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Complexities of Crumbs Function and Regulation in Tissue Morphogenesis

Minireview

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Establishing and maintaining epithelial polarity is crucial during development and for adult tissue homeostasis. A complex network of evolutionarily conserved proteins regulates this compartmentalization. One such protein is Crumbs, a type I transmembrane protein initially shown to be an important apical determinant in *Drosophila*. We discuss recent studies that have advanced our understanding of the function and regulation of Crumbs. New findings obtained in flies and fish, reporting homotypic interactions of the extracellular domain and retromer-mediated recycling, shed light on the regulation of Crumbs levels and activity. These results — obtained in different organisms, tissues and developmental stages — point to more complex functions and regulation than previously assumed.

Introduction

The establishment and maintenance of epithelial polarity is essential throughout development and adult life. Early studies in *Drosophila* and *Caenorhabditis elegans* identified three protein complexes — the Par, Crumbs and Scribble complexes — that are crucial for these processes [1]. Subsequent work elucidated some of the molecular pathways associated with these complexes and revealed that, rather than functioning as discrete protein complexes, an interconnected network of polarity determinants acts to delicately balance apical, lateral and basal components [1,2]. In this minireview we discuss work on recently identified mechanisms involving recycling and homophilic adhesion that regulate the levels of the apical transmembrane protein Crumbs (Crb), a crucial regulator of epithelial development and tissue homeostasis.

Crb – Polarity and Beyond

Crb, a type I transmembrane protein conserved from *C. elegans* to humans, was initially identified as an apical determinant in *Drosophila* embryonic epithelia [3–5] and follicular epithelial cells [6,7], and later implicated in photoreceptor cell morphogenesis and retinal degeneration [8–10], as well as cell proliferation and survival [11–14]. The highly conserved intracellular domain contains two conserved elements, a PDZ (PSD-95/Discs large/ZO-1)-binding, carboxy-terminal ERLI motif and a membrane proximal FERM (4.1/ezrin/radixin/moesin)-binding domain. The former can recruit a plasma-membrane-associated protein scaffold, composed of Stardust (Sdt), DPatJ and DLin7 [15] or Par6 and atypical protein kinase C (aPKC) [16,17]. Both of these complexes are essential for the polarity establishment and maintenance functions of Crb in embryonic epithelia, adult epithelia and the retina. The Sdt–DPatJ complex is required in almost every cell type studied to stabilize Crb at the membrane and it is tempting to speculate that this complex forms a membrane-associated scaffold that recruits additional factors. In several *Drosophila* tissues, it has also been shown that recruitment of Par6–aPKC by Crb is essential for its polarity maintenance functions because the resulting phosphorylation of Baz (the *Drosophila* orthologue of Par3) by aPKC excludes Baz from the apical surface and restricts it to the adherens junction, where it maintains cell–cell adhesion [6].

The FERM-binding domain is dispensable for apico-basal polarity in *Drosophila* embryonic epithelia, but is required for amnioserosa integrity during dorsal closure [18]. It is involved in the recruitment of cytoskeletal elements, such as spectrin and moesin. Both in flies and zebrafish, the FERM protein Yurt/Mosaic Eyes negatively regulates Crb by preventing its stabilization/accumulation at apical sites [19,20]. More recently, the FERM-domain containing protein Expanded has been shown to bind to the Crb intracellular domain. Expanded is an upstream component of the Hippo hyperplastic tumour suppressor pathway, responsible for the regulation of epithelial size. In the absence of Crb, Expanded is no longer recruited to the apical membrane and this mislocalisation results in the downregulation of the Hippo pathway and consequently leads to overproliferation. Interestingly, the Crb–Expanded interaction not only localizes Expanded, but also promotes phosphorylation and degradation of Expanded. It has therefore been proposed that Crb plays two roles in regulating the Hippo pathway: firstly, activation of Hippo through apical restriction of Expanded; and secondly, targeting of Expanded for degradation to prevent excess Hippo signaling in a feedback loop of activity refinement (readers interested in the link between Crb and the Hippo pathway are directed to recent extensive reviews [13,21]).

While numerous studies have aimed at understanding the downstream effects of Crb, regulation of Crb activity itself remained less well understood. Formation of the Crb complex provides one aspect of regulation of Crb levels and localization, as in several tissue types the stabilization and localization of the members of the Crb complex are interdependent [22–25]. Additionally, mutual antagonism between different polarity complexes is critical for restricting Crb to the apical domain [26,27]. The majority of these functions could be assigned to the short cytoplasmic tail.

In contrast, structure–function analyses based on overexpression were unable to detect any major function in the embryo of the extracellular portion of Crb, a large domain composed of numerous EGF-like and laminin-A G-like repeats [5]. It is interesting to note that, while *Drosophila* contains only one *crb* gene, mice and humans contain three. One of these, *Crb3/CRB3*, encodes a protein that contains the conserved transmembrane and cytoplasmic domain, but carries only a vestigial extracellular domain that lacks EGF- and laminin-A G-like repeats [15]. This raises the possibility that different domains of Crb mediate various functions that have been separated during evolution by gene duplication and mutation. In contrast, the single *crb* gene of flies has to accomplish all functions, which may explain why in *Drosophila* some tissues are dependent on the intracellular domain, whereas other tissues also require the extracellular domain [10,12,28].

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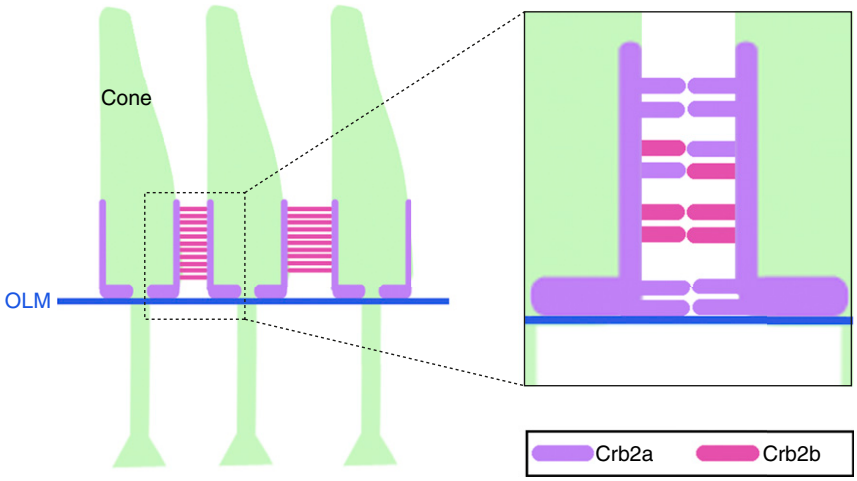

Figure 1. Crb interactions in the zebrafish retina.

Crb2a (purple) is enriched in regions apical to the outer limiting membrane (OLM, blue) and inner segment of cones. Crb2b (magenta) is restricted to the inner segments between adjacent green, red and blue cones. Crb2a–Crb2a homodimers maintain photoreceptor adhesion at the level of the OLM, whereas there remain three possibilities for Crb dimer formation between cones at the level of the inner segment (zoom box): Crb2a–Crb2a homodimers; Crb2a–Crb2b heterodimers; and Crb2b–Crb2b homodimers [36].

The extracellular domain of Crb was reported to regulate *Drosophila* head size in a Notch-dependent manner [29]. It was proposed that this is achieved by a combination of regulating Notch and Delta endocytosis and through the inhibition of γ-secretase activity. This latter function has previously been attributed to the extracellular domain of Crb [30]. In addition, the extracellular domain of Crb was shown to be required for Crb's ability to prevent light-dependent photoreceptor degeneration [10,31]. This parallels the observation that most of the mutations in human *CRB1* that result in blindness reside in the extracellular domain [32]. However, a molecular mechanism explaining this function remains elusive. Much of this lack of understanding stems from the dearth of verified interaction partners associated with the extracellular domain of Crb.

Regulating Crb Levels and Activity by Homodimerization

Previous speculations on Crb homo- or heterodimerization were based on clonal analyses, where membranes between wild-type and *crb* mutant cells were devoid of Crb protein [33,34]. While suggestive of direct homodimerisation, such evidence is far from conclusive, in particular since the generation of clones creates an *in vivo* situation that is rarely found in nature. Several papers published over the past year now suggest different modes of interaction and functions for Crb homotypic binding both in flies and in fish [34–37].

Using zebrafish, whose genome encodes five Crb orthologues (*crb1*, *crb2a*, *crb2b*, *crb3a* and *crb3b*), Zou *et al.* [36] have shown that in the adult retina Crb2a is expressed broadly in photoreceptors and Müller cells, whereas Crb2b is confined to a subset of photoreceptors, the green, red and blue cones, which form stereotypically organized cell clusters called cone mosaics. Both proteins are localized on the inner segments of photoreceptors and/or apical processes of Müller cells. The different distribution of Crb isoforms led to the hypothesis that *trans* interactions might be required for adhesion within the cone clusters. Using GST pull-down and cell aggregation assays, the extracellular domains of Crb2a and Crb2b were shown to interact homo- and heterotypically [36].

The authors found that zebrafish Crb2a is absolutely required for retinal development because *Crb2a/oko meduzy* mutants display early defects in retinal integrity due to loss of adherens junctions. Consequently, cones are scattered throughout the retina and fail to cluster. Overexpression of Crb2a in differentiated cones of Crb2a mutants did not restore epithelial integrity, but restored clustering of these cones at ectopic positions and high levels of Crb2a at the interfaces between these cells (Figure 1). These data provide compelling evidence that Crb–Crb interactions between neighboring cells (*trans* interactions) are essential for adhesion-based cell sorting in cone mosaics during development. However, this study does not rule out the possibility that Crb2b–Crb2a heterophilic interactions maintain mosaic formation (Figure 1). The authors propose that the subcellular localization of different isoforms of Crb is also critical; Crb2a, which localizes apically to adherens junctions in the retinal epithelium, is responsible for maintaining the overall integrity of the retinal epithelium by assuring E-cadherin-mediated cell–cell adhesion, whereas Crb2b, which localizes to the inner segment of differentiated cells, is involved in maintaining the specific arrangement of cone clusters. Morphogenesis of the retina may therefore prove an excellent model for studying the differential adhesion hypothesis, first posited fifty years ago [38] and, with the development of new techniques and biophysical models, now the subject of renewed interest [39].

Using mathematical modeling, Fletcher *et al.* [35] sought to address the question of how mutual antagonism between apical and basolateral protein complexes generates and maintains polarity. These authors came to the conclusion that mutual antagonism alone was insufficient to generate or maintain polarity. By introducing a positive feedback loop (Figure 2), the model could generate spontaneous polarization that was rather insensitive to the number of molecules in the system. Through a series of experiments combined with published findings, Fletcher and colleagues proposed a model in which Crb molecules, complexed with Par6–aPKC–Sdt, can recruit uncomplexed Crb molecules via hypothesized *cis*-homodimerisation of their extracellular domains. This then allows phosphorylation of the uncomplexed Crb by aPKC and subsequently recruitment of the FERM-domain protein Expanded and its partner Kibra, thereby regulating Crb levels by preventing Crb endocytosis (Figure 2). While the means by which Expanded and Kibra stabilize Crb localization at the apical membrane is not identified, the authors show that it requires both proteins working in concert and that both therefore contribute to the positive feedback loop. Investigations into an interaction

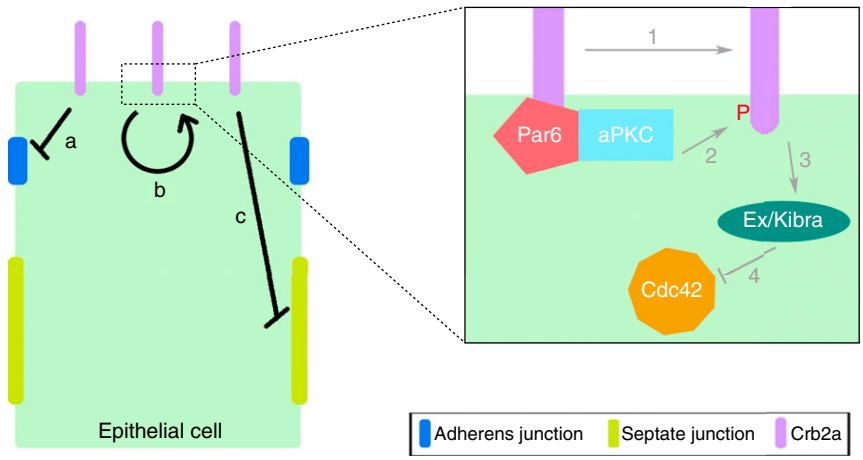

Figure 2. Three functions of Crb in epithelial cells.

Mutual antagonism between apically localized Crb and adherens junction (blue) components (a). Positive feedback (b). Mutual antagonism between Crb and basolateral septate junction components (c). Positive feedback is proposed to occur via recruitment of an uncomplexed Crb molecule by a Crb complexed with Par6 and aPKC (zoom, arrow 1) by homodimerization. aPKC phosphorylates the uncomplexed Crb (2), which drives recruitment of expanded (Ex) and Kibra (3). Ex and Kibra recruitment in turn inhibits association of Cdc42 (4), which has been shown to drive Crb removal from the plasma membrane by endocytosis [35].

between Expanded and/or Kibra with proteins known to be involved in Crb endocytosis, such as Cdc42 [40], might help clarify this.

While designed to address a fundamental aspect of polarity generation, this paper by Fletcher *et al.* [35] poses some very important questions that will influence future studies. Firstly, to what extent do mutual antagonism and positive feedback influence one another? Are they in fact mutually exclusive? A model that proposes that Crb *cis*-interactions are sufficient to stabilize Crb at the plasma membrane is indeed at direct odds with the observed loss of Crb at clonal boundaries. Secondly, are distinct pools/forms of the same protein involved in the different processes, as described in the zebrafish retina? In the case of Crb, are those Crb molecules involved in positive feedback unable to inhibit basolateral components or vice versa? Thirdly, is positive feedback the natural consequence of mutual antagonism? By inhibiting the ability of basolateral proteins to occupy the apical domain, are apical determinants creating more space for newly synthesized/recycled apical proteins that are specifically targeted there? Finally, if the balance between the contributions of these pathways is critical, does this vary during development or in different cell or tissue types? If so, is this variation responsible for the transition from initial establishment of polarity to subsequent maintenance of polarity?

Together, these papers propose that the extracellular domain of the polarity determinant Crb regulates distinct processes — adhesion, cell polarity and tissue morphogenesis, thereby significantly advancing our current understanding of the way in which polarity determinants function. Given the fact that the studies were performed in different tissues and in different organisms, it appears that these are highly conserved functions. Future studies will now need to address to what extent these context-dependent functions act via specific regions of the extracellular domains, differential modification and/or additional partners.

Anisotropic Distribution of Crb Drives Morphogenesis

If Crb molecules on adjacent membranes can strengthen adhesion between these cells, what happens at the boundary, where cells with high Crb levels abut cells with low Crb levels? This phenomenon was investigated in two recent papers and shown to result in strikingly different outcomes. Using *Drosophila* embryos, Katja Röper analyzed the invagination of the salivary gland placode, the anlage of the salivary gland [37]. Cells of the placode express high levels of Crb, which accumulates on the cell membrane when in contact with cells expressing high Crb levels. However, placode cells abutting non-placode epithelial cells expressing lower Crb levels exhibit anisotropic plasma membrane distribution of Crb. Röper suggests that reduced Crb levels in the membrane at the placode boundary results in accumulation of myosin II inside the boundary (Figure 3). This myosin II contributes to a 'supracellular' actomyosin cable that surrounds the placode. Low levels of Crb at the placode boundary are associated with a concomitant reduction in aPKC, which the author concludes relieves Rho kinase inhibition, which, in turn leads to activation of myosin and the formation of the actomyosin cable [37].

Using *Drosophila* eyes and imaginal wing discs, Hafezi *et al.* [34] used clonal analyses to assess the effect of Crb anisotropy at the borders between wild-type tissue and Crb mutant/overexpressing tissue [34]. At such boundaries, increased apoptosis was observed in cells expressing higher levels of Crb. Again, this function was attributable to the extracellular domain of Crb and the authors hypothesized that Crb's ability to form *trans*-dimers and regulate members of the Hippo pathway is responsible for the activation of the apoptotic machinery [34].

Again, we see diverse functions attributed to Crb at different developmental stages and in different epithelial tissues. This strengthens the argument that expression of tissue-specific co-factors are somehow modulating Crb function, enabling it to control an extraordinary range of processes. Obvious candidates for future studies would be known interaction partners, such as the Sdt–dPatJ and Par6–aPKC complexes, or interactions with the Hippo pathway.

Regulating Crb Levels by Recycling

Regulating Crb protein levels is also crucial if polarity is to be maintained correctly; Crb overexpression leads to basolateral domains adopting apical characteristics [5], whereas loss of Crb often results in the loss of apical identity. The recent discovery that Crb is targeted for retrograde recycling by retromer has uncovered an important step in Crb regulation [41,42]. Retromer is an evolutionarily conserved trafficking machinery that mediates early endosomal sorting of proteins and their transport back towards the Golgi [43]. The Crb intracellular domain interacts with

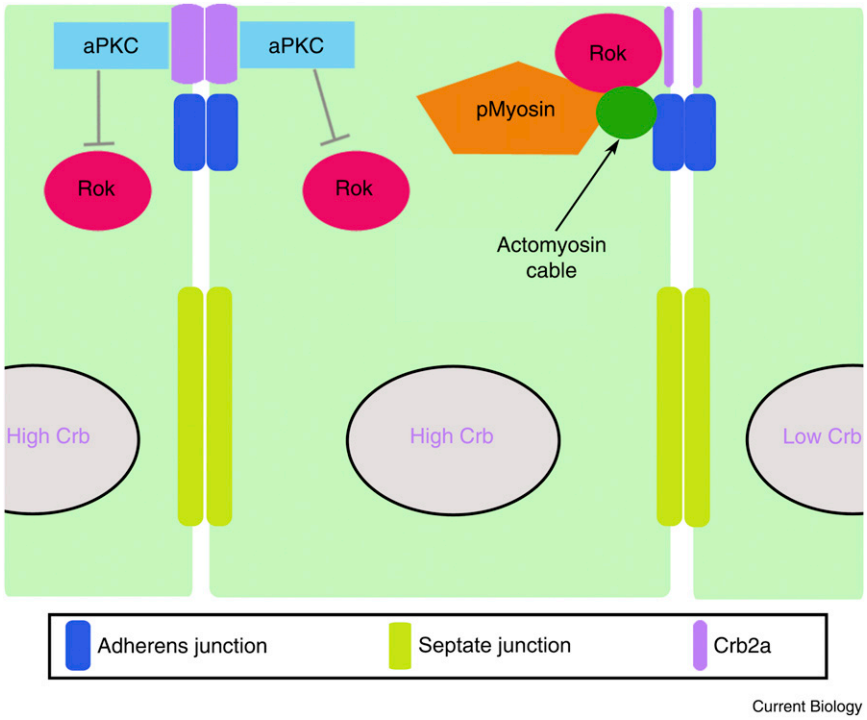

Figure 3. Crb asymmetry in the salivary placode.

Asymmetric distribution of Crb results in localized inhibition of Rho kinase (Rok) at regions where cells with high Crb levels interact and the formation of an actomyosin cable at regions of interaction between high Crb-expressing and low Crb-expressing cells in the *Drosophila* salivary placode [37].

a cargo-selective subunit of retromer, Vps35 [41]. Epithelial cells mutant for Vps35 show a reduction in Crb levels and display Crb mutant phenotypes, such as multilayering, defective cuticle secretion and reduced levels of apical aPKC and Par6 [41,42]. These data indicate that Crb levels are regulated by recycling decisions made at the early endosome. Unlike many retromer cargoes, which have a steady-state localization at the *trans*-Golgi network (TGN) [44,45], Crb is predominantly observed at the apical plasma membrane. In this context, it is interesting to note that, while Crb is seen to traffic through the TGN following internalization from the plasma membrane [41], it does not accumulate there and instead rapidly travels back to the cell surface via as yet unidentified routes. The path taken by recycled Crb back to the cell surface may include transport via Rab11 endosomes, as previous reports have shown that Rab11 is required for Crb transport to the plasma membrane [46]. However, it remains to be established whether 'new' and recycled Crb take the same route.

The consequences of this recycling are of great interest. The specific transport of Crb to the TGN raises the possibility that there are some novel functions of Crb that require passage through the TGN, perhaps the co-transport of other apically localized proteins or the delivery of apically secreted molecules that might bind to the extracellular domain of Crb. Precedent for the latter has been reported in *Drosophila* for the secretion of Wingless (Wg), a Wnt isoform that is bound by its transport receptor Wntless in the TGN and trafficked as a complex through the secretory pathway. After Wg release, Wntless is then retrieved from the plasma membrane by endocytosis and recycled by retromer back to the TGN, where the cycle of Wg secretion can occur again. The idea that Crb might be involved in the delivery of apically localized proteins fits well with the apicalisation observed upon Crb overexpression; however, until 'cargoes' for Crb are discovered, this will remain just an interesting hypothesis.

Perspectives: Emerging Polarity-Independent Roles for Crb in Cell Proliferation and Survival

An emerging theme of very recent studies has been the involvement of key polarity determinants in additional pathways, such as the regulation of cell proliferation and cell survival (reviewed in [14,47]). It remains unclear whether or not all of these roles are indeed truly independent of the classical roles for these determinants in maintaining polarity, as has been reported for Notch-mediated overgrowth phenotypes [29]. Addressing this experimentally is far from trivial, as disrupting polarity itself can lead to a huge variety of phenotypes, depending on the mode of disruption and the cell type under investigation. Various polarity defects can drive cancer initiation and progression, and therefore inevitably lead to global changes in cellular behavior, including heightened cell survival and proliferation signals [14]. It will be important for future studies to carefully dissect the temporal sequence of events in these cases and identify the primary defects caused by loss of polarity determinants and how these then lead to the multitude of downstream effects.

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