Regulated Crb accumulation controls apical constriction and invagination in Drosophila tracheal cells

Annalisa Letizia1, Sol Sotillos2, Sonsoles Campuzano3 and Marta Llimargas1,*

1Institut de Biologia Molecular de Barcelona, CSIC, Parc Científic de Barcelona, Baldini Reixac, 10-12, 08028 Barcelona, Spain
2Centro Andaluz de Biología del Desarrollo, CSIC/UPO, Ctra de Utrera Km 1, 41013 Sevilla, Spain
3Centro de Biología Molecular Severo Ochoa (CSIC-UAM), C/ Nicolás Cabrera 1, Cantoblanco, 28649 Madrid, Spain

*Author for correspondence (mlcbmc@ibmb.csic.es)

Summary

Many epithelial tissues undergo extensive remodelling during morphogenesis. How their epithelial features, such as apicobasal polarity or adhesion, are maintained and remodelled and how adhesion and polarity proteins contribute to morphogenesis are two important questions in development. Here, we approach these issues by investigating the role of the apical determinant protein Crumbs (Crb) during the morphogenesis of the embryonic Drosophila tracheal system. Crb accumulates differentially throughout tracheal development and is required for different tracheal events. The earliest requirement for Crb is for tracheal invagination, which is preceded by an enhanced accumulation of Crb in the invagination domain. There, Crb, acting in parallel with the epidermal growth factor receptor (Egfr) pathway, is required for tracheal cell apical constriction and for organising an actomyosin complex, which we propose is mediated by Crb recruitment of moesin (Moe). The ability of a Crb isoform unable to rescue polarity in crb mutants to otherwise rescue their invagination phenotype, and the converse inability of a FERM-binding domain mutant Crb to rescue faulty invagination, support our hypothesis that it is the absence of Crb-dependent Moe enrichment, and not the polarity defect, that mainly underlies the crb invagination phenotype. This hypothesis is supported by the phenotype of lethal giant larvae (lgl); crb double mutants. These results unveil a link between Crb and the organisation of the actin cytoskeleton during morphogenesis.

Key words: Crumbs, tracheal system, apical constriction, invagination, morphogenesis, subapical region

Introduction

Apicobasal polarity and intercellular adhesion are key features of epithelial tissues and regulate many cellular activities, such as intracellular trafficking, secretion, transepithelial barrier, tissue integrity and homeostasis. Polarity and adhesion are established and maintained by several evolutionarily conserved epithelial protein complexes. In epithelial cells, the adherens junctions (AJs) form an adhesion belt that separates apical from basolateral membranes. In Drosophila epithelial cells, septate junctions (SJs) form basally to AJs and establish the transepithelial diffusion barrier. In an apical position to the AJs, the subapical region (SAR) is required to establish and maintain apicobasal polarity (Knust and Bossinger, 2002; Tepass et al., 2001). Interestingly, apicobasal polarity and adhesion are established at early stages of embryonic development and maintained by the cells throughout organogenesis (Bilder et al., 2003; Harris and Peifer, 2004; Harris and Peifer, 2005; Hutterer et al., 2004; Tanentzapf and Tepass, 2003). How adhesion and polarity complexes are maintained and remodelled and whether they play active and instructive roles during morphogenesis have become major questions over recent years. It has long been known that DE-cadherin (DEcad), the principal adhesion molecule of AJs, is required to maintain tissue integrity during morphogenetic movements (Tepass, 1996; Uemura et al., 1996). More recently, an active role for AJs in driving tissue remodelling has also been described (for a review, see Wirtz-Peitz and Zallen, 2009). The transmembrane protein Crumbs (Crb), a component of the SAR, is considered to be a key apical determinant, as it is able to ‘apicalise’ the membrane upon overexpression, and, in its absence, many tissues lose polarity and adhesion, leading to major epithelial disorganisation (for a review, see Assemat et al., 2008; Gibson and Perrimon, 2003; Tepass et al., 2001). Crb is required to maintain the epithelial polarity during remodelling both of the Malpighian tubules and in the ectoderm undergoing germ band extension (Campbell et al., 2009). However, whether it plays an active role in the dynamic processes of morphogenesis has yet to be investigated.

We have addressed this question in the embryonic tracheal system – a particularly well-suited model in which to investigate the remodelling of epithelial complexes and their contribution to morphogenesis. Remarkably, specific roles for both the AJ and SJ regions during morphogenesis have been found previously. AJs maintain tracheal epithelial integrity (Cela and Llimargas, 2006) but, in addition, play an instructive role during tracheal cell rearrangements (Shaye et al., 2008). Similarly, SJs maintain the transepithelial diffusion barrier of tracheal tubes but also regulate the size of the tracheal tubes (for a review, see Swanson and Beitel, 2006; Wu and Beitel, 2004).

Here, we ask whether, as it is the case for AJs and SJs, the SAR is modulated during tracheal development and whether it contributes to specific functions during this process. Constituents of the apical Par signalling network (Atypical protein kinase C (aPKC)–Bazooka protein (Baz)–Par-6–Cdc42) and the Crb complex, formed by Crb–Stardust (Sdt)–Patj, which interact with and regulate each other (for a review, see Assemat et al., 2008;
Bulgakova and Knust, 2009; Gibson and Perrimon, 2003; Tepass et al., 2001), accumulate in the SAR. We have found that Crb accumulation is finely modulated throughout tracheal development and that this modulation is a specific feature of the SAR proteins. The functional analysis of crb indicates that it is required for different tracheal morphogenetic events, which correlate spatiotemporally with its observed pattern of accumulation. In addition to two recent reports that described a role for crb during tube elongation (Forster et al., 2010; Laprise et al., 2010), we show here an earlier requirement of crb for tracheal cell invagination and intercalation. We have focused our analysis mainly on the requirement for crb during tracheal invagination. In this, crb is required for the ordered invagination of tracheal cells. In the absence of crb, apical constriction of tracheal cells, which in the wild-type embryo precedes their internalisation, is impaired, suggesting a role for crb in the control of cell shape. Our results suggest that the invagination defects found in crb mutants are not a mere consequence of a polarity defect. Thus, in crb mutants, neither is the apical actomyosin ring, formed in wild-type embryos around the invagination edge (Brodu and Casanova, 2006), assembled properly, nor is active Moesin (pMoesin, pMoe) enriched at the apical part of the tracheal cells that undergo invagination. Furthermore, we show an essential requirement for Pd recruitment by Crb during the process of invagination. Finally, concomitant removal of crb and lgl that restores to a great extent epithelial polarity (Bilder et al., 2003; Tanentzapf and Tepass, 2003) does not preclude the abnormal tracheal invagination. Interestingly, crb appears to be functioning in parallel to the epidermal growth factor receptor (Egfr) signalling pathway to promote ordered invagination. Altogether, our results point to an active role for Egfr (and most probably other SAR components) during tracheal invagination besides maintaining apicobasal polarity and to additional requirements for Crb at later stages of tracheal development.

Results
Crb accumulation is dynamically modulated during tracheal development
Double staining of Crb and the nuclear tracheal marker tracheiless (Trh) (Isaac and Andrew, 1996; Wilk et al., 1996) revealed a modulation of Crb accumulation during tracheal development. We have termed aspects of this modulation the early-, mid- and late-tracheal pattern (Fig. 1A–D).

The early pattern, observed during stage 10 and early stage 11, revealed an increased accumulation of Crb in the dorsal-most part of the epidermis in comparison with the ventral region. Remarkably, some of the cells displaying higher levels of Crb corresponded to a subset of tracheal cells occupying the dorso-central part of each tracheal placode (Fig. 1A,A'). At stage 10, the tracheal cells, which are already specified (Ghabrial et al., 2003), form a flat epithelium with the surrounding epidermal cells. Therefore, this enhanced accumulation of Crb in the tracheal placodes is observed before the invagination of tracheal cells (Brodu and Casanova, 2006; Nishimura et al., 2007). This early pattern is very transient and rapidly evolves to give rise to the mid-tracheal pattern, observed from mid stage 11 to stage 14 and characterised by high levels of Crb in the apical part (i.e. the part facing the lumen) of all tracheal cells (Fig. 1B,B',C,C'). (see also Tepass et al., 1990). This high pan-tracheal Crb accumulation starts during tracheal invagination and lasts for the period of major branching and outgrowth (Samakovlis et al., 1996). The late-tracheal pattern is detected from late stage 14, when the levels of Crb appeared lower in the tracheal cells than in other structures such as the Malpighian tubules or the hindgut (Fig. 1D,D').

To determine the origin of Crb protein modulation, we analysed the transcriptional pattern of crb. As previously reported (Knust et al., 1987; Li et al., 2008; Tepass et al., 1990), crb transcripts were abundant in the apical region of the tracheal cells. The transcripts were clearly detectable in the trachea from mid stage 11 until stage 14 (Fig. 1F,G) and decreased afterwards (Fig. 1H). This transcription pattern correlates with the mid and late pattern of protein accumulation, and we observed that it depended on the combinatorial activity of several tracheal inducers [trh and ventral veinless (vvl)], which act downstream of the JAK–STAT pathway (supplementary material Fig. S1), an already known regulator of crb expression in the posterior spiracles (Lovegrove et al., 2006).

By contrast, we did not detect a differential transcription of crb that could be correlated with the early pattern of protein accumulation (Fig. 1E). This observation points to a dependence of the early pattern of Crb on posttranscriptional regulation. To address this point, we overexpressed a wild-type full-length form of crb (UAS-CrbVT) with a heterologous and general promoter in an otherwise protein-null mutant, crb11A22 (Fig. 1J,J'), and found that, indeed, the exogenously provided Crb was more enriched in the dorsal-most cells of the placodes than in their ventral half (Fig. 1I,I').

Accumulation of SAR proteins is specifically modulated during tracheal development
As we detected a modulation of Crb during tracheal development, we asked whether other SAR components followed the same pattern. We analysed the pattern of protein accumulation of aPKC (Fig. 2A–D), Patj (Fig. 2E–H), Par-6 and Sdt (see below; and data not shown) and found a similar early-, mid- and late-tracheal pattern as with Crb, indicating that these SAR polarity proteins are finely modulated during tracheal development. In addition, we detected similar transcription patterns for aPKC and crb, suggesting a common transcriptional regulation (supplementary material Fig. S1).

To investigate whether this modulated expression was specific to SAR proteins, we stained for the AJ markers DEcad and β-Catenin [officially known as Armadillo (Arm)]. Contrary to those of SAR proteins, the levels of the AJ markers were homogeneous in the ectoderm at stage 10 to early stage 11 (Fig. 1A', Fig. 2I'). Paralleling the mid-tracheal pattern of SAR proteins, accumulation of AJ markers in the tracheal cells increased during stage 11 (Fig. 1B',C', Fig. 2J,K). In contrast to the pattern of SAR proteins, AJ markers were maintained at high levels in tracheal cells during late embryonic stages (Fig. 1D', Fig. 2L). Altogether, these results indicate that the accumulations of SAR and AJ proteins in the tracheal cells are differentially controlled.

crb is required for ordered tracheal invagination
The high accumulation of SAR proteins in the dorso-central cells of the tracheal placodes, precisely where invagination starts, raised the possibility that SAR complexes play a role in this morphogenetic event. To address this point, we performed a functional analysis of crb, as a central component of the SAR.

We analysed the invagination process by staining embryos with an antibody against phospho-tyrosine (pTyr), to visualise the apical surface (Harden et al., 2002), and a nuclear tracheal marker. In wild-type embryos, tracheal cell invagination is a highly ordered morphogenetic event (Brodu and Casanova, 2006; Nishimura et
The process initiates at late stage 10 to early stage 11 (start-invagination step) when a small group of tracheal cells, positioned in the dorso-central part of the tracheal placode, constrict their apical surface, adopt a bottleneck shape and internalise (Fig. 3A). During mid stage 11 (mid-invagination step), a cavity forms (Fig. 3B) while waves of concentric circles of tracheal cells that organise in arcs by a process of cell intercalation undergo internalisation (Nishimura et al., 2007). By late stage 11 (end-invagination step), orthogonal sections show the formation of an initial tube with a well-defined finger-like shape (Fig. 3C), where the cells of the dorsal part of the placode completely rotate their axis while the ventral ones slide beneath (Brodu and Casanova, 2006).

Tracheal invagination occurs early enough in embryonic development to allow us to determine the contribution of \(\text{crb}^{11A22}\) before \(\text{crb}\)-null embryos collapse. In \(\text{crb}^{11A22}\) embryos, we detected strong defects in invagination in comparison with wild-type controls. At the start-invagination step, we detected a 100% penetrant phenotype of absence of apical constriction or cell shape changes (Fig. 3D). Only sporadically could we detect scattered cells showing a partial constriction of their apical surface (data not shown). During the mid-invagination step, and despite the absence...
of apical constriction, tracheal cells were able to internalise to a certain extent, but usually without forming an obvious cavity. In fact, the tracheal cells internalised in a disorganised manner (Fig. 3E), suggesting an inefficient invagination. Occasionally, we observed the formation of a cavity wider and broader than that of the wild-type embryos (data not shown). By the end-invagination step, an initial presumptive tube formed, but several cells were misplaced and occupied the lumen (Fig. 3F). This strong phenotype (hereafter termed ‘severe’) is observed in 60% of the initial tubes analysed, whereas 38% of them showed a milder phenotype (hereafter termed ‘moderate’), with only one or two cells mispositioned in an apical plane or inside the presumptive tube (Fig. 3J).

**Fig. 2. Pattern of protein accumulation of SAR and AJ components during tracheal development.** (A–L) Projections of confocal sections showing two or three tracheal metameres of wild-type embryos at the indicated stages of development stained for the indicated proteins. Note the increased levels of aPKC and PATJ (A′,E′) in the dorsal part of the tracheal placode (arrows in A,E,I) at early stages, whereas Arm shows a homogeneous pattern (I). Note the low levels of aPKC and PATJ in the tracheal cells (arrows in D,H,L) in comparison with Malpighian tubules (arrowheads in D,H,L) or Arm. Scale bars: 5 μm.

end-invagination step, most initial tubes showed a moderate (Fig. 3I) or severe phenotype (Fig. 3J).

Altogether, the analysis of **crb** mutants indicates that invagination is affected already at the start-invagination step, at the onset of apical constriction, and continues being defective in the following steps, leading to an abnormal formation of the initial tube. The strong invagination phenotypes observed in **crb** mutants, even in the hypomorphic mutants (which show an organised epidermis), suggest a key role for **crb** in the morphogenetic process of tracheal invagination.

**crb** is required for proper organisation of the actomyosin complex and apicobasal polarity during tracheal invagination

We asked about the mechanism by which Crb regulates tracheal invagination. As Crb has been shown to maintain apicobasal polarity and cell adhesion (Grawe et al., 1996; Tepass, 1996; Tepass et al., 1990), we analysed the accumulation of several apicobasal markers at the onset of tracheal invagination in **crb** mutants. In agreement with these previous reports, we found that, in the tracheal placodes and adjacent tissues of **crb**-null mutants at stages 10–11, SAR apical markers were not properly localised (Fig. 3I), and we also found examples with more than one region in the placode showing partial apical constriction. During the mid-invagination step, **crb** hypomorphic mutant embryos display a normal appearance and reach late stages of development, showing only mild defects of dorsal closure and germband retraction (Harden et al., 2002). Despite the hypomorphic nature of this allele, **crb** hypomorphic mutant embryos displayed clear defects of invagination (in more than 75% of placodes analysed; Fig. 3J) that were very similar, although milder, than those of **crb** null mutants. Apical constriction was affected in most placodes: although it was often absent or scattered, in other placodes, apically constricted cells were mispositioned (Fig. 3G), and we also found examples with more than one region in the placode showing partial apical constriction. During the mid-invagination step, **crb** hypomorphic mutant embryos display a normal appearance and reach late stages of development, showing only mild defects of dorsal closure and germband retraction (Harden et al., 2002).
indicate that polarity and adhesion start to be compromised at the onset of tracheal invagination.

Tracheal invagination has been shown to depend on the organisation of a cortical population of actin and myosin II that forms an apical ring around the invagination edge (Brodu and Casanova, 2006; Llimargas and Casanova, 1999) (Fig. 4). To determine whether Crb activity is required for the assembly of this actomyosin complex, we analysed the accumulation of actin in crb loss-of-function mutants. There, we detected a loss or strong reduction of the distinct apical actin accumulation that forms a ring lining the invagination site, although a cellular actin cytoskeleton still organises (Fig. 4E–G). This observation suggests that, besides maintaining apicobasal polarity, crb plays a role in invagination by regulating the distinct actomyosin apical enrichment.

### crb organises pMoesin accumulation during tracheal invagination

Crb contains in its cytoplasmic domain a consensus binding motif for FERM proteins (Klebes and Knust, 2000; Medina et al., 2002). Crb is able to recruit Moe, the single ERM protein in Drosophila.
through this domain (Medina et al., 2002). Interestingly, Moe represents a crosslink between the actin cytoskeleton and the apical membrane through its interaction with both F-actin and Crb (Medina et al., 2002; Polesello et al., 2002). Therefore, a possible mechanism by which Crb could regulate actin accumulation during tracheal invagination would be by recruiting Moe. The active conformation of Moe is generated by phosphorylation (Polesello et al., 2002; Speck et al., 2003), and, for this reason, we analysed the accumulation of the activated phosphorylated Moe (pMoe) during tracheal invagination. In wild-type embryos, we observed an increased accumulation of pMoe during the start-invagination and mid-invagination steps at the apical part of the tracheal cells that undergo apical constriction (Fig. 4H). By the end-invagination step, we detected a ring of increased pMoe around the invagination edge (Fig. 4K), as is the case for actin and myosin II (Brodu and Casanova, 2006; Llimargas and Casanova, 1999), which might reflect the high levels of apical pMoe. In crb-null mutants, no increased apical accumulation of pMoe was observed during the process of invagination (Fig. 4I,L). Consistent with a role for Crb in Moe recruitment, in crb-hypomorphic conditions, the apical levels of pMoe were not as increased as in the wild-type embryos (Fig. 4J,M).

To determine whether Moe recruitment can account for Crb function in invagination, we investigated whether Moe downregulation produced a similar invagination phenotype to that of crb mutants. The strong maternal contribution of Moe and the sterility of females whose germ line is mutant for Moe (Polesello et al., 2002) precludes the proper analysis of Moe loss-of-function. Thus, we used a dominant-negative construct of mammalian ezrin, another member of the ERM family, which has been shown to downregulate Moe activity in flies (Pilot et al., 2006). Ubiquitous expression of this construct caused severe or moderate invagination defects (Fig. 3J, Fig. 4N,O), similar to those found in crb mutants. In addition, as observed in crb-hypomorphic conditions, the expression of this construct caused a broad-cavity phenotype (14%, n=175; Fig. 4N). This result suggests that Moe can mediate Crb function during tracheal invagination.

Recruitment of Moe is essential to mediate the role of Crb in invagination

Crb is a multidomain protein able to interact with several other proteins that provide for the Crb complex a wide functional diversity (Bulgakova and Knust, 2009). To discern which one of the activities of Crb, as an apical determinant or by its ability to recruit Moe, is crucial for the role of Crb in the invagination of the tracheal cells, we analysed the ability of different Crb constructs to rescue the crb mutant-invagination phenotype. We overexpressed either the full-length (UAS-CrbWT) or the cytoplasmic domain of Crb (UAS-CrbiWT), which have been shown to produce a similar overexpression phenotype and a similar rescue of the crb mutant phenotype (Wodarz et al., 1995). The ubiquitous overexpression of

---

**Fig. 4.** The Crb protein regulates the organisation of the actomyosin complex during invagination. (A–D) Confocal projections showing two tracheal metameres (outlined by dotted lines) of embryos of the indicated genotypes at the early-invagination step stained to visualise the indicated proteins. Note the defects in the localisation of apical and adhesion markers in crb mutants (B,D). The lower parts of panels C,D display a reconstruction of a Z-section. (E–M) Confocal sections showing one tracheal metamere of embryos of the indicated genotypes at the mid-invagination step (E–J) or end-invagination step (K–M) stained to visualise the indicated proteins. The conspicuous apical enrichment of actin and pMoe associated with invagination observed in wild-type embryos (E,H,K) is decreased or almost missing in crb mutants (F,G,I,L,M). Arrows point to the invagination site. The lower parts of panels H–J display a reconstruction of a Z-section. (N,O) Z-sections of a single metamere at the end-invagination step ubiquitously expressing a dominant-negative ezrin construct. Broad cavities and severe invagination defects are detected. Scale bars: 25 μm.
Finally, we also tested a mutant form of Crb protein that lacks the FERM-binding domain, the ERLI domain is not essential to ensure the proper recruitment of Moe (UAS-CrbiY10A) as it contains a mutation in the FERM-binding domain (Klebes and Knust, 2000; Medina et al., 2002). As expected, we found that this construct could not trigger the assembly of the pMoe ring when it was expressed ubiquitously in a crb mutant background (Fig. 5B,K). In addition, that construct was completely unable to rescue the invagination phenotype of these crb mutants (Fig. 3J, Fig. 5K), although it showed a faint, but reproducible, rescue of apical markers and AJ organisation (Fig. 5E,H). These results indicate that the FERM-binding domain of Crb is absolutely required for Crb activity in the invagination process and that Moe recruitment might be an essential aspect of crb activity during invagination.

We next tested the activity of a Crb construct that should be defective in recruiting Moe (UAS-CrbiY10A) as it contains a mutation in the FERM-binding domain (Klebes and Knust, 2000; Medina et al., 2002). As expected, we found that this construct could not trigger the assembly of the pMoe ring when it was expressed ubiquitously in a crb mutant background (Fig. 5B,K). In addition, that construct was completely unable to rescue the invagination phenotype of these crb mutants (Fig. 3J, Fig. 5K), although it showed a faint, but reproducible, rescue of apical markers and AJ organisation (Fig. 5E,H). These results indicate that the FERM-binding domain of Crb is absolutely required for Crb activity in the invagination process and that Moe recruitment might be an essential aspect of crb activity during invagination.

Fig. 5. Crb function in tracheal invagination depends on its FERM-binding domain. (A–L) Rescue experiments. X–Y (A–I) or Z (J–L) sections of a single tracheal metamere (marked with trh or outlined by dotted lines) of stage 11 crb41A22 mutant embryos expressing different Crb constructs stained for the indicated proteins. The generalised expression of wild-type crb rescues the invagination defects of crb-null mutants (J) and restores the normal accumulation of apical markers such as Sdt or aPKC (Fig. 5F; and data not shown) nor a proper distribution of ZA components (Fig. 5I). Strikingly, we detected a consistent rescue of the invagination phenotype (Fig. 3J, Fig. 5L) when we expressed UAS-CrbiERLI in crb mutants. This rescue was accompanied by a rescue of pMoe enrichment in the invaginating placode (Fig. 5C,L). These results indicate that, in sharp contrast with the FERM-binding domain, the ERLI domain is not essential to ensure the proper activity of Crb in invagination.

The above results point to a function for Crb in tracheal invagination independent of its activity in maintaining apicobasal polarity. Apicobasal polarity is restored in crb mutant embryos that are in addition mutant for any of the components of the basolateral complex comprising Scribble (Scrib), Lgl and Dlg (Bilder et al., 2003; Tanentzapf and Tepass, 2003). Interestingly, in lgl;crb double-mutants, while we found the described rescue of epithelial polarisation (data not shown), apical constriction of tracheal cells was absent (Fig. 5M), and the placodes showed a severe (Fig. 5N) or moderate invagination phenotype (Fig. 3J). These results support the hypothesis that it is the absence of Crb and the consequent lack of pMoe enrichment, and not the absence of apicobasal polarity, that underlies the tracheal invagination phenotype of crb mutant embryos.

Crb can induce pMoe cortical enrichment and promote epidermal depressions

As previously described by Medina and colleagues (Medina et al., 2002), the overexpression of Crb in the pattern of enegraden (en) triggers an enrichment of pMoe and actin (Fig. 6B–C), indicating that Crb directly or indirectly modulates pMoe and actin apical accumulation. The gene en is expressed in ectodermal stripes (DiNardo et al., 1985) that, at stage 11, overlap with the most-anterior part of the tracheal placodes (Fig. 6A,A’,D). Interestingly, mutant embryos that are in addition mutant for any of the components of the basolateral complex comprising Scribble (Scrib), Lgl and Dlg (Bilder et al., 2003; Tanentzapf and Tepass, 2003). Interestingly, in lgl;crb double-mutants, while we found the described rescue of epithelial polarisation (data not shown), apical constriction of tracheal cells was absent (Fig. 5M), and the placodes showed a severe (Fig. 5N) or moderate invagination phenotype (Fig. 3J). These results support the hypothesis that it is the absence of Crb and the consequent lack of pMoe enrichment, and not the absence of apicobasal polarity, that underlies the tracheal invagination phenotype of crb mutant embryos.

Crb can induce pMoe cortical enrichment and promote epidermal depressions

As previously described by Medina and colleagues (Medina et al., 2002), the overexpression of Crb in the pattern of enegraden (en) triggers an enrichment of pMoe and actin (Fig. 6B–C), indicating that Crb directly or indirectly modulates pMoe and actin apical accumulation. The gene en is expressed in ectodermal stripes (DiNardo et al., 1985) that, at stage 11, overlap with the most-anterior part of the tracheal placodes (Fig. 6A,A’,D). Interestingly,
when Crb<sup>WT</sup> is overexpressed in the en pattern, we often detected an expansion of the tracheal pits towards the ectopic expression domain of crb. This leads to an abnormal formation of broad cavities due to the simultaneous internalisation of many tracheal cells (Fig. 6E), in contrast with the narrow tracheal opening formed at the invagination site in the wild-type embryos (Fig. 6D). Remarkably, we also detected the formation of precocious and/or ectopic epidermal depressions outside the tracheal tissue, particularly at the confrontations between cells overexpressing Crb and wild-type cells, and often these depressions became continuous with the enlarged tracheal pits (Fig. 6F). These results indicate that high levels of Crb are able to promote the internalisation of ectodermal cells.

Crb acts independently of the Egfr pathway during tracheal invagination

The invagination phenotype of crb mutants is similar to that described for mutants of the Egfr pathway, in which apical constriction is impaired, a broader cavity forms and no proper actomyosin complex is observed (Brodi and Casanova, 2006; Nishimura et al., 2007). We therefore asked whether Crb mediates the Egfr signalling or regulates Egfr activity.

We detected a normal accumulation of Crb in Egfr<sup>2</sup> mutants (Fig. 6K), indicating that the Egfr pathway does not regulate the early Crb tracheal pattern. By contrast, the activity of the Egfr pathway measured by diphosphorylated ERK staining (Gabay et al., 1997) was normal in crb mutants (Fig. 6G–H), indicating that the Egfr signal is not under the control of crb. Furthermore, dp-ERK did not accumulate ectopically when crb was overexpressed in the en pattern (Fig. 6I).

The Egfr regulates tracheal invagination through the RhoGAP protein crossveinless-c (encoded by cv-c); however, it was also proposed that effectors other than cv-c act downstream of Egfr to regulate the actomyosin complex (Brodi and Casanova, 2006). Thus, we asked whether Egfr also regulated the apical accumulation of pMoe during tracheal invagination. This is not the case as, in contrast to crb<sup>11A22</sup> mutants, Egfr<sup>2</sup> mutants showed a normal enhancement of pMoe around the invagination edge (Fig. 6L). Taken together, these results indicate that the two inputs, Egfr and Crb, might act in parallel to promote invagination.

crb is required after invagination for proper tracheal development

The modulated pattern of Crb and other SAR components after invagination is suggestive of additional tracheal requirements for these polarity determinants. We reasoned that the high levels of Crb from mid stage 11 to stage 14 (the mid-tracheal pattern) could indicate a requirement during the process of branch elongation. Thus, we analysed the process of tracheal cell intercalation. In wild-type embryos, cell intercalation occurs during the elongation of most tracheal branches and ensures that cells originally positioned in pairs end-up positioned in rows. This cell rearrangement transforms the original intercellular junctions attaching paired tracheal cells into autacellular junctions sealing a lumen encircled by a single tracheal cell; these autacellular junctions are interrupted by small rings of intercellular junctions, which attach the cells such that they be positioned end-to-end. DEcad labelling allows visualisation of these types of junctions (Ribeiro et al., 2004) (Fig. 7A). We perturbed crb activity by expressing in all tracheal cells a Crb intracellular domain construct (crbi<sup>T6AT9A</sup>), which is non-phosphorylatable in vitro by aPKC and...
was shown to act as a Crb dominant-negative in the embryonic ectoderm (Sotillos et al., 2004). This appears to be also the case in the tracheal tubes as its expression with generalised and early drivers causes invagination defects similar to those of crb-hypomorphic mutants (supplementary material Fig. S3). Upon expression of this construct by btl-gal4, we detected the presence of long stretches of intercellular junctions in branches that should display autocellular junctions, such as the dorsal branches (DBs; Fig. 7B). The DB-specific marker zygotic gap protein Knirps (Kni) was expressed normally, indicating no defects in branch specification (Fig. 7C). This phenotype was very reproducible (80% of branches analysed; n=80). We have previously shown that intercalation correlates with a differential accumulation of DEcad in tracheal branches, with higher DEcad levels in branches that do not undergo intercalation (Fig. 7D) (Shaye et al., 2008). DEcad was still accumulated differentially in embryos expressing crbiT6AT9A in the trachea (Fig. 7E), indicating that crb might not be directly regulating this circuit. Consistent with the phenotype of crbiT6AT9A, crbS010409 and crb11A22 mutants also displayed strong defects in tracheal intercalation (Fig. 7F–G’). As previously described (Tepass et al., 1990), crb11A22 mutants display an unpatterned tracheal system where most cells survive, form aggregates and secrete cuticle. All the rudimentary tracheal branches formed maintained intercellular junctions, indicating that the intercalation process is blocked (Fig. 7G,G’).

We also assayed the effect upon intercalation when increasing Crb activity by a mild overexpression of a crbi or full-length crb (UAS-CrbWT-Weak) in tracheal cells. We detected the presence of intercellular junctions in branches that should undergo intercalation and in which the cells maintained their side-by-side organisation instead of positioning in rows (Fig. 7H; supplementary material Fig. S2E). Overall, the results indicate that both loss and gain of crb activity result in a block of intercalation. Thus, a precise control of crb activity appears to be required to ensure intercalation.

**Discussion**

The functional relevance of Crb modulation during tracheal development

The tracheal tissue is a dynamic structure that undergoes a huge repertoire of changes at the cellular level (Affolter and Caussinus, 2008). Tracheal cells might require a balanced turnover of basic cellular activities such as polarity, or adhesion, to, on the one hand, maintain their epithelial function and, on the other, allow all the cellular changes needed.

In this work, we show that the pattern of expression and protein accumulation of crb and other SAR-related genes in tracheal cells is highly modulated and we describe an early-, mid- and late-tracheal pattern. This pattern is suggestive of special requirements for these polarity determinants in tracheal morphogenesis. Consistently, the functional analysis of crb indicates that Crb is required for tracheal invagination and cell rearrangements (described in this work) and for the control of tube size (Forster et al., 2010; Laprise et al., 2010) (A.L. and M.L., unpublished). Considering the strong spatio-temporal correlation between the pattern of Crb protein accumulation and the phenotypes observed,

---

**Fig. 7. crb modulates tracheal intercalation.**

All panels show projections of confocal sections. Embryos of the indicated genotypes stained for DEcad to visualise the apical junctions and state of intercalation. (A) At stage 15, autocellular junctions are visualised as straight lines interrupted by rings of intercellular junctions (arrows in A), and intercellular junctions as a mesh (arrowhead in A). (B,C,F–G’) crb loss-of-function gives rise to a partial (arrows in B,C,F) or complete blockage (arrows in G’) of intercalation, although DB cells are specified correctly (C). (D,E) At stage 13, crbiT6AT9A overexpression does not affect the differential accumulation of DEcad as higher levels are still detected in the DT (arrows in D,E). (H) Tracheal tubes overexpressing Crb also display intercalation defects (arrows) and cells remain side by side (asterisks). Scale bars: 25 μm (A–F,H); 10 μm (G).
we propose that the fine modulation of SAR during tracheal development is functionally relevant.

The earliest tracheal defect in \( crb \) mutants is detected at the onset of invagination, when, although the tracheal cells are specified correctly, they are unable to constrict their apical side and to internalise in an ordered manner. This phenotype correlates with the Crb early-tracheal pattern. The area of increased Crb levels covers the dorsal-most cells of each tracheal placode, precisely where the invagination process starts. Several observations indicate that this pattern is not due to an optical effect produced by the shrinkage and concentration of apical membrane during apical constriction. First, we detect the enhanced accumulation of Crb before apical constriction takes place and also in dorsal ectodermal cells outside the tracheal placode. Second, we do not detect such a pattern when analysing other apical markers, such as DEcad and Arm. We also find that this early pattern of Crb is posttranscriptionally regulated. We propose that the transient increase of Crb and other SAR proteins, by a currently unknown mechanism, in the dorsal-most cells of the placodes, would facilitate apical constriction and subsequent internalisation. We hypothesise that this could be a widely used mechanism to promote apical constriction. In this respect, it is worth noting that a role for \( crb \) and the Par network in apical constriction has also been observed during morphogenesis of the amnioserosa (David et al., 2010; Harden et al., 2002; Wodarz et al., 1995). Interestingly, Crb could play a similar role in other morphogenetic events as Crb is similarly upregulated just before posterior spiracle invagination (Lovegrove et al., 2006) and in the region of invagination in the salivary glands (A.L. and M.L., unpublished) (Myat and Andrew, 2002). In support of our hypothesis, we have observed that the overexpression of \( crb \) leads to enlarged tracheal pits, where more cells initiate internalisation, and to precocious and ectopic epidermal depressions. These observations are consistent with a role for \( crb \) in promoting internalisation of epithelial cells.

From mid stage 11 to stage 14, Crb accumulates at high levels at the apical part of all tracheal cells (mid-tracheal pattern). This mid-tracheal pattern coincides with a requirement for Crb in tracheal cell intercalation. We therefore propose that the high levels of Crb observed at mid stages of tracheal development are required to ensure ordered cell rearrangements. Crb is required for convergent extension in Malpighian tubules, a process comparable to tracheal intercalation. In the former process, Crb is required to maintain polarity during cell rearrangement (Campbell et al., 2009). Crb could play a similar role during tracheal cell intercalation and be required to maintain polarity during the processes of disassembly of intercellular junctions between old neighbours, cell rearrangements and establishment of new contacts (intercellular and autocellular contacts) during and after repositioning. In this respect, Crb could act as an apical determinant for membrane remodelling, for targeting of recycling vesicles of AJ components (Campbell et al., 2009) and/or for maintaining cell polarity during morphogenesis to preserve the proper localisation and reassembly of apical markers (Bilder et al., 2003; Campbell et al., 2009; Grawe et al., 1996; Tanentzapf and Tepass, 2003; Tepass, 1996). However, our data indicate that cell adhesion (judged by accumulation of DEcad) and polarity (judged by accumulation of aPKC (A.L. and M.L., unpublished)] are not strongly affected in our \( crb \) loss-of-function analysis. This raises the possibility that \( crb \) is also required for a previously undescribed more direct function during intercalation. Further work will be required to determine properly the mechanism, or mechanisms, by which \( crb \) affects tracheal cell intercalation.

It is known that \( crb \) controls apical membrane expansion in several tissues (Izaddoost et al., 2002; Laprise et al., 2006; Myat and Andrew, 2002; Pellikka et al., 2002; Wodarz et al., 1995). In agreement with this expected function, we and others (Forster et al., 2010; Laprise et al., 2010) have found longer and wider tracheal tubes after mild overexpression of \( crb \). In this scenario, we hypothesise that the decrease observed during the late-tracheal pattern reflects a downregulation of Crb, after tube expansion has initiated (Tsarouhas et al., 2007), required to prevent excessive growth.

The role of Crb in invagination

Our results show a previously undescribed role for \( crb \) in apical constriction and invagination of the tracheal cells. Our results also indicate that this Crb activity is mediated by a specific recruitment of Moe and not exclusively by maintaining apicobasal polarity during the process. The use of different Crb constructs has allowed us to separate genetically and molecularly the requirement for \( crb \) in maintaining polarity and in recruiting Moe during this invagination process. We find that a Crb construct defective in Moe recruitment (Klebes and Knust, 2000; Medina et al., 2002) is completely unable to rescue the invagination defects of \( crb \)-null mutants, indicating that Moe recruitment is an essential aspect of Crb activity in invagination. Strikingly, this result is in contrast with the partial rescue we find when a Crb construct that lacks the ERLI domain, which is required for Sdt association (Klebes and Knust, 2000), is expressed. Both the ERLI- and the FERM-protein-binding domains of Crb have been shown previously to be required for the rescue of the \( crb \) epithelial polarity mutant phenotype (Klebes and Knust, 2000). Our results indicating that the ERLI domain is at least partially dispensable in tracheal invagination suggest that the loss of apicobasal polarity is not the primary cause of the defects in apical constriction and tracheal invagination of \( crb \) mutants. Recently, a role for \( crb \) in salivary gland invagination has been reported. There, it was proposed that \( crb \) regulates the process by maintaining polarity (Xu et al., 2008). Nevertheless, it was not addressed whether \( crb \), besides maintaining polarity during invagination, also exerts a more direct, polarity-independent, role in salivary gland invagination. The similarities between the tracheal and salivary gland tissue (Kerman et al., 2006), together with the fact that Crb protein also displays an increased accumulation in the salivary gland placode before invagination (Myat and Andrew, 2002; Xu et al., 2008) (A.L. and M.L., unpublished), raise the possibility that Crb also plays a more direct role during salivary gland invagination.

We propose that Crb exerts its role in invagination by, in part, maintaining apicobasal polarity but, in addition, exerting a more direct role on cytoskeleton reorganisation. Several observations allow us to suggest a molecular mechanism for such a requirement. We observed that Crb activity correlates with the proper accumulation of an apical actomyosin ring, which has been proposed to facilitate invagination (Brodu and Casanova, 2006; Llimargas and Casanova, 1999). In addition, we have found that the active phosphorylated form of Moe also accumulates at high levels around the invagination site. Moe has been proposed to interact with actin (for reviews, see Hughes and Fehon, 2007; Miller, 2003; Polesello and Payre, 2004; Tepass, 2009) and to be recruited to the apical membrane by Crb (Medina et al., 2002). In agreement with this, we found that the accumulation of pMoe during invagination is \( crb \) dependent and that pMoe is upregulated by Crb overexpression (this work) (Medina et al., 2002). Therefore,
we propose that the high levels of Crb in the dorsal-most cells of the placode recruit Moe that, in turn, helps to recruit and organise an apical actomyosin complex that promotes invagination. Interestingly, a role in actomyosin contractility has already been reported for CRB in the apical actomyosin complex that promotes invagination by regulating the RhoGAP cv-c (Brodu and Casanova, 2006). By contrast, Moe antagonises the activity of Rhodo1 in epithelial cells (Speck et al., 2003). Therefore, the Crb and Egfr signals could both impinge on Rhodo1 activity to ensure proper actin organisation.

In summary, we find that the pattern of accumulation of Crb (and other SAR proteins) is regulated spatially and temporally during tracheal development and that this regulated pattern correlates with a reiterative requirement for crb in tracheal formation. Furthermore, we find that Crb accumulation, by a posttranscriptional mechanism, at the dorsal-most ectoderm overlapping the tracheal placodes, is specifically required and instructive for the ordered invagination of the tracheal cells. In this process, we find that Crb acts in parallel to the invagination-promoting activity of the Egfr pathway. This activity of Crb in tracheal cell invagination is mediated through its FERM-binding domain. We propose a molecular mechanism whereby the ability of Crb to recruit Moe through this domain underlies the requirement for Crb for apical constriction and invagination of the tracheal cells. Furthermore, our results, in agreement with those of others (Ling et al., 2010; Robinson et al., 2010), stress the ability of the different domains of the short intracellular domain of Crb to perform independent functions in development.

Materials and Methods

Drosophila strains

Strain yw118 was used as the wild-type strain. Mutant strains used for the study were: crbΔU2, crbΔU2αCR, crbΔU2λ, rh1Δ, rh1Δ, Egfr2, Df(2R)crb18, crb S1010, trh 10512, vvl GA3, Egfr f2, Df(1)os1A2, lgl 4. To select the homozygous mutant embryos, we used appropriate blue- or GFP-marked balancer stocks and crosses. These stocks are available from the Bloomington Stock Center. We are grateful to the members of the Llimargas and Casanova labs for helpful discussions. We thank J. Casanova, K. Campbell and J. C.-G. Hombria for critically reading the manuscript. A.L. acknowledges a contract from the ‘Juan de la Cierva’ programme and S.S. a contract from the ‘Ramón y Cajal’ programme. This work was supported by funds from the Ministerio de Educación y Ciencia to M.L. (BFU2006-09515/BMC, PIE2007010919) and to S.C. (BFU2008-03762) and from the Programme Consolider 2007 (CSD2007-00008) project. An institutional grant from Fundación Ramón Areces to the CBMSO is acknowledged.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/124/2/240/DC1

References

Affolter, M. and Caussinus, E. (2008). Tracheal branching morphogenesis in Drosophila: new insights into cell behaviour and organ architecture. Development 135, 2055-2064.

Assemat, E., Bazellieres, E., Paradowski-Pachoud, E., Le Bivic, A. and Massey-Harroche, D. (2008). Polarity complex proteins. Biochim. Biophys. Acta 1778, 614-630.

Bachmann, A., Schneider, M., Theilenberg, E., Grawe, F. and Knust, E. (2001). Drosophila Stardust is a partner of Crumbs in the control of epithelial cell polarity. Nature 414, 638-643.

Bazellieres, E., Assemat, E., Arsanto, J. P., Le Bivic, A. and Massey-Harroche, D. (2009). Crumbs proteins in epithelial morphogenesis. Front. Biosci. 14, 2149-2169.

Bilder, D., Schobes, M. and Perrimon, N. (2005). Integrated activity of PDZ protein complexes regulates epithelial polarity. Nat. Cell Biol. 7, 53-58.

Brodu, V. and Casanova, J. (2006). The Rhodopsin Crumbs complex: from epithelial to polar cell polarity to retinal degeneration. J. Cell Sci. 122, 2587-2596.

Campbell, K., Knust, E. and Skaer, H. (2009). Crumbs stabilises epithelial polarity during tissue remodelling. J. Cell Sci. 122, 2604-2612.

Camps-Ortega, A. J. and Hartenstein, V. (1998). The Embryonic Development of Drosophila Melanogaster. pp. 10-84 New York: Springer-Verlag.

Cela, C. and Llimargas, M. (2006). Egfr is essential for maintaining epithelial integrity during tracheal remodelling in Drosophila. Development 133, 3115-3125.

David, D. J., Tikhon, A. and Harris, T. J. (2010). The PAR complex regulates paxillin actomyosin contractions during amnioserosa apical constriction in Drosophila. Development 137, 1645-1655.

DiNardo, S., Kuner, J. M., Theis, J. and O’Farrell, P. H. (1985). Development of embryonic pattern in D. melanogaster as revealed by accumulation of the nuclear engrailed protein. Cell 43, 59-69.

Fehon, R. G., McClatchey, A. I. and Bretscher, A. (2010). Organizing the cell cortex: the role of ERM proteins. Nat. Rev. Mol. Cell Biol. 11, 276-287.

Forster, D., Armbruster, K. and Luschnig, S. (2010). Sec24-dependent secretion drives cell-autonomous expansion of tracheal tubules in Drosophila. Curr. Biol. 20, 62-68.

Gabay, L., Seger, R. and Shilo, B. Z. (1997). MAP kinase in situ activation atlas during Drosophila embryogenesis. Development 124, 3535-3541.

Giraldez, A. J., Luschnig, S., Mestecky, M. M. and Krasnow, M. A. (2003). Branching morphogenesis of the Drosophila tracheal system. Annu. Rev. Cell Dev. Biol. 19, 623-647.

Grave, W., Karsch, T. and Dovaz, T. (1997). MAP kinase in situ activation atlas during Drosophila embryogenesis. Development 124, 3535-3541.

Harden, N., Ricos, M., Yee, K., Sanny, J., Langmann, C., Yu, H., Chia, W. and Lim, L. (2002). Drac1 and Crumbs participate in amnioserosa morphogenesis during dorsal closure in Drosophila. J. Cell Sci. 115, 2119-2129.
Harris, T. J. and Peifer, M. (2004). Adherens junction-dependent and -independent steps in the establishment of epithelial cell polarity in Drosophila. J. Cell Biol. 167, 135-147.

Harris, T. J. and Peifer, M. (2005). The positioning and segregation of apical cues during epithelial polarity establishment in Drosophila. J. Cell Biol. 170, 813-823.

Hombria, J. C., Brown, S., Hader, S. and Zeidler, M. P. (2005). Characterisation of Upa2, a Drosophila IAK/STAT pathway ligand. Dev. Biol. 288, 420-433.

Hong, Y., Stronach, B., Perrimon, N., Jan, L. Y. and Jan, Y. N. (2003). A role for moesin in polarity. Nature 421, 178-183.

Kerman, B. E., Cheshire, A. M. and Andrew, D. J. (2004). From fate to function: the power of genetics finally brought to bear. Curr. Opin. Genet. Dev. 14, 571-582.

Ling, C., Zheng, Y., Yin, F., Yu, J., Huang, J., Hong, Y., Wu, S. and Pan, D. (1999). EGF signalling regulates cell invagination as Hippo signaling by binding to Expanded. J. Cell Biol. 145, 340-350.

Pilot, F., Philippe, J. M., Lemmers, C. and Lecuit, T. (2006). Spatial control of actin organization at adherens junctions by a synaptopodin-like protein Bstr. Nature 442, 580-584.

Polesello, C. and Payre, F. (2004). Small is beautiful: what flies tell us about ERM protein function in development. Trends Cell Biol. 14, 294-302.

Ribeiro, C., Neumann, M. and Affolter, M. (2004). Genetic control of cell intercalation during tracheal morphogenesis in Drosophila. Curr. Biol. 14, 2197-2207.

Robinson, B. S., Huang, J., Hong, Y. and Moberg, K. H. (2010). Crumbs regulates Salvador/Warts/Hippo signaling in Drosophila via the FERM-domain protein expanded. Curr. Biol. 20, 582-590.

Swanson, L. E. and Beitel, G. J. (2006). Tubulogenesis: an inside job. Curr. Biol. 16, R51-R53.

Tepass, U., Tanentzapf, G., Ward, R. and Fehon, R. G. (2009). FERM proteins in animal morphogenesis. Annu. Rev. Genet. 43, 75-99.

Tepass, U. (2003). Epithelial polarity proteins regulate tracheal tube size in parallel to cell intercalation in the Drosophila trachea. Curr. Biol. 13, 214-225.

Wilk, R., Weisman, I. and Shilo, B. Z. (1996). Tracheal size control in Drosophila embryo. Nat. Cell Biol. 10, 659-671.

Wu, V. M. and Beitel, G. J. (2004). A junctional function of apical proportions: epithelial tube-size control by septate junctions in the Drosophila tracheal system. Curr. Opin. Cell Biol. 16, 493-499.

Xu, N., Keung, B. and Myat, M. M. (2008). Rho GTPase controls invagination and cohesive migration of the Drosophila salivary gland through Crumbs and Rho-kinase. Dev. Biol. 321, 88-100.