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# A functional role of the extracellular domain of Crumbs in cell architecture and apicobasal polarity

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## Summary

Regulated cell shape changes in epithelial cells, which contribute to most organs and tissues, are at the basis of morphogenesis. Crumbs (Crb) is an essential apical determinant controlling epithelial apicobasal polarity. Here we provide evidence for a novel role of Crb apical localisation and stabilisation in controlling cell shape through apical domain organisation and adherens junction positioning. We find that Crb apical stabilisation requires the extracellular domain. *In vivo* results from *Drosophila* suggest that the extracellular domain assists Crb apical stabilisation by mediating Crb–Crb interactions at opposing cell membranes. We further confirm Crb–Crb extracellular interactions by showing that the extracellular domain of Crb is sufficient to promote cell aggregation *in vitro*. Furthermore, we report that Crb apical stabilisation mediated by the extracellular domain is also required for maintenance of Crb apicobasal polarity. Our results provide new insights into the mechanisms of apicobasal polarity and the cellular mechanisms of tissue architecture.

**Key words:** Crumbs, *Drosophila melanogaster,* Cell architecture*,* Morphogenesis

## Introduction

Morphogenesis and organogenesis rely on the capacity of cells to coordinately change shape. Shape changes in epithelial cells, which are present in most tissues and organs, greatly contribute to tissue remodelling. Epithelial cells show a marked apicobasal polarity, which is established and maintained by the components of the subapical region (SAR) (Knust and Bossinger, 2002; Tepass et al., 2001). The evolutionarily conserved protein Crb is a major apical polarity determinant and the only known transmembrane component of the SAR (Assémat et al., 2008; Bazellieres et al., 2009; Bulgakova and Knust, 2009). Crb contains one single transmembrane domain, a short cytoplasmic domain (Intra), and a long extracellular (Extra) domain (Tepass et al., 1990). The Intra domain interacts with different proteins to organise a membrane-associated core-complex defining apicobasal polarity with Stardust (Sdt), Pals1-associated tight junction protein (Patj) and Lin-7, and establishes links with apical βH-spectrin, actin cytoskeleton and basolateral polarity complexes. In addition, Crb participates in other cellular events through interactions with, for example, the Par polarity network (aPKC/Par6) and the Hippo pathway (Bazellieres et al., 2009; Bulgakova and Knust, 2009). It has been postulated that Crb Intra is sufficient to rescue the absence of *crb* in several instances (Johnson et al., 2002; Klebes and Knust, 2000; Wodarz et al., 1995), suggesting that Crb Extra can be dispensable. In contrast to Crb Intra, the function of the Extra domain, which contains 29 EGF-like and four laminin A G-like repeats, is less well understood. In spite of the fact that its organisation is conserved from flies to mammals (Richard et al., 2006), and that many mutations in the Crb1 human homolog responsible for severe retinal dystrophies map at this protein domain (Bujakowska et al., 2012), no general role of Crb Extra in Crb activity has been established. Based on indirect data some reports suggested the ability of Crb molecules to interact with other Crb molecules (Chen et al., 2010; Fletcher et al., 2012; Pellikka et al., 2002; Wei et al., 2006) or other proteins (Herranz et al., 2006; Richardson and Pichaud, 2010). Moreover, a recent study describes adhesion between zebrafish Crb molecules (Zou et al., 2012).

Given the importance of Crb both in physiological and pathological conditions, it is crucial to understand Crb requirements in morphogenesis. Here we provide experimental evidence for a role of the Extra domain in protein stabilisation required for Crb control of apicobasal polarity and cell shape.

## Results and Discussion

### Crb apical localisation controls cell architecture

We previously documented a differential accumulation of Crb in the ectoderm, identifying groups of cells with increased Crb levels at stages 9–11, that correspond to dorsal epidermis and tracheal placodes (Fig. 1A,A′). This increased posttranscriptional accumulation in tracheal placodes correlates with the apical constriction that precedes invagination (Letizia et al., 2011). In this study we aimed to further explore the correlation between Crb levels and cell architecture.

At confocal resolution we observed that increased Crb accumulation in dorsal epidermis or tracheal placode cells correlates with a narrower apical domain compared to cells with lower Crb levels at the ventral epidermis of stage 10 embryos (Fig. 1A′,B). In addition, in dorsal cells, SAR markers labelled a longer domain along the apicolateral membrane while adherens junctions (AJs) occupied a more basal position (Fig. 1B′–B‴). High-resolution ultrastructural analyses using transmission electron microscopy (TEM) revealed different

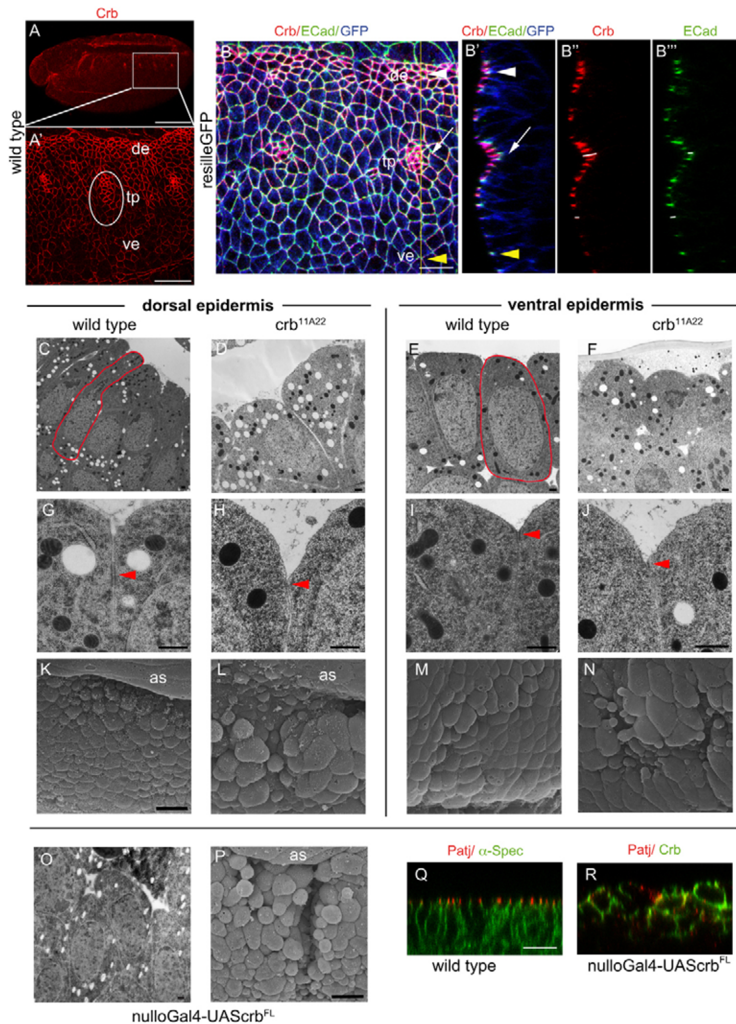


**Fig. 1. A role for Crb in cell shape.** (**A**,A′) Stage 10 embryo labelled for Crb. Note increased Crb levels in the dorsal epidermis (de) and tracheal placodes (tp) compared with ventral epidermis (ve). (**B**) Stage 11 embryo. *z*-sections show that regions of increased Crb (white arrowhead and arrow) have a longer SAR and a basal shift of AJs as compared to ventral epidermis (yellow arrowhead). Whereas the SAR enlarges (line in B″), AJs do not (line in B‴). (**C–J**) TEM images showing cell shape (C–F), AJ localisation (red arrowheads) and organisation of the apical membrane (G–J). (**K–N**) SEM analysis. (**O–R**) TEM (O), SEM (P) and confocal analysis (Q,R) show defects in shape of dorsal cells when Crb is uniformly expressed. as, amnioserosa. Scale bars: A, 100 μm; A′, 25 μm; B, 10 μm; C–J,O, 500 nm; K,P, 9 μm.

organisation of these cell types. Dorsal epidermal cells were columnar or wedge-shaped and the apical membrane facing the exterior (apical-free domain) was smooth and dome-shaped (Fig. 1C). AJs were present at the lateral membrane beneath an extended junction-free region where adjacent membranes were in contact (apicolateral domain) (Fig. 1G). We speculate that this apicolateral domain corresponds to the elongated SAR where high levels of Crb accumulate. In contrast, ventral cells were cuboidal, with a flat apical-free domain and with AJs positioned more apically, resulting in a short apicolateral domain (Fig. 1E,I). Scanning electron microscopy (SEM) confirmed differences between the apical domain of dorsal and ventral epidermal cells (Fig. 1K,M). These results indicate that, in wild-type embryos, different levels of Crb correlate with different cell morphologies.

In agreement with a role of *crb* in cell morphology, our analyses revealed defects in cell shape in *crb* mutants (Fig. 1D,H,L,F,J,N). Tracheal (Letizia et al., 2011) and dorsal cells appeared rounded (Fig. 1D) and with bulged and protruded apical domains (Fig. 1L). AJs, when present, occupied a more apical position (Fig. 1H), as compared to wild type (Fig. 1G). Interestingly, confocal and ultrastructural analyses indicated that uniform accumulation of Crb, as observed when Crb is overexpressed, also induced dorsal cells to adopt an unpolarised shape (Fig. 1O–R). Since both the absence and the mislocalisation of Crb affect cell shape it suggests that Crb needs to be properly localised to control cell architecture.

## Crb Extra stabilises Crb apically

To evaluate Crb requirement in cell morphology we sought to understand its mechanism of apical localisation. Although Crb targeting and stabilisation to the apical membrane is not fully understood, both Crb Extra and Intra domains may participate in the process (Fletcher et al., 2012).

A mechanism by which Crb Extra may stabilise Crb apically is through protein interactions in trans, as previously suggested. We and others (Chen et al., 2010; Hafezi et al., 2012; Pellikka et al., 2002) observed that in *crb* mutant clones the accumulation of Crb and Crb-interacting proteins is lost in mutant cells but also at cell–cell contacts of wild-type cells facing mutant cells (clone border, Fig. 2C). To test for Crb Extra interactions we expressed Crb$^{extra}$ (a membrane-bound GFP-tagged form of Crb that lacks the Intra domain; Fig. 2B) (Pellikka et al., 2002), in *crb* mutant clones and assayed for Crb accumulation at the clone border by analysing Patj accumulation [Crb antibody recognises both Crb$^{extra}$ and wild-type Crb (Tepass and Knust, 1993)]. Strikingly, we found a complete rescue of Patj at the clone border (Fig. 2D; supplementary material Fig. S1A). As Crb$^{extra}$ cannot rescue Patj accumulation in mutant cells, its accumulation

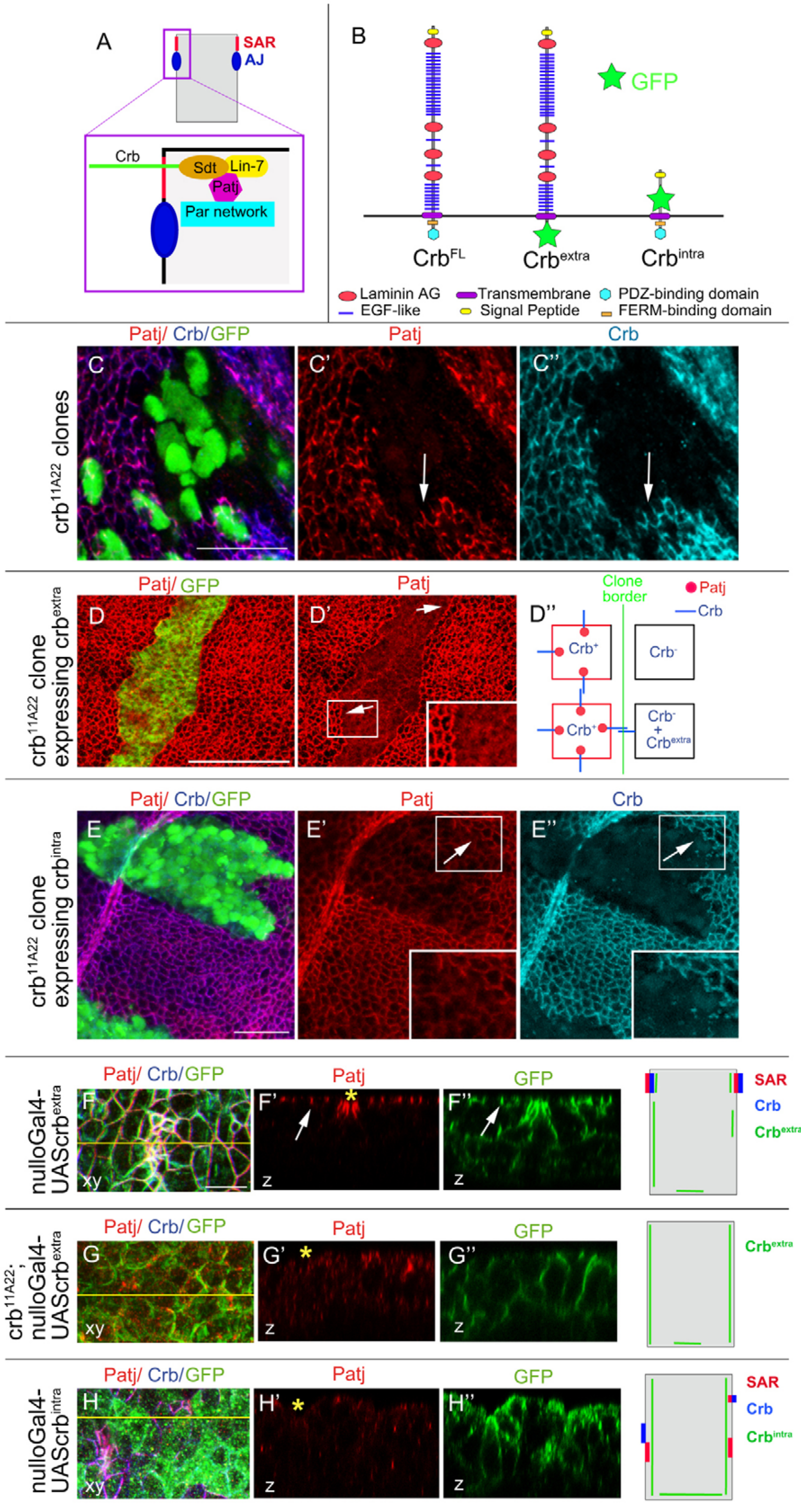


**Fig. 2. The role of Crb domains in Crb apical stabilisation.** (**A**,**B**) Scheme of the apical region of an epithelial cell (A) and of the Crb constructs used (B). (**C–E″**) Wing imaginal discs with *crb* mutant clones marked with GFP. Crb and Patj are absent in clones and at borders (arrows in C′,C″). Crb$^{extra}$ (D′) but not Crb$^{intra}$ (E′) rescues clone border Patj accumulation. (D″) Scheme of clonal analysis. (**F–H**) Embryonic epidermis of stage 11 embryos reared at 18℃ (H) or 25℃ (F,G). The yellow line in the xy plane shows the position of the z-sections. The yellow asterisks mark tracheal placodes. (F) Crb$^{extra}$ localises around the membrane and in the SAR where Patj localises (arrows). (G) In *crb* mutants, Crb$^{extra}$ is not stabilised apically. (H) Crb$^{intra}$ does not localise apically and mislocalises Patj. Scale bars: C,E, 10 µm; D, 25 µm; F, 7,5 µm.

at the border must be due to the presence of full-length Crb in wild-type cells. This demonstrates that Crb Extra is able to stabilise Crb in trans in neighbouring membranes (Fig. 2D″).

In contrast to Crb$^{extra}$, expression of Crb$^{intra}$ (a membrane-bound GFP-tagged form of Crb that lacks the Extra domain; Fig. 2B) in *crb* mutant clones did not rescue Patj accumulation at

clone border (Fig. 2E), indicating that the Extra domain is necessary for Crb apical stabilisation.

In support of these observations we find that Crb Extra, but not Crb Intra, localises apically in the presence of wild-type Crb. When Crb$^{extra}$ is expressed in the embryonic epidermis we detected accumulation along the apicobasal membrane. Crb$^{extra}$ also accumulated in a distinct apicolateral region that corresponds to the SAR, as visualised by Patj accumulation (Fig. 2F, arrows). Crb$^{extra}$ is stabilised apically only if full-length (FL) Crb is present, as the distinct apical accumulation was lost in the absence of endogenous Crb (*crb$^{11A22}$* mutants; Fig. 2G). This loss was not due to a general polarity defect as Crb$^{extra}$ also localised in an unpolarised pattern in tissues where *crb* is not required for polarity (supplementary material Fig. S1A,B). On the other hand, Crb$^{intra}$ was unable to distinctly localise apically in any condition tested. When expressed in the embryo or wing discs, Crb$^{intra}$ was present around the whole cell membrane (Fig. 2H; supplementary material Fig. S1C; Fig. S2C), as observed for other Crb$^{intra}$ tagged versions (Klebes and Knust, 2000; Muschalik and Knust, 2011; Wodarz et al., 1995). This mislocalisation is not due to an overexpression effect, since, in contrast, comparable levels of Crb$^{FL}$ (supplementary material Fig. S2F) are able to localise properly (supplementary material Fig. S2B,E).

Altogether our data provides experimental confirmation for a role of Crb Extra in mediating interactions between Crb molecules, that are required for Crb apical stabilisation *in vivo*. Although both Crb Extra and Intra domains are needed for Crb apical localisation, the Extra domain can stabilise Crb apically whereas the Intra alone cannot, presumably due to the absence of its extracellular domain.

### *Drosophila* Crb Extra is sufficient to mediate cell adhesion

To determine whether, as suggested by our *in vivo* analysis, Crb Extra molecules are able to mediate cell adhesion we performed cell aggregation assays *in vitro*. We expressed full-length Crb [Crb$^{FL}$ (Wodarz et al., 1995)], Crb$^{extra}$ and Crb$^{intra}$ using an actinGal4VP16 promoter. Cells expressing Crb$^{FL}$ formed aggregates (Fig. 3A,A′), indicating that Crb is able to mediate cell–cell interactions. Cells expressing Crb$^{intra}$ showed GFP uniformly around the cell membrane but did not consistently aggregate (Fig. 3B,B′,F). We observed on occasion the formation of small clusters of Crb$^{intra}$ expressing cells (Fig. 1B,G). Nonetheless, this clustering is Crb independent since Crb$^{intra}$ and Crb$^{FL}$ expressing cells are not able to adhere to each other (Fig. 1H). In contrast, aggregation assays with mixed populations showed that cells expressing Crb$^{extra}$ adhered to Crb$^{FL}$ cells (Fig. 1E,H). Furthermore, cells expressing Crb$^{extra}$ alone aggregated, and the majority of Crb$^{extra}$ expressing cells were part of aggregates (Fig. 1C,C′,F,G). These results indicate that the Extra domain of Crb is required and sufficient to mediate cell adhesion.

Our results support previous reports suggesting that *Drosophila* Crb may engage in homophilic interactions (Fletcher et al., 2012; Pichaud and Desplan, 2002; Tepass et al., 2001), although they do not rule out indirect Crb–Crb interactions mediated by a partner. Importantly our data are in line with recent studies showing that zebrafish Crb2a and Crb2b (Zou et al., 2012) and *Drosophila* Crb

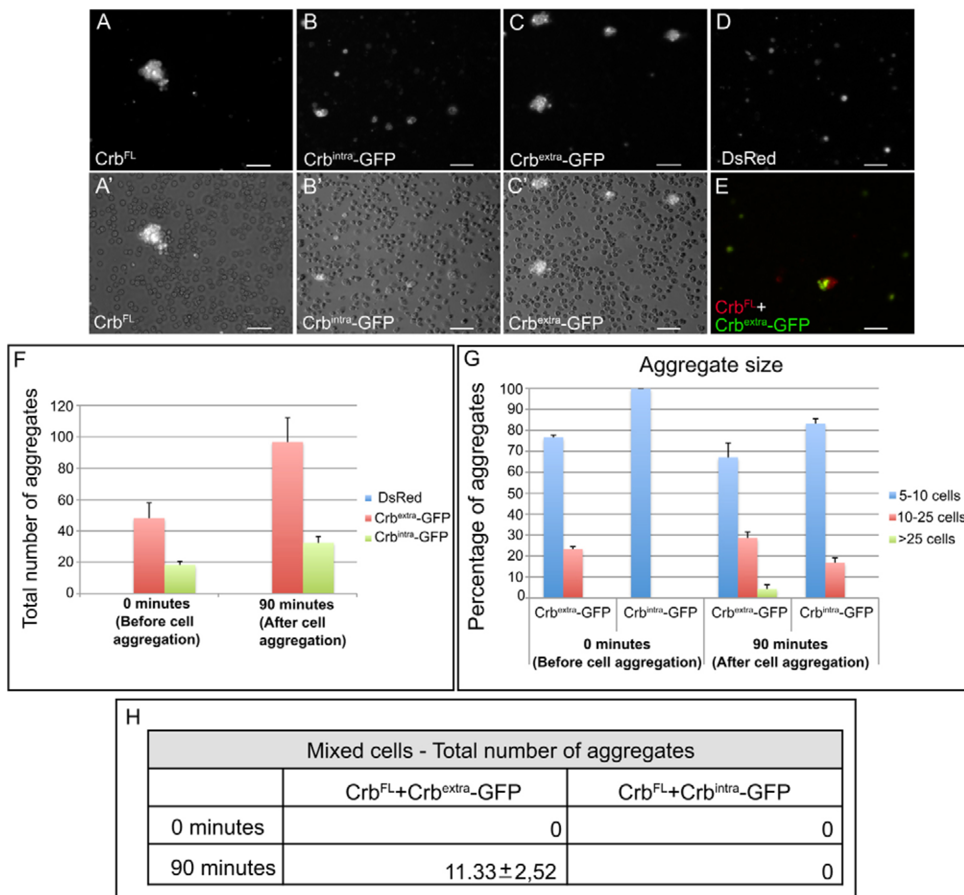


**Fig. 3. Crb Extra is necessary and sufficient to mediate cell–cell adhesion.** (**A–D**) S2 cells expressing the indicated constructs, after cell aggregation. (A′–C′) Brightfield and fluorescence channels shown. (**E**) Mixed populations of Crb$^{extra}$ and Crb$^{FL}$ cells, after aggregation. (**F**) Total number of aggregates in cells expressing the constructs, before and after aggregation. (**G**) Quantification of aggregate size in cells expressing Crb$^{extra}$ and Crb$^{intra}$, before and after aggregation. (**H**) Total number of aggregates in mixed populations. Scale bars: 50 µm. Error bars represent s.d. of two independent experiments, performed in duplicate or in triplicate.

(Röper, 2012) engage in cell–cell interactions, suggesting a universal capacity of Crb proteins to mediate adhesion.

### Crb apical stabilisation mediated by Crb Extra is required for cell morphology and apicobasal polarity

We find that Crb Extra is critical for Crb apical stabilisation. On the other hand our analysis indicate that Crb apical accumulation plays a role in cell shape. Therefore, we expected the Extra domain to be required for cell architecture. In agreement with this we find a defective rescue of cell morphology when Crb$^{intra}$ is expressed in *crb* mutants (Fig. 4D; supplementary material Fig. S4), in contrast to the rescue obtained with Crb$^{FL}$ (Fig. 4C; supplementary material Fig. S4). In addition, confocal z-sections show that in contrast to Crb$^{FL}$, Crb$^{intra}$ is unable to fully rescue AJs positioning and SAR enlargement (supplementary material Fig. S5). Although a certain rescue of cell morphology could be expected by providing Crb$^{extra}$, such rescue was very mild (Fig. 4E; supplementary material Fig. S4), probably due to the inability of this construct to localise apically in the absence of endogenous Crb.

The requirement of Crb Extra in Crb apical stabilisation predicts a role of this domain also in maintaining the general apicobasal polarity. However, it has been suggested that the Extra domain of Crb is dispensable for cell polarity (Klebes and Knust, 2000; Wodarz et al., 1995). To evaluate the sufficiency of Crb Intra in apicobasal polarity we compared the ability of Crb$^{intra}$ and Crb$^{FL}$ to rescue *crb* mutant defects in polarity, AJs formation, and embryonic patterning. Mild overexpression of Crb$^{FL}$ rescued *crb* defects (Fig. 4H,M,R; supplementary material Fig. S4). Under these conditions we did not detect gross dominant defects and Crb was apically localised (supplementary material Fig. S2B,E). In contrast, Crb$^{intra}$ could only partially rescue Crb activity (Fig. 4I,N,S; supplementary material Fig. S4). This partial rescue might reflect the activity of some Crb$^{intra}$ molecules

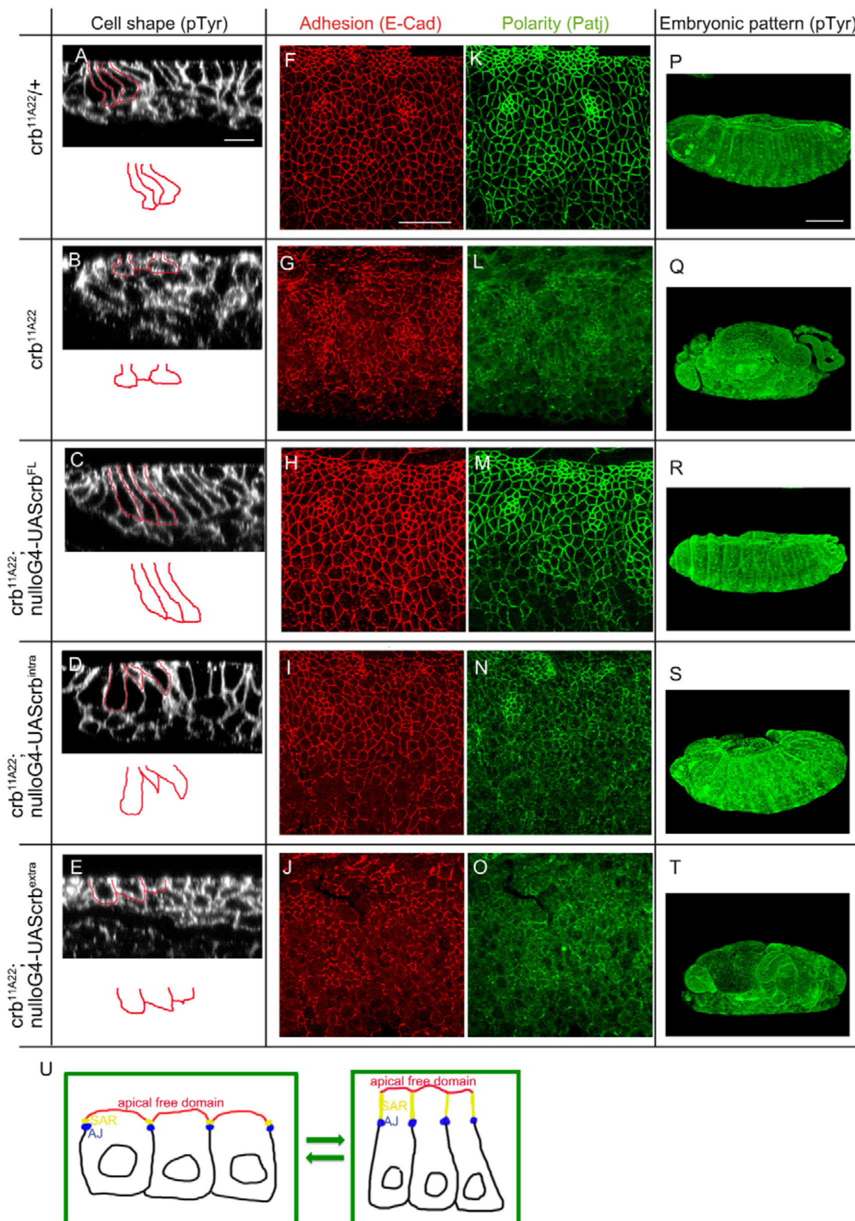


**Fig. 4. Analysis of the contribution of Crb domains to Crb activity.** Phenotypes of embryos of the indicated genotypes reared at 18°C (Crb$^{FL}$ and Crb$^{intra}$ rescues), 25°C (heterozygous and homozygous *crb* embryos) or 29°C (Crb$^{extra}$ rescues). Crb$^{FL}$ rescues *crb* mutant defects compared to control, whereas Crb$^{intra}$ is less efficient. Crb$^{extra}$ shows no rescue. (**A–E**) Z-sections of dorsal cells in contact with amnioserosa (left). (**F–O**) Epidermis of stage 10 embryos stained to visualise adhesion and cell polarity. (**P–T**) Stage 16 embryos. (**U**) Model for a role of Crb in cell architecture. Scale bars: A, 7,5 µm; F, 25 µm; P, 75 µm.

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that randomly accumulate in apical regions, which would interact with partners preferentially accumulated there. This would compensate for the absence of Crb$^{intra}$ apical stabilisation. Finally, the expression of Crb$^{extra}$ in *crb* mutants did not rescue polarity and adhesion (Fig. 4J,O,T).

Our results indicate that both the Extra and Intra domains of Crb are required to fully rescue Crb requirements in embryonic tissues, highlighting the need of protein integrity. We propose a two-step mechanism in which once Crb reaches the apical membrane it becomes stabilised through interactions in trans, and also possibly in cis as proposed (Fletcher et al., 2012), mediated by Crb Extra. In a second step, the apical localisation would be reinforced or fixed by interactions with other proteins through the Intra domain, as also proposed recently (Fletcher et al., 2012). The Intra domain would also mediate downstream effects (e.g. on polarity, adhesion and cell morphology) through interactions with these partners (Bulgakova and Knust, 2009).

## Conclusions

We report the role of Crb Extra in assisting protein stabilisation in the SAR, likely by mediating interactions between Crb molecules in neighbouring membranes. This stabilisation is functionally relevant as it is critical to maintain cell polarity. In addition, we describe a new specific Crb-dependent mechanism controlling cell shape changes. We propose that localised Crb stabilisation plays a role in cell architecture by the organisation of the apical domain and positioning of AJs. Our results are consistent with a model (Fig. 4U) in which increased levels of Crb in the SAR enlarge the apicolateral domain at the expense of the apical-free domain, leading to the narrowing of the apical area. In addition, AJs become positioned more basally, likely through interactions mediated by the Intra domain (Bilder et al., 2003; Grawe et al., 1996; Harris and Peifer, 2004; Tanentzapf and Tepass, 2003; Tepass, 1996). These morphological changes confer a columnar or constricted shape to the cell. In contrast, lower Crb levels allow an expanded apical-free domain and an apical positioning of AJs, promoting a cuboidal morphology. Crb–Crb interactions between neighbouring membranes mediated by Crb Extra, besides stabilising Crb apically, could help to stabilise cell contacts above AJs conferring to the SAR a previously undescribed junctional character. This could represent a rapid and reversible mechanism of cell shape change.

Interestingly, a similar mechanism has been recently described, where the positioning of AJs by Par1/Par3 apicobasal polarity drives epithelial folding (Wang et al., 2012). This suggests that the positioning of AJs controlled by apicobasal polarity can be a general mechanism for apical narrowing (Sumigray and Peifer, 2012). Furthermore, as Crb is also upregulated in other structures that undergo a similar apical narrowing, such as salivary glands (Myat and Andrew, 2002), posterior spiracles (Lovegrove et al., 2006) or segmental grooves (our unpublished observations) it would be tempting to speculate that in these tissues Crb accumulation also controls cell shape changes. Finally, our results are also in line with a recent report proposing a role for the Crb Extra domain in an anisotropic localisation of Crb that determines actomyosin contractility (Röper, 2012).

## Materials and Methods

### *Drosophila* strains

The following lines were used: *$y^1w^{118}$*, *$crb^{11A22}$*, FRT82B-GFP and hsFlp; tubulin-Gal4,FRT82Btubulin-Gal80, described in FlyBase (http://flybase.org/), *resilleGFP* (Morin et al., 2001), *UASCrb$^{intra}$* (*UASCrb$^{intra+transmembrane}$-GFP*, see below), *UASCrb$^{extra}$* (*UASCrb$^{extra+tranmembrane}$-GFP*; a gift from U. Tepass) and *UASCrb$^{FL}$* (a gift from E. Knust). *nulloGal4* drives ubiquitous expression in the ectoderm and *salPEGal4* in the wing pouch of imaginal discs.

### Generation of *UASCrb$^{intra}$-GFP*

The FLAG tag of UAS-Crb$_{FLAGintra}$ [a gift from E. Knust (Richard et al., 2009)] was replaced by a GFP sequence into a *Bgl*II restriction site. The *UAS-Crb$^{intra}$* construct was introduced into *y $w^{1118}$* embryos by standard P-element transformation (Ashburner, 1989). Different lines were analysed, showing a comparable GFP accumulation.

### Immunohistochemistry

Embryos were staged as described previously (Campos-Ortega and Hartenstein, 1985) and imaginal discs obtained by dissecting third instar larvae. We followed standard protocols for immunostainings. The primary antibodies used were: anti-Crb (Cq4) 1:10, anti-Ecad (DCAD2) 1:100 and alpha-Spectrin (3A9) 1:5 from DSHB, anti-aPKC ζ C-20 1:500 (Santa Cruz Biotechnology), anti-Patj 1:1000 (H. Bellen), anti-pTyr 1:1000 (Upstate), anti-GFP 1:600 (Molecular Probes and Roche), anti-βGal 1:600 (Abcam). Cy3-, Cy2- and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:300.

Confocal images were obtained with Leica TCS-SPE or TCS-SP5 system. Images were imported into ImageJ and Adobe Photoshop and assembled using Adobe Illustrator.

### Mosaic analysis

Imaginal wing discs with positively or negatively *$crb^{11A22}$* marked clones were generated by FLP-mediated mitotic recombination (Duffy, 2002). Second instar larvae were heat shocked for 1 hour at 37℃.

### Ultrastructural analyses

For TEM embryos were prepared following the protocol established (Moussian and Schwarz, 2010). The samples were analysed by a Philips CM10 electron microscope at 60 kV. SEM was performed as described (Moussian et al., 2006).

### Cell culture and transfections

*Drosophila* S2 cells were transiently transfected using Effectene reagent (Qiagen). Constructs were co-transfected with actinGal4VP16 (gift from J. Treisman). As Crb$^{FL}$ is not tagged we co-transfected Crb$^{FL}$ and DsRed (gift from R. Axel) to visualise expressing cells and confirmed membrane Crb expression with Crb antibody (supplementary material Fig. S3). Cells were used for aggregation assays after 3 days of expression.

### Cell aggregation assay

Aggregation assays were carried out on transfected cells, with comparable transfection efficiency, as described previously (Snow et al., 1989). For mixed cell aggregation, half of each individual cell population was mixed together at the time of the assay. Aggregation was imaged and quantified using a TE-200C Nikon inverted microscope coupled with a CoolSNAP camera (Photometrics) and the images were processed in ImageJ.

### Quantitative real-time PCR

Reactions were carried out using SYBR Green Universal PCR Master Mix (Applied Biosystems) at an annealing temperature of 59℃. StepOnePlus Real-Time PCR System was used according to manufacturer's instructions. Gene expression levels were normalised using endogenous α-Tubulin and differences in *crb* gene expression were determined using the StepOne 2.2 software.

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## Author contributions

A.L. and M.L. conceived the experimental design, and analysed and interpreted the data. S.R. performed the cell aggregation assays. B.M. performed TEM and SEM experiments. N.M. generated UASCrbintra-GFP. A.L. performed the rest of the experiments. M.L. wrote the manuscript with the help of S.R.

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