (sense) and 5'-GATCGTGCAGACTCAGAAGGATCTGAC-3' (antisense). Human *MPP4* was synthesized with primers 5'-GATCCCGGGCCATCAGAAGGAGGACAG-3' (sense) and 5'-GATCGTGCAGACTCAGAAGGACAG-3' (antisense), and mouse *Mpp4*...
with 5'-GATCCCGGGCCATCAGTCTAGATGTCGAGGAAACC-3' (sense) and 5'-GATCGTCGAC-3' (antisense). A 3xFLAG epitope tag was created at the N terminus of human MPP4 with the following primers 5'-GACTACAAAGACCATGACGTTACATCGATGCTAGGAGAGCAG-3' (sense), and 5'-GTACAGCTTGTCATCGTCTTATAAATCGATGTACATGCTTATAATCAC GTCATGGTCTTTGTAGTC-3'(antisense).

The following constructs were made by PCR using the a cloning system (Gateway; Invitrogen, Groningen, The Netherlands), according to the manufacturer’s procedures, using full-length constructs as a template: the intracellular domains of bovine CRB1 (bCRB1\textsubscript{intra}) and human CRB1 (hCRB1\textsubscript{intra}); the full-length human MPP5 (MPP5\textsubscript{FL}); the SH3 and HOOK domains of human MPP5 (MPP5\textsubscript{SH3+HK}); the C terminus of human MPP5 from the HOOK domain (MPP5\textsubscript{HK-end}); the PDZ domain of human MPP5 (MPP5\textsubscript{PDZ}); the coiled-coil domain of human MPP5 (MPP5\textsubscript{CC}); the GuK domain of human MPP5 (MPP5\textsubscript{GuK}); full-length human MPP4 (MPP4\textsubscript{FL}); the PDZ domain of human MPP4 (MPP4\textsubscript{PDZ}); the C terminus of MPP4 containing the E domain and GuK domain (MPP4\textsubscript{Edom-end}); the C terminus of MPP4 (MPP4\textsubscript{365-637}); the SH3 and HOOK domains of human MPP4 (MPP4\textsubscript{SH3+HK}); and the C terminus of MPP4 from the SH3 domain (MPP4\textsubscript{SH3-end}). Gene-specific primers that were used to make these constructs are listed in Table 1. The attB1 and attB2 linkers were attached to the 5' end of, respectively, the sense and antisense primers.

The commercially adapted destination vectors pBD-GAL4/DEST and pAD-GAL4/DEST (Gateway; Invitrogen) were created by insertion of the blunt-ended reading frame (Rf) cassette B into, respectively, the EcoRI and SalI sites of pBD-GAL4-2.1-Cam (Stratagene, Amsterdam, The Netherlands) and the BamHI and SalI sites of pAD-GAL4-2.1 (Stratagene),\textsuperscript{32} with sticky ends previously filled in using Klenow (Invitrogen). The destination vectors pDest-15 (N-GST fusion tag) and pDest-17 (N-6xHis fusion tag) were purchased from Invitrogen. The pDest566 was constructed by introduction of a reading frame cassette (Gateway; Invitrogen) into a modified version of pET-43a (Novagen, Madison, WI) containing an amino terminal His6-maltose-binding protein tag (Esposito D, Hartley J, unpublished data, 2003). All novel constructs were verified by nucleotide sequencing.

**Yeast two-hybrid**

A GAL4-based yeast two-hybrid system (Hybrizap; Stratagene), with yeast strain PJ69-4a, was used to identify proteins that interact with CRB1\textsubscript{intra} and MPP5. The pBD-hMPP5\textsubscript{CC}, pBD-hMPP5\textsubscript{GuK}, and pBD-hMPP5\textsubscript{SH3+HK} constructs were used as baits on an oligo-dT primed human retina cDNA library, representing 2.1 x 10\textsuperscript{6} primary cDNA clones. In total, 8.2 x 10\textsuperscript{5}, 1 x 10\textsuperscript{6}, and 6.8 x 10\textsuperscript{5} clones were plated, respectively. The human and bovine pBD-CRB1\textsubscript{intra} constructs were used to screen respectively a pretransformed oligo-dT primed human and a randomly primed bovine retina cDNA library by yeast cell-to-cell mating, resulting in screening 1.0 x 10\textsuperscript{7} and 1.4 x 10\textsuperscript{7} clones.\textsuperscript{33} In subsequent yeast two-hybrid experiments, different domains of MPP5 and -4 were
tested for interaction by cotransformation into the YRG-2 yeast strain. Interactions were quantified in a liquid ortho-nitrophenyl-\(\beta\)-D-galactopyranoside (ONPG) assay for \(\beta\)-galactosidase activity.\textsuperscript{34}

**Antibodies**

Bacterially expressed full-length MPP5 protein was used for immunization of chicken. The yolk was processed with an IgY Purification Kit (Eggcellent Chicken; Pierce Biotechnology, Rockford, IL) according to the manufacturer’s protocol, and IgY antibodies were consequently affinity purified on a protein-coupled NHS-activated HP column (Hi-Trap; Amersham Biosciences, Roozendahl, The Netherlands).

Production AK2, AK5, AK7, AK4 and AK8 antibodies have been described.\textsuperscript{24} Anti-c-myc monoclonal mouse antibodies (clone 9E10) were purchased from Roche; anti-rat MUPP1 (clone 43) and anti-\(\beta\)-catenin (clone 14) mouse monoclonal antibodies from BD Biosciences (Leiden, The Netherlands); anti-6x His antibody from Santa Cruz Biotechnology (Heerhugowaard, The Netherlands); anti-FLAG monoclonal mouse antibody (clone M2), monoclonal anti-chicken IgG (clone CG-106), and rat monoclonal anti-uvomorulin (clone Decma-1) from Sigma-Aldrich (Amsterdam, The Netherlands).

Secondary antibodies conjugated to Alexa 488, Cy3, and Cy5 were obtained from Molecular Probes (Leiden, The Netherlands) and Jackson ImmunoResearch Laboratories (West Grove, PA). Secondary antibodies conjugated to horseradish peroxidase, were purchased from Sigma-Aldrich and Zymed (Uden, The Netherlands).

**Cell culture**

Human embryonic kidney (293HEK) and Madin-Darby canine kidney type II (MDCKII) cells were grown in DMEM (Invitrogen) containing 1% penicillin/streptomycin and 10% fetal bovine serum. Stably transfected MDCKII/CRB1 clones were generated by transduction of MDCKII cells with pBABE-CMV-Puro-CRB1 retroviruses, and subsequently selected with 2 \(\mu\)g/mL puromycin.

**GST-pull down, co-immunoprecipitation, and Western blotting**

Arabinose inducible BL21-AI cells were transformed with GST-hCRB1\textsuperscript{intra}/pDest15, or His-MBP-hMPP5\textsuperscript{PDZ}/pDest566 and IPTG-inducible BL21-DE3 cells with GST-hMPP5\textsuperscript{SH3+HK}/pDest15 or His-MBP-hMPP4\textsuperscript{HK-end}/pDest566. BL21-DE3 cell lysates were prepared according to a 1.5% sarkosyl protocol with DNase added before centrifugation.\textsuperscript{35} BL21-AI cell lysates were prepared (B-PER; Pierce Biotechnology, Etten-Leur, The Netherlands), with a protease inhibitor cocktail (Roche, Almere, The Netherlands) plus 1 \(\mu\)g/mL pepstatin A and 5 mM dithiothreitol (DTT). For GST pull downs, equal amounts of blocked (1.5 mg/mL BSA) glutathione Sepharose beads (4B; Amersham Pharmacia, Uppsala, Sweden) with glutathione S-transferase (GST), beads with GST fusion proteins, or beads alone were incubated with 0.5 mL of lysates containing His-MBP-fusion proteins for 2 hours at 4°C. After several washes with lysis buffer and TBS containing 1% Triton X-100 and 2 mM DTT, the beads were boiled, and proteins were resolved on SDS-polyacrylamide gels.
MPP5 recruits MPP4 to the CRB1 complex in photoreceptors

For coimmunoprecipitation experiments, 293HEK cells were transfected with pBabe-CMV-Puro/Hygro-CRB1/MPP5/MPP4, with a commercial reagent (Fugene 6; Roche) or calcium phosphate. After 48 hours, cells were lysed in 50 mM HEPES (pH 7.4), 150 mM sodium chloride, 10% glycerol, 0.5% Triton X-100, 1.5 mM magnesium chloride, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor cocktail (Roche), and 10 µg/mL aprotinin (Sigma-Aldrich). Either protein LA-agarose (Sigma-Aldrich) was used to bind the primary antibodies after incubation of precleared supernatants with 10 to 15 µg antibody for 4 to 16 hours at 4°C, or antibodies were precoupled to protein G beads (Dynabeads; Dynal Biotech ASA, Oslo, Norway) before incubation of supernatants for 2 hours at 4°C. For immunoprecipitation with anti-MPP5 antibody SN47, mouse monoclonal anti-chicken IgG was precoupled to the protein G beads (15 µg/reaction), followed by a second round of coupling of chicken anti-MPP5 antibody SN47 (10 µg/reaction) and incubation with cell lysates for 2 hours at 4°C. The beads were washed three times in 10% glycerol/PBS or lysis buffer, respectively and boiled in sample buffer with β-mercaptoethanol, and the immunocomplexes were resolved by SDS-PAGE. For Western blot analyses, proteins were electrophoretically transferred onto nitrocellulose membranes, which were then blocked, incubated with primary and secondary antibodies (conjugated to horseradish peroxidase) in 0.3% to 5% milk powder/TBS, and washed in TBS. The bands were visualized with a chemiluminescence reagent (ECL; Amersham Biosciences).

N-glycosylation experiments

After they reached 70% confluence, stable clones of MDCKII cells expressing hCRB1 were cultured for 1 to 5 days in DMEM supplemented with 1% penicillin/streptomycin, 10% fetal bovine serum, and 5 µg/mL tunicamycin dissolved in dimethylsulfoxide (DMSO) or only DMSO for the control cells.

Expression profiles

Total RNA was isolated from different human tissues and from an ARPE-19 cell-line (Dunn KC, et al. IOVS 1995;36:ARVO Abstract 766). For the semiquantitative RT-PCR, 3.1 µg RNA was reverse transcribed using random hexanucleotides. A touchdown PCR was performed for 28 and 33 cycles on 62 ng cDNA for CRB1, MPP5, MPP4, and the housekeeping gene GAPDH, which served as a standard. The following primer pairs were used: 5'-ACCAATGTATTCAACAGGGACC-3' (sense) and 5'-TCGTTTCCGTGTAGTGTTCTCC-3' (antisense) for CRB1, 5'-GTATGGAACTAGCATAGATTCTG-3' (sense) and 5'-CAAGATCGGAATTCAATTCGCC-3' (antisense) for MPP5, 5'-CACCTGTATGGCACTAGTGTGG-3' (sense) and 5'-CATACACCTCATATTGATGGC-3' (antisense) for MPP4, and 5'-ACCACAGTCCATGCCCATCAC-3' (sense) and 5'-TCCACCACCTGTGCTGTA-3' (antisense) for GAPDH.

Immunohistochemistry

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Eight human postmortem retinas, with enucleation times of 8 to 24 hours, were obtained from the cornea bank in Amsterdam and treated in accordance with the guidelines of the Declaration of Helsinki for the use of human tissue in research. Frozen human retina sections, 10 \( \mu \)m thick, were treated essentially as described previously using PBS buffer and 1% BSA. Sections were imaged on a confocal laser-scanning microscope (model 501; Carl Zeiss Meditec, Jena, Germany).

**Immunoelectron Microscopy**

Immunoelectron microscopy on mouse retina sections was performed as described previously.\(^{37}\) Ultrathin sections were examined and photographed (model 201 electron microscope; Phillips, Eindhoven, The Netherlands).

**Molecular modeling of MPP4 and -5**

The amino acid sequences of MPP4 (Swiss Prot accession number Q96JB8; http://www.expasy.org; provided in the public domain by Swiss Institute of Bioinformatics, Geneva, Switzerland) and MPP5 (accession number Q8N3R9) were submitted to the 3D-PSSM fold recognition server\(^{38}\) to search the protein data base (PDB) for homology modeling templates. The best hit in both cases (E-value 0.07) was PDB file 1KJW, the SH3-GuK module of Postsynaptic Density Protein 95 (PSD-95), solved at 1.8-Å resolution.\(^{39}\) With 40% sequence identity in the aligned regions, the modeling template PSD-95 can be expected to be very similar to the target structures,\(^ {40}\) except for structurally divergent loop regions. Consequently, homology models for MPP4 and -5 were built with What If,\(^ {41}\) using a backbone-dependent rotamer library\(^ {42}\) (see Fig. 3B, left side). Flexible HOOK residues (blue in Fig. 3B) were deleted, and the independent SH3 and GuK domains of MPP4 and -5 were arranged in all four possible permutations. Finally, the side-chain rotamers at the domain interface were optimized with YASARA (Yasara Biosciences, http://www.yasara.org/index.html) by minimizing the NOVA force field energy.\(^ {43}\) The parameters of the NOVA energy function have been optimized based on known high-resolution x-ray structures, so that the function has stable minima as close as possible to these structures. The relative domain-binding energies of the four models were then calculated as described previously.\(^ {44}\) Coordinate files of the models are available from the authors on request.
MPP5 recruits MPP4 to the CRB1 complex in photoreceptors

**Fig.1 Alignment of Stardust homologues**

Stardust was individually aligned with its closest human homologue MPP5 and with human and mouse MPP4. The percentages of identical and similar amino acid sequence of the conserved PDZ, SH3, and GuK domains are shown in the respective boxes. Stardust and MPP5 contain an additional HOOK domain. Strands A and D flanking the SH3 domain and E and F flanking the GuK domain were identified according to homology to PSD-95.
Fig. 2 Identification and confirmation of interactions between MPP5 and MPP4.

(A) Determination of the relative binding affinity between MPP5 and -4. YRG-2 yeast cells were cotransformed with the bait (SH3-HOOK region of MPP5) and different clones of MPP4 from the yeast two-hybrid screen. Activation of the LacZ reporter gene was determined by a liquid ONPG assay (β-galactosidase activity, black bars). In this assay, the pBD-GAL4 domain, fused to the intracellular domain of CRB1, which has no binding affinity for the PDZ domain of MPP4, was used as a negative control, showing basal levels of reporter gene activation. (B) Interaction between MPP5 and -4 was confirmed in a GST-MPP5<sub>SH3-HK</sub> pull-down from bacterial lysates expressing His-MBP-MPP4<sub>365-637</sub>. (C) Anti-FLAG antibody communoprecipitated MPP5 from cells overproducing 3xFLAG-MPP4 and MPP5, as well as from cells overproducing 3xFLAG-MPP4, MPP5, and CRB1-myc. Asterisk: an endogenous 70-kDa form of MPP5, commuinoprecipitated from all 3xFLAG-MPP4-overexpressing cells. (D) Anti-MPP5
antibody (SN47) coimmunoprecipitated MPP4 from cells overproducing 3xFLAG-MPP4 and MPP5, as well as from cells overproducing 3xFLAG-MPP4, MPP5, and CRB1-myc. (E) The anti-MPP4 antibody (AK4) immunoprecipitated MPP4 from cells overproducing 3xFLAG-MPP4 or CRB1-myc and 3xFLAG-MPP4. *Asterisk*: an unspecific band of 92 kDa. (F) Anti-MPP4 antibody (AK4) coimmunoprecipitated CRB1-myc from cells overproducing CRB1-myc and 3xFLAG-MPP4. (G) Anti-myc antibody coimmunoprecipitated 3xFLAG-MPP4 from cells overproducing CRB1-myc and 3xFLAG-MPP4. (H) N-glycosylation of CRB1. Incubation of stable MDCKII/CRB1 cells with tunicamycin shifted the molecular weight from more than 220 kDa toward the calculated 154 kDa, indicating extensive N-glycosylation of CRB1.
Fig. 3 Analysis of the interacting domains of MPP4 and -5.

(A) For quantification of the intermolecular interaction between MPP4 and -5 (above dotted line), the YRG-2 yeast strain was cotransformed with the SH3-HOOK region of MPP5, together with two different deletion constructs of MPP4. The minimal region of MPP4 that is needed for interaction contains the E-GuK-F region. For determination of the intramolecular interaction of MPP5 as well as MPP4 (below dotted line), yeast cells were cotransformed with two constructs of MPP5 (the pBD-GAL4 domain fused to the SH3-HOOK region and the pAD-GAL4 domain fused to the HOOK-GuK domain), as well as two constructs of MPP4 (the pBD-GAL4 domain fused to the GuK domain and the pAD-GAL4 domain fused to the SH3 domain). Interactions were quantified by determining the activation of the LacZ reporter gene in a liquid ONPG assay (β-galactosidase activity, black bars). As a negative control, the pBD-GAL4 domain, fused to CRB1 intra, and the pAD-GAL4 domain, fused to MPP4 PDZ, was used. (B) Homology modeling of MPP4 and -5. The initial models are shown on the left, covering the SH3 (yellow, green) and GuK domains (red, light blue). By swapping the domains, one obtains a heterodimer, half of which is shown in the middle: the SH3 domain of MPP5 bound to the GuK domain of MPP4. Energy calculations predicted a high binding energy for this interaction (Table 2), due to several salt bridges, shown in the close-up (right): the triad Glu 414\textsubscript{MPP4}-Lys 351\textsubscript{MPP5}-Asp 596\textsubscript{MPP4}, Lys 375\textsubscript{MPP5}-Glu 588\textsubscript{MPP4}, and (not shown) Arg 418\textsubscript{MPP4}-Glu 395\textsubscript{MPP5}. Note that an essential tyrosine (Y413) in the GUK domain of MPP4 is present in the core of the SH3 domain of MPP5.
MPP5 recruits MPP4 to the CRB1 complex in photoreceptors

Fig. 4 mRNA expression profiles of CRB1, MPP5, and MPP4 in human tissues and an RPE cell line (ARPE) determined by RT-PCR

Lane 1: retina; lane 2: brain; lane 3: skeletal muscle; lane 4: heart; lane 5: lung; lane 6: testis; lane 7: kidney; lane 8: liver; lane 9: placenta; lane 10: ARPE; lane 11: RPE; lane 12: fetal eye; lane 13: fetal cochlea; and lane 14: negative water control. GAPDH served as a positive control.
Fig. 5 Distribution of MPP4, MPP5, CRB1, and β-catenin in adult human retina.
Confocal images of human retinas stained with antibodies against β-catenin (A, C, D, E, G, H, M, O, Q), CRB1 (B–D, I, K, L), MPP5 (F–H, J–L), MPP4 (N-Q), or control secondary antibodies (P). Anti-β-catenin antibody strongly stained the adherens junction (D, H, Q), whereas anti-CRB1 antibody AK2 (D, L), anti-MPP5 SN47 (H, L), and anti-MPP4 AK4 (N-Q) stained the SAR in the OLM. MPP5 and CRB1 colocalized at the SAR (L). AK4 stained the OPL (N, O) and the OLM (O, Q), whereas secondary antibodies (P) produced some background staining in the photoreceptor inner and outer segments. IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments; INL, inner nuclear layer. Scale bars: (A–C, H, L, P, Q) 10 μm; (D, E–G, I–K, M–O) 20 μm.
MPP5 recruits MPP4 to the CRB1 complex in photoreceptors

Fig. 6 Immuno-electron microscopy of mouse Mpp4 in retina.
(A) In the OLM, Mpp4 was located at the plasma membrane apical to and at the zonula adherens contacts (arrowhead and arrow, respectively). There was also staining of the trans-Golgi network (*). (B) In rod photoreceptors, the lateral plasma membranes of the spherules were strongly stained (*). Mpp4 staining was also found associated with vesicles proximal to the presynaptic membrane (arrow). (C) Mpp4 was found at the basal side of cone pedicles, where it concentrated at the contacts with horizontal cell processes and bipolar cell dendrites (arrowhead) and also at the plasma membrane of the lateral side of the pedicle membrane (*). As in rods, Mpp4 staining was also associated with vesicles proximal to the presynaptic membrane (arrow). (D) Detailed view of the staining of the Golgi area in the inner segment of a rod. (E) Magnification of the adherens junction showing the presence of the signal at the photoreceptor side (arrow), not in the Müller glia cell. (F) MPP4 was located at the presynaptic plasma membrane and proximal vesicles (arrow). (G) At the cone synapse, the signal was associated with the presynaptic membrane. R, rod photoreceptor; C, cone photoreceptor; H, horizontal cell; M, Müller glia cell. Scale bar, 0.5 μm.
Table 1. Sense and Antisense Primer Sequences for GATEWAY Constructs

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Table 2. Binding Energies (in kcal/mol) of Four SH3/GuK Module Combinations, Predicted from Homology Models

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References


MPP5 recruits MPP4 to the CRB1 complex in photoreceptors


MPP5 recruits MPP4 to the CRB1 complex in photoreceptors


MPP3 is recruited to the MPP5 protein scaffold at the retinal outer limiting membrane

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Chapter 4

Abbreviations

CRB1 Crumbs homologue 1;
HEK Human embryonic kidney
INL inner nuclear layer;
IP immunoprecipitation(s);
IS inner segments;
MAGUK membrane-associated guanylate kinase protein;
MPP membrane-associated palmitoylated protein;
OLM outer limiting membrane;
ONL outer nuclear layer;
OPL outer plexiform layer;
OS outer segments;
PDZ postsynaptic density 95 / discs large / zonula occludens 1;
PPRPE retinitis pigmentosa with preservation of para-arteriolar retinal pigment epithelium;
RP retinitis pigmentosa;
SAR subapical region;
SH3 Src-homology-3; MRE, MAGUK recruitment element;
Summary

Mutations in the human Crumbs homologue 1 (CRB1) gene are a frequent cause of various forms of retinitis pigmentosa. The CRB1-MPP5 protein complex is thought to organize an intracellular protein scaffold in the retina that is involved in maintenance of photoreceptor-Müller glia cell adhesion. This study was focused on the binding characteristics and subcellular localization of MPP3, a novel member of the MPP5 protein scaffold at the outer limiting membrane (OLM), and of the DLG1 protein scaffold at the outer plexiform layer of the retina. MPP3 localized at the photoreceptor synapse and at the subapical region adjacent to adherens junctions at the OLM. Localization studies in human retinas revealed that MPP3 colocalized with MPP5 and CRB1 at the subapical region. MPP3 and MPP4 colocalized with DLG1 at the outer plexiform layer. Mouse Dlg1 formed separate complexes with Mpp3 and Mpp4 in vivo. These data implicate a role for MPP3 in photoreceptor polarity and, by association with MPP5, pinpoint MPP3 as a functional candidate gene for inherited retinopathies. The separate Mpp3/Dlg1 and Mpp4/Dlg1 complexes at the outer plexiform layer point towards additional yet unrecognized functions of these membrane associated guanylate kinase proteins.

Introduction

Polarized cells, like epithelia, photoreceptors and other neurons, establish and maintain unequal distribution of proteins [1,2], which is vital for their proper function. Polarization has been an area of intense study in the recent years, helping us to understand the pathological pathways in the retina that are triggered by mutations in genes encoding components of such complexes. MAGUKs are localized at the membrane–cytoskeleton interface of cell–cell junctions, and appear to have both structural as well as signaling roles [3]. MAGUK proteins also play an important role at synaptic junctions by regulating the release of neurotransmitters from synaptic vesicles [4]. This protein family is characterized by a specific set of protein-binding domains, consisting of one or more PDZ domains, a SH3 domain and a GuK domain [5,6]. A subset of this protein group also has a domain found to bind mLIN7, and named the L27 domain [7]. This includes all seven members of the MPP subfamily of MAGUKs, excluding MPP1. The strong structural conservation as well as their matching subcellular localizations in different animals suggests a functional conservation of MAGUK proteins. Moreover the phenotype of a mutation in a MAGUK coding gene in transgenic flies can often be rescued by some of the mammalian homologues [8,9].

The Drosophila MAGUK protein Stardust is the homologue of MPP5 (PALS1) in mammals. Loss of Stardust induces an eye phenotype in Drosophila, characterized by a shortened stalk membrane and altered rhabdomere morphogenesis resembling the loss of Crumbs phenotype [10–12]. Stardust was found to co-localize with Crumbs and directly interact with the C-terminus of Crumbs via its PDZ domain [13].

The Drosophila Crumbs protein and the human homologue CRB1, contain similar conserved protein motifs. Mutations have been identified in the CRB1 gene in individuals...
with Leber congenital amaurosis, retinitis pigmentosa (RP) type 12 with preservation of para-arteriolar retinal pigment epithelium (PPRPE), RP with Coats-like exudative vasculopathy, early onset RP without PPRPE, and pigmented paravenous chorioretinal atrophy [14-20]. Mouse Crb1 is involved in maintenance and integrity of the retinal outer limiting membrane [21,22]. Moreover it prevents loss of adhesion between photoreceptors and Müller glia cells and prevents death of retinal neurons [22]. MPP5 and CRB1 interact physically. The PDZ domain of MPP5 binds the C-terminal ERLI motif of CRB1 [23].

The GuK domain of MPP4, another MPP subfamily member, binds the SH3/HOOK domain of MPP5 in 293 human embryonic kidney cells [24]. MPP4 and MPP5 both localize at the outer limiting membrane (OLM), suggesting a role for these proteins in photoreceptor polarity [22,24]. Mpp4 is also present at the pre-synaptic photoreceptor membrane in the outer plexiform layer (OPL) [24], implying it’s involvement in functional aspects of synaptic transmission.

MPP3 belongs to the same protein subfamily as MPP4 and MPP5. MPP3 has been found to associate directly with DLG1 (SAP97) in the brain. This interaction was mediated by the MAGUK recruitment (MRE) domain of DLG1 and both L27 domains of MPP3. DLG1 was shown also to bind to MPP2, but not MPP6, two other members of the MPP subfamily of MAGUK proteins [25].

In this study, we examined the retinal subcellular localization and protein interactions of MPP3. We demonstrate the presence of MPP3 at the OLM and its interaction to MPP5. We demonstrate separate Mpp3/Dlg1 and Mpp4/Dlg1 complexes at the photoreceptor synapse.

Results

Cloning of human retinal MPP3 isoforms

Primer were designed from the human MPP3 brain cDNA sequence (NM_001932) to amplify 2 kb MPP3 cDNA products from a human retinal cDNA library. Alignments of the MPP3 cDNA with the human genome database indicated that the open reading frame was split into 18 exons. Sequence analysis of the cDNA products revealed that there are two 2 kb products due to alternate splicing of exon 11 comprising 21 base pairs. In 15 retinal cDNA products tested, 2 cDNAs (accession number AM050144) contained exon 11 and encoded a full length MAGUK protein of 585 amino acids, identical to the reported brain cDNA. The 13 other cDNAs (accession number AM050145) lacked exon 11 and encoded a shorter protein of 315 amino acids due to premature truncation of the open reading frame (Fig. 1E). The shorter version (MPP3ΔGuK) lacked the GuK domain. MPP4 and MPP5 were more similar to MPP3 than to each other. Homology comparisons between MPP3 and other MAGUKs are shown in table 1 and figure 1. MPP5 and Stardust contain a HOOK domain between the SH3 and GuK domains. This domain contains a conserved putative protein 4.1 binding site, which is not present in MPP3 and MPP4.
Detection of MPP3 in human retina and expressing cells.
A chicken (SN45) and a rabbit polyclonal antibody (CPH8) against human MPP3 were raised using recombinant full length human MPP3 purified from E. coli. To verify the specificity of the antibodies we performed Western blot and immunoprecipitation analysis. On Western blots, the two antibodies recognized a 75 kDa recombinant full length purified MPP3 protein, and MPP3 or MPP3ΔCuK expressed in 293 HEK cells (Fig. 2A and B). CPH8 antibody recognized a 75 kDa band in human retina, while SN45 showed in addition unspecific bands not present in the pre-immune serum (Fig. 2C and D). Human MPP3 protein immunoprecipitated by CPH8 from retinal lysates was detected on Western blots by the two independent antibodies SN45 and CPH8 as 75 kDa product (Fig. 2C and D respectively). MPP3ΔCuK predicted to be predominantly expressed was undetectable, suggesting that the 37 kDa band observed in the input retina is unspecific. Moreover this band was not visible in mouse retinal lysates (Fig. 2E). CPH8 and SN47 did not cross-react with MPP5 (data not shown) or MPP4 (Fig. 6B lane 2 and data not shown).

MPP3 co-localizes with MPP5 at the OLM in human retina.
In between the retinal pigment epithelium and the outer limiting membrane of the retina resides the so-called subretinal space, which is a lumen. The apical side of the retinal pigment epithelium faces the subretinal space. The inner and outer segments adjacent to the outer limiting membrane are the most apical side of photoreceptors and extend into the subretinal space. The outer limiting membrane contains a so-called subapical region (SAR) adjacent to the adherens junctions (AJs) between photoreceptors and Müller glia cells. At the outer plexiform layer, the most basal side of photoreceptors form synapses with bipolar and horizontal cells.

Rabbit anti-MPP3 (CPH8) detected MPP3 at the OLM and OPL of human retina (Fig. 3B). CPH8 detected Mpp3 at the OLM, OPL and IPL of mouse retina (data not shown). The pre-immune serum was used as a control, and gave a weak and diffuse staining in the retina with no specific pattern. Affinity purified anti-MPP3 SN45 gave staining patterns similar to the corresponding pre-immune yolk and was not used for further immunohistochemical studies (data not shown).

Immunohistochemistry and confocal laser scanning microscopy were used to determine the subcellular protein localization of human MPP3 relative to the MAGUK protein MPP5. Direct co-localization studies using anti-MPP3 and CRB1 could not be performed because both are rabbit antibodies. Anti-MPP3 (CPH8) detected the protein in a region apical to β-catenin, which is a marker for adherens junctions (Fig. 3A, C and D). When retina was co-stained for MPP3 and MPP5 the two signals overlapped at the OLM (Fig. 3M-Q). Thus taking into account our previous results that showed co-localisation of MPP5 with CRB1 at the subapical region (SAR) of the OLM [22,24] we deduce that MPP3, MPP5 and CRB1 co-localize at the SAR.

MPP3 co-localizes with DLG1 at the photoreceptor synapse in human retina.
In the OPL, the MPP3 signal partially overlapped with the staining for human DLG1 (Fig. 3G, H). Using monoclonal antibodies against human DLG1 we observed immunoreactivity in the OPL similarly to that described for the rat retina [26], but we detected no staining in the inner plexiform layer. DLG1 was occasionally detected at the OLM, but
this staining was inconsistent, possibly due to low level of expression or dynamic localization. DLG1 immuno-reactivity pattern in the OPL showed also partial overlap with MPP4 (Fig 3K-L). Anti-MPP4 showed a very strong signal in the OPL and a relatively weak signal at the OLM under standard immunohistochemical conditions (OPL staining shown in Fig. 3J, OLM staining not shown). Direct co-localization studies using anti-MPP3 and MPP4 could not be performed because both are rabbit antibodies.

**MPP3 forms a complex with CRB1 via MPP5 in 293 cells**
MPP3 and MPP5 have very similar secondary structures and are both localized at the OLM. The PDZ domain of MPP5 interacted directly with the C-terminal ERLI motif of CRB1[23], whereas the SH3/HOOK domains interacted directly with the GuK domain of MPP4 [24].

Human embryonic kidney cells (293 HEK) express endogenous MPP3 [27] and MPP5 [24] at low level, but not MPP4 or CRB1[24]. To test for the presence of a protein complex containing MPP3 and CRB1, we used 293 HEK cells expressing FLAG- and/or myc-tagged proteins. Anti-FLAG antibody immunoprecipitated FLAG-tagged MPP3 or MPP3△GuK, but not the non-FLAG-tagged MPP3 or MPP3△GuK from overproducing cells (data not shown), and did not co-immunoprecipitate detectable amounts of CRB1 from cells co-expressing FLAG-tagged MPP3 or MPP3△GuK and CRB1-myc (Fig. 4A). In a reciprocal experiment, anti-myc antibody immunoprecipitated CRB1 from CRB1-myc overproducing cell lines (data not shown), but no co-immunoprecipitation of MPP3 or MPP3△GuK from cells co-expressing MPP3 (or MPP3△GuK) and CRB1-myc was detected (Fig. 4B). In control experiments, anti-myc co-immunoprecipitated MPP5 efficiently from cells co-expressing MPP5 and CRB1-myc (data not shown). Based on the homology of MPP3 and MPP4, and in analogy to the putative CRB1-MPP5-MPP4 complex we described previously [22,24], we hypothesized that MPP5 could link MPP3 to CRB1. The endogenous MPP5 in 293 cells either was in insufficient amount or did not link MPP3 to CRB1. To discriminate between these two possibilities we expressed MPP5 in cells over-expressing CRB1 and one of the two forms of MPP3 (full length or lacking the GuK domain). Indeed confirming our hypothesis, upon co-expression of these three proteins interaction between CRB1 and MPP3 was detected, suggesting a bridging role for MPP5 (Fig. 4C and D). Note that in lanes 3 and 4 of Fig. 4D the endogenous MPP3 was detected, which is clearly specific as it can not be detected in lanes 1 and 2, which do not have MPP5 overexpressed, and thus serve as negative controls. As MPP3 without the GuK domain could not be detected in complex with CRB1 it appears that this domain is essential in linking MPP3 to CRB1 via MPP5 in 293 cells.

**MPP3 forms a complex with MPP5 at the OLM**
To test for a physical interaction between MPP3 and MPP5 we used 293 HEK cells expressing FLAG-tagged MPP3 or MPP3△GuK, and/or MPP5 in pull-down experiments. Anti-FLAG antibody co-immunoprecipitated over-expressed as well as endogenous MPP5 from cells that overproduced MPP3-FLAG (Fig. 5A), but not from cells expressing non-FLAG-tagged MPP3 or MPP3△GuK with or without FLAG-tag (lanes 1-4). These results confirmed interaction of MPP5 with MPP3, and that this interaction was specific, and required the GuK domain (Fig. 5A lanes 3 and 4). The endogenous MPP5 was detected mainly as a 70 kDa band in cell lysates, but upon co-immunoprecipitation with
MPP3 (Fig. 5A lanes 5 and 6) it was visible as a double band of 70 and 80 kDa, due to enrichment of the 80 kDa form [24 and Annex]. The recombinant MPP5 has a molecular weight of 80 kDa. The interaction between MPP3 and MPP5 occurred in the presence as well as absence of CRB1. MPP3 had strong affinity for MPP5, as it co-immunoprecipitated endogenous MPP5 from cells transfected only with MPP3-FLAG at similar levels as cells that expressed recombinant MPP5 (Fig. 5A lanes 5 and 6). Interestingly, we observed previously that the level of endogenous MPP5 co-immunoprecipitated by CRB1-myc was much lower than when MPP5 was overexpressed (see annex). This together with the observed strong association between MPP3-MPP5 independently of CRB1 gives an indication that not all of the endogenous MPP5 available for binding to MPP3 is linked to CRB1. For that reason the level of MPP5 should be elevated in order to detect the MPP3-MPP5-CRB1 complex (Fig. 4C lane 6). In a reverse experiment we immunoprecipitated MPP5 with SN47 antibody and tested for co-precipitation of endogenous MPP3 and/or exogenous MPP3 or MPP3ΔGuK. SN47 efficiently pulled down MPP3 along with MPP5 only from cells overexpressing MPP3, but not MPP3ΔGuK, confirming the results described above. Endogenous MPP3 could not be co-precipitated to detectable levels (data not shown).

The interaction between Mpp3 and Mpp5 was confirmed by immunoprecipitation of Mpp3 with CPH8 antibody from mouse retinal lysates. We detected efficient co-immunoprecipitation of Mpp5 (Fig. 5B). Crb1 was below detection level in the Mpp3 immunoprecipitate. The latter may be explained by 1) the relatively low level of Crb1 in the retinal lysate, 2) a partial association of the Mpp3-Mpp5 complex with Crb1 as suggested by the experiments performed in 293 cells, and 3) the abundant localization of Mpp3 at the OLM, OPL and inner plexiform layer of the mouse retina (data not shown) whereas Mpp5 and Crb1 are only localized at the OLM, and 4) steric hindrance in the CPH8-Mpp3-Mpp5-Crb1 complex..

Mpp3 does not interact with Mpp4 in retina in vivo

As both MPP3 and MPP4 bound MPP5 we aimed to investigate if these could be found in a complex. 293 HEK cells expressing MPP3 or MPP3ΔGuK, and/or MPP4-FLAG were used in pull-down experiments. Anti-FLAG antibody co-immunoprecipitated MPP3 from all cells overproducing MPP4-FLAG (Fig. 6A lanes 1-3). Unlike in the case of MPP5, MPP3ΔGuK was detected in a complex with MPP4. This suggests that the GuK domain of MPP3 is not necessary for the binding to MPP4. In a reverse experiment the FLAG tag was placed on MPP3 and MPP3ΔGuK; we precipitated MPP3 with anti-FLAG antibody and checked if MPP4 was present in the complex. While full length MPP3-FLAG co-precipitated MPP4, surprisingly MPP3ΔGuK did not (data not shown). The position of the tag or/and the antibody binding may preclude the interaction between MPP3ΔGuK-FLAG and MPP4.

Upon pull down of Mpp4 from mouse retinal lysates using AK4 antibody we checked for the presence of co-immunoprecipitated Mpp3. Both AK4 and normal IgG immunoprecipitation lanes were negative, while Mpp3 was easily detected in the input as a triple band (Fig. 6B). In a reverse experiment we immunoprecipitated Mpp3 with CPH8 and tested for co-immunoprecipitation of Mpp4. CPH8 pre-immune serum was used as a control. Whereas we could detect Mpp5 in the anti-Mpp3 immunoprecipitate (Fig. 5B), we could not detect Mpp4 although it was readily identified in the retinal lysates (Fig.
These data suggest that there are no in vivo Mpp3-Mpp4 complexes in the retina. The difference in the MPP3-MPP4 association seen in vitro vs. in vivo can be explained by the possible existence of a protein that mediates this interaction in 293 HEK cells by opening up the structure of the molecules and allowing their intermolecular binding. This mediator might be missing in the retina or is competed out by another protein that does not facilitate the binding of Mpp3 and Mpp4. Alternatively, Mpp3 and Mpp4 are transported to different membrane subdomains in vivo, or are recruited to the synapse by a protein that can bind either Mpp3 or Mpp4 but not both.

**Dlg1 and Mpp4 exist in a complex at the photoreceptor synapse.**
The partial colocalization of MPP4 and DLG1 suggested the existence of a complex between the two proteins. This hypothesis was tested by immunoprecipitation of Mpp4 from mouse retinal lysates using AK4 antibody. Dlg1 was visualized as a double band of 100 and 140 kDa in the retinal lysate. Only the 100 kDa band was co-precipitated along with Mpp4 (Fig. 7A). Double or triple bands corresponding to Dlg1 have been described before [28,29] and was in some cases due to alternative splicing [30]. We also performed anti-Dlg1 pull down on mouse retinal lysates with monoclonal anti-Dlg1, and normal mouse IgGs as control. The membranes with separated proteins were probed with anti-Mpp4 and a positive signal was visualized only in the Dlg1 immunoprecipitation lane (lane 2 in Fig. 7B).

**Dlg1 and Mpp3 exist in a complex at the photoreceptor synapse.**
DLG1 partially overlapped with MPP3 in the OPL. To test for a Dlg1–Mpp3 complex we performed anti-Dlg1 pull-down on mouse retinal lysates. We used monoclonal anti-Dlg1, and normal mouse IgGs as control. The membranes were probed with anti-MPP3 and a positive signal was observed only in the lane of Dlg1 immunoprecipitation and not in the control IgGs. All three bands of Mpp3 detected in the lysates were co-immunoprecipitated (Fig. 7C). In a reverse experiment we immunoprecipitated Mpp3 with CPH8, while CPH8 pre-immune serum served as a control. We detected Dlg1 in the CPH8 immunoprecipitate (Fig. 7D), but not in the control pre-immune serum, confirming the Mpp3-Dlg1 specific association. Interestingly, a 140 kDa Dlg1 protein was co-immunoprecipitated by Mpp3 (Fig. 7D), whereas a 100 kDa Dlg1 protein was immunoprecipitated by Mpp4 (Fig. 7A). Similar experiments were performed using human retinal lysates. A human DLG1 positive signal of 120 kDa was detected only in CPH8 immunoprecipitation and input lanes (Fig. 7E).

To summarize the described above: the data suggests that retinal Mpp3-Mpp4 complexes do not exist in vivo; both Mpp3 and Mpp4 associate with Dlg1, but with different Dlg1 isoforms of 140 and 100 kDa, respectively. All this together suggest that Mpp3 and Mpp4 form separate complexes with Dlg1 at the photoreceptor synapse.

**Discussion**

Two main retinal cDNA products of MPP3 were identified. One encoded full-length MPP3 protein, the other a protein truncated after the SH3 domain (MPP3ΔGuK). The latter transcript was more abundant, but we did not detect MPP3ΔGuK protein in the retina. Probably the mRNA or the resulting protein has a relatively short half-life, as
indicated by consistently lower levels of expression of MPP3\(\Delta\)GuK in cell lines compared to MPP3 full length upon transfection with equal or higher amounts of DNA. Also, relatively often we could observe degradation products of the MPP3\(\Delta\)GuK form. The instability of MPP3\(\Delta\)GuK protein in retina could be due to unfeasible intra-molecular interaction between the SH3 and GuK domains[31]. One can speculate that similarly to DLG1 [32], the different splice forms of MPP3 could have different localizations, but because of MPP3\(\Delta\)GuK levels below the detection level we could not elaborate further on it.

In human retina, MPP3 was detected at the SAR adjacent to adherens junctions at the OLM, and at the OPL. In mouse retina, Mpp3 was detected at the SAR of the OLM, and at the OPL and IPL. Here we showed that MPP3 forms protein complexes and colocalizes with MPP5 at the SAR of the OLM. We also showed that MPP3 does not bind directly to CRB1. We and others showed previously that MPP5 interacts directly to the C-terminal ERLI motif of CRB1[24,33]. In addition, previous results showed that MPP5 forms protein complexes and colocalizes with CRB1 at the SAR of the OLM[22,24]. These data indirectly suggest that MPP3, MPP5 and CRB1 colocalize at the SAR. In 293 cells we detected tripartite complexes of MPP3-MPP5-CRB1 suggesting that MPP5 recruits MPP3 into the CRB1 complex in cellulo, but these complexes were below detection levels in retinal lysates. Therefore, our data suggests the existence of MPP3-MPP5 complexes but do not exclude the existence of MPP3-MPP5-CRB1 complexes at the SAR.

In 293 cells, MPP3 efficiently bound endogenous MPP5. Our previous experiments showed that only part of CRB1 is associated with endogenous MPP5, as the amounts of MPP5 co-precipitated with CRB1 increased dramatically upon MPP5 over-expression [24]. Here we showed that MPP5 recruited MPP3 into the CRB1 complex in 293 cells. The MPP3-MPP5 interaction appeared to be independent of CRB1 and did not affect the association of CRB1 with MPP5. In addition, MPP3-MPP5 interaction requires the GuK domain of MPP3, indicating a mechanism for binding similar as described for MPP4 and MPP5 [24].

MPP3 is capable of binding MPP4 in 293 HEK cells independently of the GuK domain suggesting different interaction modes or intermediators involved. However, we did not detect interaction of Mpp3 and Mpp4 in retinal lysates. Lack of in vivo interaction between Mpp3 and Mpp4 may be due to transport to different membrane subdomains in vivo, or recruitment to the synapse by proteins (e.g. Dlg1) that can bind either Mpp3 or Mpp4 but not both. Here we showed separate associations of Mpp3 and Mpp4 with different Dlg1 isoforms, suggesting involvement in different functional complexes at the photoreceptor synapse. It remains to be shown whether these complexes are redundant or have unique functions.

In the OPL, MPP3 partially overlaps and interacts with DLG1. In the rat brain, DLG1 binds GluR1-containing AMPA receptors in the endoplasmic reticulum and delivers them to the synapse where the complex dissociates [34]. In addition, in rat brain, DLG1 and MPP3 are binding partners of the Kir2.2 potassium channel, along with PSD-95, PSD-93, SAP102, CASK, MPP2, and MPP6, two isoforms of Veli (1 and 3), Mint1, and actin-binding LIM protein. Some of the MAGUKs identified bind directly to the channel, like DLG1 and Veli [35,36] while others are recruited via binding to another MAGUK, like
for example CASK binds DLG1 or Veli [36]. These MAGUKs regulate the intracellular trafficking and modulate the activity of the channel [37]. The interaction of Kir2 channels with class I PDZ domain-containing proteins is regulated by PKA phosphorylation on the PDZ binding motif [35,38]. This indicates that MAGUKs can form complex networks of interactions with other MAGUKs and transmembrane proteins, including channels, thus providing fine tuning of their clustering, trafficking and function.

The SH3 domain can engage in MAGUK intermolecular and intramolecular interactions with the GUK domain via a mechanism that does not involve the usual proline rich recognition site for SH3 domains. The SH3-GUK intramolecular association, which predominates over the intermolecular association, has been shown to regulate intermolecular binding of MAGUKs and the clustering of PDZ binding proteins including DLG1 and PSD95 [39-43]. As MPP4 has been described to be involved in such interaction [24], MPP3 and MPP4 might play a similar role in targeting or retention of the DLG1 complex at the plasma membrane or vesicles. MAGUK complexes are believed to link to channels or receptors, therefore retinal MPP3 and/or MPP4 may be involved in channel or receptor positioning, stability at the membrane and its function.

The co-localization and interaction of MPP3 with MPP5 (and CRB1) at the OLM suggests a role for MPP3 in the maintenance of retinal integrity by regulation of cell adhesion between photoreceptors and Müller glia cells. Based on the recruitment of MPP3 to the MPP5 protein scaffold at the OLM, the involvement of MPP5 in the CRB1 protein scaffold, the disruption of retinal laminar observed in Crb1 knock-out mice[22] and in the zebrafish MPP5 homologue *Nagie oko*[44], we propose that MPP3 is a functional candidate gene for inherited retinal degenerations.

**Materials and Methods**

**Cloning and analysis of human retina MPP3 cDNA**

Human retina Marathon Ready cDNA (Clontech laboratories, Woerden, The Netherlands) was used to amplify MPP3. Primer pair 5’ GATCCCCGGCCAGCATGCCAGTGCTATCGGAAGG 3’ (sense) and 5’ GATCGTGACTTACCTGACCCAACTAACAGG 3’ (antisense) were designed from the human MPP3 cDNA sequence (NM_001932). Underlined are the start and stop codons of the gene. Alternatively, sense primer 5’ GATCCCCGGCCACCATGGAGCTTCAATACCACCTCCAC 3’ in combination with the antisense primer was used. The full-length PCR products were sub-cloned into pGEM-T for sequencing.

Two main cDNA products of 2 kb were identified. One coding for full length protein, another encoding a short MPP3 lacking the GuK domain (MPP3ΔGuK) due to skipping the 21 bp of exon 11. Primer pairs 5’ AGCCTTGTGACAAGAGACC 3’ (sense) and 5’ GAAGGCCGCGAGAGCGGCCA 3’ (antisense) were used on individual cDNA clones to determine by PCR the frequency of occurrence of exon 11. The correct cDNAs coding for either full length MPP3 or MPP3ΔGuK were SmaI/SalI cut out of pGEM-T and sub-cloned into BamHI/SalI opened retroviral cDNA expression vectors pBabe-CMV-Neo or pBabe-CMV-Hygro.
A FLAG epitope tag was created at the N-terminus of human MPP3 by annealing the following primers: 5’-GACTACAAAGACCACGGTGATTATAAAAGATCATGACACATCGATTACAAGG
ATGACGATGACAAAGCTCATG-3’ (sense), and 5’-GTACAGCTTGTCATGCATCTCTTGTAAATCGATGTCATGATCTTTATAATCAG
CGTCATGTCTTTTGTAGTC -3’ (antisense), and ligated into a blunted SphI site in MPP3 (introduced in the cloning primer) followed by sequencing to determine the vectors with correct insert orientation. This resulted in insertion of the epitope at the very amino terminal end.

Protein purification and antibody production
For the purification of full length MPP3 protein, cDNA was amplified by PCR from pGEM-T-MPP3 using 5’ GGTGGTTGCTCTTCCAACATGCCAGTGCTATCGGAGG
3’ (sense) and 5’ GATCGTCGAC TTA CCTGACCCAACTAACAGG 3’ (antisense) primer pair. After sequencing a SapI/SalI cut PCR product was subcloned into SapI/SalI opened pTYB11 vector (New England BioLabs, Leusden, The Netherlands). Protein was expressed in E. coli strain ER2566 and purified essentially following the manufacturer’s protocol (IMPACT-CN manual New England BioLabs).
MPP3 protein was used for immunization of chickens and rabbits. The yolk was processed with Eggcellent™ Chicken IgY Purification Kit (Pierce, Etten-Leur, The Netherlands) according to the manufacturer’s protocol. All antibodies/sera were consequently affinity purified on protein or peptide coupled Hi-Trap NHS-activated HP column (Amersham Biosciences, Roosendaal, The Netherlands).

Cell culture
Human embryonic kidney (HEK) 293 cells were grown in DMEM (Invitrogen, Breda, The Netherlands) containing 1% penicillin/streptomycin and 10% fetal bovine serum.

Transfection, co-immunoprecipitation experiments and Western blotting
Vectors pBabe-CMV-Puro-CRB1-myc, pBabe-CMV-Hygro-MPP5 and pBabe-CMV-Puro/Hygro-MPP4 with or without FLAG were as described before [24]. Cells were transfected with pBabe-CMV-Puro-CRB1-myc, pBabe-CMV-Hygro-MPP5, pBabe-CMV-Puro-MPP4 with or without FLAG pBabe-CMV-Hygro-MPP3 with or without FLAG pBabe-CMV-Hygro-MPP3ΔGυK with or without FLAG or combinations of these vectors using calcium phosphate. At 48 hours after transfection, cells were homogenized in lysis buffer: 50 mM HEPES pH 7.4, 150 mM sodium chloride, 10% glycerol, 0.5% Triton X-100, 1.5 mM magnesium chloride, 1 mM EGTA, 1 mM PMSF, Protease inhibitors cocktail (Roche) and 10 µg ml⁻¹ aprotinin (Sigma, Zwijndrecht, The Netherlands). For retinal lysates the tissue was homogenized in extraction buffer: 10 mM HEPES pH 7.9, 10 mM NaCl, 3 mM MgCl₂, 1 mM dithiotreitol 1 mM PMSF, 1 mM Na₃VO₄, 1x Complete protease inhibitors (Roche, Woerden, The Netherlands), centrifuged at 1000g, and after discarding the nuclear fraction centrifuged at 20000g. The cytosolic fraction was discarded and the membrane fraction was dissolved in the lysis buffer described above. Alternatively, proteins were extracted from tissues or cells with NP-40 lysis buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 10% glycerol, 1% NP-40, 1 mM EDTA, supplemented by Complete Protease Inhibitor Cocktail and 0.8 mM Pefabloc SC.
PLUS (Roche). Material from 12 animals (males and females, 6-8 weeks old), or one 10 cm culture dish (for cells) were used per tube. Every immunoprecipitation was repeated 2-7 times. Animals were treated in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

All lysates were clarified by centrifugation for up to 30 min, 20000g at 4°C. Supernatants were incubated for 2 hours at 4°C with antibodies pre-coupled to Dynabeads® protein G (Dynal Biotech ASA, Breda, The Netherlands) following manufacturer’s protocol. For the immunoprecipitations with anti-MPP5 SN47, we pre-coupled mouse monoclonal anti-chicken IgG to Dynabeads® protein G (15 µg/reaction). This was followed by a second round of coupling of chicken SN47 antibody (10 µg/reaction). Dynabeads were washed three times with lysis buffer, boiled in sample buffer with β-mercaptoethanol, and the material was resolved on 8% SDS-PAGE. Proteins were electrophoretically transferred onto nitrocellulose membranes.

Membranes were blocked, incubated with primary and secondary antibodies (conjugated to horseradish peroxidase) in 0.3-5% milk powder/TBS, washed in TBS, and the bands were visualized using ECL reagent (Amersham Biosciences).

**Antibodies**

Production of and conditions for anti-Crb1 (AK2, AK5, AK7), anti-MPP4 (AK4 and AK8) and anti-MPP5 (SN47) antibodies have been described [22,24]. Anti-c-myc monoclonal mouse antibodies (clone 9E10) were purchased from Roche, anti-Dlg1 (clone 12), and anti-β-catenin (clone 14) mouse monoclonal antibodies from BD Transduction laboratories (Alphen aan den Rijn, The Netherlands), anti-FLAG monoclonal mouse antibody (clone M2), monoclonal anti-chicken IgG (clone CG-106) and normal mouse and rabbit IgG from Sigma. The following dilutions of antibodies were used for immunodetection: anti-MPP3 CPH8 (1:500 – 1:1000), anti-MPP3 SN45 (1:500), anti-Dlg1 (1:500).

Secondary antibodies conjugated to Alexa 488, Cy3, and Cy5 were obtained from Molecular probes (Leiden, The Netherlands) and Jackson ImmunoResearch Laboratories (Amsterdam, The Netherlands). Secondary antibodies conjugated to horseradish peroxidase were purchased from Sigma.

**Immunohistochemistry**

Eight human post-mortem retinae, from 5 males and 3 females age 33-51, with postmortem times of 8-24 hours, were obtained from the cornea bank in Amsterdam and treated in accordance with the Declaration of Helsinki for the use of human tissue in research.

Frozen human retina sections, 10 µm thick, upon paraformaldehyde or aceton fixation were treated as described previously [22] with the difference of using PBS buffer and 1% BSA instead of PB buffer and 0.1% BSA. Each immunohistochemical staining was performed on 3 different donor retinae 2-7 times. Sections were imaged on a Zeiss 501 confocal laser scanning microscope (Jena, Germany).
Table 1. MPP3 was individually aligned with MPP4, MPP5, DLG1 and Stardust (STD). The identities and similarities in amino acid sequence were compared between individual domains and the full length protein.

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<td>28/51;25/47</td>
<td>37/62</td>
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Figure 1. Protein structures of MPP3 and MPP3ΔGuK homologues.
All membrane palmitoylated protein family members have very similar protein structures consisting of two L27 domains, one PDZ, SH3 and GuK domain. In addition, MPP5 has a coiled-coiled region at the amino terminus. Stardust also has coiled-coiled region, and together with DLG1 and MPP5 comprises a HOOK domain situated between SH3 and GuK domains.
Figure 2. Immuno-reactivity of MPP3 antibodies.

(A) CPH8 antibody tested on 293 HEK expressing MPP3 full length or MPP3ΔGuK (lanes 1 and 2, respectively). MPP3 full length is detected as bands of 75 and 70 kDa, most likely due to posttranslational modification. MPP3ΔGuK is detected as a band of 35kDa (note the breakdown products visible below the 35kDa band). In the control cells an unspecific band of 73 kDa can be detected upon longer exposure (lane 3). (B) Western blots of SN45 antibody tested on 293 HEK expressing MPP3 or MPP3ΔGuK (lanes 1 and 2, respectively). MPP3 full length is detected as a single band of 78 kDa. Some breakdown products are visible below the full length products. MPP3ΔGuK is detected as a band of 35 kDa. (C) Immunoprecipitation was performed on human retinas with anti-MPP3 CPH8 antibody and normal rabbit IgGs as control. The material was probed with anti-MPP3 SN45, which readily recognizes the recombinant and immunoprecipitated MPP3 (lanes 3 and 1, respectively), while in the input human retina many unspecific bands were visualized (lane 4). (D) Immunoprecipitation was performed with CPH8 and normal rabbit IgGs as control. The material was probed with the CPH8 affinity purified antibody. Note the background band of 50 kDa corresponding to the heavy chains of the IgGs used for the pull down detected by the secondary goat anti-rabbit antibody. An unspecific band of 39 kDa was recognized by CPH8 in the human retinal input material (asterisk), but was not immunoprecipitated. The 39 kDa band was also detected by the pre-immune serum (data not shown). E. Detection of Mpp3 in mouse retina. Note that unlike in the case with human retinal lysates, the 37 kDa band is not detected.
MPP3 is recruited to the MPP5 protein scaffold at the retinal outer limiting membrane

Figure 3. Localization of MPP3, MPP4, MPP5, DLG1 and β-catenin in adult human retina.

(A-Q). Confocal images of human retinae stained with antibodies against β-catenin (A, C, D), MPP3 (B-D, F-H, M, O, Q), MPP4 (J-L), MPP5 (N, O, Q), and DLG1 (E, G-I, K, L). Anti-β-catenin antibody strongly stained the adherens junction (A, C, D), whereas anti-MPP3 CPH8 (B-D) stained the region just apical to the outer limiting membrane (OLM) (D) and parts of the outer plexiform layer where synapses are formed between the photoreceptors and bipolar cells. (OPL) (F-H). MPP5 and MPP3 colocalize (O, Q). Anti-DLG1 antibody stained the OPL (E, I), where it partially colocalized with MPP3 (G, H) and MPP4 (K, L). In (J) antibody-epitope retrieval was not used, therefore levels of MPP4 at the OPL are well detectable but at the OLM are not[22,24]. IS, inner segments; OS, outer segments; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer. Scale bar represents 20 µm, excluding the detail inserts where it is 10 µm.
Figure 4. Interactions between MPP3 and CRB1.

(A) Pull down with anti-FLAG antibody did not co-immunoprecipitate CRB1 from cells overproducing FLAG-tagged MPP3 or MPP3ΔGuK and CRB1-myc. Lanes 1-4 serve as controls for unspecific binding. (B) Pull down with anti-myc antibody did not co-immunoprecipitate MPP3 or MPP3ΔGuK from cells overproducing MPP3 or MPP3ΔGuK and CRB1-myc, indicating lack of direct interaction. Anti-myc co-immunoprecipitated endogenous MPP5 (data not shown). Lanes 1-3 serve as controls for unspecific binding. (C) Anti-FLAG antibody co-immunoprecipitated CRB1 from cells overproducing MPP3-FLAG, CRB1-myc and MPP5 (lane 6), but not from cells overproducing MPP3ΔGuK-FLAG, CRB1-myc and MPP5 (lane 5) or Flag-tagged MPP3ΔGuK or MPP3 and CRB1-myc (lanes 3 and 4, respectively). Lanes 1 and 2 serve as controls for unspecific binding. (D) Overexpression of MPP5 is required to incorporate endogenous or overexpressed MPP3 into a complex with CRB1 (lanes 3-5). Pull down with anti-myc antibody immunoprecipitated MPP3 from cells overproducing MPP5, CRB1-myc and/or MPP3, suggesting a bridging role of MPP5 in binding of MPP3 and CRB1. Anti-myc co-immunoprecipitated endogenous (lanes 3 and 4) and over-expressed MPP3 (lane 5) but not MPP3ΔGuK (lane 4) in the presence of elevated levels of MPP5. The levels of MPP3Δ were well detectable in cells overexpressing MPP3Δ (lane 9), but co-precipitation of MPP3Δ with CRB1 could not be detected even when examined on very long exposures, suggesting that full length MPP3 does, but MPP3Δ does not, interact with CRB1 (lane 4).
MPP3 is recruited to the MPP5 protein scaffold at the retinal outer limiting membrane

Figure 5. Interactions between MPP3 and MPP5
(A) Anti-FLAG antibody co-immunoprecipitated endogenous and/or recombinant MPP5 from cells expressing MPP3-FLAG (lanes 5 and 6) but not from cells expressing MPP3ΔGuK-FLAG (lanes 3 and 4). Note that endogenous MPP5 can be detected as 70 kDa band in cell lysates, but upon co-immunoprecipitation with MPP3 it is visible as double band of 70 and 80 kDa, due to enrichment of the 80 kDa band. Over-expressed MPP5 is detected as 80 kDa protein (last lane in the right). Lanes 1 and 2 serve as controls for unspecific binding. "e/r" stands for endogenous/recombinant.
(B) Anti-MPP3 CPH8, co-immunoprecipitated Mpp5 protein from mouse retinal lysates (lane 1), while the control pre-immune serum did not (lane 2), indicating specific interaction of Mpp3 and Mpp5.

Figure 6. Interactions between MPP3 and MPP4
(A) Anti-FLAG co-immunoprecipitated recombinant MPP3 or MPP3ΔGuK from cells overproducing MPP4-FLAG and MPP3 (lane 2) or MPP3ΔGuK (lane 1). Anti-FLAG co-immunoprecipitated endogenous MPP3 from cells overproducing MPP4-FLAG (lanes 1 and 3). Lanes 4-6 serve as controls for unspecific binding. The FLAG tag is indicated as "f", the deletion of the GuK domain as Δ, and all CRB1 molecules used in these experiments are myc-tagged; IP, immunoprecipitation.
(B) Mpp3 was not co-immunoprecipitated upon Mpp4 pull down.
(C) Anti-MPP3 CPH8, did not co-immunoprecipitate Mpp4 protein from mouse retinal lysates (lane 1), while the signal was easily detectable in the input (lane 3).
Figure 7. Immunoprecipitation on mouse and human retinal tissue.
Immunoprecipitations from mouse (A-D) or human (E) retinal lysates were blotted and incubated with the antibodies indicated. (A) Dlg1 was co-immunoprecipitated with polyclonal anti-Mpp4 AK4, from retinal lysates (lane 2), but not with control normal rabbit IgGs (lane 1), indicating specific interaction of Mpp4 and Dlg1. *The input lane in this picture is taken from a longer exposure, as it was invisible on the film with the IP lanes shown here. (B) Mpp4 was co-immunoprecipitated specifically with anti-Dlg1 (lane 2), but not with control normal mouse IgGs (lane 1), indicating specific association. Note that the input signal was not detectable at this exposure. (C) Mpp3 was co-immunoprecipitated with Dlg1 (lane 2), but not with control normal mouse IgGs (lane 1) from retinal lysates, indicating specific interaction of Mpp3 and Dlg1. (D) Polyclonal anti-MPP3 CPH8, co-immunoprecipitated Dlg1 protein from mouse retinal lysates (lane 1), while the control pre-immune serum did not (lane 2), indicating specific interaction of Mpp3 and Dlg1 IP, immunoprecipitation. (E) Polyclonal anti-MPP3 CPH8, co-immunoprecipitated DLG1 protein from human retinal lysates (lane 1), while the control pre-immune serum did not (lane 2), indicating specific interaction of MPP3 and DLG1.
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References


Chapter 5

Mpp4 recruits Psd95 and Veli3 towards the photoreceptor synapse.

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Chapter 5

Abbreviations

Cask  calcium-calmodulin-dependent serine kinase
Crb1  Crumbs 1
Dlg1  disc large 1
GCL  ganglion cell layer.
IGL  intergranular layer
INL  inner nuclear layer
IPL  inner plexiform layer
LCA  leber congenital amaurosis
MAGUK  membrane-associated guanylate kinase
ML  molecular layer
Mpp4  membrane palmitoylated protein 4
OLM  outer limiting membrane
ONL  outer nuclear layer
OPL  outer plexiform layer
Patj  PALS1 associated tight junction protein
PCL  Purkinje cell layer
PPCA  pigmented paravenous chorioretinal atrophy
Psd95  post synaptic density protein 95
RP  retinitis pigmentosa
RPE  retinal pigment epithelium
SAR  subapical region
Abstract
Membrane-associated guanylate kinase (MAGUK) proteins function as scaffold proteins contributing to cell polarity and organizing signal transducers at the neuronal synapse membrane. The MAGUK protein Mpp4 is located in the retinal outer plexiform layer (OPL) at the presynaptic plasma membrane and presynaptic vesicles of photoreceptors. Additionally, it is located at the outer limiting membrane (OLM) where it might be involved in OLM integrity. In Mpp4 knockout mice, loss of Mpp4 function only sporadically causes photoreceptor displacement, without changing the Crumbs (Crb) protein complex at the OLM, adherens junctions or synapse structure. Scanning laser ophthalmology revealed no retinal degeneration. The minor morphological effects suggest that Mpp4 is a candidate gene for mild retinopathies only. At the OPL, Mpp4 is essential for correct localization of Psd95 and Veli3 at the presynaptic photoreceptor membrane. Psd95 labeling is absent of presynaptic membranes in both rods and cones but still present in cone basal contacts and dendritic contacts. Total retinal Psd95 protein levels are significantly reduced which suggests Mpp4 to be involved in Psd95 turnover, whereas Veli3 proteins levels are not changed. These protein changes in the photoreceptor synapse did not result in an altered electroretinograph. These findings suggest that Mpp4 coordinates Psd95/Veli3 assembly and maintenance at synaptic membranes. Mpp4 is a critical recruitment factor to organize scaffolds at the photoreceptor synapse and is likely to be associated with synaptic plasticity and protein complex transport.

Introduction
Six classes of neurons build up the complex neuronal network of the retina. Light of various wavelengths is absorbed by rod and cone photoreceptors, followed by the integration of the signal through bipolar, amacrine and horizontal cells and further transmitted to the brain by ganglion cells (1). Polarity of neuronal cells is essential for the retinal integrity. To maintain photoreceptor polarity, protein complexes are recruited towards particular subcellular locations, including the outer limiting membrane (OLM), which consists of the adherens junction and a region with similarity to tight junctions, the so-called subapical region (SAR) (2). The OLM contributes to retinal integrity by connecting photoreceptors to Müller glia cells. As in other polarized cells, members of the membrane associated guanylate kinase (MAGUK) family are found in complexes at the plasma membrane of several subcellular compartments. Here, these scaffold proteins play an important role in targeting, clustering, and anchoring of other proteins. They can assemble combinations of cell adhesion molecules, cytoskeletal proteins (3), receptors, ion channels, and their associated signaling components at specific membrane sites (4,5). MAGUK proteins contain multiple protein-protein interaction domains (at least one PSD95, Dlg and ZO-1 (PDZ), a Src homology 3 (SH3) and a guanylate kinase homolog (GUK) domain (5,6)) required for their scaffolding function. MAGUK proteins can be divided in the Dlg, p55, Lin-2 and ZO-1 subfamilies based on their number of PDZ
domains, additional domains and sequence similarity (5,7). Within the p55 subfamily, seven membrane palmitoylated proteins (MPP) have been identified (Mpp1 to 7) of which Mpp4 is present in photoreceptors (8). Mpp4 is a protein of 637 amino acids containing two N-terminal L27 domains, a PDZ domain, a SH3 domain and a C-terminal GUK domain (8). Although its mRNA is also present in heart, spleen, liver and cerebellum, Mpp4 is preferentially expressed in photoreceptors of the mammalian retina and of main interest for this study (8,9).

In photoreceptors, Mpp4 is localized at presynaptic vesicles and the plasma membrane of the photoreceptor synapse at the outer plexiform layer (OPL). Additionally, some antibodies directed against Mpp4 detect protein at the connecting cilia (the region between the inner and outer segments) and the OLM (9,10). At the OLM, Mpp4 is localized at the SAR and so is the Crb complex (11). Crb1 is associated with retinal disease, since mutations in the CRB1 gene lead to a variety of retinal degenerative diseases such as Leber’s Congenital Amaurosis (LCA), Retinitis Pigmentosa type 12 (RP), Retinitis Pigmentosa with Coats-like exudative vasculopathy, and Pigmented Paravenous Chorioretinal Atrophy (12-15). These human retinal degenerative diseases can be mimicked in part by the Crb1<sup>168</sup> and Crb1<sup>-/-</sup> mouse models showing severe retinal disturbances and retinal degeneration (16,17). Moreover, in Drosophila both mutations in the Stardust (Mpp5; Crb1 interaction partner) and the Crumbs gene gave rise to the same rough eye phenotype, which includes an altered rhabdomere morphogenesis resulting in shortened rhabdomeres and changed stalk membranes (18-20). In vitro, Mpp4 interacts, via its GUK domain, with the SH3 domain of Mpp5 (11).

This phenomenon of heterodimerization has been described for several MAGUK family members, eventually linking them to the cell cytoskeleton or the C-terminus of transmembrane proteins and ion channels (7,21). Psd95 and Dlg1 are known to heterodimerize via their L27 domains with other MAGUK’s such as calcium-calmodulin-dependent serine kinase (Cask) (22,23). In the OPL, several members of the Dlg subfamily, including Dlg1, Psd95, Psd93, and SAP102 are localized at the neuronal synapse (24), but it has not been identified which of these MAGUK proteins are part of a Mpp4 assembly.

Other potential binding partners of MAGUK proteins are Veli1, Veli2 and Veli3, which are mammalian homologues of C. elegans Lin-7 (25). Lin-7 is part of the complex consisting of Lin-2 (Cask), Lin-7 (Veli) and Lin-10 (Mint) (26) and its homologue complex Cask/Veli/Mint is detected in mammalian brain (27). The interactions are mediated by the N-terminal L27 domains of p55-like MAGUKs and the L27 domain located at the N-terminus of the Veli proteins (28,29). Both Veli1 and Veli3 are expressed in the retina, where the expression pattern of Veli3 and Mpp4 partially overlaps. Interaction between the L27 domains of Mpp4 and Veli3 was demonstrated, recently (30).

In order to unravel the dual function of Mpp4 at the OLM and photoreceptor synapse, and to investigate whether the gene for Mpp4 is causal to retinal disease, we generated and analyzed Mpp4 knockout mice (Mpp4<sup>-/-</sup>). We have demonstrated that loss of Mpp4 causes sporadically photoreceptor displacement. No structural differences were detected at the OLM or photoreceptor synapse as visualized by light and electron microscopy. No alterations were observed in the overall retinal activity determined by electroretinography, or in the overall retinal structure examined by scanning laser
ophthalmology, suggesting that $Mpp4$ is a candidate gene causal for mild retinopathies only. This study shows that Mpp4 is essential to maintain Psd95 and Veli3 at the photoreceptor presynaptic membrane. Psd95 protein levels were strongly reduced, whereas total retinal Veli3 protein levels remained unchanged, suggesting an essential role for Mpp4 in regulating the Psd95 turnover at the photoreceptor synapse. We propose that Mpp4 functions as a critical recruitment factor to organize signal transducers at the photoreceptor synapse.

Results

Mpp4 knockout mice

Homozygous $Mpp4^{-/-}$ mice were obtained by generating a mutant $Mpp4$ allele that introduced a frame shift in the beginning of exon 7 encoding the PDZ domain of Mpp4 (figure 1A). The recombined allele was visualized on southern blot (figure 1B). $Mpp4^{-/-}$ mice are indistinguishable from their wild-type littermates; they grew and bred normally. Twenty-two heterozygote intercrosses resulted in 167 pups, with an average of 7-8 pups per litter, of which 29% was genotyped as knockout, 47% as heterozygote and 23% as wild-type. The homozygous as well as the wild-type mouse stocks were maintained as a cross of C57BL/6 and 129/Ola (50%/50%). In $Mpp4^{+/+}$ animals, Mpp4 staining using immunohistochemical analysis was observed in the OLM and OPL. Both signals were not detectable in $Mpp4^{-/-}$ retinas (figure 3A).

Histological morphology of the $Mpp4^{-/-}$ retina

Under normal light/dark cycle the $Mpp4^{-/-}$ retina develops and remains histologically normal up to 12 months of age (figure 2A-F), except for sporadically displaced photoreceptors. Only in 1 $Mpp4^{-/-}$ animal out of 5 studied, a small area of displaced photoreceptors was observed (figure 2C). Continuous exposure to white light of 3000 lux for 3 days did not significantly increase the number of displaced photoreceptors in retinas at 3 months of age. Small areas of photoreceptor displacement were observed in 4 out of 7 animals, showing one example in figure 2D. However, this phenomenon was not observed at an earlier time point of 1 month or at the later time points of 6 and 12 months of age (figure 2E,F). No photoreceptor displacement was observed in all wild-type animals studied. No morphological defects were found in $Mpp4^{-/-}$ retinas developed in complete darkness when compared to $Mpp4^{+/+}$ retinas at the age of 1 and 3 months (data not shown). Moreover, in vitro culture of retinas isolated at day 0-1 and cultured up to 1 month showed normal development of the retinal layers in $Mpp4^{-/-}$ and $Mpp4^{+/+}$ retinas (data not shown).

Scanning-laser ophthalmoscopy and electroretinography

At the age of 9 and 18 months, fundus visualization with scanning-laser ophthalmoscopy (SLO) did not reveal any major changes or signs of retinal degeneration in $Mpp4^{-/-}$ compared to $Mpp4^{+/+}$ retinas (figure 6). Additionally, a group of six wild-type
and six Mpp4−/− animals were exposed to light at 3 months of age and SLO was used to visualize the fundus. Photoreceptor displacement as observed on histological sections could not be detected by SLO (data not shown). The analysis of the retinal vasculature using angiography revealed no changes in the inner retina (figure 6 FLA) nor in the choroid (figure 6 ICGA).

To investigate the effect of the Mpp4 deletion on retinal function, ERGs were recorded from Mpp4−/− and Mpp4+/+ mice (9 and 18 months of age) under scotopic and photopic conditions. Under both conditions, no significant differences were observed between the retinal electric signals obtained from Mpp4−/− and Mpp4+/+ retinas at 9 and 18 months (figure 7 A-C).

**Mpp4 is not required for correct localization of the Crb complex**

Immunostainings for several proteins located at the OLM were performed. Important members of the Crb-complex such as Crb1 (figure 3F), Crb2, Crb3, and the multiple PDZ proteins Mupp1 and Patj (PALS1 associated tight junction protein); the MAGUK proteins Mpp5 (Pals1) and Mpp3, and the polarity protein Par3 were all detectable at the OLM, as well as proteins connected to the adherens junction (ZO-1, β-catenin) or inner segments (F-actin). Staining patterns of all these proteins were identical in Mpp4−/− and Mpp4+/+ retinas (figure 8 and data not shown).

**Down regulation of Psd95 and mislocalization of Veli3**

To unravel the function of Mpp4 in the OPL, we investigated proteins that are expressed in the OPL and putatively colocalize with Mpp4. Several of them remained unchanged in their expression and localization. These include the MAGUK proteins: Dlg1 (figure 3C) and Mpp3, synaptic vesicle connected proteins such as synaptogyrin, synaptotagmin and clathrin, snare protein 25 (Snap-25), and the vesicle transporter proteins VGlut1 and VGAT1 (data not shown). However, intensity of Veli3 and Psd95 staining at the OPL were substantially reduced (figure 3B,E). In the Mpp4+/+ retina, Psd95 protein was observed in the photoreceptor synapses of the OPL and weakly in the IPL, as shown in figure 3E (and data not shown). In Mpp4−/− retinas, only the IPL staining remained detectable. Colocalization of Mpp4 and Psd95 is depicted in figure 3G. Whether the cerebral Psd95 expression was also influenced by the loss of Mpp4 from the cerebellum was checked by comparing Mpp4−/− and Mpp4+/+ cerebral sections (figure 5C). In the Mpp4+/+ cerebellum, Mpp4 is observed in the Purkinje cell layer (PCL) and does not colocalize with Psd95. In the Mpp4−/− cerebellum, the staining for Mpp4 disappeared from the PCL without influencing the Psd95 staining (figure 5C).

The Veli3 antibody produced an intense staining of the photoreceptor synapses in the OPL, whilst less intense Veli3 staining was found at the OLM, cone photoreceptors and a subset of bipolar cells, in accordance with Stöhr et al (30). In the Mpp4−/− retinas, despite the reduced signal for Veli3 in the OPL, the signal remained present at the OLM, cones and bipolar cells (figure 3B). Merged signals for Veli3 and Psd95 show colocalization in figure 3H. The staining patterns for the MAGUKs Dlg1 and Cask were unaltered in Mpp4−/− and Mpp4+/+ retinas (figure 3C,D). In contrast to colocalization shown for Veli3
and Psd95, Cask and Psd95 did not colocalize (figure 3I). In summary, Mpp4 is essential to recruit Psd95 and Veli3 to the presynaptic plasma membrane of photoreceptors.

**Altered localization of Psd95 visualized by electron microscopy**

Using electron microscopy, no structural differences in rod and cone terminals were observed between $Mpp4^{-/-}$ and $Mpp4^{+/+}$ retinas. Psd95 was detected at the plasma membrane of $Mpp4^{+/+}$ rod and cone spherules. In the rod spherules, the Psd95 signal was concentrated at the lateral plasma membrane and synaptic vesicles (figure 4A). In the cone pedicles, strong association of the signal with the basal contacts between photoreceptor and horizontal and bipolar cells was observed, in addition to its association with the lateral plasma membrane (figure 4B). In the IPL, Psd95 was also detected at the plasma membrane of ganglion cells concentrated at dendritic profiles which contain neurotubuli and localize postsynaptically to the ribbon synapses structures of bipolar cells (figure 4C). Psd95 was not present at presynaptic vesicles or at the plasma membrane of rod and cone synapses of $Mpp4^{-/-}$ retinas, whereas Psd95 labeling at the cone basal contacts and IPL were similar for $Mpp4^{-/-}$ and $Mpp4^{+/+}$ animals. Previous results on immuno-EM staining of Mpp4 demonstrated Mpp4 positive signals in the rod spherules and cone pedicles located especially at the lateral membrane and membranes of presynaptic vesicles (11). Thus, Psd95 positive signals became highly reduced at parts of the rod and cone presynaptic membranes corresponding to the Mpp4 localization.

**Loss of Mpp4 causes increased turnover of Psd95**

The association between Mpp4, Psd95, Dlg1, Veli3 and Cask was investigated by immunoprecipitation (IP). Psd95 was coimmunoprecipitated with Mpp4 from mouse retina lysates (figure 5A). Input samples for IP showed significantly reduced Psd95 protein levels in $Mpp4^{-/-}$ retina lysates compared to protein levels from $Mpp4^{+/+}$ retina lysates, which is confirmed by western blot of total retina lysates (figure 5B). The reduction in Psd95 was not universal as confirmed by unchanged Psd95 protein levels found in cerebral cortex (data not shown) and cerebellum (figure 5A). The interaction between the MAGUK proteins Dlg1 and Mpp4 was demonstrated by coimmunoprecipitation of the two proteins from $Mpp4^{+/+}$ retina lysates (figure 5A). In addition, coimmunoprecipitation of Mpp4 and Veli3 confirmed the interaction of Veli3 and Mpp4 as described recently by Stöhr et al (30). However, in contrast to the reduction in Psd95 protein levels found in the input samples, Veli3 retinal protein levels remained comparable in $Mpp4^{-/-}$ and $Mpp4^{+/+}$ retina lysates (figure 5A). Since Dlg1 was not detectable in the input samples, separate western blotting was performed to reveal that Dlg1 protein levels were also comparable in $Mpp4^{-/-}$ and $Mpp4^{+/+}$ total retina lysates (figure 5B). Interaction between Mpp4 and Cask was not detectable by IP and no changes were observed in protein levels of Cask (figure 5A). In summary, Mpp4 is connected to Psd95, Dlg1 and Veli3 at the photoreceptor synapse, but has a unique influence on the Psd95 protein turnover.
Discussion

The function of MAGUK protein Mpp4 was investigated because this protein is preferentially expressed in the mammalian retina (10) and might be connected to the Crumbs (Crb) complex via Mpp5 (11). These two characteristics provide a reason to hypothesize that Mpp4 is a candidate gene causal to retinal disease. However, aside from sporadic focal morphogenetic alterations around 3 months of age, Mpp4 knockout mice did not show retinal degeneration and remained structural and functional normal up till the age of 18 months. Additional light exposure or complete darkness did not consistently induce morphogenetic alterations or severe retinal degeneration. The minor morphological effects suggest that Mpp4 might be a candidate gene for mild human retinopathies only. MPP4 mutational studies have been performed on some cohorts of retinal disease patients but no disease-causing mutations were identified (31). By studying the function of Mpp4 in the photoreceptor synapse we discovered that Mpp4 is involved in the recruitment of Psd95 and Veli3 at presynaptic membranes. Moreover, Mpp4 has a crucial role in Psd95 turnover.

Localization of Crb complex members such as Crb1, Crb2, Crb3, Mpp5, Patj, and Mupp1 at the outer limiting membrane (OLM) is independent of Mpp4, because localization and immunofluorescent reactivity of Crb complex proteins were not affected by the loss of Mpp4. In a previous study (11), we identified Mpp4-immunoreactivity at electron microscopic level just above the adherens junction at the OLM. At light microscopic level, the Mpp4 immunoreactive signal was observed at the OLM and in the outer plexiform layer (OPL). Stöhr et al. (10) did not detect Mpp4 at the OLM, but detected Mpp4 at the cilium, which we were not able to confirm. This discrepancy can be due to the different antibodies and antibody epitope retrieval techniques used. Rosettes, retinal folds and severe retinal disturbances as observed in the Crb1-/- (17) and Crb1rd8 (16) mutant mice were only sporadically found in the Mpp4-/- mice but not in Mpp4+/+ mice. Also, scanning-laser ophthalmoscopy (SLO) at 9 and 18 months of age revealed no major differences between the retinas of the Mpp4-/- and Mpp4+/+ mice. These results suggest that the supposed docking capacity of Mpp4 for the Crb complex is not an essential function at the site of the OLM or that this function might easily be fulfilled by other (MAGUK) protein(s). Although the precise role of Mpp4 at the OLM is not resolved by studying the Mpp4-/- mouse, it is clear from the same mouse model that Mpp4 has a major role in homing at least two proteins; Psd95 and Veli3, at the photoreceptor synapse.

At the OPL, Mpp4 colocalizes with several other MAGUK proteins and synaptic vesicle-binding proteins. By comparing the staining patterns of these proteins in Mpp4+/+ mice with their patterns in Mpp4-/- mice, two proteins showed a remarkable reduction in intensity. Staining of Psd95 (SAP90), a MAGUK protein homologue to Drosophila DLG, is normally observed in the OPL and IPL (24). A significant reduction in Psd95 in the OPL without changes in the IPL was observed in the Mpp4-/- retina. Electron microscopic immunostaining of Psd95 confirmed the disappearance of labeling from the rod and cone presynaptic vesicles and plasma membrane, whereas the labeling was still detectable at the cone basal contacts and dendritic contacts in the IPL. Previous results on immuno-EM staining of Mpp4 demonstrated Mpp4 positive signals in the rod spherules and cone pedicles located especially at the lateral membrane and membranes of presynaptic vesicles (11). To confirm that Mpp4 and Psd95 are associated, we immunoprecipitated
Mpp4 from retina lysates and found Psd95 to coimmunoprecipitate with Mpp4. Input signals demonstrated that Psd95 protein levels in Mpp4−/− retinas are significantly reduced, whereas its protein levels in the cerebellum, where colocalization with Mpp4 is absent, remained unchanged. The decrease in the amount of retinal Psd95 protein was not due to suppression of transcription by Mpp4, since mRNA levels as detected by quantitative PCR were unaltered (data not shown). The results from this Mpp4−/− mouse model, suggest a major role for Mpp4 in the localization, stabilization and regulation of Psd95 protein turnover in the rod synaptic terminal. In the cone pedicles these mechanisms are partly independent of Mpp4, whereas in ganglion dendrites these mechanisms are fully independent of Mpp4. The function of Psd95 has mainly been investigated in other tissues than the retina. At the postsynaptic density, Psd95 is thought to be involved in assembling signal transduction complexes and in regulating synaptic plasticity. In vitro, Psd95 is connected to subunits of N-methyl-d-aspartate (NMDA)-type glutamate receptors (32,33), Shaker K+-channels (34) and neuronal nitric oxide synthase (nNOS) (35). Lack of Psd95 results in altered synaptic transmission in the hippocampus without obvious changes in morphology and NMDA-receptor currents but causing a substantially lower learning index (36). In Mpp4−/− retinas, photoreceptor synapses with highly reduced Psd95 protein levels were morphologically indistinguishable from those in Mpp4+/+ retinas (36). Although changes in ERG could not be detected, insufficient amounts of Mpp4 and Psd95 might alter the synaptic composition of signal transduction complexes and synaptic plasticity, influencing the synaptic signal transmission on a more subtle scale not detectable by ERG. Moreover, studying the interaction between Mpp4 and Psd95 in rod synapses may unravel a general mechanism involved in the stabilization of Psd95.

The correct localization of Veli3 seems to be dependent on Mpp4 as well. This homologue of Lin-7 (25), previously demonstrated to be a binding partner of Mpp4 (30), was no longer detectable at the presynaptic area of the OPL, while its pattern in the cone bodies, OLM and subset of bipolar cells remained unchanged. Although Veli3 was no longer localized at the synapses of rod and cones in the absence of Mpp4, its retinal protein level was still comparable between Mpp4+/− and Mpp4−/− mice. Whereas, Mpp4 functions in regulating the turnover of Psd95 and membrane targeting at the photoreceptor synapse, Mpp4 only functions in membrane targeting of Veli3. At the OLM, Veli3 is still correctly localized in Mpp4−/− retina, where it is able to bind Mpp5 (30), a member of the retinal Crumbs complex (17). Our data strengthens the hypothesis that Veli proteins are part of several protein complexes involved in polarization of different cell types and an aid in the assembly of signal transduction complexes (28).

It has been shown that Veli is accompanied by Cask and Mint1, to create the Cask/Mint/Veli (Lin2/Lin10/Lin7) complex present in neurons (26). They bind together in a complex leaving the PDZ domains free to recruit cell adhesion molecules, receptors, or other MAGUK proteins. Only two members of this complex are expressed in the photoreceptors cells, Cask and Veli3. The third member, Mint1, was not detectable at the OPL, but was detectable at the synaptic contacts of the IPL (data not shown). Veli3 localization at the OPL is Mpp4 dependent, while Cask localization is Mpp4 independent. The latter can be explained by the lack of direct binding between Cask and Mpp4 in retina lysates and different cellular locations observed for Cask and Mpp4. These results
suggest that the Cask/Mint/Veli complex observed in other types of neurons does not exist in photoreceptors. The localization ofDlg1, a protein closely related to Psd95, and the localization of Mpp3 were not affected by the absence of Mpp4. Immunoprecipitation demonstrated that Dlg1 is able to bind to Mpp4 and earlier results showed that Mpp3 (Dlg3) interacts with Dlg1 (SAP97) in the brain (37). We are currently investigating the existence of two complexes; one containing Dlg1 and Mpp4, which exists separate from the second containing Dlg1 and Mpp3 (data not shown). In the neuromuscular junction, Cask is also known to bind Dlg1 (22,38), suggesting that Cask might be involved in the correct localization of Dlg1, however, their staining patterns in the photoreceptor terminals are very different. So, in Mpp4-/- retina either Mpp3 is able to take over the homing of Dlg1 at the synaptic terminal, or Dlg1 is functioning as an anchor to Mpp4 and Mpp3 containing complexes ready to get connected to the membrane. The last suggestion leads to the hypothesis that Dlg1, bound to the membrane, serves as an anchor for Mpp4 (or Mpp3) to recruit different complexes. So, at the photoreceptor synapse, a unique Mpp4/Psd95/Veli3 protein complex probably homed by Dlg1 involved in the retention of proteins to the membrane. Our hypothesis is in agreement with previous studies indicating that Veli proteins and Psd95 are clustered together to recruit receptors towards the membrane (39). Moreover, our data suggest that Mpp4 is essential for Psd95 stabilization and maintenance of Psd95 at the rod and cone synaptic membrane. So far, the function of the protein complex containing Mpp4, Psd95 and Veli3 is unclear. Putative complex members and binding partners of Mpp4, Veli3 and Psd95 at the presynaptic photoreceptor terminal, such as PMCA, a critical regulator of the calcium homeostasis (40), will be studied. Research on the complex interactions, together with electrophysiological experiments on Mpp4-/- retina compared to the Mpp4+/- retina, might unravel the putative role of this complex in visual perception or synaptic plasticity.

Materials and Methods

Generation of Mpp4-/- mice

Gene targeting was performed as described previously (41). Primers JW54 (5'-GCCTTG CTGAGTGCCCATG-3') and JW55 (5'-GATCACTTGCTCAGGGTCC-3') were used to amplify a 291 bp fragment from a full length mouse Mpp4 cDNA (11). The cDNA fragment encoding the PDZ domain of Mpp4 was used to screen a λEMBL3 genomic 129/Ola DNA phage library. Forward primer JW95 (5'-GCGGCCGCGGATCCCGGG-3') in the multiple cloning site and primer JW96 (5'-GACGCTTGATGGTTGCTCC-3') in exon 7 of Mpp4 were used to amplify a 5.2 kb 5'-targeting arm using a long distance PCR kit (Advantage 2; Clontech). Primer JW97 (5'- ACCCTGAGCAAGTGATCC-3') in exon 8 of Mpp4 and primer JW100 (5'-CTCGGGTGGATTGAGGC-3') in exon 11 were used to amplify a 3.0 kb 3'-targeting arm. A targeting vector was constructed by assembling the 5'-arm, a hygromycin resistance gene driven by the mouse phosphoglycerate kinase (PGK) promoter in the opposite orientation, and the 3'-arm. Correct targeting deleted 2.3 kb of Mpp4 sequence,
MPP4 recruits Psd95 and Veli3 towards the photoreceptor synapse

removed intron 7 and 123 bp of exons 7 and 8, encoding part of the PDZ domain, thereby removing the splice donor site of exon 7 and splice acceptor of exon 8. The targeting efficiency in ES cells was 5.4%. The insertion of the hygro cassette introduced 7 additional amino acids followed by a stop codon after amino-acid 169 in the PDZ domain (aa 153-234). Two ES clones with normal karyotype were injected into C57BL/6 mouse blastocysts to generate chimaeric mice. Chimaeric mice were crossed with C57BL/6 mice to generate Mpp4+/− heterozygous mice on mixed genetic background (50% 129/Ola : 50% C57BL/6). Heterozygous mice were intercrossed to generate homozygous Mpp4−/− mutant and control wild-type mice on mixed genetic background (50% 129/Ola : 50% C57BL/6). Mpp4−/− mutant and control mice were maintained on mixed genetic background (50% 129/Ola : 50% C57BL/6) by crossing homozygous Mpp4−/− mutant or control wild-type mice, respectively. The mouse stocks were kept at a 12 hours dark / 12 hours dimmed light cycle (100 lux).

For Southern blot analysis of the 5′-flanking region, a 767-bp PCR fragment was generated using primers JW155 (5′-CTGGATATCTTCAATGAGACC-3′) and JW156 (5′-ATCAGATAGGGACTAATATCC-3′). For PCR genotype analysis, the wild-type Mpp4 allele was detected by PCR using primers JW150 (5′-ACACTGAAGCTGGTTAATGTGC-3′) and JW151 (5′-TTGCCAAAC AAAGCAAGGC-3′). These primer pairs amplify a 420-bp fragment from intron 7 of wild-type Mpp4. The mutant allele was amplified using forward primer T1 (5′-CCACTT GTGATCGCCAAGT-3′) in the PGK promoter and reverse primer JW92 (5′- TGGATCCTTGCTCAGGG-3′) in exon 8. These primer pairs amplify a 180-bp fragment from the Mpp4 mutant allele. All animals were treated according to guidelines established at the institutions in which the experiments were performed.

Histological analysis, light exposure and complete darkness

Animals were kept at normal light cycle of 12 hours dark/12 hours dimmed light (100 lux) and had access to food and tap water ad libitum. With and without the exposure to additional light, the eyes of both genotypes were histologically examined and compared at the age of 1, 3, 6, 9 and 12 months. At each time-point, 4-8 age-matched female animals were used per genotype. The light experiment starts with a dark period of 14-16 hours. Thereafter, the animals were placed in a white box and continuously exposed to diffuse white light of 3000 lux for 72 hours (TLD-18W/33tubes, Philips; 350-700nm) without pupillary dilation. Immediately after these 72 hours of light exposure the animals were sacrificed by inhalation of CO2 and cervical dislocation. The eyes were enucleated and a blue spot (Alcian blue) was placed on the superior side of each eye for orientation, followed by paraformaldehyde (4% in PBS) immersion-fixation for 30 minutes. The left eye was cryo-protected with sucrose (5% and 30%) or dehydrated in an ethanol series and embedded in paraffin for immunohistochemical analysis. The right eye was dehydrated in an ethanol series and stored in Technovit (Kultzer, Wehrheim, Germany). The whole right eye was cut in 3 μm sections, stained with 1% toluidine blue and analyzed for the presence of retinal changes. The same procedure of enucleation and fixation was followed for animals raised in complete darkness. Pregnant females of both genotypes (Mpp4+/− and Mpp4−/−) were kept in complete darkness and their offspring was raised in
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complete darkness up till the age of 1, 3 or 6 months, followed by the histological comparison of the retinas.

**Immunohistochemical analysis**

Cryosections (7 μm) and paraffin sections (4 μm) were used for immunohistochemical analysis of several proteins located in the OLM and OPL. Epitopes used to raise antibodies against Crb1, Crb2, Mpp3 and Mpp4 are described by van de Pavert et al. (17) and Kantardzhieva et al. (42). The anti-Mpp4 (AK4 and AK8) antibodies used in Western blotting, immunohistochemistry and immunoprecipitation were raised against aa 345-359 and aa 252-268, respectively. For correct antibody epitope retrieval for anti-Mpp4 staining on paraffin sections, the slides were incubated with glycine (0.75 M) at 35°C for 15 minutes prior to blocking. Primary antibodies against Psd95 and Cask were purchased from Affinity Bioreagents, Dlg1, Mupp1, p120, synaptogyrin, synaptotagmin, clathrin, Snap-25, and β-catenin from BD Transduction laboratories, VGlut from Synaptic Systems, VGat from Chemicon and Veli-3 from Zymed. Secondary antibodies were IgGs conjugated to Cy3, Alexa488, FITC, or TRIC (Jackson ImmunoResearch and Invitrogen). In short, cryosections were rehydrated in PBS and blocked for 1 hour in 10% serum, 0.4% Triton X-100 and 1% BSA in PBS, followed by the incubation overnight at 4 °C with primary antibody in 0.3% serum, 0.4% Triton X-100 and 1% BSA in PBS. Sections were washed three times with PBS and incubated with secondary antibody diluted in 0.1% serum and 1% BSA in PBS at room temperature for 1 hour. Paraffin sections were deparaffinized. Sections were boiled for 7 minutes in EDTA buffer (4 mM Tris, 1mM EDTA, pH 8.0) using the microwave and slowly cooled to room temperature (2 hours). After 1 hour of blocking with 10% serum and 1% BSA in PBS, the primary antibody (in 1% BSA in PBS) was applied, followed by overnight incubation at 4 °C. The sections were incubated with secondary antibody (in 0.1% BSA in PBS) and incubated at room temperature for 1 hour. The sections were visualized by confocal laser scanning microscopy (Zeiss 501) and pictures were made with Zeiss LSM image browser v3.2. All stainings were repeated at least three times using four different animals per genotype at two different time-points (3 and 6 months of age).

**Electron microscopy and Immunohistochemistry**

Two Mpp4+/+ and two Mpp4−/− female animals at the age of 6 and 12 months were sacrificed with an overdose of pentobarbital, the chest was opened and a needle was placed in the left cardiac ventricle for retrograde perfusion-fixation (1 minute: 0.1 M sodium cacodylate in saline; pH 7.4; 3-5 minutes: 1% paraformaldehyde, 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer; pH 7.4). Eyes were enucleated and fixed in 4% paraformaldehyde, 0.1 M sodium cacodylate in saline for 30 minutes followed by overnight incubation in 0.1 M sodium cacodylate buffer. The next day, the eyes were washed once in 0.1 M sodium cacodylate buffer and osmium-fixed for at least one hour. Subsequently the eyes were dehydrated for 5-10 minutes in ethanol 30% (twice), ethanol 50%, 70%, 96%, and ethanol 100% (twice for 15 min). The eyes were washed with acetone three times for 15 minutes and impregnated with epoxy resin/acetone (30/70, 15 min; 50/50, 30 min; 80/20, 30 min). Finally, the eyes were
impregnated with epoxy resin 100% for 30 minutes followed by embedding and polymerization at 35 °C for 16 hours, 45 °C for 8 hours and 65 °C for at least 24 hours.

For immunohistochemistry, animals were retrograde perfusion-fixed as described above. Eyes were enucleated and fixed in 4% paraformaldehyde in saline for 30 minutes followed by cryo-protection with sucrose (5% and 30%) and stored at −80 °C. Frozen sections 30-40 µm thick were cut on the cryostat and collected in phosphate buffer (PB).

Sections were incubated for 96 hours with primary antibody (Psd95, Affinity BioReagents). After rinsing the sections with PB, they were incubated with the secondary antibody ImmunoVision Poly-HRP-Goat Anti-mouse IgG (ImmunoVision Technologies Co., Daly City, CA). A Tris-HCl diaminobenzidine (DAB) solution containing 0.03% H2O2 was used to visualize the peroxidase present on the secondary antibody and intensified by the gold-substituted silver peroxidase method (43). Sections were rinsed in sodium cacodylate buffer (0.1 M; pH 7.4) and postfixed for 20 minutes in 1% osmium supplemented with 1% potassium ferricyanide in sodium cacodylate buffer (0.1 M; pH 7.4). After a second wash with sodium cacodylate buffer, the material was dehydrated and embedded in epoxy resin as described above. Ultrathin sections were cut from both immuno-stained and non-stained sections, and photographed using the FEI TECHNAI electron microscope. Pictures were collected with the ImageView soft imaging system and processed using Adobe photoshop (44).

**Immunoprecipitation and immunoblotting**

For protein detection, brains of Mpp4−/− (n=6; 6 months of age) and Mpp4+/+ (n=6; 6 months of age) mice were isolated. The cerebellum was separated from the rest of the brain, washed in PBS and immediately snap-frozen in liquid nitrogen. Tissues were homogenized in SDS-sample buffer (0.125 M Tris-HCl, 21% glycerol, 4% SDS; pH 6.8) through short ultra sonification (twice) and prolonged shaking at 14 °C until tissues were fully homogenized. Samples were stored at −20 °C and used as material for immunoblotting.

For immunoprecipitation, eyes of twelve animals per genotype at the age of 3 months were enucleated and after the removal of the anterior segment, lens and vitreous, the retina was isolated as described previously (11). Up to 12 retinas were pooled and homogenized in extraction buffer (10 mM HEPES, 10 mM NaCl, 3 mM MgCl2, 1 mM dithiotreitol (DTT), 1 mM PMSF, 1 mM Na3VO4, 1 x Complete protease inhibitors (Roche); pH 7.9). Lysates were centrifuged at 1,000g and the obtained nuclear fraction was discarded. Supernatants were centrifuged again at 20,000g to separate the cytosolic and membrane fractions. The membrane fraction was dissolved in lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 1 mM PMSF, 1 x Complete protease inhibitors (- EDTA, Roche), 10 µg/ml aprotinin (Sigma); pH 7.4). All lysates were clarified by centrifugation at 20,000g at 4 °C for 30 min. Before precipitation, supernatants were incubated with Dynabeads® protein G (Dynal Biotech ASA) coupled to the antibody of interest (coupled to the beads according to the manufacturer) for 2 hours at 4 °C. Thereafter, the Dynabeads® were washed three times with lysis buffer, followed by boiling in sample buffer supplemented with β-mercapto-ethanol or alternatively by elution in glycine (pH 1.5) at 37 °C for 5 minutes. The obtained proteins were loaded onto a 9% SDS-PAGE gel and after electrophoresis
transferred onto nitrocellulose membranes. Membranes were blocked (1% milk, 1% BSA in Tris buffered saline) and protein detection was performed using primary and secondary antibodies (conjugated to horseradish peroxidase) followed by visualization of the bands by using ECL reagent (Amersham Biosciences).

**Electroretinography and scanning-laser ophthalmoscopy**

Electroretinography (ERG) and scanning-laser ophthalmoscopy (SLO) were performed according to previously described procedures (45,46), figures 6 and 7). Both Mpp4−/− (n=6; at the age of 9 months and 18 months) and Mpp4+/+ (n=4; at the age of 9 months, n=5; at the age of 18 months) female mice were dark-adapted overnight and anaesthetized with ketamine (66.7 mg/kg) and xylazine (11.7 mg/kg). The pupils were dilated and single flash ERG recordings were obtained under dark-adapted (scotopic) and light-adapted (photopic) conditions.

Light adaptation was accomplished with a background illumination of 30 cd/m² starting 10 minutes before recording. Single white-flash stimulation ranged from 10⁻⁴ to 25 cd*s/m², divided into ten steps of 0.5 and 1 log cd*s/m². Ten responses were averaged with an inter-stimulus interval (ISI) of either 5 seconds or 17 seconds (for 1, 3, 10, and 25 cd*s/m²). Band-pass filter cut-off frequencies were 0.1 and 3000 Hz.

Scanning-laser ophthalmoscopy was performed with a Heidelberg Retina Angiograph (HRA, Heidelberg Engineering, Germany), a confocal scanning-laser ophthalmoscope (SLO). The HRA features two argon wavelengths (488 nm and 514 nm) in the short wavelength range and two infrared diode lasers (795 nm and 830 nm) in the long wavelength range. Laser wavelengths used for fundus visualization were: 830 nm (infrared channel), 514 nm (red-free channel), and 488 nm (for autofluorescent images). Additionally, the 488 nm and 795 nm lasers were used for fluorescein (FLA) and indocyanine green (ICG) angiography, respectively. FLA was performed using an s.c. injection of 75 mg/kg body weight fluorescein-Na (University pharmacy, University of Tübingen, Germany), and ICGA following an s.c. injection of 50 mg/kg body weight ICG (ICG-Pulsion, Pulsion Medical Systems AG, Munich, Germany).
Figure 1. Targeted disruption of Mpp4. (A) Mpp4 disrupted by insertion of the targeting vector. Correct homologous recombination deleted the PDZ domain of the Mpp4 gene. (B) XbaI/SpeI Southern blot analysis using a 767 bp PCR fragment probe in the 5’ flanking region (a 14.2 kb fragment for Mpp4+/+ DNA and a 10.5 kb fragment for Mpp4−/− DNA). (C) EcoRI Southern blot analysis using a 256 bp 3’ probe in the 3’ flanking region (a 5.6 kb fragment for Mpp4+/− DNA and a 5.1 kb fragment for Mpp4−/− DNA). Scale bar: 1 kb.
Figure 2. Retinal phenotype of Mpp4−/− mice. Technovit sections stained with toluidine blue from Mpp4+/+ (A) and Mpp4−/− (B) retina at 3 months of age. Mpp4+/+ retina at 3 months (C), 6 months (E) and 12 months (F) of age. Note the sporadically mislocalized photoreceptors in Mpp4−/− retina (arrow in panel C). Morphology of Mpp4−/− retina after light exposure to 3000 lux for 72 h at 3 months of age showing sporadic rosette formation (D). RPE, retinal pigment epithelium; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 100 μm.
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Figure 3. Confocal images of 3 months old Mpp4+/+ and Mpp4−/− retinas. Retinal localization of Mpp4, Veli3, Dlg1, Cask, Psd95 and Crb1. (A) After glycine treatment of paraffin sections, Mpp4 was detected at the OLM and OPL of Mpp4+/+ but not of Mpp4−/− retina. The background staining in the INL was not observed in frozen section but the signal at the OLM was weaker (17). No signal was detected in the cilia of wild-type mice. (B) Reduced Veli3 signal in the OPL of Mpp4−/− retinas. Although Veli3 signal is changed in the OPL, staining of the OLM and cones in the ONL remained unchanged. (C, D) Dlg1 and Cask staining at the OPL was comparable between Mpp4−/− and Mpp4+/+ retinas. (E) Reduced Psd95 signal in the OPL of Mpp4−/− retinas. (F) Crb1 localization at the OLM is unaltered. (G-I) Merged pictures of the OPL signals for Mpp4 and Psd95 (upper), Veli3 and Psd95 (middle) and Cask and Psd95 (bottom) depicting the colocalization of Mpp4, Veli3 and Psd95 at the plasma membrane of the synaptic terminal, while Cask is localized at the tip of the terminal. Figure 3. A-F and G-I are made at similar magnifications. Scale bars: 10 μm.
Figure 4. Immuno-electron-microscopic detection of Psd95. Vertical cryo-sections of the retina immunolabeled with Psd95 show: (A) Rod spherules receiving invaginating processes (stars) of unlabeled bipolar or horizontal cell dendrites. *Mpp4*<sup>+/+</sup> rod spherules immunolabeled for Psd95 (left) at the plasma membrane and presynaptic vesicles. Loss of Psd95 from the *Mpp4*<sup>−/−</sup> rod spherules (right). (B) Cone pedicles receiving invaginating processes (stars) of unlabeled bipolar or horizontal cell dendrites. *Mpp4*<sup>+/+</sup> labeled cone pedicle base (left, top half) showing Psd95 labeling at the plasma membrane, basal contacts and presynaptic vesicles. Loss of Psd95 signal from the plasma membrane and presynaptic vesicles of the *Mpp4*<sup>−/−</sup> cone pedicle leaving only the cone basal contacts positive (arrowheads right). (C) Positive Psd95 labeling in both *Mpp4*<sup>+/+</sup> and *Mpp4*<sup>−/−</sup> postsynaptic processes of ganglion cells in the IPL. M, mitochondrion; R, Rod; C, Cone and G, Ganglion cell. Scale bars: 500 nm.
Figure 5. Immunoprecipitation of Mpp4 detecting Psd95 and Veli3. (A) Anti-Mpp4 (AK4) coimmunoprecipitated Psd95 (95 kDa), Dlg1 (100 kDa) and Veli3 (22 kDa) but not Cask (110 kDa) from retinal membrane fractions. 2% input is 2% of retinal lysates prior to immunoprecipitation. Note the reduced Psd95 and Mpp4 protein levels in the Mpp4−/− retinal lysates. No changes were observed in Psd95 cerebral protein levels (repeated experiment; n=3). (B) Dlg1 protein levels (140 & 100 kDa) in Mpp4−/− and wild-type total retinal lysates. Psd95 protein levels (95 kDa) in Mpp4−/− and wild-type total retinal lysates. (C) Absence of colocalization of Mpp4 and Psd95 in Mpp4−/− and wild-type cerebral sections. Mpp4 staining in Mpp4+/+ cerebellum is observed in the Purkinje cells. ML; molecular layer, PCL; Purkinje cell layer, IGL; intergranular layer. Scale bars: 50 µm.
Figure 6. Retinal SLO images from wild-type and Mpp4–/– mice at 9 and 18 months of age. The wavelengths shown are infrared (830 nm, IR), red-free (514 nm, RF) and autofluorescence (488 nm, AF). Visualization of the vasculature was performed using ICG angiography (795 nm, ICGA) and fluorescein angiography (488 nm, FLA).
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Figure 7. Electroretinographic data from wild-type and Mpp4<sup>−/−</sup> mice at 9 and 18 months of age. Scotopic and photopic b-wave amplitude or latency vs. log intensity function. Boxes indicate the 25% and 75% quantile range, whiskers indicate the 5% and 95% quantiles and the asterisk indicates the median of the Mpp4<sup>−/−</sup> data. The normal range is delimited by solid lines indicating the 5% and 95% quantile of the wild type data. No sign of impaired retinal function was found in the Mpp<sup>−/−</sup> mice (A). Dark-adapted (scotopic, B) and light-adapted (photopic, C) single-flash ERG intensity series of a Mpp<sup>+/+</sup> mouse and a Mpp<sup>−/−</sup> mouse. Stimulus intensities are indicated in the panels. Vertical line crossing each trace shows the timing of the light flash.
Figure 8. Confocal images of 3 months old \textit{Mpp4}^{+/+} and \textit{Mpp4}^{-/-} retinas. Retinal localization of Crb proteins and Crb-complex related proteins. Localization and signal intensity of the Crb proteins Crb1 (A), Crb2 (B), Crb3 (C) were comparable between \textit{Mpp4}^{-/-} and \textit{Mpp4}^{+/+} retinas. Immunostainings show that signals for the Crb proteins; Mpp5 (D) and Mupp1 (E) were unaltered. All pictures are made at similar magnification.
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References


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We generated a mouse model for LCA by disruption of the \textit{Crb1} gene. The phenotype observed in \textit{Crb1}-/- mice deficient for Crb1 was very different from the function described for \textit{Drosophila} Crumbs in ectodermal epithelia, and partially overlapped with the phenotype in \textit{Drosophila} retina. In the invertebrate Crumbs-deficient epithelia spot adherens junctions (AJs) do not form a zonula adherens (ZA), and the basolateral polarity is compromised.\textsuperscript{1} Crumbs has an essential role in rhabdome shape, ZA integrity, and stalk membrane formation in fly photoreceptors. The loss of Crumbs in the fruit fly eye causes displacement of interacting partners and death of pigment cells\textsuperscript{2,3}. In the mammalian retina Crb1 is not responsible for the assembly of the AJ, neither does it cause gross polarity defects in unaffected regions. In \textit{Crb1}-/- mouse retinae the photoreceptor outer and inner segments, corresponding to the fly rhabdomeres and stalk membrane, respectively, are not changed. Death of retinal pigment epithelium cells was neither observed in unaffected regions, and markers for the subapical region (SAR) and AJs localized as in wild type retinae.

Whereas only one Crb isoform is expressed in \textit{Drosophila}, three CRB isoforms (CRB1, CRB2, and CRB3) exist in mouse and humans.\textsuperscript{4,5} All three members of the CRB family are expressed in the mammalian retina and localize at the SAR, as described in chapters 2 and 3. It is possible that the Crb family members have redundant functions in the assembly of AJs.

The function of Crumbs in protecting the retina from light damage is partially preserved between flies and mammals.\textsuperscript{6,7} No apoptosis was observed in 3 month-old mutant retinae, but a sharp increase in the formation of retinal disorganization was noted, indicating that Crb1 protects against light-accelerated damaging effects in the retina. The extracellular domain of \textit{Drosophila} Crumbs plays an important role in the protection against light.\textsuperscript{6} Currently, there is no proof for a similar function of the extracellular domains of Crb1 and Crb2. The endogenous levels of Crb2 at the outer limiting membrane were however not sufficient to suppress the light-accelerated phenotype.

Crb1 is required to maintain adhesion between photoreceptors and Müller glia cells. It is currently not known how Crb1 localized at the SAR regulates cell adhesion mediated by the cadherin/catenin complex localized at the AJ. Maintenance of cell adhesion may also be mediated via the extracellular domains of Crb1 and Crb2 either in homo- or heterophilic fashion with molecules present on opposite cell membranes. The extracellular domains of Crb1 and Crb2 contain laminin G-like domains that might interact with integrins. Some integrins are specifically expressed at the OLM\textsuperscript{8} and might interact with the laminin domains of Crb1 thus stabilizing the contacts in the vicinity of the AJ. There are currently two different \textit{Crb1} mutant mouse models, the \textit{rd8} mouse which is a natural occurring mutant, and the \textit{Crb1}-/- mice described in chapter 2. The \textit{rd8} mutation results in a truncated protein without transmembrane or intracellular part, and lacks the last four EGF domains and one laminin A G-like domain. In addition, it has an
altered C-terminal region containing 47 novel amino acids.\(^9\) Crb1\(-/-\) mice lack any detectable Crb1 protein and show loss of cell adhesion between Müller glia cells and photoreceptors, demonstrated by protrusion and ingression of photoreceptor nuclei into the subretinal space and outer plexiform layer, respectively. In Crb1\(-/-\) mice in limited regions all neurons die. Rd8 mice show loss of structural integrity of the outer limiting membrane, and loss of photoreceptors without loss of other retinal neurons. The differences observed between the Crb1\(-/-\) mice and the rd8 mouse could be due to partially retained (or abnormal) function of the truncated Crb1\(^{rd8}\) protein. It can not be excluded that Crb1\(^{rd8}\) is toxic to cells as the phenotype is observed as early as 4 weeks of age (vs. 3 month in Crb1\(-/-\) mice). A dominant negative effect is however excluded since the presence of a single WT allele is able to fully rescue the negative effect of the rd8 mutation. The phenotype in rd8 mice may however also be due to a different genetic background of the mice.

\(\text{Mpp4}\) is a candidate gene for retinal degenerations, as it is predominantly expressed in the retina, and the Mpp4 protein was shown in chapters 2-5 to interact with different MAGUK proteins localized at the SAR and synapses of photoreceptors. The Mpp4 gene is located in a chromosomal region associated with retinitis pigmentosa 26, which recently was shown to be due to mutations in another gene at that locus.\(^10\) The transient and rather mild phenotype observed in our Mpp4\(-/-\) animals implied that Mpp4 is a candidate gene for only mild retinopathies. The phenomena of a critical time window for development of rosettes, spontaneously or upon light exposure, could be explained by functional redundancy of other proteins that are expressed after and/or before 3 month of age. Hormonal influence can not yet be excluded as the mice used in the experiments were young adult females. Knockout of other MAGUK genes also showed milder phenotypes than expected.\(^11,12,13\) Mpp3 and Mpp5 are ubiquitously expressed during early development and might therefore be essential genes (Agnes van Rossum and Serge van de Pavert, unpublished results). In order to reveal redundancy in MAGUK proteins, double, triple and quadruple knockout mice may turn out to be necessary to uncover the function of individual MAGUK members in synaptic protein complex formation and maintenance. Although no synaptic structural phenotype has been observed in Psd95\(-/-\) mice, learning and memory are affected probably through altered activity of AMPA receptors.\(^13\) Additional functional tests will be needed to evaluate further the effect of Mpp4 loss in the retina. Mpp4 interacts with Veli3, and Veli proteins have been shown to bind subunit NR2B of the NMDA receptor in the brain, and the protein complex includes PSD95.\(^14,15\) Subunit NR1C2 of the NMDA receptor is expressed in rods and cones in the retina, and it would be worthwhile to test whether it is bound by Veli3, and forms a NR1C2/Veli3/PSD95/Mpp4 complex.\(^16\)

We showed the presence of several MAGUK proteins (Mpp3, Mpp4, Dlg1, Psd95) at the photoreceptor synapse. Whereas these proteins can associate in cellulo, they do not necessarily do so in photoreceptors in the retina. Mpp3 and Mpp4 have similar protein structures, and both of them exhibit dual localization patterns in the retina: OLM and OPL. We demonstrated by immunoprecipitations that Mpp3 and Mpp4 do not associate in the retina, and that these proteins might participate in alternative or competing protein complexes. We demonstrated in chapter 4 that Mpp3 and Mpp4 interact with different isoforms of Dlg1. This observation was further strengthened by the fact that in Mpp4\(-/-\)
photoreceptor synapses the localization of Dlg1 was not altered, whereas the localization of Veli3 and Psd95 was. In addition, the localization of Mpp3 was not altered. Veli3 staining did not disappear from the OLM in *Mpp4*/*-* retinae, which is most likely due to its interaction with Mpp5 present at the OLM.\(^{14}\) Moreover, in *Drosophila* it has been shown that Stardust (the Mpp5 homolog) is responsible for retention of DLin-7 (the Veli3 homolog) at the SAR, whereas Dlg1 is required for postsynaptic localization of DLin-7.\(^{17}\) In *Mpp4*/*-* retinae, Psd95 is lost from the photoreceptor synapse except from basal contacts in cone photoreceptors, but can still be detected postsynaptically in the dendrites of ganglion cells that do not contain Mpp4. We could not detect Mpp3-Veli3 or Mpp3-PSD95 interactions in retinal lysates (A.K., unpublished data), which might explain the inability of Mpp3 to retain the Psd95 and Veli3 proteins at the photoreceptor synapse. We therefore hypothesize that Mpp3 fulfills a different recruitment function at the photoreceptor synapse than Mpp4. Mpp4-Psd95 interaction, co-localization and retention dependence is an interesting example of tissue specific association of proteins. In the cerebellum, these two proteins do not localize in the same cells, do not depend on each other for their localization and most likely do not interact. In photoreceptors, Mpp3 and Mpp4 both localize to photoreceptor synapses, but are most likely themselves recruited into different protein complexes on similar vesicular and plasma membrane domains. This might explain the discrepancy between demonstrated Mpp3-Mpp4 interactions in 293 HEK cells and absence of interactions in retina.

In the retina, Mpp3 binds Dlg1 similar as in the brain.\(^{18}\) The N-terminal domain of Dlg1 has been shown to be essential for its proper targeting.\(^{19}\) Karnak et al. and Wu et al. showed that the L27 domain of Dlg1 can interact with L27 domains of CASK and Mpp3\(^{18,20}\), suggesting that in retina Dlg1 could bind Mpp3 and Mpp4 through L27 domains as well. Interestingly, CASK and Dlg1 appear to interact as well via GuK-SH3 intermolecular binding, although intramolecular interactions predominate in vitro.\(^{21}\) Similar GuK-SH3 interactions have been described by us in chapter 3 for Mpp4 and Mpp5. We cannot exclude a GuK-SH3 binding mode for Dlg1-Mpp3 and Dlg1-Mpp4. The SH3-GuK module shares a homology with voltage gated calcium channel β-subunits.\(^{22,23,24,25}\) This channel has a α1 membrane pore-forming subunit, a disulfide-linked α2δ complex and a cytosolic β-subunit, which interacts with the α1 subunit to promote channel membrane trafficking and affects its gating.\(^{26,27,28,29}\) Thus Dlg1, Mpp3 and Mpp4 could be involved in a similar interaction with yet unidentified channels or receptors at the photoreceptor synapse.

The predominance of PDZ domain-containing proteins in metazoans suggests that these specialized structures are developed in response to the increased needs to organize complicated signalling complexes. MAGUK proteins assemble large complexes by forming multiple interactions, which as shown here can differ in composition depending on the microenvironment, and thus probably providing alternative functions by usage of rather limited number of individual protein units.

Our work is an example of complicated interactions and redundancy of protein families (e.g CRB and MPP) in mammals, often resulting in phenotypes different than the ones described in more distant (and simple) genetic model organisms like *Drosophila*. This puts a stress on the need of developing proper vertebrate/mammalian models to study human diseases (e.g their pathogenesis and therapeutic strategies).
References


Annex to chapter 3

Interaction between MPP5 and CRB1
Transfection of 293 HEK cells and immunoprecipitations were done as described in chapter 3.

Interaction between MPP5 and CRB1
Anti-Myc antibody co-immunoprecipitated MPP5 from cells overproducing CRB1-Myc and MPP5 (lane 3) or CRB1-Myc only (lane 5). Note that in lane 5 endogenous MPP5 is co-precipitated. The 80 kDa form of MPP5 is not detected in the input of cells not expressing recombinant MPP5 (lane 2).
Fig. 2 Organization of Tight junctions / Subapical region and Adherens Junctions in epithelial and photoreceptor/Müller glia cells.
**Fig. 6 Domain organization of junctional and polarity proteins**

RA, Ras association domain; FHA, Forkhead associated domain; SynN, Syntaxin N-terminal domain; t-SNARE, Helical region found in soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs); Sorb, Sorbin homologous domain; Rho, Ras homology domain; ARM, Armadillo/β-catenin-like domain; LRR, Leucine rich repeats; CA, cadherin repeat; VIN, Vinculin binding region; IG-v-type, Immunoglobulin V-type; IG, Immunoglobulin like domain; WD40, WD (Trp-Asp) 40 repeats; C1, Protein kinase C conserved region 1; PB1, Bem1 protein domain; TM, transmembrane domain; PDZ, Postsynaptic density 95/Discs large/Zonula occludens domain; SH3, Src homology 3 domain; GuK, Guanylate kinase domain; L27, domain found in Lin2 and Lin7 proteins; HOOK, variable hinge (flexible region). CRB proteins are depicted in Fig. 4.
**Fig. 7. Developmental stages of Drosophila photoreceptors**

A. Third-instar eye disc. Apical domain of photoreceptors (orange) is separated from the baso-lateral membrane by the AJ (green).
B. 37% pupal development. Apical domains turn and face each other.
C. 67% pupal development. The rhabdomere (blue) and the stalk membrane form.
D. Schematic representation of a tangential section of an adult ommatidium. Rhabdomeres in blue; AJ in green; stalk membrane in orange; bristle in beige; secondary pigment cells in purple; tertiary pigment cells in grey.
Figure 1. Confocal images of 3 months old wild-type mice and human retinas. These images are high power insets of the OLM. (A) Crb1 is confined to the SAR, while β-catenin localized more basally at the AJ. (B) Crb1 localized at the basal part of the F-actin localization in the PRC inner segments. Mpp4 (C), Pals1 (D), Mupp1 (E) and Patj (F) localized at the SAR, compared to the location of β-catenin at the AJ. (G) aPKC co-localized with Crb1 to the SAR. (H-I) Crb2 and Crb3 localized to the SAR. A similar staining was detected in Crb1-/- retina. (J) CD44 localized in the MGC apical villi, but did not co-localize with Crb1 in the SAR. Par6, moesin or ZO-3 were not detected in the OLM (data not shown). Claudin-1 to –5 and occludin were not detected in the retina, but claudin-2 and occludin were detected in the retinal pigment epithelium (RPE). (K) CRB1 localized at the SAR of the human retina, apical to β-catenin in the AJ. (L) MUPP1 localized at the SAR of the human retina. (M) Schematic diagram of the localization for the different proteins at the SAR or AJ. Bars represent 2.5 μm.
Figure 3. Generation of Crb1−/− mice and confocal images of mouse retinas.

(A) Crb1 disrupted by insertion of the targeting vector. E1, exon 1; E2, Exon 2; pA, polyadenylation signal; PGK, phosphoglycerate kinase promoter; B, BamHI; RV, EcoRV; Bg, BgIII. (B-C) Deletion of the exon encoding the N-terminal signal peptide prevents the production of Crb1 protein with C-terminal transmembrane and intracellular domains. (B) EcoRV Southern blot analysis using a 750 bp BgIII-Accl fragment probe in the 3′ flanking region. (C) Immunoprecipitation of Crb1, with AK7, from lysate of wild-type but not of the Crb1−/− retina. As positive control 293/Crb1 cell lysates were used. Crb1 was stained using AK2. Asterisks represent cross-reacting bands with AK2 in 293 cells. (D-G) Localization of Crb1 (red) in the OLM and staining of cone segments and pedicles by peanut agglutinin (green) in the retinas of wild-type (D) and Crb1−/− (F) mice. Detail of the localization of Crb1 at the SAR for the wild-type (E) and Crb1−/− retina (G). Bars represent 30 μm.
Figure 5. Retinal phenotype of 3 months Crb1<sup>−/−</sup> old mice exposed to 72 hours 3000 lux. (A) Representative experiment indicating number of protrusions, ingrowths and total amount in cycled light (12 hours dark/12 hours 100 lux) vs. 72 hours 3000 lux exposed wild-type and Crb1<sup>−/−</sup> retinas. Error bars represent SEM. Asterisks indicate statistical difference (P<0.02) between the cycled and 72 hours 3000 lux exposed groups. (B) Numerous ingrowths areas through the OPL (arrow) and protrusions through a distorted OLM (arrowhead) in the Crb1<sup>−/−</sup> retina. (C) OLM present in rosette (arrow), no segments are present on the disorganized PRCs (arrowhead). (D-J) Fluorescence microscopy images of degenerated areas. Nuclei are stained with Hoechst (blue). Note that in unaffected areas, adjacent to the ingrowths areas, localization of the proteins is normal. In both cycled and 72 hours 3000 lux of light exposed wild-type mice GFAP localized near the inner limiting membrane, at the MGC end-feet and in horizontally radiating MGC rootlets in the OPL (data not shown). (E) Detail of (D), strong staining of GFAP in the ONL and through OLM. (F) Areas of protrusions (arrowheads) and ingrowths (arrows) where Mpp4 is lost at the OLM and OPL as well as mislocalized into the ONL (asterisk). (G) β-Catenin mislocalized throughout the ONL in ingrowths areas (arrows). (H) ZO-2 localization perturbed in a protrusion and ingrowth area. (I-J) Mislocalized and loss of Patj or Mupp1 in affected areas (arrowheads). (K) Apoptotic cells are rarely present in WT retinas. (L) Slightly increased apoptosis in Crb1<sup>−/−</sup> mice around ingrowths areas. Bars represent 50 μm. (M) SLO image (514.5 nm) of a 3 months old 72 hours 3000 lux exposed Crb1<sup>−/−</sup> mouse fundus. The multiple dots (arrowhead) indicate areas of rosette formation in the inferior temporal quadrant of the retina.
Figure 7. Electrophysiology of 9 months old Crb1<sup>−/−</sup> mice. (A) Scotopic b-wave amplitude vs. log Intensity (VlogI) function. Boxes indicate the 25% to 75% quantile range, whiskers the 5% and 95% quantiles, and the asterisk the median of the Crb1<sup>−/−</sup> data. The normal range is delimited by solid lines indicating the 5% and 95% quantile of the control group. There is no sign of impaired retinal function in Crb1<sup>−/−</sup> mice. (B) Typical scotopic intensity series in a wild-type (right column) and a Crb1<sup>−/−</sup> mutant mouse (left column). Log light intensities (from top to bottom) were -4, -3, -2, -1.5, -1, -0.5, 0, 0.5, 1, 1.5 log cd*s/m². No differences in amplitude or waveform are visible. (C-D) Electrophysiological results in 3 months old light exposed Crb1<sup>−/−</sup> mice. Also under these conditions, there is no sign of impaired retinal function in Crb1<sup>−/−</sup> mice. No differences in amplitude or waveform are visible. Calibration marks: Vertical 200 μV/div., horizontal 40 ms/div.
Fig. 3 Analysis of the interacting domains of MPP4 and -5.
(B) Homology modeling of MPP4 and -5. The initial models are shown on the left, covering the SH3 (yellow, green) and GuK domains (red, light blue). By swapping the domains, one obtains a heterodimer, half of which is shown in the middle: the SH3 domain of MPP5 bound to the GuK domain of MPP4. Energy calculations predicted a high binding energy for this interaction (Table 2), due to several salt bridges, shown in the close-up (right): the triad Glu 414\textsubscript{MPP4}-Lys 351\textsubscript{MPP5}-Asp 596\textsubscript{MPP4}, Lys 375\textsubscript{MPP5}-Glu 588\textsubscript{MPP4}, and (not shown) Arg 418\textsubscript{MPP4}-Glu 395\textsubscript{MPP5}. Note that an essential tyrosine (Y413) in the GUK domain of MPP4 is present in the core of the SH3 domain of MPP5.
**Fig. 5 Distribution of MPP4, MPP5, CRB1, and β-catenin in adult human retina.**
Confocal images of human retinas stained with antibodies against β-catenin (A, C, D, E, G, H, M, O, Q), CRB1 (B–D, I, K, L), MPP5 (F–H, J–L), MPP4 (N–Q), or control secondary antibodies (P). Anti-β-catenin antibody strongly stained the adherens junction (D, H, Q), whereas anti-CRB1 antibody AK2 (D, L), anti-MPP5 SN47 (H, L), and anti-MPP4 AK4 (N–Q) stained the SAR in the OLM. MPP5 and CRB1 colocalized at the SAR (L). AK4 stained the OPL (N, O) and the OLM (O, Q), whereas secondary antibodies (P) produced some background staining in the photoreceptor inner and outer segments. IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments; INL, inner nuclear layer. Scale bars: (A–C, H, L, P, Q) 10 μm; (D, E–G, I–K, M–O) 20 μm.
Figure 3. Localization of MPP3, MPP4, MPP5, DLG1 and β-catenin in adult human retina.

(A-Q). Confocal images of human retinae stained with antibodies against β-catenin (A, C, D), MPP3 (B-D, F-H, M, O, Q), MPP4 (J-L), MPP5 (N, O, Q), and DLG1 (E, G-I, K, L). Anti-β-catenin antibody strongly stained the adherens junction (A, C, D), whereas anti-MPP3 CPH8 (B-D) stained the region just apical to the outer limiting membrane (OLM) (D) and parts of the outer plexiform layer where synapses are formed between the photoreceptors and bipolar cells. (OPL) (F-H). MPP5 and MPP3 colocalize (O, Q). Anti-DLG1 antibody stained the OPL (E, I), where it partially colocalized with MPP3 (G, H) and MPP4 (K, L). In (J) antibody-epitope retrieval was not used, therefore levels of MPP4 at the OPL are well detectable but at the OLM are not[22,24]. IS, inner segments; OS, outer segments; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer. Scale bar represents 20 µm, excluding the detail inserts where it is 10 µm.
Figure 3. Confocal images of 3 months old **Mpp4^{+/+}** and **Mpp4^{--}** retinas. Retinal localization of Mpp4, Veli3, Dlg1, Cask, Psd95 and Crb1. (A) After glycine treatment of paraffin sections, Mpp4 was detected at the OLM and OPL of **Mpp4^{+/+}** but not of **Mpp4^{--}** retina. The background staining in the INL was not observed in frozen section but the signal at the OLM was weaker (17). No signal was detected in the cilia of wild-type mice. (B) Reduced Veli3 signal in the OPL of **Mpp4^{--}** retinas. Although Veli3 signal is changed in the OPL, staining of the OLM and cones in the ONL remained unchanged. (C, D) Dlg1 and Cask staining at the OPL was comparable between **Mpp4^{--}** and **Mpp4^{+/+}** retinas. (E) Reduced Psd95 signal in the OPL of **Mpp4^{--}** retinas. (F) Crb1 localization at the OLM is unaltered. (G-I) Merged pictures of the OPL signals for Mpp4 and Psd95 (upper), Veli3 and Psd95 (middle) and Cask and Psd95 (bottom) depicting the colocalization of Mpp4, Veli3 and Psd95 at the plasma membrane of the synaptic terminal, while Cask is localized at the tip of the terminal. Figure 3. A-F and G-I are made at similar magnifications. Scale bars: 10 µm.
Figure 5. Immunoprecipitation of Mpp4 detecting Psd95 and Veli3. (A) Anti-Mpp4 (AK4) coimmunoprecipitated Psd95 (95 kDa), Dlg1 (100 kDa) and Veli3 (22 kDa) but not Cask (110 kDa) from retinal membrane fractions. 2% input is 2% of retinal lysates prior to immunoprecipitation. Note the reduced Psd95 and Mpp4 protein levels in the Mpp4<sup>-/-</sup> retinal lysates. No changes were observed in Psd95 cerebral protein levels (repeated experiment; n=3). (B) Dlg1 protein levels (140 & 100 kDa) in Mpp4<sup>-/-</sup> and wild-type total retinal lysates. Psd95 protein levels (95 kDa) in Mpp4<sup>-/-</sup> and wild-type total retinal lysates. (C) Absence of colocalization of Mpp4 and Psd95 in Mpp4<sup>-/-</sup> and wild-type cerebral sections. Mpp4 staining in Mpp4<sup>+/+</sup> cerebellum is observed in the Purkinje cells. ML; molecular layer, PCL; Purkinje cell layer, IGL; intergranular layer. Scale bars: 50 µm
Samenvatting

De prevalentie van erfelijke netvlies degeneratie bedraagt ongeveer 1 op 3000 levend geboren kinderen. Patienten met retinitis pigmentosa (RP) lijden aan een voortschrijdend verlies van het donkeradaptatie- en het gezichtsvermogen waarvan de eerste symptomen vaak ontstaan op jongvolwassen leeftijd. Mutaties in het Crumbs homoloog 1 (CRB1) gen zijn ontdekt in patienten met verschillende vormen van RP waaronder Leber’s aangeboren blindheid (congenitale amaurosis, LCA). Het wegvalen van de CRB1 functie veroorzaakt LCA of andere progressieve vormen van RP, afhankelijk van de resterende CRB1 activiteit en de overige genetische achtergrond. In dit proefschrift staat het muizen CRb1 eiwit centraal evenals de mogelijke hiermee geassocieerde membraan gepalmitoyleerde eiwitten -3 (membrane palmitoylated protein 3, Mpp3), -4 (Mpp4) en -5 (Mpp5, identiek aan Pals1) die behoren tot de membraan geassocieerde guanylaat kinase (MAGUK) familie.

Een literatuur overzicht van de huidige kennis over Crb1, de eiwitten waarmee Crb1 een interactie aangaat en hun vermoede functie in verschillende diermodellen staat in hoofdstuk 1.

Om de functie van het Crb1 eiwit in de preventie van retinale degeneratie te analyseren hebben we met behulp van genetische modificatie een muizenmodel gemaakt waarin het Crb1 eiwit ontbreekt, verwachtend dat deze muizen een model zouden zijn voor het ziektebeeld LCA. Het maken en karakteriseren van deze zogenaamde knockout muizen (Crb1−/− muizen) is beschreven in hoofdstuk 2. De Crb1−/− muizen waren gezond, vruchtbaar en vertoonden geen duidelijke afwijkingen. Het expressiepatroon van het Crb1 eiwit werd bestudeerd in niet-gemodificeerde (zogenaamde wild-type) dieren, gebruikmakend van de Crb1−/− muizen als vergelijkingsmateriaal. Crb1 was zowel bij de muis als mens, met name te vinden in de buitenste begrenzende membraan (outer limiting membrane, OLM) van het netvlies, een gebied dat de aanhechtingpunten ("adherens junctions") tussen Müller cellen en fotoreceptoren bevat. Crb1 en de verwante eiwitten Crb2 en Crb3 werden gelocaliseerd in het gebied net boven deze ‘adherens junctions’, de zogenoemde subapicale regio (SAR). We toonden aan dat de SAR veel eiwitten bevat die normaal gevonden worden in nauwe aanhechtingpunten (‘tight junctions’) in één enkele cellaag van gepolariseerde epitheel cellen. Op basis van electronenmicroscopische waarnemingen bleken nauwe aanhechtingpunten (tight junctions) echter te ontbreken in het netvlies en in overeenstemming hiermee werden eiwitten zoals claudines niet waargenomen in de OLM. Verder onderzoek liet zien dat het meervoudige PDZ eiwit Mup1 een complex kan aangaan met Mpp5/Pals1, Mpp4 en Crb1.

Drie maanden oude Crb1−/− muizen ontwikkelden locale gebieden in het netvlies waar de ordening van Müller cellen en fotoreceptoren veranderd was zonder dat er cellen verloren gingen. In het Crb1−/− netvlies waren de kernen van de lichtgevoelige cellen (fotoreceptoren) verplaatst naar de zogenaamde subretinale ruimte tussen het retinaal pigment epitheel (RPE) en de buitenste kernlaag (outer nuclear layer, ONL), en naar de onderliggende buitenste (“outer”) plexiforme laag (OPL). Dit wijst op het verlies van een adhesie tussen de Müller cellen en fotoreceptoren ontstaan door veranderingen in de OLM. De veranderingen in het netvlies konden versterkt worden door blootstelling aan gemiddelde niveaus wit licht. In de niet-aangedane gebieden was de localisatie van de Crb interacterende eiwitten niet significant veranderd. Dit kan betekenen dat Crb1 niet
Samenvatting

noodzakelijk is voor een correcte localisatie van Crb interacterende eiwitten in de OLM. Daarnaast zijn Crb2 en Crb3 nog altijd aanwezig in de OLM, wat suggereert dat deze eiwitten de rol van Crb1 niet geheel kunnen overnemen. De conclusie uit deze experimenten luidde dat Crb1 noodzakelijk is voor het behoud van adherens junctions in de OLM gedurende blootstelling aan wit licht en niet voor het ontstaan van de adherens junctions. Na 6 tot 9 maanden, vertoonh het Crb1\(^{-/-}\) netvlies focale degeneratie waar de fotoreceptoren evenals interneuronen en ganglion cellen verdwijnen. Activatie van de Müller cellen is te zien aan de toegenomen interferilamenten. Opmerkelijk was dat de netvliesdegeneratie minder dan 25% van het totale netvliesoppervlak besloeg en met name in het buiten-onderste gedeelte zat. Dat kan de reden zijn dat het electroretinogram (ERG) niet verschillend was tussen Crb1\(^{-/-}\) and wild-type netvliezen. Deze waarneming verschilt belangrijk met de situatie in de mens: bij LCA patienten met CRB1 mutaties is het ERG binnen het eerste levensjaar beduidend verslechterd. De reden voor deze tegenstelling is onduidelijk. Recente experimenten (van Rossum et al, niet gepubliceerd) laten zien dat Crb1\(^{-/-}\) netvliezen, 36 uren na de geboorte uitgenomen en die drie weken in kweek worden gehouden, voor 60-80% van organisatie veranderen. Dit toont aan dat groefactoren en bijbehorende receptoren een mogelijke rol spelen in het voorkomen van degeneratie van de Crb1\(^{-/-}\) retina.

Bij onze speurtocht naar eiwitten die een interactie aangaan met het humane CRB1-bindend eiwit MPP5/PALS1 hoopten wij nieuwe eiwitten in het CRB1 protein complex te vinden. In hoofdstuk 3 hebben we de interactie tussen MPP5/PALS1 en MPP4 onderzocht. We gebruikten het SH3-HOOK gedeelte van MPP5 in een gist twee-hybride systeem en vonden dat het gebonden werd aan het GUK gedeelte van MPP4. Voor immuno-precipitaties werden de volledige cDNAs van MPP4 en MPP5 gekloneerd en tot overexpressie gedracht in humane embryonale niercellen (293 HEK). Co-immunoprecipitatie van beide eiwitten bevestigde de interactie tussen het volledige MPP4 en MPP5. In cellen waarin de drie eiwitten CRB1, MPP5 en MPP4 tot expressie kwamen, ontstonden CRB1/MPP5/MPP4 complexen. Hieruit volgt de conclusie dat MPP4 onderdeel kan zijn van het CRB1 complex door een directe verbinding van het MPP4 GUK gedeelte met het MPP5 SH3 gedeelte. Gecomputeriseerde molecular dynamische analyse toonde interacties tussen homo- en heterodimers van MPP4 en MPP5 via een 3-dimensioneel domein uitwissel mechanisme. Dit mechanism laat toe dat de eiwitten dimeren of zelfs meervoudige oligomeren vormen met behulp van de uitwisseling van gelijkende structuurdeel. We maakten antilichamen tegen CRB1, MPP4 en MPP5 en gebruikten die voor het localiseren van deze eiwitten in het netvlies. De eiwitten waren alle drie te vinden in de OLM van het menselijke netvlies in de subapicale regio (SAR) direct naast de aanhechtingspunten (adherens junctions). Immuno-electronenmicroscopie liet zien dat muizen Mpp4 terug te vinden was nabij de synapsmembraan van de fotoreceptoren, in de synapsblaasjes in de OPL, en in de fotoreceptoren nabij de apicale plasma membraan ter hoogte van de aanhechtingspunten tussen fotoreceptoren en Müller cellen. MPP5 kwam tot expressie in veel organen, terwijl MPP4 en CRB1 met name tot expressie kwamen in het netvlies. Deze resultaten wijzen erop dat MPP4 betrokken kan zijn bij fotoreceptor polariteit en het functioneren van de synaps. Daarnaast zijn de associatie met MPP5 en z’n weefsel-specifieke expressie aanleiding om MPP4 te zien als een kandidaat voor erfelijke afwijkingen aan het netvlies.
Naast de voorafgaande twee MPPs, kwam in de retina nog een derde MPP tot expressie: MPP3. Onze experimenten hadden als doel de mogelijke interactie van MPP3 met componenten van het CRB1 complex te onderzoeken. **Hoofdstuk 4** beschrijft de volledige klonering van cDNA van *MPP3* en de karakterisering van een cDNA dat koeide voor een MPP3 variant dat het GUK gedeelte mist (MPP3ΔGU). Het volledige MPP3 eiwit is gevonden in het netvlies van muis en mens. In de huidige experimenten kon het MPP3ΔGU eiwit niet in het netvlies gevonden worden. Dit kan worden veroorzaakt doordat het eiwit niet stabiel is door gebrek aan goede vouwing, hoewel dit laatst opnieuw bevestigd moet worden. In het netvlies is MPP3 te samen met CRB1 en MPP5 te vinden in de SAR van de OLM direct naast de aanhechtingspunten. In de OPL wordt MPP3 gedeeltelijk samen met MPP4 en DLG1 gevonden. We brachten MPP3, MPP3ΔGU, MPP4, MPP5 en CRB1 in 293 HEK cellen tot overexpressie voor co-immunoprecipitatie experimenten. MPP3ΔGU was in staat te binden aan MPP4 maar niet aan MPP5, wat verschillende manieren van interactie suggereert. Het volledige MPP3 kon binden met MPP4 en MPP5; met CRB1 was deze binding zwak en alleen in aanwezigheid van MPP5. Eiwitcomplexen van muisen Mpp3 en Mpp5 werden gevonden in oplossingen van netvliescellen, echter bevatten de eiwitcomplexen geen Crb1. Op basis hiervan concludeerden we dat Mpp3 door Mpp5 naar de SAR van de OLM direct boven de aanhechtingspunten wordt gebracht. Een grote hoeveelheid Mpp4 werd waargenomen in de OPL, maar complexen van Mpp3 en Mpp4 konden niet geprecipiteerd worden uit oplossingen van muisennetvliescellen. Dit suggereert dat Mpp3 en Mpp4 in het netvlies niet aan elkaar binden. Deze laatste waarneming werd versterkt door het feit dat Mpp4 en Mpp3 in deze celoplossingen afzonderlijk werden gevonden in precipitaties met verschillende vormen van Dlg1. Deze resultaten wijzen op een rol van Mpp3 in fotoreceptor polariteit en bestempelen Mpp3 vanwege zijn binding aan Mpp5 ook als kandidaatgen voor erfelijke netvliesafwijkingen.

Het laatste gedeelte van dit proefschrift is gewijd aan de fysiologische rol van Mpp4 in de muis. De bijdrage van Mpp4 aan het CRB1 eiwitcomplex in de OLM en aan het DLG1 eiwitcomplex in de OPL werd onderzocht. Daartoe maakten we muisen die geen Mpp4 tot expressie brengen. Deze *Mpp4-/−* muisen waren gezond, vruchtbaar en vertoonden geen duidelijke afwijkingen. Scanning laser oogspiegelen (SLO) bracht geen verschijnselen van een verstoorde organisatie of degeneratie van het netvlies aan het licht. Tot de leeftijd van 18 maanden vertoonden het electroretinogram geen afwijkingen. Het expressiepatroon van het Mpp4 eiwit werd bestudeerd in wild-type dieren, gebruikmakend van de *Mpp4+/−* muizen als negatieve controle. Mpp4 kwam tot expressie in de OPL en OLM. In *Mpp4+/−* muizen vonden we soms gebieden met netvlies disorganisatie, zoals netvliesplooien en de vorming van rosetten, zelfs nog in een veel lagere frequentie dan in *Crb1+/−* muizen. Er was geen verschil tussen *Mpp4+/−* en wildtype muizen in localisatie van de merkers voor de SAR of de aanhechtingspunten in de OLM. Evenmin vonden wij aanwijzingen voor de betrokkenheid van Mpp4 in het Crb1 eiwitcomplex. De structuur van de fotoreceptor synaps was niet veranderd en de meeste synaps eitwitten, geselecteerd op basis van hun vermeende functie, eitwistructuur en in de literatuur vermelde interacties, waren normaal gelocaliseerd, met uitzondering van Psd95 en Veli3. De immuno-positieve signalen voor Psd95 en Veli3 waren significant verminderd in de synapsen van staafjes en kegeltjes. Het totale eitwtniveau van Veli3 was onveranderd, terwijl het eitwtniveau van Psd95 significant was verminderd. Dit
Inherited forms of retinal dystrophy affect approximately 1 in 3000 individuals world-wide. Patients with retinitis pigmentosa (RP) experience progressive night blindness and visual loss with a wide range of expression from blindness at birth to a start late in adulthood. Mutations have been identified in the Crumbs homolog 1 (CRB1) gene in individuals with various forms of RP and Leber's congenital amaurosis (LCA). Loss of CRB1 function may cause among others the eye disease LCA or other forms of progressive RP, depending on the amount of residual CRB1 activity and the genetic background. The work in this thesis focuses on the mouse Crb1 protein and putatively associated members of the membrane associated guanylate kinase (MAGUK) protein family, membrane palmitoylated proteins-3 (Mpp3), -4 (Mpp4), and -5 (Mpp5, also called Pals1).

A literature review is provided describing the current knowledge about Crb1, its associated proteins, and their functions in a number of animal models (chapter 1). To study the protective function of CRB1 against retinal degeneration we generated and analyzed mice lacking Crb1, and we hypothesized that these mutant mice would be a mouse model for LCA. The generation and characterization of Crb1-/- mice is described in chapter 2. The Crb1-/- mice are viable, fertile and show no overt phenotype. Using the Crb1-/- mice as null controls we studied the distribution of Crb1 in wild type mice. We detected Crb1 specifically at the outer limiting membrane (OLM), a region that contains adherens junctions between photoreceptors and Müller glia cells. Crb1, and the family members Crb2 and Crb3, are localized at a subapical region (SAR) adjacent to the adherens junctions. We could show that the subapical region contains many proteins normally detected in tight junctions of polarized monolayers of epithelial cells. In accordance with the lack of tight junction structures in the neural retina observed with electron microscopy, essential tight junction proteins such as claudins were absent at the OLM. Further studies showed that the multiple PDZ domain protein Mupp1 formed complexes with Mpp5/Pals1, Mpp4 and Crb1.

The Crb1-/- mice developed at 3 months of age disorganization of photoreceptors and Müller glia cells at foci in the retina, without significant retinal degeneration. The mutant retina showed abnormal localization of photoreceptor nuclei in the outer plexiform layer (OPL), and in the so-called subretinal space between the retinal pigment epithelium (RPE) and the outer nuclear layer (ONL), indicating loss of cell adhesion between Müller glia cells and photoreceptors due to loss of integrity at the OLM. Retinal disorganization was significantly increased upon exposure to moderate levels of white light. Nevertheless, in unaffected regions of the mutant retina, Crb1-interacting proteins were not significantly dislocalized, indicating that Crb1 is not required to recruit the Crb1-interacting proteins to the OLM. Crb2 and Crb3 were still detected at the OLM, suggesting that these proteins can not efficiently rescue the phenotype in Crb1-/- retinas. These studies led us to conclude that Crb1 is required to maintain adherens junctions upon exposure to white light, and not for the assembly of these junctions. At 6-9 months of age, Crb1-/- mice showed focal retinal degeneration, focal death of photoreceptors but also interneurons and ganglion cells, and an increase in intermediate filaments indicating activation of Müller glial cells. Surprisingly, retinal degeneration in the Crb1 mutant mouse occurred in less than 25% of the retina, in a lateral inferior region. Therefore
Summary
electroretinography (ERG) recording did not detect differences between mutant and wild type retinas. The ERG findings are an important difference with human LCA patients lacking CRB1 in which ERG’s are significantly reduced within the first year of life. It is still unclear what the reasons are for these discrepancies. Whereas Crb1<sup>−/−</sup> mice develop retinal disorganization at 3 months of age, Crb1<sup>−/−</sup> retinas isolated at 1.5 day postnatally and cultured for 3 weeks, develop disorganization and degeneration in 60-80% of the retina (van Rossum et al., unpublished), suggesting that growth factors or their signaling receptors play a role in prevention against retinal degeneration in Crb1<sup>−/−</sup> retinas.

Our screening studies for proteins that interact with the CRB1-interacting protein MPP5/PALS1 aimed at identification of novel proteins in the CRB1 protein complex. In chapter 3 we studied the molecular interaction of MPP5 with MPP4. We used the SH3-HOOK domain of MPP5 in yeast two-hybrid studies and identified the guanylate kinase(GuK) domain of MPP4 as binding partner. We cloned the full length cDNAs of human CRB1, MPP4 and MPP5 and overexpressed them in human embryonic kidney (293 HEK) cells for immunoprecipitation studies. Interaction between full length MPP4 and MPP5 was identified since the proteins co-immunoprecipitated. Cells that overexpressed CRB1, MPP5, and MPP4 showed formation of CRB1/MPP5/MPP4 complexes. We found therefore that MPP4 exists in a complex with CRB1 through direct interaction of its GuK domain with the SH3 domain of MPP5. Computer-based molecular dynamics analysis showed in cellulo interaction of homo-and heterodimers of MPP4 and MPP5 through a 3 dimensional domain swapping mechanism. The latter allows proteins to assemble dimers or higher order oligomers by a complementary substructure exchange. Antibodies generated against CRB1, MPP4 and MPP5 were used to determine the localization of the proteins in the retina. The three human proteins co-localized at the OLM of human retina at the subapical region (SAR) adjacent to the adherens junctions. Immuno-electron microscopy showed localization of mouse Mpp4 at the membranes of photoreceptor synaptic terminals and membrane-proximal vesicles at the outer OPL, as well as at the plasma membrane apical to and at the adherens junction contacts. MPP5 was expressed in many organs, whereas MPP4 and CRB1 were predominantly expressed in the retina. These data implicated a role for MPP4 in photoreceptor polarity and synapse function and, by association with MPP5 and its tissue-restricted expression, pinpointed MPP4 as a functional candidate gene for inherited retinopathies.

The retina expresses at least a third MPP protein, MPP3. The studies reported here for MPP3 were aimed at studying putative interactions of MPP3 with components of the CRB1 complex. In chapter 4 we cloned the full-length cDNA for MPP3 and identified a cDNA that encoded a variant MPP3 that lacks the GuK domain (MPP3ΔGuK). Antibodies against MPP3 were generated and used in interaction and localization studies. The full length MPP3 was present in human and mouse retina. In the current studies the (MPP3ΔGuK) variant could not be detected in these retinas. This might be due to instability of the protein since it likely can not fold intramolecularly, but the latter needs to be validated. In the retina, MPP3 co-localized at the OLM with CRB1 and MPP5 at the subapical region adjacent to the adherens junction. At the OPL, MPP3 partially co-localized with MPP4 and Discs Large (DLG1). We overexpressed MPP3, MPP3ΔGuK, MPP4, MPP5 and CRB1 in 293 HEK cells for immunoprecipitation studies. MPP3ΔGuK interacted with MPP4 but not with MPP5, suggesting different modes of interaction. The full length MPP3 interacted with MPP4 and MPP5; with CRB1
this interaction was weak and detectable only in the presence of high levels of MPP5. Complexes of Mpp3 with Mpp5 could be precipitated from mouse retinal lysates, but no Crb1 could be detected in the immunoprecipitate. We suggested therefore that Mpp3 is recruited to the Mpp5 protein complex at the subapical region adjacent to adherens junctions. The highest levels of Mpp4 were detected at the OPL. Complexes of Mpp3 with Mpp4 could not be precipitated from mouse retinal lysates, suggesting that they do not interact in the retina. The latter observation is strengthened by the finding that Mpp3 and Mpp4 co-immunoprecipitated different isoforms of Dlg1 from retinal lysates. These data implicated a role for MPP3 in photoreceptor polarity and, by association with MPP5, pinpoint MPP3 as a functional candidate gene for inherited retinopathies.

The last section of this thesis focused on the physiological function of mouse Mpp4. To further study the contribution of Mpp4 to the Crb1 protein complex at the OLM, and the Dlg1 protein complex at the OPL, we generated mice lacking Mpp4 (chapter 5). The Mpp4−/− mice were viable, fertile and showed no overt phenotype. Scanning laser ophthalmoscopy revealed no retinal disorganization or degeneration, and registration of the light-induced electrical responses from the retina by electroretinography up to 18 month of age revealed no loss of overall retinal function. Mpp4−/− mice showed very limited focal retinal disorganization such as retinal folding and formation of rosettes of photoreceptors, and to an even lesser extent than observed in Crb1−/− mice. At the OLM, markers for the subapical region and its adjacent adherens junction were not differently localized compared between Mpp4−/− and wild type mice, and we did not find further indications for involvement of Mpp4 in the Crb1 complex. The synapse structure was not altered and most synaptic proteins tested (chosen on the basis of their protein structure, putative functions and interactions described in literature) were localized as normal, except for Psd95 and Veli3 that showed significant reduction of protein levels at the rod and cone photoreceptor synapse. Total retinal protein levels of Veli3 remained unchanged, whereas levels of Psd95 were significantly reduced, suggesting a role for Mpp4 in the prevention of Psd95 degradation. Some Psd95 protein remained localized in the Mpp4−/− retina at the basal contacts in cone photoreceptors, suggesting that in limited subdomains of cone photoreceptors Mpp4 is not required for Psd95 recruitment and complex stabilization. Further studies showed co-localization of Mpp4, Dlg1, Psd95 and Veli3 at the OPL. Complexes of Mpp4/Dlg1, Mpp4/Psd95 and Mpp4/Veli3 could be immunoprecipitated from retinal lysates, but Dlg1 did not show altered localization in Mpp4−/− retinas.

The described data resulted in the hypothesis that membrane-associated Dlg1 retains Mpp4/Veli3/Psd95 complexes at pre-synaptic vesicles and plasma membranes. The described data in this thesis suggest that Mpp4 could be a critical recruitment factor to organize signal transducers at the photoreceptor synapse and could be associated with synaptic plasticity and protein complex transport.
Thank word

It has been a pleasure and challenge for me to carry my dissertation thesis in the Netherlands Institute for Neurosciences over the last 4.5 years. I would like to use this opportunity to express my gratitude to many people who supported me on my way. Many thanks to my supervisor and co-promoter Dr. Jan Wijnholds for the patience, discussion time and guidance provided by you to my research and writing skills. I am happy I had the opportunity to carry this project in a lab never short of equipment, supplies and helping hand. I am grateful to my promoters Paulus de Jong and Anton Berns for the wake up calls, contributions to my development, and patience with reading my papers in preparation. The work has been helped by friendly and sharp colleagues in the group (in alphabetical order): Agnes, Anna, Carla, Dounia, Inge, Jan, Serge, Svetlana, and Wendy, who have shared their scientific experience and views with me. Many people around the institute, and KNAW have eased my time here by assisting me with practical advice, PR or legal issues, reagents for research or sociable attitude throughout the years (in alphabetical order): Alexander, Anneke, Arthur, Bart, Ben, Christiaan, Christina, Cor, Damian, Ernita, Frederike, Hadi, Heidi, Jacoline, Jan, Joep, Leny Luyendijk, Lidy, Luuk, Maarten, Marcia, Rikesh, Simone, Svetlana, Theo, Ton, Wil, William and Xiaofeng. Many thanks to the members of my thesis committee for reading this work. No PhD student can survive without the love and support of friends and family. I will take the opportunity to thank my family for their believe in me, constant understanding, and protection from misfortunes in life. My father did not live to see me making the final steps of this trip, but through the invisible link he left in his daughter, he could navigate me in the last harsh moments. I am also very fortunate to have devoted friends in Bulgaria, here in Holland, and around the world, who have shared with me the difficult as well as the happy moments, the hopes and disappointments and numerous discussion on various life and science related topics. Thank you very much (in alphabetical order): Albena Jordanova, Areezoo, Asad, Atanas, Bob, Borislava, Boryana, Dimitar, Dobri, Ilia, Ivailo, John, Krasimira, Lyubomira, Martijn, Monika, Nevena, Nikola, Nikolai, Pavlina, Plamen, Roland, Rumen, Sancho, Sergei, Sridhara, Stefan, Stela, Stoyan, Tanya, Vasili, Vesna, Zlatina. Frustrations and worries are inherent to his period, but you have always found the right way to comfort and encourage me.
Curriculum Vitae

The author of this thesis was born on 28\textsuperscript{th} of December in 1976 in nowadays Turkmenistan, which was at that time part of USSR. She graduated in Bulgaria in 1994 from a Russian language school. Albena started her university study in 1995 and in 2000 obtained a master degree in biotechnology from University of Sofia “Kliment Ohridsky”. She did her master thesis work in the Laboratory of Molecular Pathology at the University Hospital of Obstetrics and Gynaecology under the supervision of Prof. Ivo Kremenski and Dr. Albena Jordanova. The topic of that work was “Molecular diagnostics of Bulgarian patients with Charcot-Marie-Tooth type 1 disease (CMT1) using polymorphic DNA markers”. In 2001 Albena began a PhD study in the Division of Development, Aging and Genetic Diseases at The Netherlands Ophthalmic Research Institute Amsterdam under the supervision of Dr. Jan Wijnholds. The work concentrated on the role of Crumbs and associated Postsynaptic density 95/Discs large/Zonula occludens (PDZ) proteins in retinal dystrophies. That led to a change of field from human genetics to protein work and analysis of murine disease models.

For her future scientific development Albena is interested in obtaining knowledge in the field of stem cells, including fundamental science concerning culturing, differentiation and possible genetic modification of the cells, with later application for regeneration.
List of publications


* Indicates equal contribution