Crumbs controls epithelial integrity by inhibiting Rac1 and PI3K

François J.-M. Chartier, Émilie J.-L. Hardy and 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, G1R 2J6, Canada

*Author for correspondence (Patrick.Laprise@crhdq.ulaval.ca)

Accepted 16 June 2011
Journal of Cell Science 124, 3393–3398

doi: 10.1242/jcs.092601

Summary

Drosophila Crumbs (Crb) and its mammalian ortholog CRB3 control epithelial polarity through poorly understood molecular mechanisms. Elucidating these mechanisms is crucial, because the physiology of epithelia largely depends on the polarized architecture of individual epithelial cells. In addition, loss of CRB3 favors tumor cell growth, metastasis and epithelial to mesenchymal transition (EMT). Using *Drosophila* embryos, we report that Rac1 sustains PI3K signaling, which is required for Rac1 activation. Crb represses this positive-feedback loop. Notably, this property confers to Crb its ability to promote epithelial integrity in vivo, because attenuation of either Rac1 or PI3K activity rescues the *crb* mutant phenotype. Moreover, inhibition of Rac1 or PI3K results in Crb-dependent apical membrane growth, whereas Rac1 activation restricts membrane localization of Crb and interferes with apical domain formation. This illustrates that Crb and the Rac1–PI3K module are antagonists, and that the fine balance between the activities of these proteins is crucial to maintain epithelial organization and an appropriate apical to basolateral ratio. Together, our results elucidate a mechanism that mediates Crb function and further define the role of PI3K and Rac1 in epithelial morphogenesis, allowing for a better understanding of how distinct membrane domains are regulated in polarized epithelial cells.

Key words: Epithelial morphogenesis, Crumbs, Rac1, PI3K, Epithelial polarity

Introduction

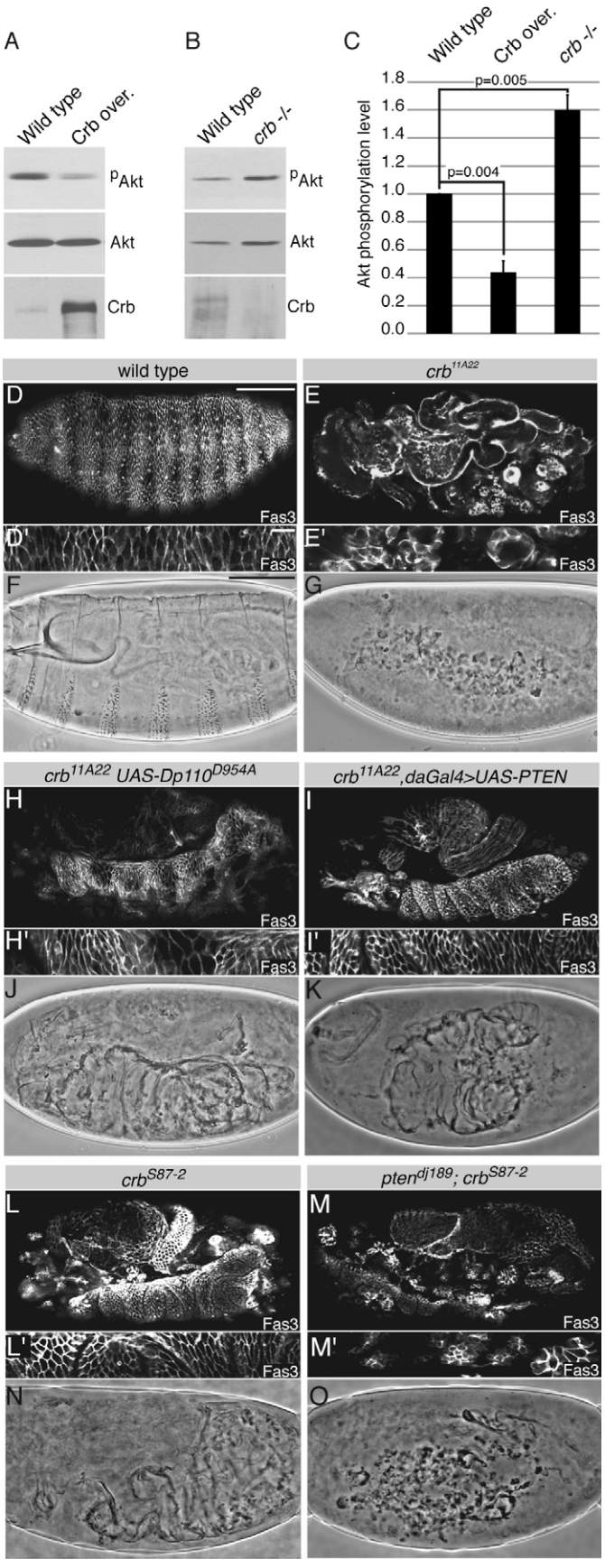
The polarized architecture of epithelial cells is crucial for the development and physiology of most organs. Epithelial polarity is controlled by evolutionarily conserved proteins, including the apical transmembrane protein Crumbs (Crb) (Bazellieres et al., 2009; Bulgakova and Knust, 2009). Fly embryos lacking Crb show epithelial polarity defects and lose epithelial tissue integrity (Bulgakova and Knust, 2009; Tepass et al., 1990). Overexpression of Crb leads to an expansion of the apical domain (Wodarz et al., 1995), illustrating that Crb is a central apical determinant. Identification of the molecular mechanisms involved downstream of Crb is important to understand epithelial polarity regulation and human diseases, including retinal dystrophies and tumor growth (Bazellieres et al., 2009; Bulgakova and Knust, 2009; den Hollander et al., 1999; Karp et al., 2008).

The phosphoinositide 3-kinase (PI3K) pathway is another important regulator of cell polarity (Gassama-Diagne et al., 2006; Martin-Belmonte and Mostov, 2007). PI3K produces mainly phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], which activates downstream effectors of the PI3K pathway, including Akt that is phosphorylated in response to production of PtdIns(3,4,5)P_3 (Yuan and Cantley, 2008). The tumor suppressor PTEN inactivates the PI3K pathway by dephosphorylating PtdIns(3,4,5)P_3 to generate PtdIns(4,5)P_2 (Leslie et al., 2008). In polarized epithelial cells, PTEN is enriched apically leading to concentration of PtdIns(4,5)P_2 at the apical membrane (Martin-Belmonte et al., 2007; von Stein et al., 2005). By contrast, PtdIns(3,4,5)P_3 is mainly associated with the basolateral domain (Gassama-Diagne et al., 2006; Martin-Belmonte et al., 2007). Forced insertion of PtdIns(4,5)P_2 in the plasma membrane generates ectopic apical membrane domains (Martin-Belmonte et al., 2007), whereas PtdIns(3,4,5)P_3confers a basolateral character to the plasma membrane (Gassama-Diagne et al., 2006). Therefore, the fine-tuning of the PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 ratio and their appropriate localization is crucial for epithelial polarity. However, the upstream regulators of PI3K and PTEN in the context of epithelial polarity need to be further defined. Here, we report that Crb controls epithelial integrity by counteracting a Rac1–PI3K module, which restricts Crb activity. Thus, the equilibrium between these mutually antagonistic polarity regulators is crucial to maintain epithelial organization.

Results and Discussion

Crb controls epithelial integrity by modulating the PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 ratio

PtdIns(4,5)P_2 and Crb are important regulators of apical membrane formation (Martin-Belmonte et al., 2007; Wodarz et al., 1995). By contrast, PtdIns(3,4,5)P_3 confers a basolateral character to the plasma membrane (Gassama-Diagne et al., 2006). This raises the intriguing possibility that Crb could promote apical membrane identity by increasing the concentration of PtdIns(4,5)P_2 and/or restricting the formation of PtdIns(3,4,5)P_3. To test this possibility, we assessed the effect of modulating Crb expression on Akt phosphorylation levels in vivo. Overexpression of Crb in *Drosophila* embryos reduced Akt phosphorylation (Fig. 1A,C). Moreover, Akt phosphorylation was increased in *crb* mutants compared with wild-type embryos



(Fig. 1B,C), suggesting that Crb normally functions to repress the PI3K–Akt pathway. To verify the functional importance of the Crb-dependent inhibition of PI3K signaling on epithelial tissue integrity, we counteracted PtdIns(3,4,5)P_3 production in *crb* mutant embryos (null allele crb^{11A22}) by expressing a dominant-negative form of PI3K ($Dp110^{D954A}$) or overexpressing wild-type PTEN. Whereas loss of Crb resulted in epithelial breakdown and formation of isolated cysts of epithelial cells (Fig. 1E) (Tepass et al., 1990), *crb* mutant embryos expressing $Dp110^{D954A}$ or increased amount of wild-type PTEN produced continuous sheets of well-organized epithelial cells (Fig. 1H,I). Rescue of the *crb* mutant phenotype was also evident in the organization of the cuticle, which is secreted by the underlying epidermis and reflects its integrity. The cuticle was severely fragmented and appeared as isolated granules in the absence of Crb (Fig. 1G) (Tepass et al., 1990), whereas uninterrupted layers of cuticle are formed upon reduction of PI3K activity or increased PTEN expression in *crb* mutant embryos (Fig. 1J,K). In addition, *pten* mutation enhanced epithelial collapse associated with the hypomorphic allele crb^{S87-2} (Fig. 1L–O), further demonstrating the functional relationship between phosphoinositide metabolism and Crb. Collectively, these data show that the ability of Crb to increase the PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 ratio is critical for epithelial integrity in vivo.

Epithelial morphogenesis requires Crb-dependent Rac1 inhibition

Although the crucial function of PtdIns(3,4,5)P_3 in epithelial polarity regulation is well established, it is still unclear how this lipid acts in this context. It was previously shown that PI3K inhibition restricts lateral membrane growth, thus reducing epithelial cell height (Gassama-Diagne et al., 2006; Jeanes et al., 2009; Laprise et al., 2002). Interestingly, inhibition of Rac1 has a similar outcome (Jeanes et al., 2009). This suggests that Rac1 and PI3K could act in a common pathway to organize the lateral domain. In support of this hypothesis, PI3K signaling activates Rac1 in many mammalian cell types (Rivard, 2009). Similarly, an active form of PI3K ($Dp110^{CAAX}$) increased the amount of active GTP-bound Rac1, whereas expression of $Dp110^{D954A}$ reduced Rac1 activation in *Drosophila* embryos (Fig. 2A). This suggests that PI3K activity is required for full activation of Rac1 in vivo. Our data further propose that Rac1 signals back to PI3K and activates this kinase, as expression of a

Fig. 1. Crb regulates PI3K signaling to maintain epithelial integrity. (**A**) Akt activation levels assessed by western blot (for phosphorylated Akt) in stage 9–12 wild-type embryos and embryos overexpressing Crb (Crb was overexpressed in all embryos). Total Akt and Crb levels were also assessed as controls. (**B**) Measurement of Akt activation in stage 8–10 wild-type embryos and *crb* mutant embryos (*crb* mutant embryos used were obtained from *crb* germline clone females crossed to *crb*/+ males, thus half the embryos were fully null for *crb*, and the other half had a mutated maternal *crb* allele and a wild-type paternal copy of *crb*). (**C**) Quantification of Akt phosphorylation relative to total Akt upon modulation of Crb expression. Values are means + s.e.m. (**D**–**O**) Fasciclin 3 (Fas3) staining of late-stage whole embryos (D,E,H,I,L,M), a close-up of epidermal cells of the same Fas3-labeled embryos (D′,E′,H′,I′,L′,M′) or a cuticle preparation (F,G,J,K,N,O) for the following genotypes: wild type (D,F), crb^{11A22} (E,G), crb^{11A22} *UAS-Dp110*^{D954A} (*crb* mutant expressing $Dp110^{D954A}$) (H,J), crb^{11A22} *daGal4/UAS-PTEN;* crb^{11A22} (crb^{11A22} mutant overexpressing PTEN) (I,K), crb^{S87-2} (L,N) or $pten^{dj189}$; crb^{S87-2} double mutants (M,O). Scale bars: 100 μm (D,E,H,I,L,M and F,G,J,K,N,O); 10 μm (D′,E′,H′,I′,L′,M′).

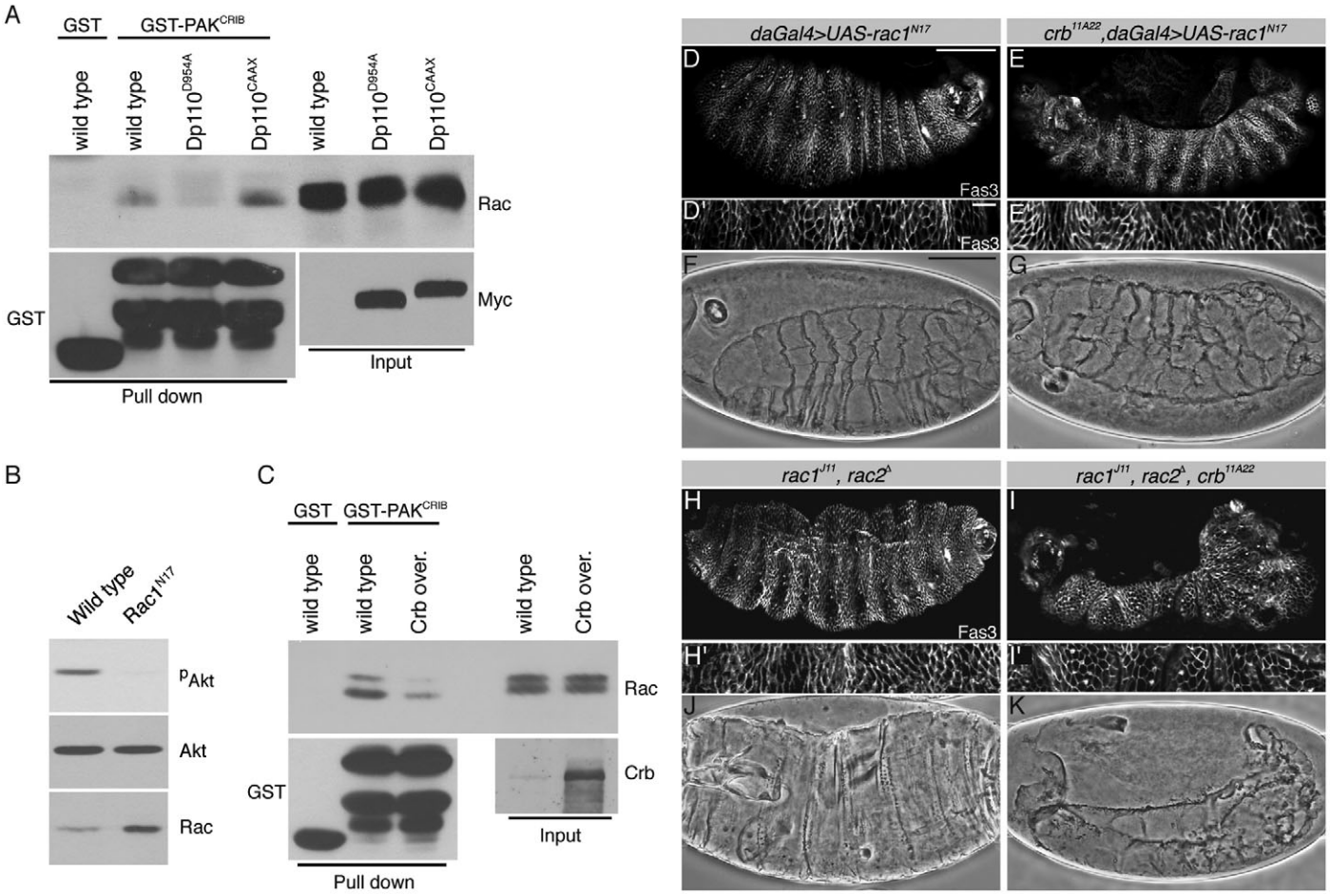


Fig. 2. Repression of Rac1 by Crb is crucial for epithelial integrity. (**A**) Pull-down of active Rac1 detected by western blot. $Dp110^{D954A}$ and $Dp110^{CAAX}$ are Myc-tagged and their expression was detected with an anti-Myc antibody. Embryos were collected at stage 9–12, and they all expressed $Dp110^{D954A}$ or $Dp110^{CAAX}$. (**B**) Analysis of Akt phosphorylation in stage 8–10 wild-type embryos and $Rac1^{N17}$-expressing embryos ($Rac1^{N17}$ was expressed in all embryos). (**C**) Determination of Rac1 activation levels in stage 9–12 wild-type embryos and embryos overexpressing full-length Crb (Crb was overexpressed in all embryos). (**D**–**K**) Fasciclin 3 (Fas3) staining of late-stage whole embryos (D,E,H,I), a close-up of epidermal cells of the same Fas3-labeled embryos (D′,E′,H′,I′) or a cuticle preparation (F,G,J,K) for the following genotypes: *daGal4/UAS-rac1*^{N17} (expression of $Rac1^{N17}$ in a wild-type background) (D,F), *crb*^{11A22}, *daGal4/crb*^{11A22}, *UAS-rac1*^{N17} (*crb*^{11A22} mutant expressing $Rac1^{N17}$) (E,G), *rac1*^{J11}, *rac2*$^{\Delta}$ (H,J) and *rac1*^{J11}, *rac2*$^{\Delta}$, *crb*^{11A22} triple mutant (I,K). Scale bars: 100 μm (D,E,H,I and F,G,J,K); 10 μm (D′,E′,H′,I′).

dominant-negative form of Rac1 ($Rac1^{N17}$) suppressed Akt phosphorylation (Fig. 2B). Rac1 also activates PI3K during MDCK cell cyst morphogenesis (Liu et al., 2007). This shows that PI3K and Rac1 are part of a positive-feedback loop in embryos, and suggests that Crb-dependent inhibition of PI3K signaling impacts Rac1 activity. Accordingly, overexpression of Crb in embryos reduced the level of GTP-loaded Rac1 (Fig. 2C), showing that Crb is a negative regulator of Rac1 activation. Based on the rescue of the *crb* mutant phenotype by $Dp110^{D954A}$ and the reciprocal positive relationship linking PI3K and Rac1 that we demonstrated, it is possible to predict that increased Rac1 activity is involved in the deleterious effects associated with the loss of Crb. To validate this hypothesis, we expressed $Rac1^{N17}$ in a *crb* mutant background. In contrast to what is observed for embryos lacking Crb (Fig. 1E), *crb* mutants expressing $Rac1^{N17}$ formed a well-organized epidermal epithelium and cuticle covering a large fraction of their surface (Fig. 2E,G). Similar results were observed when the *crb* mutation was combined with *rac1* and *rac2* mutant alleles to reduce cellular Rac activity (Fig. 2I,K), showing that the counteraction of Rac1 activation by Crb has a physiological role in maintaining epithelial integrity in vivo. Thus, our study establishes that Crb controls epithelial organization by counteracting a positive-feedback loop in which Rac1 and PI3K activate each other, and defines Crb as a membrane protein that regulates intracellular signaling pathways.

The equilibrium between Crb activity and the Rac1–PI3K module specifies apical membrane size

Next, we directly investigated the role of Rac1 and PI3K in epithelial cell polarity and organization in vivo. Staining of the apical marker Crb in the epidermis of stage 16 embryos expressing $Rac1^{N17}$ or $Dp110^{D954A}$ revealed long protrusions (Fig. 3B,C, arrowheads) and deep invaginations (Fig. 3B,C, arrows), which indicate increased apical membrane length. Indeed, individual epithelial cells showed an enlarged dome-shaped apical membrane upon inhibition of Rac1 or PI3K (Fig. 3G,H, arrows). These features are typical of the phenotype resulting from Crb overactivation (Laprise et al., 2006; Laprise et

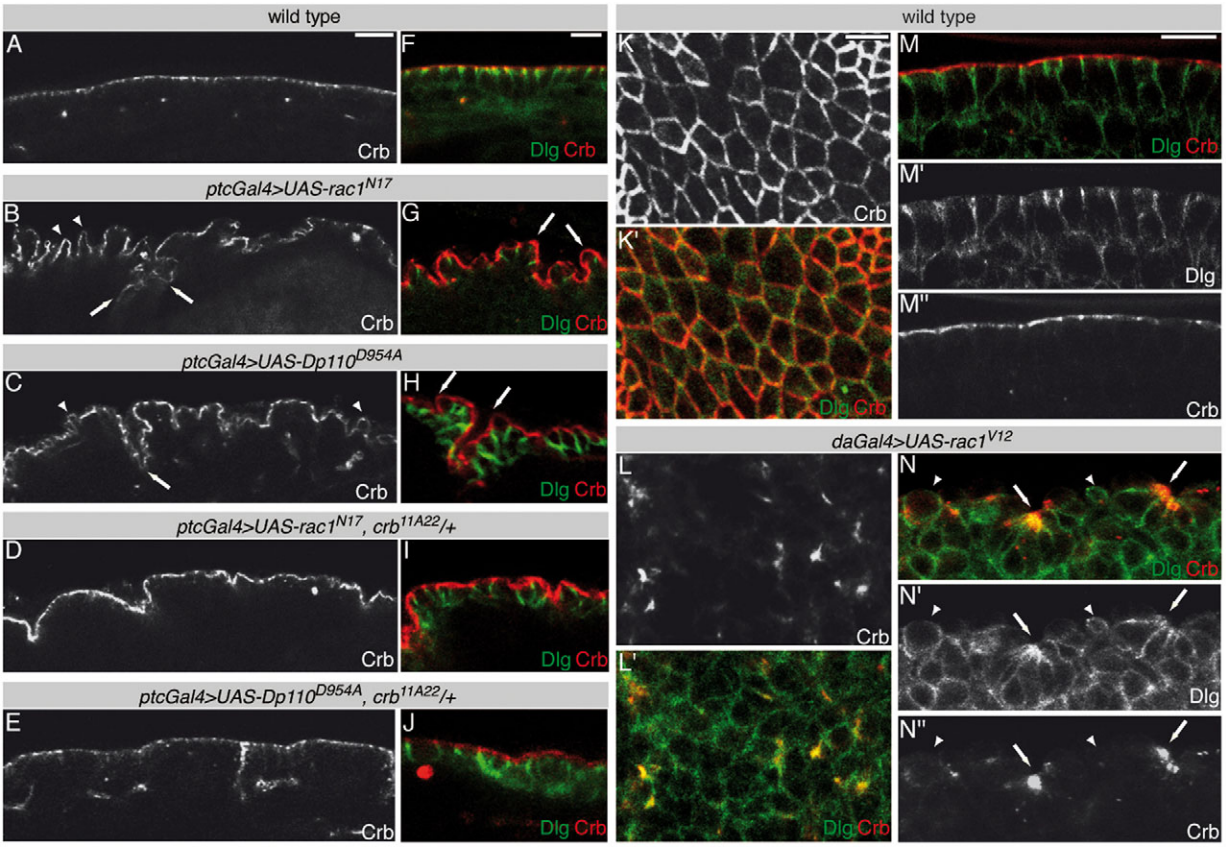


Fig. 3. Rac1 and PI3K repress Crb-dependent apical membrane growth. (**A**–**E**) Crb staining in the ventral epidermis of a wild-type embryo (A), an embryo expressing $Rac1^{N17}$ (B), an embryo expressing $Dp110^{D954A}$ (C), an embryo expressing $Rac1^{N17}$ and heterozygous for the null allele crb^{11A22} (D) or an embryo expressing $Dp110^{D954A}$ and heterozygous for the null allele crb^{11A22} (E) (embryos were at stage 16). The arrowheads in B and C point to extensions of the epidermis, whereas arrows indicate invaginations of the epidermis. (**F**–**J**) Immunolabeling of the apical marker Crb (red) and of the lateral marker Dlg (green) in the ventral epidermis of a wild-type embryo (F), an embryo expressing $Rac1^{N17}$ (G), an embryo expressing $Dp110^{D954A}$ (H), an embryo expressing $Rac1^{N17}$ and heterozygous for the null allele crb^{11A22} (I) or an embryo expressing $Dp110^{D954A}$ and heterozygous for the null allele crb^{11A22} (J) (embryos were at stage 16). The arrows in G and H point to extended apical domains. (**K**,**L**) Surface view of the ectoderm of a stage 11 wild-type embryo (K) or a stage 11 $Rac1^{V12}$-expressing embryo (L) stained for Crb (red) and Dlg (green). (**M**,**N**) Lateral view of the ectoderm of a stage 11 wild-type embryo (M) or a stage 11 $Rac1^{V12}$-expressing embryo (N) stained for Crb (red) and Dlg (green). Scale bars: 20 μm (A–E); 10 μm (F–J and K–N).

al., 2009; Wodarz et al., 1995), suggesting that Crb is involved in $Rac1^{N17}$- and $Dp110^{D954A}$-induced phenotypes. Accordingly, loss of one copy of *crb* suppressed the enlargement of the apical domain associated with the inhibition of Rac1 or PI3K signaling (Fig. 3D–E,I–J). Additionally, Crb is severely reduced in stage 11 embryos expressing the constitutively active $Rac1^{V12}$, as shown by a surface view of the ectoderm (Fig. 3, compare K with L). Lateral view revealed that residual Crb forms tight aggregates corresponding to the contact point between apexes of several epithelial cells. These cells show a highly constricted apical domain compared with that observed in wild-type embryos (Fig. 3M,N). In addition, the basolateral protein Dlg colocalizes with Crb in cells showing contracted apexes (Fig. 3N, arrows) or is found around the periphery of cells where Crb expression is almost absent (Fig. 3N, arrowheads). This demonstrates that activation of Rac1 causes defects in the formation and organization of the apical domain. Together, these results show that the PI3K-Rac1 module represses Crb-dependent apical membrane growth. Expression of an activated form of Rac1 also decreases Crb level in salivary glands of *Drosophila* embryos (Pirraglia et al., 2010). In addition, it was recently shown that β_H-spectrin, which is physically and functionally linked to Crb (Medina et al., 2002; Pellikka et al., 2002), represses Rac1 activity (Lee and Thomas, 2011). Moreover, Pak1, an important Rac1 effector, decreases apical spectrin and Crb levels, thus destabilizing the apical domain (Conder et al., 2007). These observations further support the notion that the Crb complex and Rac1 signaling are mutually antagonistic to sustain epithelial polarity.

Collectively, our data show that Rac1 and PI3K are part of a positive-feedback loop in fly embryos. Crb breaks this loop and represses activation of Rac1 as well as PI3K signaling. Reciprocally, the Rac1–PI3K module restricts Crb function and apical membrane growth, showing that the antagonistic functional relationship linking these proteins is bidirectional. In normal epithelial cells, Crb activity inhibits the Rac1–PI3K cassette at the apical membrane. In this way, epithelial cells establish proper apical domain and epithelial tissue integrity is preserved (Fig. 4). In *crb* mutant embryos, the Rac1–PI3K pathway is activated. This contributes to the *crb* mutant phenotype, as we have shown that reduction of Rac1 or PI3K activity rescues the phenotype associated with the loss of Crb. In addition, activation of Rac1 has a similar outcome to the absence of Crb, including defects in apical domain formation. By contrast,

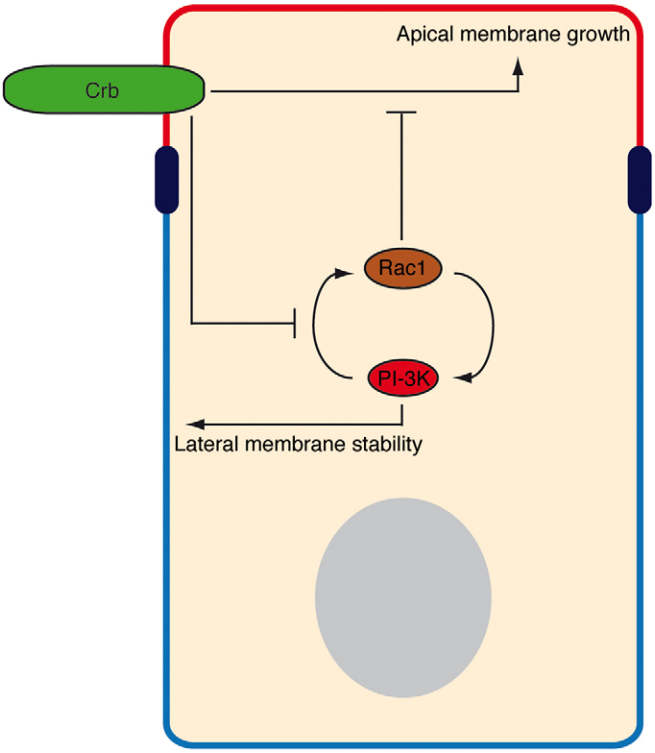


Fig. 4. Mutually antagonistic interactions between Crb and the PI3K-Rac1 module define apical membrane length and maintain epithelial organization. Crb antagonizes the Rac1–PI3K module at the apical membrane to maintain this domain. In return, Rac1 and PI3K limit Crb activity to preserve the lateral membrane and avoid extension of the apical membrane.

overexpression of Crb or reduction of Rac1 or PI3K activity results in a Crb-dependent increase of apical membrane size. Thus, the fine balance between Crb and the Rac1–PI3K module is crucial to establish proper apical to basolateral ratio in epithelial cells and to sustain epithelial tissue organization. Our study contributes to defining the molecular mechanisms that regulate epithelial polarity, which is crucial for the morphogenesis and physiology of epithelial tissues.

Materials and Methods

Drosophila genetics

Fly stocks used in this study were: crb^{11A22}, $crb^{S87\text{-}2}$, $rac1^{j11}$, $rac2^{\Delta}$, $pten^{dj189}$, UAS-$rac1^{N17}$, UAS-$Dp110^{D954A}$, UAS-*PTEN2*, UAS-crb^{wt2e}, *da-GAL4* (all alleles and transgenic lines are described in Flybase; ><http://flybase.org/). Overexpression of $Rac1^{N17}$ in a crb^{11A22} mutant background was achieved by crossing crb^{11A22}, *da-GAL4* animals to crb^{11A22}, UAS-$rac1^{N17}$ flies at 18°C. Crb, $Dp110^{D954A}$, PTEN2, $Rac1^{N17}$ and $Rac1^{V12}$ were expressed in embryos by crossing the corresponding UAS lines with *da-Gal4* flies at 25°C or *ptc-Gal4* at 29°C. *crb* mutant embryos used were obtained from *crb* germ line clone females crossed to *crb*/+ males.

Immunofluorescence

Drosophila embryos were fixed as described previously (Tepass et al., 1990). The primary antibodies anti-Fas3 (7G10) and anti-Dlg (4F3) (Developmental Studies Hybridoma Bank) were used at a dilution of 1:50 and 1:10, respectively. Anti-Crb antibody (Pellikka et al., 2002) was diluted 1:500. Secondary antibodies were conjugated to Cy3 (Jackson Immunoresearch Laboratories) or Alexa Fluor 488 (Molecular Probes).

Western blotting

Western blotting was performed as described (Laprise et al., 2002). Primary antibodies used were: rabbit anti-phospho Akt Ser473 (Cell Signaling), 1:1000 dilution; rabbit anti-Akt (Cell Signaling), 1:1000; mouse anti-Rac1 (clone 102; BD transduction Laboratories), 1:1000; rat anti-Crb (Pellikka et al., 2002), 1:5000; rabbit anti-GST (provided by J.-Y. Masson, Laval University), 1:10,000. HRP-conjugated secondary antibodies were from GE Amersham and used at a 1:1000 dilution.

Determination of Rac1 activation levels

The protocol used was based on the method described by Picard and colleagues (Picard et al., 2009). Briefly, embryos were homogenized in lysis buffer and part of extracts was kept to monitor the total amount of Rac1 in each sample (input). Then, GTP-bounded Rac1 was pulled down from 1 mg of the same samples with 20 µg of the CRIB domain of PAK fused to GST (45 minutes, 4°C). GST alone was used as negative control. The beads were then washed twice with washing buffer. Pulled-down Rac1 levels or the total amount of Rac1 present in cellular extracts was determined by western blotting.

Acknowledgements

We are grateful to S. Beronja for critical reading of the manuscript. We thank U. Tepass, A. Wodarz, E. Knust, S. Leevers, N. Lamarche-Vanne, J.-Y. Masson, the Bloomington *Drosophila* Stock Center and the Developmental Studies Hybridoma Bank for reagents.

Funding

This work was supported by an operating grant from the Canadian Institute of Health Research (CIHR) [grant number 200903MOP-199572-CP-CFBA to P.L., who is a CIHR new investigator]. F.J.-M.C. was supported by a postdoctoral fellowship from CIHR.

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