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Crumbs proteins in epithelial morphogenesis

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1. ABSTRACT

Cell polarity is an essential feature of most eukaryotic cells, especially epithelial cells in multicellular animals. Polarity protein complexes that regulate epithelial organization have been identified. In this review, it is proposed to describe how the Crumbs complex acts in the process of cell polarity and epithelial organization. During the last decade, several partners of Crumbs, an apical transmembrane protein, have been identified and their direct or indirect associations with the cytoplasmic domain of Crumbs have been dissected. In addition, mutants of several of the genes encoding proteins belonging to the Crumbs network have been obtained in animals ranging from flies to mouse, which have led to a better understanding of their functions *in vivo*. These functions include polarity axis formation, stabilization of epithelial apico-lateral junctions, photoreceptor organization and ciliogenesis. Since human *CRUMBS1* mutations are associated with retina degeneration, it has become essential to define Crumbs network and to understand exactly how this network acts in polarized cells, with a view to developing suitable therapeutic approaches for treating this severe degenerative disease.

2. INTRODUCTION

Cell polarity is first characterized by the formation of a polarity axis that leads to the vectorial distribution of different proteins within the cell where they exert vectorial functions. The processes involved in establishing cell polarity are under the control of protein complexes and involve the re-organization of actin and microtubule cytoskeletons. This occurs in yeast where the tightly regulated formation of a bud leads to the coordinated accumulation of cell material in the bud to give rise to a new cell (for recent review see (1)). In animal cells, additional mechanisms are used to create transient or more stable cell sub-domains along a polarized axis. Cell to matrix adhesion mechanisms induce the formation of an apico-basal axis, while cell to cell adhesion mechanisms stabilize the lateral plasma membrane domains (for review see (2)). Similar processes occur in migrating cells and neurons, where a front to rear or an axonal to dendritic axis is also created by stabilizing membrane domains while keeping a strong plasticity. In animal cells, these polarity axes are governed by a set of genes which were first identified in *Caenorhabditis elegans* by genetic screens to identify any mutants that were unable to undergo asymmetric cell division in the early embryo (3). These genes encode Par (partition defective) proteins that are acting as two opposite groups (Par3, Par6 and aPKC in the anterior side and Par1, Par2 and Par4 in the posterior side) to polarize the early embryo. These protein complexes are conserved in the course of animal evolution, and further studies on *Drosophila melanogaster* have shown the existence of new genes which are essential for epithelial polarity. In addition to the Par system, two other genetic complexes are crucial to the establishment and maintenance of epithelial polarity. First there is the Crumbs complex (DCrumbs (DCrb), Stardust (Sdt) and DPatj), its organization and function will be described below, and the second complex Scribble consists of at least three genes: Scribble, Discs Large and Lethal Giant Larvae (which were recently reviewed in (4)). During the early stages of fly embryogenesis, all these complexes act in a coordinated fashion to localize and stabilize apico-lateral junctions providing a visible border between the apical domain facing the outside of the embryo and the lateral domain (for review, see (5)). While most of the genes controlling epithelial polarity are maternally provided and are therefore probably involved in early polarity determination, it is worth noting that in flies, two members of the Crumbs complex, DCrb and Sdt, start to be expressed at an early stage in gastrulation, which suggests that they may play a crucial role in Zonula adherens (ZA) stabilization and epithelial cohesion (6). Early gastrulation, which coincides with the first cell movements, requires strong cell-cell adhesion processes in the first epithelial layer that is provided by the ZA belt located at the apico-lateral border. In this review, we will focus on the role of a specific family of proteins, the Crumbs family, which form a network of interactors involved in epithelial and neuroepithelial morphogenesis. Crumbs proteins probably originated at an early stage in animal evolution, since they have been found to exist in nematodes, echinoderms, arthropods and vertebrates, but their first appearance in the course of evolution has not yet been determined: for this purpose, genome sequencing will have to be performed on species such as sponges and cnidarians originating from the early branching of the animal tree.

3. CRUMBS PROTEINS: EVOLUTION AND SCAFFOLDING

3. 1. Evolution of Crumbs proteins among known animal genomes

Crumbs was first identified in *Drosophila melanogaster,* where mutations in this gene produced an absent or dispersed cuticle, hence giving the name "crumbs" (DCrb in the case of *Drosophila* Crumbs) (7, 8). This phenotype indicated a role of DCrb in epithelium organization as the cuticle is secreted by the underlying epithelial epidermis. DCrb is a transmembrane protein with a small cytoplasmic domain consisting of 37 amino acids and a large extracellular domain consisting of 28 EGF-like repeats and three laminin G-like repeats (Figure 1A). A few years later, Wodarz *et al.* (9, 10) established that the cytoplasmic domain of DCrb is crucial for its function in epithelial morphogenesis. This intracellular domain shows a FERM- (4.1-ezrin-radixin-moesin) binding motif (11), a C-terminal PDZ- (PSD-95, Discs Large, ZO1) binding motif (ERLI aminoacids) and a putative DaPKC phosphorylation motif (12).

The first evidence of a conservation of Crumbs between invertebrates and vertebrates was presented 10 years later by den Hollander *et al.* (13). The authors discovered that a human gene mutated in Retinitis Pigmentosa group 12 (RP12) disease was very similar to *DCrb*. This gene was called *CRB1* and the largest protein isoform expressed has the same domain architecture as both the extra and intracellular domains of DCrb (Figure 1A). Two additional genes, *CRB2* and *CRB3*, were then discovered (14) and found to encode proteins with a highly conserved cytoplasmic domain (showing 51 and 58% of identity with CRB1) (Figure 1A). CRB3 is the only homolog exhibiting a very short extracellular part with no recognizable protein domain and a proline-rich domain in the intracellular part for CRB3A (two isoforms have been described: CRB3A and B, as shown in Figure 1A). Several splice variants have been described, which encode either proteins lacking the transmembrane and intracellular domains, which are therefore presumably secreted (CRB1s and CRB2s) (15-17) or a protein with a C-terminal cytoplasmic domain lacking the proline rich region and showing a different PDZ-binding motif (CLPI instead of ERLI, in the case of CRB3B) (18, 19) (Figure 1A and B).

Subsequent studies have shown that Crumbs is also conserved in other model species such as mouse (20), worm (11, 21) and zebrafish (22). The short intracellular domain was found to serve as the docking site for a highly conserved protein scaffold. This functional domain is frequently used to study the evolution of CRB proteins. In this review, we have compared the transmembrane and cytoplasmic sequences of CRB orthologs using a multi-alignment software programme (ClustalW) (Figure 1B), and we have generated a phylogenic tree (PHYLIP

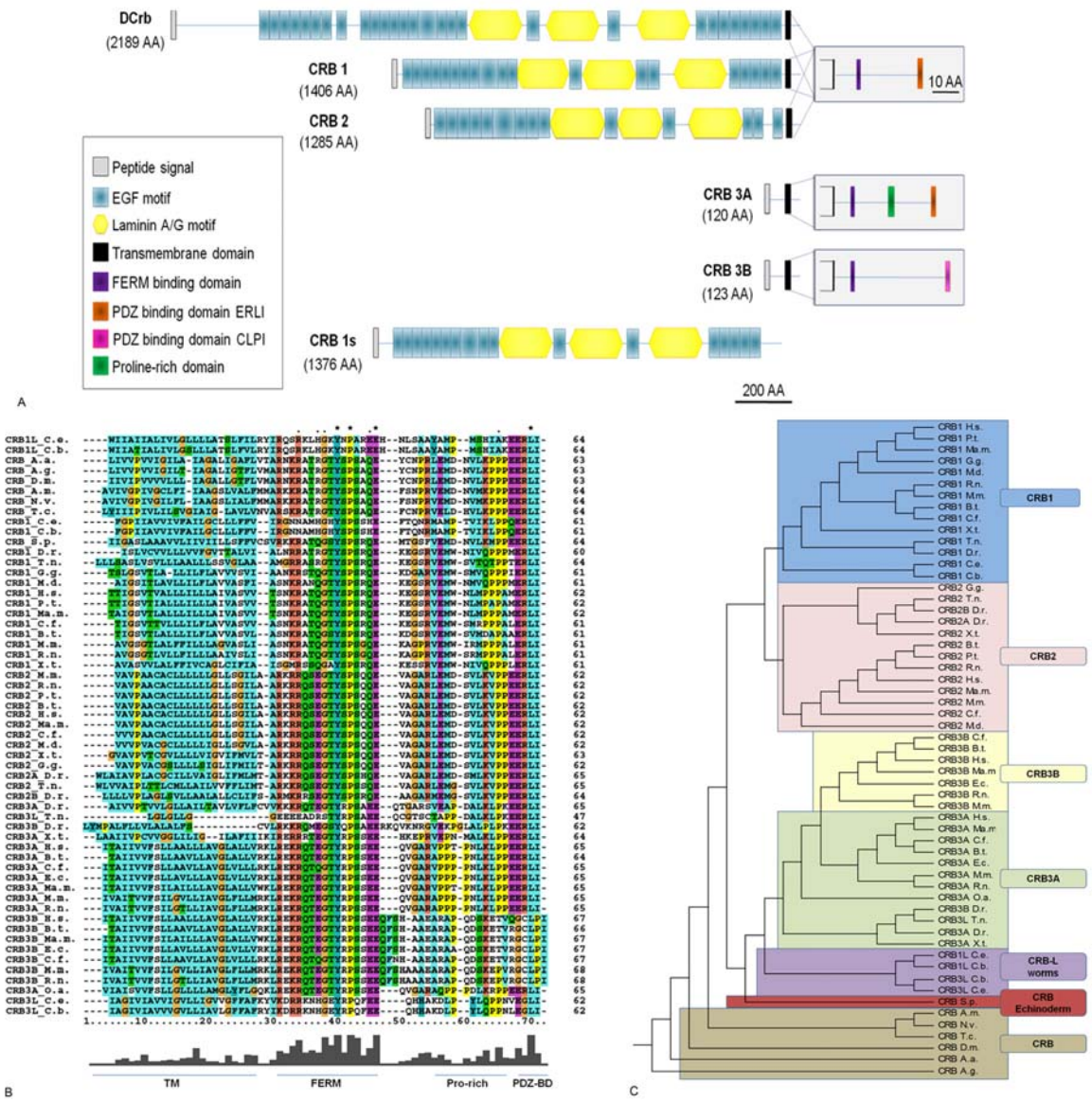

Figure 1. Evolution of Crumbs proteins. A: Schematic representation of DCrb and human CRB1, CRB2, CRB3A and B and CRB1s (secreted). Extracellular parts of DCrb, CRB1, 2 and 1s are composed of EGF and Laminin A/G domains. The small intracellular parts have been blown up (insert) and the known binding-domains are shown (FERM domain, Proline-rich region and PDZ binding domain). Domains are shown according to the SMART program (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de). B: Crumbs proteins multiple alignment on 23 species. Crumbs protein sequences (CRB) were found in NCBI (http://www.ncbi.nlm.nih.gov) and Ensembl (http://www.ensembl.org) databases. Note that most of the protein sequences are predicted. Alignment of the transmembrane and intracellular domains was obtained with the ClustaW program (http://bips.u-strasbg.fr/fr/Documentation/ClustalW/). Colors indicate conserved amino acids. A.a., *Aedes aegypti* (Yellow fever mosquito), A.g., *Anopheles gambiae* (Malaria mosquito), A.m., *Apis mellifera* (Honeybee), B.t., *Bos taurus* (Cow), C.b., *Caenorhabditis briggsae* (Roundworm), C.e., *Caenorhabditis elegans* (Roundworm), C.f., *Canis familiaris* (Dog), D.m., *Drosophila melanogaster* (Fruit fly), D.r., *Danio rerio* (Zebrafish), E.c., *Equus caballus* (Horse), G.g., *Gallus gallus* (Chicken), H.s., *Homo sapiens* (Human), M.d., *Monodelphis domestica* (Opossum), M.m., *Mus musculus* (Mouse), Ma.m., *Macaca mulatta* (Rhesus macaque), N.v., *Nasonia vitripennis* (Jewel wasp), O.a., *Ornithorhynchus anatinus* (Duck-billed platypus), P.t., *Pan troglodytes* (Chimpanzee), R.n., *Rattus norvegicus* (Rat), S.p., *Strongylocentrotus purpuratus* (Purple sea urchin), T.c., *Tribolium castaneum* (Red flour beetle), T.n., *Tetraodon nigroviridis* (Spotted green pufferfish), X.t., *Xenopus tropicalis* (Frog). C: Phylogenic tree of Crumbs proteins. The tree was obtained with Protpars and Consense Phylip programs (http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-fr.html) and drawn with the TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). CRB1, CRB2, CRB3A, CRB3B, CRB1 and insect CRB groups are shown in different colors. Two other groups, one composed of CRB-like worms' proteins (CRBL) and another one composed of echinoderm CRB (S.p.) are indicated separately.

programs) (Figure 1C). This tree shows that insect CRB form a coherent group. Vertebrate CRB1, CRB2 and CRB3 (A and B isoforms) also form compact groups, which can be subdivided into mammals and other vertebrates (zebrafish, frog, chick, etc). Interestingly, two separate clusters have been observed in this tree: one composed of sea urchin (*Strongylocentrotus purpuratus*) CRB protein and the second one of worm (*Caenorhabditis elegans* and *C. briggsae*) CRB1L and CRB3L proteins; this suggests that these proteins have evolved differently. It has been reported that the extracellular part of CRB1L has only EGF domains and lacks the laminin domains found to exist in all CRB1 and CRB2 proteins. In addition, we have observed that the GTY motif (in the FERM-binding domain) is replaced by G (H/K)Y in worm CRB1 and CRB1L proteins, although this difference does not seem to have crucial consequences at the functional level (11). Another not previously described CRB protein was found to exist in the worm databases, which was more closely related to CRB3 because of its short extracellular domain. We named this protein CRB3L (CRB3-like) because it differs from the other CRB3 proteins: the ERLI and GTY motifs are replaced in this case by EGLI and GEY, respectively. Worm CRB3L and zebrafish (*Danio Rerio*) CRB3A and B cytoplasmic domains lack the typical proline-rich region predicted to bind to SH3 (Src homology region 3)-domain proteins or WW-domain proteins, which is conserved in mammalian CRB3A proteins. Conversely, CRB3B splicing isoform, which was found to exist only in mammals, might have been acquired more recently during evolution, giving Crumbs proteins a new functional role. All in all, these findings may help us to build new hypothesis for the functional role played by Crumbs orthologs in variousspecies.

3. 2. Scaffolding of Crumbs complex in *Drosophila melanogaster* and mammals

The high level of homology found to exist between the cytoplasmic domains of Crumbs proteins suggests that the protein–protein interactions are conserved. Most of the protein-protein interactions described in this section were detected in genetic studies or studies using yeast two-hybrid or Far-Western blotting approaches and have been confirmed *in vivo* using GST pull down and/or co-imunoprecipitation methods. The Crumbs complex is a highly dynamic protein scaffold which is conserved in *Drosophila* and mammals: the first level of this scaffold, which is composed of identified partners of the backbone Crumbs complex *i.e.* DCrb/CRB, Stardust/PALS1 and DPatj/PATJ, forms an interactome (as shown in Figures. 2 and 3), which is involved in the establishment and maintenance of epithelial junctions and apico-basal polarity.

3. 2.1. Crumbs complex in *Drosophila melanogaster*

The low redundancy of insect genes makes the fly Crumbs complex a useful prototype for studying vertebrate Crumbs complex in general (as described in 3. 2.2). The Crumbs complex, which was discovered at the end of the 90s, consists of 3 proteins: DCrb, Sdt (Stardust) and DPatj (protein associated with tight junction) and this tripartite association was taken to form the backbone of the DCrb complex, hence the name DCrb/Sdt/DPatj complex, which will be described below.

DCrb is a 234kDa transmembrane protein (7, 8), which includes a large extracellular part. Since no ligands have been found so far which bind to this large extracellular part, the exact function of this part still remains to be elucidated . The Crumbs complex is formed around the short cytoplasmic part (37 amino acids) of DCrb, in which two protein-interacting domains have been identified: a FERM binding domain (GTY amino acids) and a PDZ binding domain (the last four ERLI amino acids). This PDZ binding domain binds to Sdt PDZ domain, (6, 23, 24). *Sdt* encodes at least three protein isoforms, Sdt-A, Sdt-B, and Sdt-GUK1. Two of them, Sdt-A (named Sdt-MAGUK1 in (23) and Sdt-B, are scaffolding proteins belonging to the MAGUK (membrane-associated guanylate kinase) family, which is characterized by the presence of a GUK domain (which is homologous to the previously identified guanylate kinases but without enzymatic activity) (23, 24), a SH3 domain, a hook domain, a single PDZ domain and two adjacent L27 (lin2 lin7 binding) domains (called L27N or C, depending on their position in the sequence). Sdt-A and Sdt-B differ in terms of the presence or absence of a large exon, which encodes a 433-amino acid region in the N-terminal (see Sdt-A in Figure 2.). By contrast, the third known isoform, Sdt-GUK1, lacks most of the PDZ domain, the SH3 and hook domains and part of the GUK domain (23). DPatj contains 4 PDZ domains and a L27 domain (25) that binds to the L27N domain of Sdt (26).

In addition to the core complex formed by DCrb, Sdt and DPatj, an increasing number of DCrb network partners have been identified.

3. 2.1.1. Partners of DCrumbs

The DCrb PDZ binding motif can also bind to the single PDZ domain of DPar6 (*Drosophila* Par6) (27). DPar6 consists of one PB1 (Phox/Bem 1) domain followed by a CRIB (Cdc42/Rac interaction binding) and a PDZ domain. The PB1 domain, which is located at the N-terminal part of DPar6, can bind to other PB1 domain-containing proteins such as aPKC (reviewed in (28)). DPar6 belongs to the conserved Par complex (DPar6/Bazooka (Baz)/DaPKC), which is also involved in establishing and maintaining apico-basal epithelium polarity and in generating the anterior-posterior polarity axis in *Caenorhabditis elegans* (29, 30). In addition, DCrb (see the following paragraphs) has to be phosphorylated by DaPKC to be functional, and DCrb and DaPKC must therefore interact transiently (12) (these interactions are not shown in Figure 2A). Two FERM proteins, DMoesin (31) and Yurt (32), have been found to bind to the DCrb FERM binding domain. Since DCrb is connected to beta Heavy-spectrin and apical spectrin-based membrane skeleton (SBMS) via the DMoesin protein, the DCrb present in the epithelial cell architecture participates in stabilizing Zonula Adherens (ZA) by recruiting the SBMS into the junctional region (33). By contrast, the interactions between Yurt (a basolateral cytoskeleton protein) and DCrb are part of a negative feedback loop that regulates epithelial polarity and apical membrane growth: any loss of Yurt or overexpression of DCrb result in the expansion of the

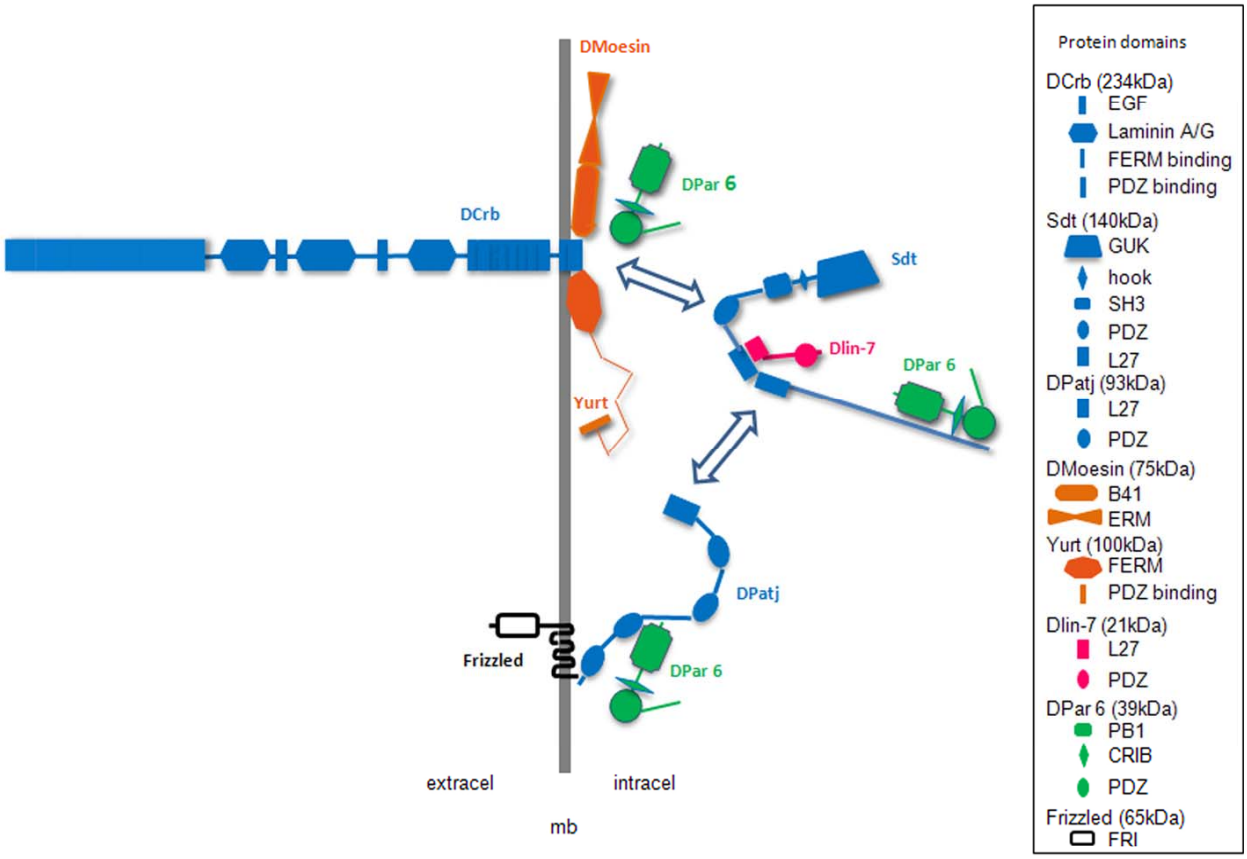

Figure 2. Crumbs complex in *Drosophila* melanogaster. The backbone of this complex is composed of DCrb/Sdt/DPatj, which are shown in blue. Only the direct partners of these three proteins have been included here. Domains are shown according to the SMART (Simple Modular Architecture Research Tool) database (http://smart.embl-heidelberg.de).

apical membrane (32). A PDZ binding domain has been found to exist at the C-terminal end of the Yurt protein, but the partner involved in the binding process has not yet been identified.

3. 2.1.2. Partners of Stardust

To our knowledge, none of the partners interacting with GUK and SH3 domains have yet been identified; whereas the L27C domain interacts with the L27 domains of DLin-7 (34, 35) and the Sdt N-terminal part (21-141 amino-acids) can bind to the CRIB and PDZ domains of DPar6 (36). It is worth noting that since the Sdt-B isoform lacks the DPar6 interaction motif Sdt-B does not interact with DPar6.

3. 2.1.3. Partners of DPatj

Among the four PDZ domains of DPatj, only the partners with which the last two interact have been identified. The third PDZ domain binds to the N-terminal PB1 domain of DPar6 (37). The fourth PDZ domain binds to the Frizzled 1 (Fz1) C-terminal domain (38). This molecular link connects apico-basal determinants (DCrb/Sdt/DPatj complex) to planar cell polarity protein complexes (Fz1 and associated signaling proteins). Fz1 belongs to the Frizzled family of receptors, which have seven transmembrane-spanning domains and are activated by the Wnt family of lipoglycoproteins (for a review see (39)). Djiane *et al* (38) have suggested that the link between DPatj and Fz1 may recruit DaPKC (possibly via DPar6 linked to DPatj), which in turn phosphorylates and thus inhibits Fz1.

It is worth noting that the three partners involved in the DCrb complex can interact with DPar6, which seems to be an essential interface between DCrb and DPar polarity complexes (40). In addition, Nam and Choi (37) have established that these two complexes depend on each other to be able to achieve their proper location in photoreceptors.

3. 2.2. Crumbs complex in mammals

In mammals, three genes denoted *CRB1*, *2*, *3* have been identified and their products have been detected in expressed sequence tag (EST) databases (33). Their patterns of distribution suggest the existence of a tissue-related functional specialization of CRB proteins in mammals despite the conserved cytoplasmic parts they have in common. Since CRB3 is mainly expressed in epithelial tissues, unlike CRB1 and 2 and since the CRB3 complex has been more fully documented in the literature than its counterparts, we will focus in this part of the review on CRB3 (17, 41-44).

The mammalian CRB core complex consists of the mammalian homologues of fly proteins, namely CRB3A/DCrb, PALS1/Sdt, and PATJ/DPatj (Figure 3A). In addition, the interacting domains are highly conserved. CRB3A differs from other CRB in that it has a very short extracellular part and its cytoplasmic part has an additional proline-rich domain (Figure 1A). The link between CRB3 (as well as CRB1 (26)) and PALS1 (proteins associated with Lin seven also called MPP5 for membrane protein palmitoylated 5: for a review, see (40)) is based on the recognition between the four ERLI N-terminal amino acids (the PDZ binding domain) of CRB3A and the PDZ domain of PALS1 (42, 45). PALS1 has the same interacting domains as Sdt: two L27, one PDZ, one SH3, one hook and one GUK domains (23, 24, 46) but it has a shorter N-terminal part than Sdt-A.

As in *Drosophila*, PALS1 and PATJ interact via their cognate L27 domains (26). The structural bases of L27 domain heteromeric complex assembly have been studied using biochemical, NMR spectroscopic and crystallization approaches (47, 48), and the results of these studies have shown the existence of a heterodimerization mechanism driven by hydrophobic interactions. More interestingly, an association between two heterodimers (PALS/PATJ) may occur in an asymmetric (47) or symmetrical tetramer (48) and it seems likely that the tetrameric structure is based on a general assembly mode involving cognate pairs of L27 domains (49). For the sake of clarity , this process of oligomerization has not been included in figure 3A. The PATJ-L27 domain present at the N-terminal is followed by up to ten PDZ domains: their number depends on the alternative splicing process producing at least 5 isoforms (50, 51) (14). In mammals, there exist two homologues of DPatj: PATJ and MUPP1 (multi-PDZ domain protein); their PDZ domain similarities and their respective partners are indicated in Figure 3B. The N-terminal of MUPP1 also has an L27 domain that can bind to PALS1 (26, 52, 53). However, unlike PATJ, MUPP1 is not generally included among the constitutive members of the CRB3A complex and has therefore not been shown in Figure 3A.

As occurs in *Drosophila*, the mammalian CRB3A/PALS1/PATJ complex constitutes the core of a cytoplasmic protein scaffold in which an increasingly large number of partners have been identified. However, the partners of CRB3A identified so far are all human homologues of those existing in the fly, whereas new partners for PALS1 and PATJ have been identified in mammals

3. 2.2.1. Partners of CRB3A

The FERM binding domains of all mammalian CRB bind to two mammalian Yurt orthologs, Epb4.1l5 (erythrocyte protein band 4.1- like 5) and EHM2 (expressed in high metastatic cells) FERM proteins (32). Interestingly, the PDZ binding domain of Crb3A binds to the PAR6PDZ domain (43). In addition, as in *Drosophila*, PAR6 (consisting of one PB1 domain followed by a CRIB and a PDZ domains) participates in building the PAR complex along with PAR3 and aPKC. The PAR complex is involved in the establishment and maintenance of epithelial tight junctions and apico-basal polarity (for a review, see (40))

3. 2.2.2. Partners of PALS1

The evolutionarily conserved N-terminal region of PALS1 is able to bind to the PAR6 PDZ domain (36). However, there is a requirement for the PALS1 L27N domain in the interaction between PAR6 and PALS1, whereas in *Drosophila*, the Sdt amino terminus lacking the L27 domain still binds to DPar6 (36). In addition, it seems likely that the relationship between PALS1-PATJ and PALS1-PAR6 interactions may be competitive rather than synergistic (36). The L27C domain of PALS1 binds to the L27 domain of Lin-7 (also known as VELI for Vertebrate lin-7, and MALS for mammalian lin-seven) (46), and this interaction stabilizes PALS1 (54). The PALS1 SH3 and hook domains can bind to another MPP protein, MPP4, via its GUK domain (55). Interestingly, the SH3 and GUK domains of PALS1 (like those of other MPP and MAGUK proteins) may be involved in intramolecular binding processes. These processes keep the SH3-GUK domains in the 'closed' state and may prevent heteromerization of PALS1 with MPP4 as well as homodimerization of PALS1 between the SH3 hook domains of one PALS1 molecule and the GUK domain of another PALS1 molecule (56). The hook domain of PALS1 can interact with Epb4.1l5 in mammals (57), as previously found to occur in zebrafish between NOK (zebrafish PALS1) and MOE (a FERM protein) (57, 58).

3. 2.2.3. Partners of PATJ

Among the 10 PDZ domains of PATJ, only four have identified partners. Extensive searches have been carried out for the partners of the 2nd and 3rd PATJ PDZ domains because of their homologies with PDZ 1 and 2 of DPatj, and several partners have been identified. First of all, 2nd and 3rd PDZ domains can bind to a tumor suppressor protein, the tuberous sclerosis 2 protein (TSC2) at its C-terminal part, including its PDZ-binding motif (TEFV) and its GAP (GTPase-activating) domain (59). The TSC2 pathway controls many cellular features such as cell size, cell survival, apoptosis and cell cycle progression (for a review, see (60-64)). In addition, these 2nd and 3rd PDZ domains can also bind to the C-terminal PDZ-binding motives of Amot (angiomotin) and MASCOT (MAGI-1-associated coiled-coil tight junction protein), whereas only the 3rd PDZ is responsible for its interaction with JEAP (junction-enriched and -associated protein) (similar results have been obtained on the 2nd and 3rd MUPP1 PDZ domains) (65). These three proteins have a very similar structure and contain a coiled-coil motif followed by a PDZ-binding motif at the C-terminal end (66-68). Amot contributes to stabilizing tight junction structure and maintaining epithelial polarity (69).

The 6th and 8th PATJ-PDZ domains can interact with the C-terminal PDZ-binding motives of ZO3 (zona occludens3, a tight junction MAGUK protein) and claudin1 (51), respectively. Likewise, MUPP1 interacts with claudin1 via its 10th PDZ domain, which is highly similar to the 8th PDZ domain of PATJ (Figure 3B) (70).

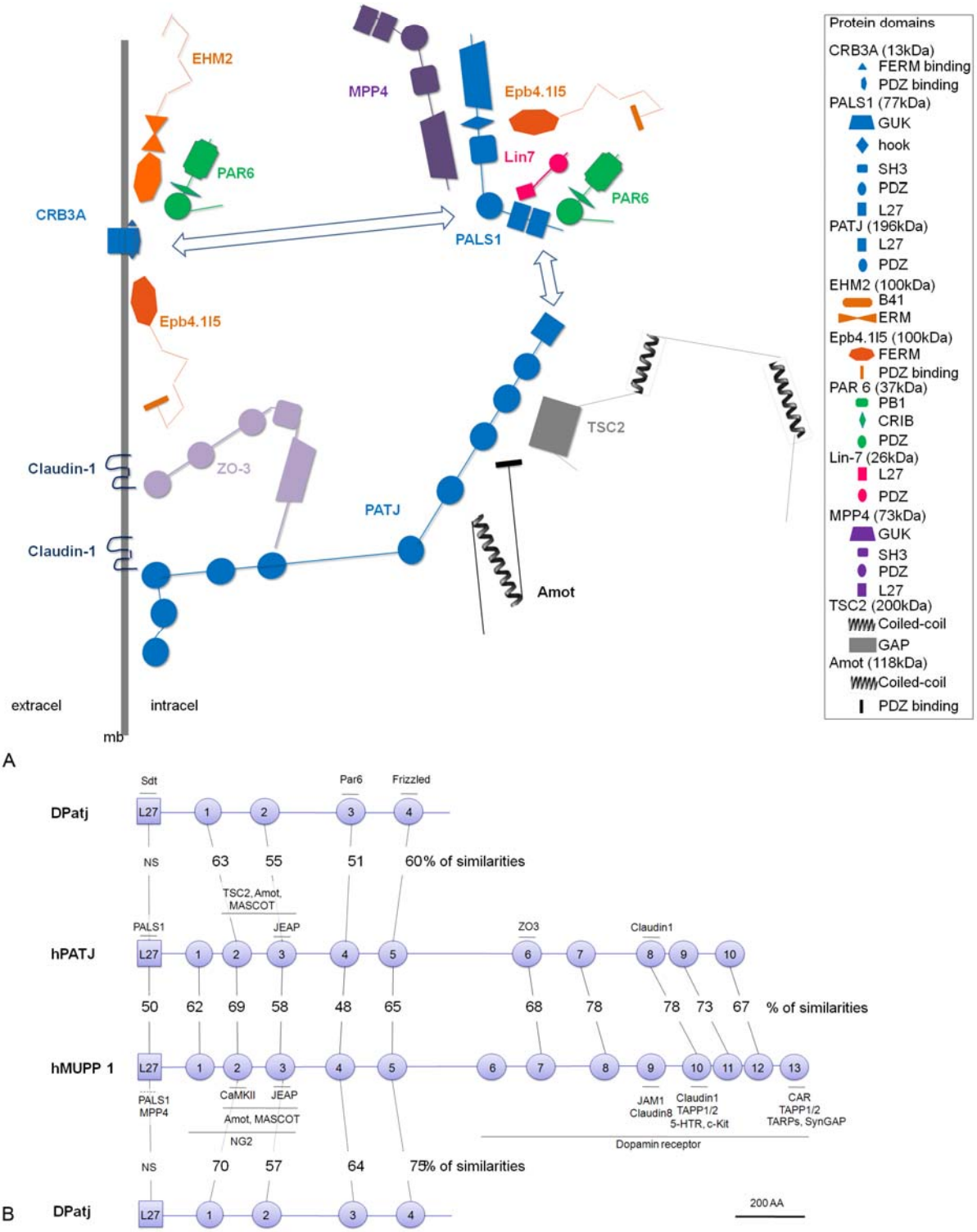

Figure 3. Crumbs3A complex in mammals. A: The backbone of this complex is composed of CRB3A, PALS1 and PATJ, which are shown in blue. Only the direct partners of these three proteins have been included here. Domains are shown according to the SMART (Simple Modular Architecture Research Tool) database (http://smart.embl-heidelberg.de). B: Aminoacid sequence similarities between DPatj, hPATJ (h for human) and hMUPP1 domains (according to the SMART database) found with the Blast2 sequences of the NCBI internet site (http://www.ncbi.nlm.nih.gov./blast/bl2seq/wblast2.cgi). All known interactors of DPatj, hPATJ and hMUPP1 are mentioned in the text except for CamKII (147), TAPP1/2 (148), TARPs (149), synGAP (147), Dopamine receptor (150), JAM1 (70), NG2 (151), Claudin 8 (152) , 5-HTR (52) , c-Kit (153), CAR (154). NS: no similarities.

Interestingly, the C-terminal PDZ-binding motif of claudin1 can also bind to the 1st PDZ domain of ZO3 and conversely, the GUK domain of ZO3 (like that of ZO1 and 2) can bind to the C-terminal fragment (consisting of 150 amino acids) of occludin (71, 72). In addition, the targeting of PATJ to tight junctions is abolished when the 6th PDZ domain binding to ZO3 is deleted, which suggests that ZO3 plays an important role in the targeting of PATJ to tight junctions. On the other hand, the deletion of the 8th PDZ domain (binding to claudin1) does not affect the tight junction location of PATJ (51).

4. THE CRUMBS COMPLEX: ITS ROLE IN DEVELOPING ORGANISMS

4. 1. Early embryogenesis and primary epithelia

During embryogenesis, the first differentiated structure formed is an epithelium. Studies on the early steps in the development of model organisms (such as the fly and mouse) therefore shed interesting light on the molecular events leading to *de novo* formation of epithelia.

4. 1.1. *Drosophila melanogaster*

After the fertilized egg has been laid, the process of development begins in *Drosophila* with the occurrence of synchronous syncytial nuclear divisions not involving cytokinesis, which results in the syncytial blastoderm. The process of cellularization results from the invagination of apical plasma membrane, which leads to the formation of the membrane furrow (reviewed in (73-75)). At this stage, only DPatj protein is present (there are no DCrb and Sdt proteins), and participates in membrane formation by moving in the basal direction along the leading edge of the invaginating membrane furrow and concentrating in the basal domain of the columnar epithelial cells. *DPatj* null mutation causes early first instar larval lethality, which indicates that DPatj, or more specifically, its N-terminal L27-PDZ1 domains, is crucial in the early stages of development (25, 76). By contrast, DPar6 is also present but does not participate in the process of cellularization, and its inactivation does not perturb this process (77). However, DPar6 recruits DPatj apically at the end of the cellularization process. During this step, spots of adherens junctions (enriched with E-cadherin/beta-catenin complexes) appear on the lateral membrane. Epithelial polarity is thus first established during the process of cellularization and requires the presence of DPatj.

DCrb and Sdt proteins are first detected apically and at the top of the lateral domain in low levels during early gastrulation. A fraction of the DPatj protein is also recruited at that time apically, while most of the remainder is still basal at this stage. The apical localization of DCrb seems to rely on a dynein dependent transport process, since in the dynein mutant, DCrb protein and mRNA were not found to be localized apically, and this deficit leads to apico-basal polarity defects (78). Likewise, the apical localization of Sdt A mRNA depends on its binding to dynein (79).

During gastrulation and shortly thereafter, the spot adherens junctions aggregate, forming the apico-lateral belt of ZA (zonula adherens). In *DCrb* and *Sdt* mutants, defects in ZA establishment occur rapidly only a few minutes after DCrb is normally expressed and precede deterioration of the epithelial cell structure (80). In addition, these mutants are characterized by the down-regulation of apical markers, loss of polarity, severe disruption of the cuticle and extensive cell death in the epiderm. Interestingly, two copies of *Sdt* in an amorphic background of *DCrb* could rescue the *Crb* phenotype indicating that Sdt acts downstream of DCrb in the complex (6). Sdt is also required for DCrb to be correctly localized and control epithelial polarity (23). Overexpression of DCrb expands the apical cell surface at the expense of the basolateral domain, which suggests that DCrb is a crucial factor defining the apical surface of epithelial cells. The overexpression of DCrb also results in the disruption of epithelial integrity due to the loss of adherens junctions, establishment without any loss of polarity (10, 80). This phenotype might result from the lack of interactions with the cortical cytoskeleton, since it resembles the phenotype induced by the loss of FERM protein Yurt (32). In wild type *Drosophila*, Yurt acts as a negative regulator of the DCrb complex, restricting its localization to the subapical region.

The establishment of epithelial polarity during *Drosophila* embryogenesis therefore results from coordinated interactions between three polarity complexes, the DCrb, DPar and Scrib complexes (see introduction and review (81)). Using genetic approaches it has been shown that DCrb and DPar complexes act in the same way while the Scrib complex antagonizes the function of DCrb and DPar complexes (82) (for a review, see (5, 40, 83)).

4. 1.2. Vertebrates

In mammals, the first epithelium is a unique single-layered tissue called the trophectoderm, which forms on the outside of the embryo during the cleavage stage and give rise at a later stage in pregnancy to the chorio-allantoic placenta. In the early amphibian embryo, the outer epithelium becomes the basic tissue involved in gastrulation and comprises progenitors responsible for all three germ layers. In both cases, this initial epithelium engages in the polarized transport processes generating the blastocœle cavity, thus providing the embryo an opportunity for regulating the composition of the internal tissues it requires for metabolic and developmental purposes and, most importantly, providing a malleable platform on which cell rearrangements and the process of morphogenesis can shape the future anatomy of the developing embryo. A role for CRB proteins in these early mammalian development stages will be likely uncovered by future studies.

In the context of vertebrate embryogenesis, epithelial polarity pathways have been most extensively studied in zebrafish. Genetic screens using this model organism showed the presence of a group of loci serving as determinants of apico-basal polarity (84). Cloning of mutant loci revealed a close relationships between vertebrate neuroepithelial polarity pathways and mechanisms regulating the polarity of fly embryo epithelia.

The five *CRB* genes identified in zebrafish show different patterns of expression, which suggests that these genes play various developmental roles. As in mouse and human studies (13, 53, 85), CRB1 knock-down in zebrafish failed to produce a clear-cut embryonic phenotype (22). By contrast, mutations in *CRB2A (*the *oko meduzy* (*OME*) mutant), and morpholino knockdown of *CRB2A* and *CRB3A* decrease or abolish the apical surface features, and are frequently associated with severe morphological defects in the developing embryo: the mechanisms involved here still remain to be elucidated, however *(*22).

In *Xenopus*, CRB3 promotes the development of the apical membrane identity and inhibits the basolateral domain expansion, as its overexpression induced the expansion of the apical region of the developing embryo (86). In developing mammalian embryos, TJs biogenesis in trophectoderm cells is usually studied in terms of the distribution of a variety of cellular markers including ZO1 (87), occludin (88), but the exact localization of CRB proteins and their contribution to this process have not yet been established. Recent studies have focused on the partners involved in CRB network interactions in the mouse embryo. The FERM protein Epb4.1l5 is an important component of the epithelial architecture and Epb4.1l5 mutant mouse embryos died at E8.5-9.0; whereas CRB localization was not affected in these mice (89). By contrast, the orthologs of Epb4.1l5 in zebrafish (MOE) and *Drosophila* (Yurt) are required for the proper localization of CRB2A and DCrb, to occur, respectively, which indicates that Epb4.1l5-Crumbs related function is not conserved among species (32, 58, 90). In addition PALS1 zebrafish ortholog (NOK) is essential not only to the proper localization of the CRB protein, but also to CRB protein stabilization, which suggests that the mechanism involved differs between NOK and MOE (58). This function of PALS1 is not conserved in mammals, since the down-regulation of PALS1 did not induce the down-regulation of CRB3 in MDCK cells (91) (see section 5. 2.2). All the data obtained in studies where the pattern of CRB expression was modified in vertebrates show the presence of mild defects in comparison with the *Drosophila* phenotype (embryonic lethality). Since in vertebrates, there exist at least 3 genes encoding different CRB proteins, we can speculate that during embryogenesis, there may exist some functional redundancy.

4. 2. Organogenesis

Crumbs is necessary not only during the earliest stage of development (section 3.1); in this part, we will discuss the role of Crumbs in the formation of differentiated epithelia such as retina and kidney.

In human, CRB1 expression was found to be restricted to the retina and brain (13, 20), whereas in mouse, it was also detected in the kidney, stomach, lung and testis (15). The mammalian paralogs CRB2 and CRB3 are expressed in a wider range of tissues, including the retina (14, 17, 42). CRB2 is mainly expressed in the retina, brain and kidney and in low levels in the lung, placenta and heart (17). CRB3 is expressed not only in the retina (53, 92), but also in the kidney, colon, lung, heart and mammary gland (42, 43). Immunolabeling studies have shown the presence of components of the CRB complex, such as PATJ and CRB3, at the most apical membrane domain of the human colonic epithelium (14, 43), more specifically at the tight junctions. On the other hand, cCRB (chickCrumbs, isoforms not fully described so far) and cPALS1 (chickPALS1), two members of the CRB complex, have been detected at the most apical membrane domain of the chick embryonic neural tube epithelium (93).

In zebrafish, highly conserved CRB orthologs were identified (see section 3.1) and analysis of their mutants showed that some of these genes are required for the normal development of structures including the retina and kidney. Since the CRB complex has been found to be mainly expressed in the retinal and renal systems, it is proposed to focus here on these systems.

4. 2.1. Retina

4. 2.1.1. *Drosophila* retina

In the fly, DCrb is a central component of a molecular scaffold controlling the assembly of ZA and regulates the size and shape of the stalk (an apical membrane subdomain connecting the rhabdomeres to the ZA), where the DCrb complex is also localized (37, 94-98) (Reviewed in (99)). Several lines of evidence have shown that loss of function of *DCrb* in *Drosophila* leads to impaired ZA integrity, stalk membrane and rhabdomere length (both shortened) and the progressive light-induced degeneration of photoreceptors (PhRs) (94, 95, 97, 99). Interestingly, similar defects to those occurring in *DCrb* mutant PhRs, including shortened stalk membranes and distorted rhabdomeres, were observed in *Drosophila* retinas lacking the DCrb binding partners Stardust (Sdt) and DPATJ (76, 97, 98, 100).

In addition to the crucial role of these DCrb-binding partners in retinal morphogenesis, *Drosophila Sdt* and *DPatj* mutations have shown that the complete absence of Sdt and DPatj expression in late pupal and adult PhRs also leads to similar progressive light-dependent retinal degeneration processes to those occurring in *DCrb* mutants (96, 100, 101). This finding provided evidence that the DCrb-Sdt-DPatj complex is indispensable for *Drosophila* eye integrity (23, 94-96, 98, 100). Recent findings of Berger *et al.* (100) showed that only a subset of *Sdt* alleles controls PhR morphogenesis or prevents light-induced retinal degeneration, which suggests that the DCrb complex may vary in its Sdt composition during development.

The proper localization and function of DCrb in *Drosophila* retina have also been found to depend on the function of DCrb-binding proteins, such as Mbt *(*mushroom bodies tiny), a protein binding to Cdc42, which is probably involved in actin cytoskeleton reorganization (102), and FERM proteins such as DMoesin (103) and Yurt (32). The dynamic distribution of DMoesin during differentiation and the impact of DMoesin gain- and loss-of-function during PhR morphogenesis suggest that this protein may play a crucial role in the organization of the rhabdomeres (103). However, DMoesin and DCrb define complementary sub-domains in the PhR apical surface, which are located in the rhabdomere base and the stalk membrane, respectively: this

finding suggests that DMoesin is not a DCrb-binding partner in PhRs (103) as reported to occur in the case of embryonic epithelia (31). Laprise *et al.* (32) have reported that Yurt mutant PhRs, without disrupting apico-basal polarity, exhibit longer apical stalks which are similar to those of the PhRs overexpressing DCrb and unlike the PhRs lacking DCrb, which display shorter stalks. The authors concluded that Yurt is a negative regulator of DCrb which is involved in the local control of apical membrane growth.

4. 2.1.2. Zebrafish retina

The zebrafish *Danio rerio* model system is highly suitable for studies on retinal development for at least two reasons: their larvae have large eyes, which can be easily manipulated experimentally, and numerous mutants in which the neuronal pattern of the eye differs from that of normal zebrafish have been identified (104). In particular, mutations induced in the *CRB2* ortholog named *CRB2A (OME* mutants) resulted in impairments affecting the epithelial shape of the retinal neuroepithelium , whereas apico-basal organization was maintained in brain neuroepithelium since both aPKC and PAR3 are correctly localized in *OME* (22, 105). Consistent with the *OME* neuroepithelial phenotype, CRB2A is localized in the retina just apically from the adherens junctions (AJs), which supports the idea that this protein is essential to the epithelial integrity of the retinal cells (22). These authors further established that *CRB2B,* an *CRB2A* paralog almost exclusively expressed in PhRs (in both the retina and the pineal gland), is a determinant of the apical surface in these cells. The *CRB2B* knockdown showed a dramatic reduction of the PhR inner segment size (22), a phenotype in zebrafish similar to that involving a decrease in the rhabdomeric membrane stalk observed in *DCrb* fly mutants (94). However, since PhR maturation was only slightly delayed in *CRB2B*-deficient animals, CRB2B may not play a general role in PhR differentiation. In addition, Omori and Malicki (22) reported that zebrafish *CRB3A*, another *CRB3* paralog, is also required for the morphogenesis of the kinetocilium in ciliated cells of the ear.

On the other hand, *nagie oko (NOK)* mutants, defective in a homolog of the *Drosophila* Stardust and mammalian PALS1/MPP5, resulted in a severe disruption of the retinal architecture, which showed that NOK is essential to zebrafish eye integrity and retina cellular patterning (106, 107). The fact that NOK protein was found to be localized in the vicinity of junctional complexes in the retinal neuroepithelial sheet suggests that nok is required for the establishment and/or the maintenance of the OLM (outer limiting membrane) (107).

Lastly, it was established that three zebrafish CRB proteins (CRB1, CRB2A, CRB2B) can interact directly with zebrafish *mosaic eyes* (*MOE*), a FERM protein (fly Yurt ortholog), which co-immunoprecipitates with CRB2A and is colocalized apically with CRB proteins (58). Genetic analyses showed that *MOE* is required for normal retinal lamination and RPE (retinal pigmented epithelium) integrity, and that the apical domain is expanded in rod PhRs lacking MOE, which suggests that MOE negatively regulates CRB protein function, and especially the size of the rod PhRs (58). Interestingly, the embryonic phenotypes of *MOE* mutants are similar to both the phenotypes of *NOK* mutants and those of *CRB2A* morphants (morpholino knockdown mutants), opening the possibility of genetic interactions between these genes (58, 90).

4. 2.1.3. Mammalian retina

In humans, mutations in the *CRB1* gene were identified as a major cause of severe inherited retinal diseases, including Retinitis Pigmentosa type 12 (RP12), an early onset form of autosomal recessive RP characterized by a preserved para-arteriolar RPE, autosomal Leber congenital amaurosis (LCA), which is the earliest and most severe form of all inherited retinal dystrophies, RP with coats-like exudative vasculopathy, and Pigmented Paravenous Chorio-retinal Atrophy ((13, 108-113) recently reviewed in (101)).

No pathological variants have been detected to date in the *CRB2* and *CRB3* genes of patients with RP12 or LCA (17). However, the *CRB2* gene is highly polymorphic, unlike the *CRB1* gene (109). It is thus possible that a more complex clinical phenotype may be associated with the loss or altered function of *CRB2* or *CRB3* in humans (101). The *CRB2* sequence variants detected in RP12 and LCA patients (17) may be involved in digenic and polygenic diseases (101).

Human retinal degenerative diseases associated with *CRB1* gene mutations can be partly mimicked by the *CRB1*^{rd8} and *Crb1-/-* mouse models (53, 85), since deficiencies in mouse *CRB1* also cause retinal abnormalities (13, 41, 101, 108, 109). The rather thick appearance of the retina in the mouse *CRB1* knockout correlates with the thick retinas of LCA patients, for instance (112). However, the retinal patterning defects occurring in the mouse are less severe than those observed in humans, although bright light also increases PhR death in *CRB1-/-* mice as in *DCrb* mutant retinas (96), without being required to induce retinal degeneration (53). Retinal disorganization, which is always most striking in older animals, has been observed in both mouse mutant models lacking CRB1 (53, 85). In mouse retina, CRB1 protein was found to be confined to the region of the ZA, referred to as the OLM, more specifically at a subapical region (SAR) located just above the AJs connecting PhRs to Müller glia cells (32, 53, 85, 92, 94) (Figure 4). As in *Drosophila,* the loss of CRB1, that plays a critical role in the maintenance of ZA, leads to defective junctional complexes, and consequently to the loss of retinal integrity in mouse mutant models (53, 85). Confocal and immunoelectron microscopy observations have shown that CRB1 is nearly restricted to the Müller glia cells, since this protein is barely expressed in PhRs, whereas CRB2, CRB3, PATJ and MUPP1 are all present in AJ-associated CRB complexes in both cell types (92). It was recently specified that loss of CRB1 in *CRB1 -/-* mouse retina results in irregularities in the number and size of the apical microvilli of Müller glia cells, which suggests that CRB1 is required to control the regular development of these microvilli (114).

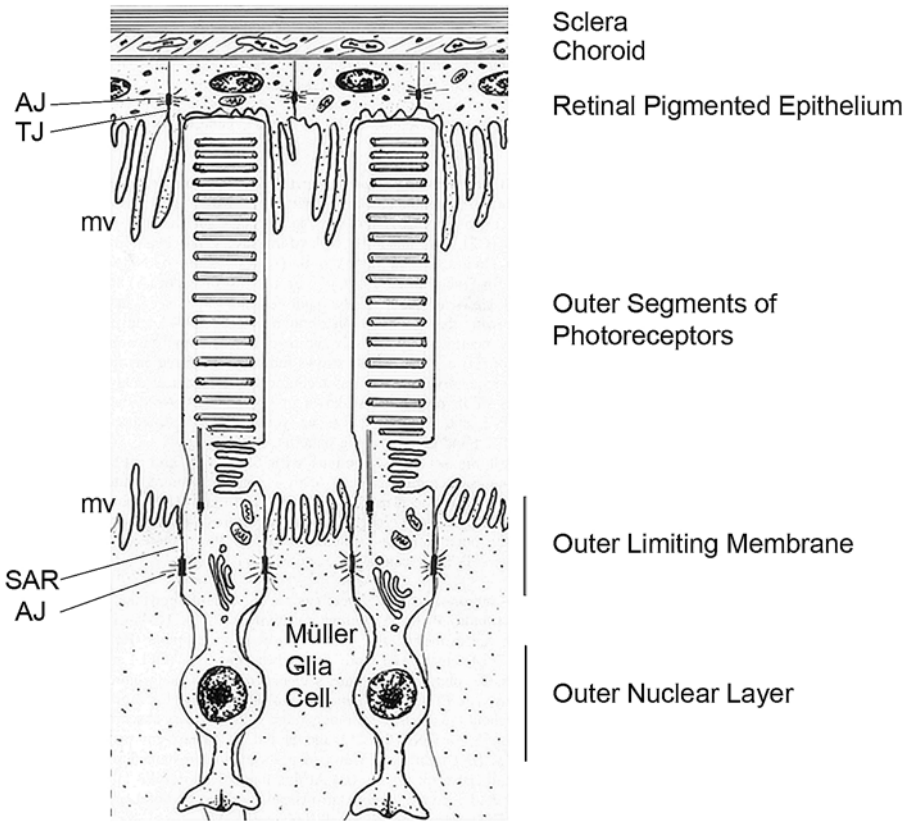

Figure 4. Adult mouse retina. Schematic diagram of the outer part of the adult mouse retina indicating the exact sites where CRB and PATJ are colocalized, *i.e.* in the tight junctions (TJ), at the apical surface of the RPE cells, and in the subapical region (SAR), just above the adherens junctions (AJ) located between the photoreceptors (PhR) and the Müller glia cells (MGC). Scl, sclera; Chor, choroid; Os, outer segment of PhR; mv, apical microvilli of the RPE or the MGC.

In addition, the CRB complex localized at the OLM in the mammalian retina contains other proteins, including MAGUK members of the MPP-family, such as MPP5/PALS1, MPP4, MPP3, MPP1/p55 (56, 110-115) and FERM proteins, such as Epb4.1l5 and EHM2 (32, 58). As in *Drosophila*, Sdt ortholog PALS1/MPP5 is required in mouse retina for the correct localization of the CRB family members (CRB1, CRB2, and to some extent CRB3), MUPP1 and Veli3 (mLin7C/MALS-3) (92) at the SAR of the OLM.

However, PALS1 was found to be exclusively confined to the SAR of the OLM (92, 115) where PALS1 recruits MPP4 and MPP3 into the CRB1 complex (55, 116, 117), while MPP4, which is expressed in high levels in the retina (118, 119), was also detected in the PhR connecting cilia (120) and in the OPL (outer plexiform layer) at the PhR terminals (115, 117, 120, 121). MPP4, which has been found to interact directly with VELI isoforms *in vitro* (115), was found to be colocalized with both VELI3 and PSD95 at the PhR presynaptic terminals (117, 121), which suggests that MPP4 coordinates the assembly of these synaptic components and plays a role in organizing protein scaffolds at the PhR synapses. However, no retinal degeneration and only a slight PhR displacement not accompanied by any changes in the CRB complex at the OLM were detected in *MPP4* knockout mice (117) and mutation analysis of the *MPP4* gene in several patients showed the occurrence of no pathologic mutations (119), which suggests that MPP4 is not involved in either the function or the localization of the CRB complex. Regarding FERM proteins found in human and mouse retina, Laprise *et al.* (32) estblished that the Yurt orthologs Epb4.1l5 (also known as Lulu or YMO1 (89)) and EHM2 can bind to the three human CRB proteins. Hsu *et al.* (58) reported that Epb4.1l5 is coexpressed with CRB1 and CRB2 in PhRs and cells of the Inner Nuclear Layer, and Laprise *et al.* (32) estblished that the Epb4.1l5 protein is colocalized with CRB2 in the inner PhR segments of the adult mouse retina. The CRB complex therefore plays an important role in both the morphogenesis and the functional role of photoreceptors and the retina.

4. 2.2. Kidney

4. 2.2.1. Zebrafish kidney

Studies on zebrafish have confirmed that the CRB complex also regulates ciliogenesis. In *CRB2B*-deficient animals, apical surface defects are associated with the formation of pronephretic cysts and with short cilia showing defective motility (22). Interestingly, these defects in the cilia can be rescued by injecting *CRB2B* mRNA, indicating that CRB2B is a determinant of cilial length and

motility in the zebrafish nephric epithelia. It is noteworthy that it is *CRB3A* rather than *CRB2B* that regulates the length of the hair cell kinocilia. This idea was supported by the shortening of the latter which occurred after knockdown of *CRB3A* gene and the fact that CRB3A protein accumulated in the basal bodies of these cilia (22).

On the other hand, most interestingly and unexpectedly, Omori and Malicki (22) also reported that the strength of the rescue process was similar with *CRB2A*, *CRB2B,* and *CRB3A* mRNA*,* which suggested that the *CRB* genes are at least partly functionally interchangeable in the zebrafish.

4. 2.2.2 Mammalian kidney

Confocal analysis of adult rat kidney sections showed that CRB3 is present at the apical membrane domain, including the primary cilia (122). MALS-3, via its L27 domain, mediates stable assembly of the apical CRB complex and the basolateral SCRIB complex. These polarity complexes were disrupted in renal epithelia from *MALS-3* knockout mice, especially those derived from metanephric mesenchyme (123). *MALS-3* mutant mice have hypomorphic kidneys characterized by cysts and fibrosis and show defective polarization of proximal tubular epithelial cells, where the tight junctions lack the CRB complex. MALS-3, which interacts with the CRB and SCRIB polarity complexes, is therefore essential for correct polarization of renal epithelia and hence, for normal kidney development to occur.

5. CRUMBS COMPLEX: ROLE IN EPITHELIAL CELL CULTURE

In this part of this review, we will focus on CRB3A, as CRB3A is highly expressed in epithelial tissues (42, 43) and this isoform has been extensively studied in *in vitro* systems. In contrast, the role of the CRB3B splicing variant has been described only during ciliogenesis (18).

5.1. Cell cultures: model systems for studying the function of the CRB3A complex

Functional studies on the role of CRB3A in cell polarization have mainly focused on tissues such as retina or kidney. However, since it is difficult to obtain homogeneous populations of differentiated cells from these tissues, detailed analyzes of the functional contribution of the CRB3A complex in epithelial polarity *in vivo* are hard to address. This can be achieved, in cultured cell lines, and the cells most commonly used for this purpose are MDCK (Madin-Darby canine kidney) cells that derived from canine kidney and Caco2 cells derived from a human colon adenocarcinoma. Both of these cell lines are still able to polarize, build tight junctions and form a transporting epithelial monolayer (124-127).

Two different methods of growing these cell lines are commonly used to study epithelial cell polarity: in two-dimension with cells grown on a filter with a predetermined free apical surface, and in three-dimension the cells are grown in collagen or Matrigel matrix, with no forced axis of polarization. In the latter case, MDCK cells form cysts, which are polarized monolayers containing an enclosed lumen and encircled by a basement membrane, tight junctions being restricted to the apico-lateral membrane boundary. The ability of epithelial MDCK cells to mimick a tissue-like organization is useful because it helps to understand how interactions between extracellular factors and the intrinsic differentiation program contribute to specifying the architecture of the epithelial tissue (128). This is not the only advantage of growing cysts *in vitro*, since this system also seems to be more sensitive than the two-dimensional monolayer culture system as a means of detecting cell polarity defects. For instance, Lemmers *et al* (43) and Roh *et al* (45) have reported that overexpression of CRB3A in MDCK cells led to a more severe phenotype when these cells were grown in collagen (instead of developing into cysts, they formed multicellular aggregates with a loss of apico-basal polarity), whereas only apical surface expansion (no loss of apico-basal polarity) occurred when these cells were grown on filters.

However, three-dimensional cell culture method has limitations: for example, it is not amenable to forward genetic analyses or for large biochemical experiments, for which two-dimensional methods are much more suitable. In two-dimensional system, MDCK cells and Caco2 cells are grown on filters and form a monolayer with the basal surface in contact with the filters and the apical surface facing the medium.

An elegant study by Saaf *et al* (129) has shown that the temporal pattern of gene expression observed during Caco2 cell polarization in two-dimensional cell cultures showed similar molecular targets to those involved in the temporal and spatial *in vivo* gene expression program of enterocyte cell differentiation along the crypt-villus axis. It is worth noting that similar parallels can be drawn between proliferating unpolarized Caco2 cells and tumorogenic colonic cells on the one hand and between fully polarized differentiated Caco2 cells and healthy human colonic cells on the other hand. Several groups have therefore used this *in vitro* approach to dissect the molecular events linking CRB3 to apical epithelial morphogenesis.

5. 2. Genetic approaches

5. 2.1. CRB3 knockdown and overexpressing cells

In cell cultures, as *in vivo*, endogenous CRB3 localizes not only at tight junctions but also to the apical membrane and to the cilium (a specialized apical structure) (42, 43, 122). Fan *et al.* (18, 122), who studied the role of CRB3A/B in MDCK cells using the knockdown approach, did not observe any polarity defects apart from those involved in cilia morphogenesis. Knockdown of splice variants CRB3A and CRB3B led to the loss of cilia, which indicates that both CRB3 variants are required for ciliogenesis in MDCK cells (122, 130), and that the PDZ binding domain of CRB3A is necessary for this variant to be able to target the cilium. The fact that PAR6, which is known to interact directly with the CRB3A cytoplasmic domain (43), and PAR3 have also been detected in the primary cilia (122) suggests that the PAR complex may also be involved in ciliogenesis (122, 130). Since

knockdown of PAR3 results in shorter cilia, the elongation/growth of cilia presumably requires PAR3 (130). The authors proposed a model in which PAR3, via PAR6, acts as a linker between the transmembrane protein CRB3A, which is essential for ciliogenesis, and the ciliary axoneme.

Like endogenous CRB3A, overexpressed CRB3A localized to the apical membrane, where it accumulates at the tight junctions along with PATJ, PALS1, ZO1 and occludin (42, 43). In MDCK cells, since overexpressed CRB3A and apical surface markers (gp135, BC11, BC44) were detected in both the apical and lateral regions, it is likely that Crb3A overexpression may result in apical membrane expansion (43, 45). Interestingly, the overall polarity of these cells was not affected. Several morphological studies using calcium-switch assay have shown that overexpression of CRB3A induces a delay in the establishment of tight junction markers (PATJ, ZO1) in a complete belt of tight junction staining (43, 45). Inulin leakage assay and transepithelial resistance measurements combined with calcium-switch methods showed the occurrence of a much slower but complete process of functional tight junction recovery. Since adherens junctions form normally under these conditions, these results suggest that CRB3A may play a specific but important role in the establishment of tight junctions and the apical membrane domain. This discrepancy between knockdown and overexpression phenotypes might be the consequence of redundant expression of CRB proteins in MDCK cells.

The results of biochemical analyses led to the conclusion that the phenotype of CRB3A overexpression in MDCK cells depends on the last four residues (ERLI), as overexpression of CRB3A missing ERLI motif has no effect on cyst and tight junction formation (43, 45), while correctly targeted to the apical membrane (43). These results strongly suggest that the defects observed may be mediated by potential interactions between PDZ proteins and the ERLI motif of CRB3A. Two partners have been reported to bind to the ERLI motif of CRB3A: PALS1 (26, 42) and PAR6 (43). It has been established that overexpression of PAR6 in MDCK cells delays the assembly of functional tight junctions (131), which show a very similar phenotype to that resulting from CRB3A overexpression. Thus, the two proteins act in the same pathway for the building of tight junctions.

5. 2.2. PALS1 knockdown cells

In the case of PALS1, no overexpression experiments have been performed so far. Roh *et al* disrupted the endogeneous interaction between PALS1 and CRB3A using a dominant negative construct containing the PALS1 PDZ domain, which binds to CRB3A (45). The same group also used PALS1 different specific siRNAs to suppress PALS1 expression in MDCK cells (91, 132). In both cases, tight junction formation was delayed as occurred with the CRB3A overexpression phenotype. Additionally in one study (91) defects in adherens junction formation were observed in PALS1 down-regulated cells. Modification of PALS1 expression does not affect the level of CRB3A expression or CRB3A localization at the apical surface and tight junctions. By contrast, reduction of PALS1 expression levels resulted in a corresponding loss of PATJ, the third member of the CRB3A complex. All in all, these results indicate that the absence of PALS1, which acts as an adaptor between CRB3A and PATJ, is essential to the establishment and maintenance of tight junctions. Since PATJ becomes downregulated in PALS1 knockdown cells, it is difficult to attribute the defects observed to the impairment of PALS1 alone. Wang *et al* (91) have reported that when exogenous PALS1 was reintroduced, the normal levels of PATJ expression and the formation of tight junctions and adherens junction were restored. Interestingly, PALS1 N-terminal region (L27 and PDZ domains) or mutated in its SH3 domain rescue PATJ expression, but these two proteins were diffusely distributed in the cells, and no recovery of the formation of tight and adherens junctions was observed. One can hypothesize that the SH3 mutation of PALS1 or the deletion of the C-terminal domain may induce changes in the protein conformation, thus preventing its interaction with CRB3A. In this case, PALS1 and PATJ will not be recruited to the apical membrane; which will prevent junctions from being properly formed. The PALS1-L27N mutant, defective in binding PATJ, cannot rescue either PATJ re-expression or the formation of tight and adherens junctions in PALS1 knockdown cells. Interestingly, a previous study by Roh *et al* (26) showed that this L27N domain of PALS1 is necessary for PALS1 to be targeted to the tight junctions via its interaction with PATJ. Taken as a whole, these results suggest that PALS1, via its L27N domain, may contribute to the stabilization of PATJ (since unbound PATJ might be destabilized and then degraded); and that not only the interaction between PALS1 and PATJ but also their localization to the apical membrane and tight junction may be essential for the tight and adherens junctions to be properly formed.

5. 2.3. PATJ knockdown cells

Several studies have focused on the role of PATJ during epithelial polarization. In non-polarized MDCK cells, PATJ was found to be localized in the vacuolar apical compartment and to target to the apical region and tight junctions during the process of polarization (133). Targeting to tight junction occurs via the interaction of the 6th domain of PATJ and ZO3 (51). Contrary to the outcome of PALS1 down-regulation, but in line with that of CRB3A overexpression, PATJ down-regulation affects only tight junction formation: the adherens junctions form normally (133, 134) and the levels of expression of PALS1 and CRB3A were not affected. It is not yet clear whether the functional integrity of tight junctions is affected by the loss of PATJ, since Michel *et al* (134) observed a normal transepithelial resistance, whereas Shin *et al* (133) did not. These phenotypic differences might point out tissue-specific defects: Michel *et al* (134) worked with Caco2 cells, which are derived from human colon while Shin *et al* (133) worked with MDCK that derived from canine kidney. In both studies, mislocalization of PALS1 was observed, as in previous studies by Roh *et al* (51) and Hurd *et al* (135) (using a PATJ dominant negative) in which PATJ was found to be responsible for restricting PALS1 to the tight

junctions. Michel *et al* (134) also observed that loss of PATJ induced occludin and ZO3 leakage along the lateral membrane and an accumulation of CRB3A to the early endosomal network. It is therefore tempting to speculate that one of the functions of PATJ in Caco2 cells might consist in restricting or stabilizing CRB3A, PALS1, occludin and ZO3 to the tight junctions. All the data available on the CRB3A/PALS1/PATJ complex therefore support the idea that this complex is involved in tight junction formation.

5. 3. CRB3A induces *de novo* tight junction formation

Fogg *et al* (44) used MCF10A cell line, derived from mammary epithelium, which lacks tight junctions and shows very low levels of endogeneous CRB3A expression. In these cells, endogeneous ZO1, occludin, claudin1, PALS1 and PATJ yielded a diffuse cytoplasmic staining pattern. In response to mycCRB3A overexpression, PALS1 and PATJ were recruited to tight junctions and were co-stained with ZO1, occludin and claudin1, and all these proteins formed a continuous belt-like pattern around the cell. The ability of CRB3A to induce tight junction formation was lost when the protein carried ERLI or FERM-binding motif mutations. These data suggest that CRB3A may act as an anchor to recruit the proteins that together are able to build tight junctions.

6. PERSPECTIVES FOR CRB FUNCTION

6. 1. CRB3A is linked to actin-binding proteins

As described above, PALS1 and PAR6 can bind to the last amino acid ERLI of CRB3A (43, 45) and are known to play an important role during cell polarization. In a recent study, a FERM protein, Epb4.1l5, was newly identified as a partner of the mammalian CRB3A complex (57). Epb4.1l5 is the first mammalian FERM protein that is known to bind to the FERM domain of CRB3 and to the hook domain of PALS1. Interestingly, overexpression of Epb4.1l5 in MDCK cells leads to defects in the tightness and formation of tight junctions indicating that similarly to CRB3A overexpression, this protein contributes importantly to cell organization. It is worth noting that the proteins belonging to the FERM-domain family serve as adaptors linking transmembrane proteins to the cortical actin cytoskeleton (136). Interestingly, in Epb4.1l5 knockout mice, Lee *et al* (89) observed an apical redistribution of F-actin with ectopic F-actin foci, and the overexpression of Epb4.1l5 in HelaS cells resulted in actin cytoskeleton reorganization. On the other hand PAR6 and PALS1, which are both partners of CRB3A, seem to be also linked to actin cytoskeleton. PAR6 is known to interact with cytoskeleton-related proteins, such as 14.3.3 protein (137) and Cdc42 (138), and the pattern of F-actin staining obtained was more diffuse in PALS1-downregulated cells (91). All these data strongly suggest that Epb4.1l5, PAR6 and PALS1 may link CRB3A to actin cytoskeleton. In addition, recruitment and reorganization of cytoskeletal elements through these proteins may contribute to tight junction formation. More studies on the role of the Crumbs complex in regulating cortical actin cytoskeleton and junction remodelling should be performed in the future, both in flies and mammals.

6. 2. CRB3A and trafficking

Exocytosis and endocytosis are thought regulate the formation of the apical membrane and tight junctions. It has been reported that the exocyst is localized to the tight junctions in polarized MDCK cells (139, 140) and that it mediates the targeting of intracellular vesicles to specific sites on the plasma membrane (141). Wang *et al* (91) have observed that Sec8, an exocyst marker, was co-localized with PALS1, and that upon PALS1 downregulation, the exocyst was mislocalized, while newly synthesized E-cadherin accumulated in punctate structures in cell periphery. These authors hypothezised that in PALS1 knockdown cells, the E-cadherin exocytic vesicles may be correctly sorted to the cell surface from the recycling endosomes, but cannot be targeted or fused with the plasma membrane. One can speculate that the formation and maintenance of tight junctions regulated by the CRB complex requires exo- and endocytic trafficking. This idea was supported by the finding that in *Drosophila* mutants such as *avalanche,* a Syntaxin encoding gene, mislocalization of DCrb and epithelial defects are observed (for review, see (142)). Further studies are therefore now required on the links between intracellular trafficking and Crumbs complex function in order to determine the exact mechanisms underlying their interdependence.

6. 3. CRB complex and cell proliferation

The CRB complex is also connected to several signaling pathways whereby it might control cell growth and proliferation. For example, PATJ binds to TSC2, a key component of cell homeostasis (59). TSC2 exhibits a GAP activity domain for the small G protein Rheb (143-145), but this enzymatic activity requires the interaction with TSC1. The TSC1/TSC2 complex has been shown to be a negative regulator of mTORC1 (Target of Rapamycin), which in turn controls cell growth and apoptosis. This data led to the hypothesis that once tight junctions have been established, a signal is sent to the mTOR pathway via PATJ and TSC, to down-regulate epithelial proliferation thus preventing over-proliferation and tumor development. Thus through the intracellular proteins binding to Crumbs cytoplasmic domain, emerges a new picture of how epithelial cell homeostasis is regulated, based on the links between apico-lateral junctions and the cell cycle and survival. While these connections between members of the Crumbs complex and the TOR pathway have still to be uncovered *in vivo* (TSC2 in flies, for example does not exhibit a typical PDZ-binding motif able to interact with DPatj, to our knowledge this potential interaction has not been tested so far); these new findings have interesting potential applications in the field of cancer (this is already the case for PAR4/LKB1 that both control polarity and cell metabolism (146). It seems likely that increasing numbers of links between polarity complexes and cell proliferation will soon be discovered, leading to a more comprehensive picture of epithelial cell biology.

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Abbreviations: 5-HTR: Serotonin receptor, AJ :adherens junctions, Amot: Angiomotin, aPKC: atypical protein kinase C, CAR: Coxsackievirus and Adenovirus Receptor, C-kit: proto-oncogene (receptor for stem cell factor), CRB: crumbs, CRIB: Cdc42 Rac interaction binding, DLG: discs large, EHM2 : expressed in high metastatic cells, Epb4.1l5: erythrocyte protein band 4.1-like 5, EST: expressed sequence tag, FERM: band 4.1 ezrin radixin moesin, JAM: Junctional Adhesion Molecule, L27: Lin2 lin7, LCA : Leber congenital amaurosis, MAGUK: membrane associated guanylate kinase, MALS:mammalian lin seven, MOE: mosaic eyes protein, MPP: membrane protein palmitoylated, MUPP1: multi PDZ domain protein, NG2: membrane-spanning proteoglycan, NOK: nagie oko protein, OLM : outer limiting membrane, OME: oko meduzy mutant, OPL : outer plexiform layer, PALS: protein associated with Lin seven, PAR: partition defective, PATJ: protein associated with tight junction, PDZ: PSD-95, Discs large, ZO1, PhR : photoreceptors, PSD95: post-synaptic density 95, RP : Retinitis Pigmentosa , RPE : Retinal Pigmented Epithelium, SAR : subapical region, Scrib: scribble, Sdt: stardust, SH3: Src homology domain 3, TAPP: tandem-PH (pleckstrin-homology) domain-containing protein, TARPs: transmembrane AMPA receptor regulated proteins, TSC: tuberous sclerosis complex, Veli : vertebrate lin seven, ZA : zonula adherens, ZO: zonula occludens

Note: *Drosophila melanogaster* and *Caenohabditis elegans gene* and Protein names are in small print, whereas vertebrate *GENE* and PROTEIN names are in capital letters. All gene names are given in italics.

Key Words: Epithelium, Polarity, PATJ,PALS1, MUPP1,PAR6, Stardust, Review

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