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Review Article

Emerging Role for Epithelial Polarity Proteins of the Crumbs Family as Potential Tumor Suppressors

Patrick Laprise

Department of Molecular Biology, Medical Biochemistry and Pathology/Cancer Research Center, Laval University and CRCHUQ-Hôtel-Dieu de Québec, 9 McMahon, Québec, QC, Canada G1R 2J6

Correspondence should be addressed to Patrick Laprise, patrick.laprise@crhdq.ulaval.ca

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Defects in apical-basal polarity regulation are associated with tissue overgrowth and tumorogenesis, yet the molecular mechanisms linking epithelial polarity regulators to hyperplasia or neoplasia remain elusive. In addition, exploration of the expression and function of the full complement of proteins required for the polarized architecture of epithelial cells in the context of cancer is awaited. This paper provides an overview of recent studies performed on *Drosophila* and vertebrates showing that apical polarity proteins of the Crumbs family act to repress tissue growth and epithelial to mesenchymal transition. Thus, these proteins emerge as potential tumor suppressors. Interestingly, analysis of the molecular function of Crumbs proteins reveals a function for these polarity regulators in junctional complexes stability and control of signaling pathways regulating proliferation and apoptosis. Thereby, these studies provide a molecular basis explaining how regulation of epithelial polarity is coupled to tumorogenesis.

1. Epithelial Polarity

Epithelial tissues cover the surface and line internal cavities of the human body. Simple epithelia act as a diffusion barrier, generate vectorial transport, and sustain spatially oriented secretion to subdivide the body into morphologically and physiologically distinct compartments. The unidirectional nature of these functions requires the asymmetric distribution of many cellular constituents, a structural organization referred to as epithelial polarity. Epithelial polarization results from the regionalization of the plasma membrane into apical, lateral, and basal domains. The apical domain faces the external environment or a lumen, the lateral domain spans across the plane of the epithelium and contacts neighboring cells, and the basal domain is attached to the basement membrane (Figure 1). Apical junctional complexes are established at the interface between the apical and lateral domains to maintain the cohesion and impermeability of epithelia. Several proteins important for epithelial polarity have been identified in recent years, mainly in model organisms like *C. elegans* and *Drosophila melanogaster* [1]. The function of these proteins is conserved from worm to man, reflecting the significance of epithelial polarity. The importance of the polarized architecture of epithelial cells is further emphasized by the fact that numerous pathologies are associated with epithelial polarity defects, including most human cancers [2, 3].

2. Roles of Crumbs Proteins in Epithelial Polarity Regulation

Epithelial polarity is organized by a complex network of evolutionarily conserved proteins, including the apical transmembrane protein Crumbs (Crb) [1, 4–6]. *Drosophila* embryos lacking Crb display apical-basal polarity defects in several epithelia, which eventually collapse [5, 7]. Overexpression of Crb dominantly extends the apical domain at the expense of the lateral domain [8], showing that Crb is an important apical determinant. Mutual antagonism between Crb and basolateral polarity modules is crucial for segregation and size control of membrane domains in epithelial cells, thus impacting on tissue morphogenesis [9–13].

The human genome encodes three Crb orthologs named CRB1, CRB2, and CRB3 [4]. CRB1 expression is restricted

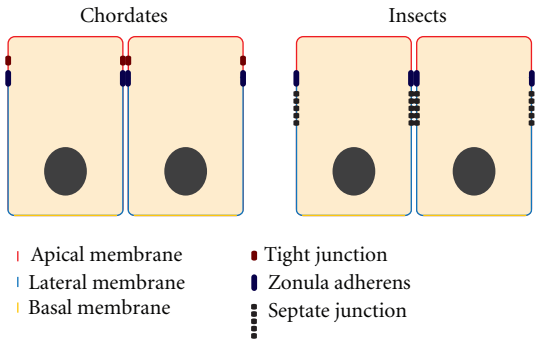


Figure 1: Organization of epithelial tissues. Epithelial cells are polarized along an apical-basal axis. The apical domain faces a lumen or the environment, the lateral domain contacts neighboring cells, and the basal domain is anchored to the basement membrane. This polarized architecture sustains unidirectional functions, such as vectorial transport. The apical and lateral domains are segregated by the zonula adherens, which is a circumferential adherens junction maintaining intercellular adhesion within epithelial tissues. In chordates epithelia, paracellular diffusion is limited by tight junctions, which sit apical to the zonula adherens. In insect, this sealing function is assumed by septate junctions that are established basal to the zonula adherens.

to the brain, cornea, and retina [14–16]. Mutations in human *CRB1* or mouse *Crb1* genes cause degenerative retinal pathologies [14, 17–19]. Similarly, loss of Crb is associated with light-induced retinal degeneration in flies [20]. CRB2 is expressed in several tissues [21], but its function remains poorly understood. CRB3 is expressed in most epithelia and exist as two splice variants. CRB3A is apically localized at the level of tight junctions [4, 22] and promotes intercellular junction formation as well as epithelial polarity [23–25]. CRB3B is associated with spindle poles in dividing cells or found in the apical cilium of polarized kidney epithelial cells to control cytokinesis and ciliogenesis, respectively [26].

Similar to Crb, CRB1 and CRB2 possess a large extracellular domain containing EFG and laminin repeats [5, 7, 14]. In contrast, CRB3 has a short extracellular domain showing no clear homology with the other Crb proteins [22]. However, *Drosophila* Crb and the entire set of human CRB proteins contain a highly conserved cytoplasmic tail [4, 5], which is characterized by the presence of a FERM (4.1, ezrin, radixin, moesin) domain-binding site that is important for Crb function and regulation [11, 27, 28]. In addition, the last four amino acids (ERLI) of the cytoplasmic tail of Crb, CRB1, CRB2, and CRB3A interact with the protein Stardust (Sdt; named PALS1 in mammals) linking Crb or human CRB proteins to *Drosophila* PATJ (dPatj) or Patj, respectively [29–31]. Mutations in *sdt* phenocopy *crb* loss-of-function [29, 32, 33]. In addition, the ability of exogenous Crb to rescue the *crb* mutant phenotype depends on the ERLI motif [27]. PALS1 has been implicated in epithelial polarity as well as cell-cell junction formation [25, 34]. dPatj is important for Crb complex stabilization at the apical membrane, and Patj controls the delivery of CRB3 to the apical domain as well as tight junction formation [35–37]. These studies suggest that Sdt/PALS1 and dPatj/Patj play crucial roles as downstream effectors and/or regulators of Crb/CRB3.

3. Polarity and Cancer

Loss of cell polarity is a typical hallmark of tumor progression in epithelial tissues. The polarized architecture of epithelial cells is compromised at early steps of epithelial to mesenchymal transition (EMT), a process also associated with loss of cell-cell adhesion and acquisition of migratory and invasive properties [38]. Thus, EMT is a critical step in carcinoma progression and metastasis. These observations predict that regulators of apical-basal polarity are fundamental to preserve epithelial homeostasis and to limit tumorogenesis. This hypothesis was first supported by studies in *Drosophila* showing that loss of any members of the lateral-promoting Scribble (Scrib) polarity module promotes epithelial tissue overgrowth and disorganization, resulting in a tumor-like phenotype [39]. The tumor suppressor function of Scrib is conserved in mice [40]. These data have inspired a number of scientists who demonstrated that the expression of many epithelial polarity regulators is altered in several cancers and that proteins required for epithelial polarization are important targets of viral oncoproteins [2, 3, 41]. Finally, mutations in the gene encoding the polarity protein Lkb1 cause a genetic syndrome associated with a high incidence of cancer [42]. Together, these recent discoveries clearly established that further characterization of proteins coordinating epithelial polarity will contribute to our understanding of cancer biology. Deciphering the molecular mechanisms by which polarity proteins act as tumor suppressors is a major issue yet to be solved in this field of research.

3.1. CRB3 and Tumor Growth. The role of the polarity protein CRB3 in cancer was not thoroughly studied yet, but increasing evidence suggests that this protein could restrict tumor progression. Gene expression profiling revealed that repression of CRB3 expression correlates with increased tumorigenic potential in mouse kidney epithelial cells. Re-expression of CRB3 restored cell-cell junctions integrity and cell polarization, while limiting cell motility and metastasis [43]. This suggests that loss of CRB3 in tumor cells is not coincidental, but plays an active role in tumorogenesis. A mechanism leading to the loss of CRB3 expression was recently elucidated. Indeed, CRB3 expression is repressed by two factors promoting EMT, namely, the transcription regulators Snail and ZEB1 [44–46]. Expression of these proteins alters cell-cell adhesion, while increasing migration, invasion, and metastasis [45, 47, 48]. Snail and ZEB1 directly bind to and repress *CRB3* promoter [44, 46]. Importantly, expression of exogenous CRB3 in Snail-expressing cells partially restores the formation of cell-cell junctions and the epithelial phenotype, suggesting that *CRB3* gene is a functional target of Snail and that its repression contributes to EMT [46]. Expression of Snail and ZEB1 is increased in many human tumors, and it correlates with dedifferentiation and invasion [47–49]. This suggests that CRB3 expression is

reduced in human cancers allowing for tumor progression, but a former demonstration of this hypothesis remains awaited. Collectively, these findings establish that it is of great interest to study CRB3 in tumor of epithelial origin, which accounts for the vast majority of human cancers.

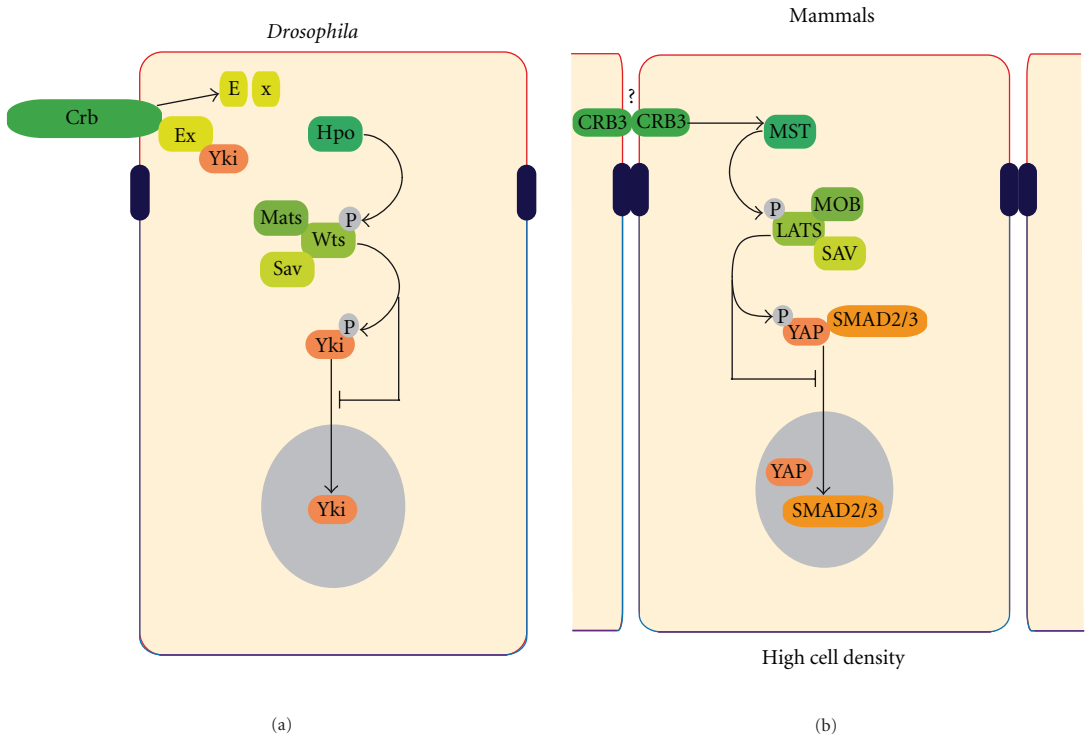
3.2. Mechanisms by Which Crb Proteins Could Act as Tumor Suppressors

3.2.1. Crb and CRB3 Activates the Tumor Suppressor Salvador/Warts/Hippo Pathway. Although it is clear that alteration of epithelial polarity contributes to tissue overgrowth, mechanistic insights into how polarity regulators act in this context are missing. Recent studies on *Drosophila* and mammalian cells have shown that Crb and CRB3 regulate signaling pathways controlling proliferation and survival, including the Salvador/Warts/Hippo (SWH) pathway. In *Drosophila*, the adaptor protein Salvador (Sav) associates with the kinase Hippo (Hpo), which phosphorylates and activates the kinase Warts (Wts) [50]. Activated Wts, in association with mob as a tumor suppressor (Mats), phosphorylates the transcriptional coactivator Yorkie (Yki). Phospho-Yki is sequestered in the cytoplasm and unable to activate its proproliferative (*cyclin E*, E2F1, and *bantam*) and antiapoptotic (*Drosophila* inhibitor of apoptosis (*diap1*)) target genes [50, 51]. Thus, inhibition of the SWH pathway leads to Yki-dependent stimulation of cell proliferation and survival, leading to tissue hyperplasia (Figure 2) [51]. The cortical FERM-domain protein Expanded (Ex) also represses Yki-dependent transcription. Ex may act by binding directly to Yki to limit its nuclear translocation [52]. Proper localization at apical junctions and function of Ex depend on the protocadherin Fat, which potentially couples extracellular signals to Yki regulation [53–55]. The SWH pathway is well conserved in mammals and contributes to cell growth regulation. Overexpression of the Yki ortholog YAP promotes EMT and uncontrolled proliferation [56]. A similar scenario prevails *in vivo* where YAP overexpression increases liver size and expands progenitor cell population in the intestinal epithelium [57, 58]. Based on these studies, it is not surprising that LATS1, MOB, and SAV (the homolog of Wts, Mats and Sav, resp.) were described as tumor suppressors [59–62]. Thus, further defining the regulation of the SWH pathway will refine our understanding of tissue growth and organ size regulation, as well as human diseases associated with proliferation and apoptosis deregulation such as cancer. In particular, identification of upstream regulators of the SWH and determination of their mechanism of action represent an important challenge.

Loss of *crb* leads to tissue hyperplasia in *Drosophila* larval epithelial discs [63–65]. Reduction of Crb amount increases organ overgrowth resulting from Yki overexpression, and a *crb* null allele enhances tissue enlargement in a hypomorphic *ex* background [64, 66]. Moreover, lack of Crb increases the expression of Yki target genes [64]. This suggests that Crb is a positive regulator of Ex and SWH pathway and contributes to limiting Yki activity. In the absence of Crb, Ex protein accumulates, but is delocalized basally to its normal location [63, 64, 66]. This suggests that Crb-dependent Ex localization is crucial for its function. The recruitment of Ex by Crb likely depends on a direct binding of these proteins [63]. Whether there is collaboration between Fat and Crb to localize Ex remains to be determined [53, 54]. Intriguingly, overexpression of Crb or expression of the membrane-bound cytoplasmic tail of Crb (Crb_{intra}) decreases Ex levels and increases cell growth through Yki activation [63, 64, 66–68]. Thus, lack of Crb or excessive amount of this protein results in a similar Yki-dependent phenotype. This could suggests that Crb overabundance has a dominant negative effect, perhaps by disrupting stoichiometry of a Crb-containing complex. However, the opposite effect of Crb_{intra} expression or loss of Crb on Ex levels argues against this hypothesis and rather suggest that Crb has a complex function within the SWH pathway. One plausible hypothesis is that Crb plays an important role in fine-tuning of the SWH pathway activity by favoring Ex function through an effect on its localization, while limiting its level (Figure 2). Collectively, these data show that Crb is a regulator of the SWH pathway, conferring to Crb the ability to control proliferation and epithelial tissue size.

In mammals, high cell density leads to a cytoplasmic retention of YAP through the activation of the SWH pathway, which thereby contributes to contact-mediated inhibition of growth [68, 69]. This implies that a receptor sensing cell density relays information to the SWH pathway to limit proliferation. Similar to YAP, members of the SMAD family, which regulate transcription downstream of TGF-β signaling [70], are enriched in the nucleus of cells cultivated at low density, whereas they are trapped in the cytoplasm of epithelial cells grown at high density [71]. Interestingly, phosphorylated YAP binds to SMAD2/3, suggesting that phospho-YAP could contribute to the cytoplasmic retention of these proteins. Accordingly, knockdown of LATS kinases results in a nuclear accumulation of both YAP and SMAD2/3 along with an increase in expression of TGF-β target genes [71]. This shows that the SWH pathway regulates TGF-β/SMADs signaling through a YAP-dependent regulation of SMAD localization. Strikingly, YAP binds to CRB3 complex components, and knockdown of CRB3 decreases YAP phosphorylation. Reduction of CRB3 correlates with YAP and SMAD2/3 nuclear accumulation and potentiates TGF-β-induced EMT [71]. This mechanism may explain how the loss of CRB3 leads to EMT in other cell types [46]. Thus, like *Drosophila* Crb, CRB3 participates in the regulation of the SWH pathway. In particular, CRB3 couples cell density to activation of this pathway. It will be interesting to investigate whether this functional relationship has a tumor suppressor function in humans. One initial step would be to correlate CRB3 expression and localization with Yki target genes' expression in cancer tissues.

3.2.2. Control of Notch Signaling by Crb. Notch proteins are evolutionarily conserved transmembrane receptors, which are activated by transmembrane ligands expressed at the surface of adjacent cells [72]. Notch-mediated short-range intercellular communication fulfills crucial roles in embryonic development and tissue renewal. Notch activation



Conserved Salvador-Warts-Hippo pathway components		
Drosophila	Mammals	Protein family/function
Yki	YAP	Transcriptional coactivators
Hpo	MST1/2	Sterile 20-like kinases
Wts	LATS1/2	DBF family kinases
Mats	MOB	Adaptor proteins
Sav	SAV	WW-repeat scaffolding proteins

Figure 2: Functional relationship linking Crb proteins to the SWH pathway. (a) In *Drosophila*, nuclear Yki promotes cell proliferation and survival by modulating gene expression. Activation of the SWH pathway leads to the phosphorylation and cytoplasmic retention of Yki. Binding to the FERM domain protein Ex also prevents the nuclear translocation of Yki. Crb restricts Yki-dependent tissue hyperplasia by contributing to Ex localization and function. Overexpression of Crb leads to degradation of Ex, suggesting that Crb plays a crucial role in the fine-tuning of the SWH activity by activating Ex, while limiting its level. The table lists the components of the SWH pathway in *Drosophila* and mammals. (b) In mammalian cells, high cell density activates the SWH pathway, which contributes to contact-mediated inhibition of growth. Phosphorylation of YAP also results in cytoplasmic sequestration of SMAD proteins, thus limiting TGF-β responsiveness. CRB3 is important to couple cell density information to the SWH, perhaps through a homophilic interaction, leading to the suppression of TGF-β signaling and epithelial to mesenchymal transition.

influences cell fate specification, proliferation, apoptosis, and differentiation. It is therefore not surprising that deregulation of Notch signaling has profound effects on tissue homeostasis and results in human pathologies, including many human cancers [73]. Following ligand binding, Notch intracellular domain (NICD) is released by proteolysis. Full proteolytic processing of NICD requires the multimeric γ-secretase complex. Processed NICD reaches the nucleus where it partakes in a transcription complex allowing for expression of Notch pathway target genes [72].

In *Drosophila* wing epithelial discs, Notch promotes the expression of Crb, which represses Notch activity [74]. Thus, Notch signaling refines itself by inducing the expression of its repressor. The function of Crb in limiting Notch signaling is not restricted to the wing disc epithelium. Loss of Crb in the developing eye imaginal disc results in Notch-dependent

overproliferation and tissue hyperplasia [65]. Several mechanisms may explain the effect of Crb on Notch signaling. First, Crb inhibits endocytosis of Notch [65], which is required for activation of Notch signaling [75, 76]. Secondly, Crb limits γ-secretase complex activity, thus interfering with Notch processing [74]. Similarly, human CRB2 inhibits γ-secretase action in cell lines and cell-free assays [77]. CRB1 and CRB3 also show a similar inhibitory effect. CRB2 associates with components of the γ-secretase complex. This interaction depends on CRB2 transmembrane domain, which is essential for γ-secretase complex inhibition [77]. This suggests that CRB2 counteracts γ-secretase-mediated proteolysis through a direct interaction. Crb family proteins are also functionally linked to Notch signaling in zebra fish. In this vertebrate organism, the extracellular domain of Crb binds to Notch and inhibits its activity in *cis* [78]. This mechanism involving a direct interaction of Crb with Notch might be conserved, as Crb-dependent Notch inhibition in flies requires the extracellular domain of Crb [65]. Finally, the Crb-dependent regulation of Notch may depend on the ability of Crb to activate the SWH pathway. Indeed, YAP1 stimulates proliferation through activation of Notch signaling [58]. In addition, the membrane localization of Notch is increased in *hpo* mutant clones [79]. However, the SWH pathway, and Notch can activate each other in *Drosophila* ovaries [80, 81]. Thus, more work is required at this point to clarify the complex functional relationship linking Crb, the SWH pathway and Notch signaling. Overall, these studies establish that Crb proteins share an evolutionarily conserved function in limiting Notch signaling. Several mechanisms have been proposed to explain how Crb family members limit Notch-modulated cell behavior (Figure 3), but further studies are required to clarify whether lack of CRB proteins favor tumor growth through activation of Notch signaling.

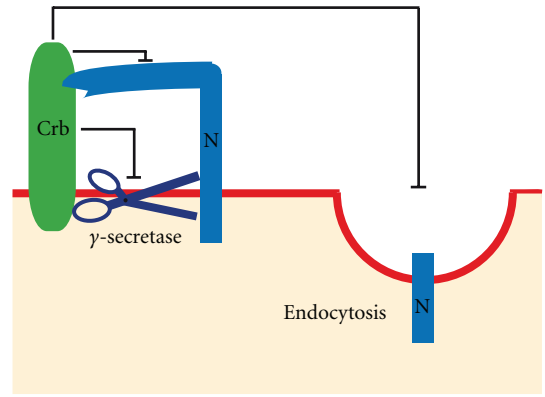


Figure 3: Crb proteins control Notch-dependent tissue growth. Crb proteins repress Notch (N) signaling in *Drosophila*, zebra fish, and mammalian cells. Different mechanisms account for the Crb-dependent inhibition of Notch signaling. *Drosophila* Crb and CRB2 inhibit γ-secretase activity. In addition, Crb limits Notch endocytosis, which is important for Notch signaling. Finally, the extracellular domain of Crb proteins binds to Notch and counteracts its activity.

3.2.3. Regulation of Apical Junctional Complexes by Crb Proteins. The barrier function of epithelia relies on different types of junctional complexes, which maintain cohesion between epithelial cells and seal the intercellular space. The zonula adherens (ZA), a belt-like adherens junction, makes prominent contribution to adhesive forces holding epithelial cells together [82]. The ZA also plays crucial roles in tissue morphogenesis and homeostasis. The homophilic adhesion receptor E-cadherin is a core component of the ZA. E-cadherin indirectly links the cortical microfilaments of neighboring cells through cytoplasmic adaptor proteins, including β-catenin [82]. *Drosophila* Crb is required for proper assembly of the ZA in epithelial and photoreceptor cells [83, 84]. Moreover, Crb contributes to precisely localizing the ZA through apical displacement of the scaffold protein Bazooka (Baz; Par3 in *c. elegans* and vertebrates), which acts as a landmark to establish the ZA at the apical-lateral border [85–88]. The capacity of Crb to promote ZA integrity may confer to this protein the ability to maintain epithelial tissue homeostasis. Indeed, E-cadherin has an important tumor suppressor function, by limiting proliferation, invasion, and metastasis [89]. Many human tumor types show a loss of E-cadherin expression. Loss of E-cadherin expression in cancer may be explained by loss of heterozygosity, epigenetic modification of the E-cadherin locus, and transcriptional silencing by EMT-promoting factors. Many mechanisms other than genetic alterations or epigenetic modifications can interfere with E-cadherin function. For instance, regulation of E-cadherin level at the plasma membrane by endocytosis is important for ZA integrity [89]. Interestingly, appropriate level of Crb resulting from the equilibrium between its delivery, endocytosis, and recycling could play an important role in stabilizing E-cadherin at the membrane, [90, 91]. However, a formal demonstration of this hypothesis is awaited. In addition, expression of E-cadherin is strongly repressed during EMT [38], which could be repressed by CRB3. Thus, it would be pertinent to investigate whether there is a correlation between CRB proteins and E-cadherin expression in human cancers and to better define CRB proteins function in the regulation of ZA integrity, especially in vertebrates.

While the cohesion of epithelial cells largely depends on the ZA, the selective permeability of epithelia relies on tight junctions (TJs) in vertebrates and septate junctions in invertebrates [92, 93]. TJs sit just apical to the ZA and restrict paracellular diffusion. Several transmembrane proteins are associated with TJ, including members of the claudin family, which are core components of TJ required for their assembly, and CRB3 [94, 95]. In the absence of CRB3, TJ integrity is compromised [95]. Structure-function analysis revealed that the C-terminal ERLI motif is required for the positive impact of CRB3 on TJ organization [43, 95]. Consistent with this observation, PALS1 and Patj, which are recruited into the CRB3 complex through the ERLI domain, are also required to establish functional TJ [34, 35]. Dysfunctions of TJ were linked to cancer over recent years [94]. Overexpression of ZO-1, a protein associated with the cytoplasmic face of TJ, blocks proliferation in cultured epithelial cells [96].

Thus, it is not surprising that ZO-1 level is decreased in several human tumors [97, 98]. The mechanism used by ZO-1 or other TJ proteins to maintain a normal epithelial phenotype remains elusive. However, increasing evidence suggests that TJ proteins have an impact on proliferation through regulation of gene expression. For instance, ZO-1 binds to and sequesters the dual-location protein ZONAB at TJ [96, 99]. Loss of ZO-1 or disruption of TJ releases this protein, which can then translocate to the nucleus where it acts as a transcription factor. ZONAB upregulates the expression of cell cycle regulators, DNA replication factors, and oncogenes [96, 99, 100]. Moreover, ZONAB binds to CDK4 to favor its nuclear accumulation, thus facilitating the G1/S transition [96]. ZO-2, a TJ-associated protein related to ZO-1, can also regulate gene expression. ZO-2 can shuttle in the nucleus to repress cyclin D1 expression [101]. Thus, it is possible that CRB3 limits proliferation by maintaining TJ integrity and sustaining TJ-dependent gene expression regulation. However, it will be important to investigate whether CRB3 has a direct impact on the regulation of TJ-associated transcription factor regulating proliferation such as ZONAB.

4. Conclusions and Perspectives

Recent studies have established that Crb/CRB proteins regulate epithelial tissue growth by acting as transmembrane proteins controlling intracellular signaling important for proliferation and apoptosis. Crb/CRB proteins are linked to many pathways through different domains, showing a complex function for these proteins in relaying growth-control signals. Indeed, Crb/CRBs inhibit Notch signaling through a direct interaction with the extracellular domain of Notch receptor in *cis*, counteract γ-secretase activity using the transmembrane domain, control Ex localization activity using the FERM domain-binding site within the cytoplasmic tail, while promoting TJ integrity through the C-terminal ERLI amino acids. These studies therefore provide mechanistic insights linking a cell polarity regulator to restriction of tissue hyperplasia. One outstanding question is whether CRB3 acts as a receptor transmitting extracellular cues inside the cell to maintain epithelial homeostasis. Although the extracellular domain of CRB3 is short, it may be involved in protein-protein interaction or may bind to lectin proteins, as it is glycosylated [22]. Identification of binding partners for the extracellular domain of CRB3 would help in addressing this question. Further investigation is required at this point to formerly establish that CRB3 is a tumor suppressor in humans, but deciphering the molecular mechanisms acting downstream of CRB3 seems a promising avenue to better understand cancer biology and to identify potential therapeutic targets.

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