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

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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 Assemet 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 Assemet 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 Moe 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 *crb* (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 trachealeless (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* (*vv1*)], 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-Crb^{WT}) with a heterologous and general promoter in an otherwise protein-null mutant, *crb^{1A22}* (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,I'). 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 phosphotyrosine (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

Fig. 1. Pattern of expression and protein accumulation of *crb* during tracheal development. (A–D'') 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 Crb in the dorsal part of the tracheal placode at early stages (arrow in A), whereas DEcad shows a homogeneous pattern (A''). The levels of Crb in the tracheal cells at late stages (arrows in D,H) are lower than those in Malpighian tubules (arrowhead in D) and in comparison with those of other junctional proteins (D''). (E–H) In situ hybridisation to whole-mount embryos shows that *crb* expression is not particularly enhanced in the tracheal system before mid stage 11 (E). From late stage 11, *crb* is highly expressed in the tracheal system (F,G), and the levels decrease from stage 14 (H). Arrows point to the fifth tracheal metamere. (I–J') Projections of confocal sections showing two tracheal metameres of *crb*-null mutants (J,J') and *crb*-null mutants overexpressing Crb (I,I'), at stages 10 or 11, stained for Crb. Scale bars: 25 μ m. In this and all figures, dorsal is up and anterior to the left.

al., 2007). The process initiates at late stage 10 to early stage 11 (start-invasion 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-invasion 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-invasion 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 *crb* before *crb*-null embryos collapse. In *crb*^{11A22} embryos, we detected strong defects in invagination in comparison with wild-type controls. At the start-invasion 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-invasion step, and despite the absence

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 μ m.

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).

crb^{S010409} 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^{S010409}* mutants displayed clear defects of invagination (in more than 75% of placodes analysed; Fig. 3J) that were very similar, although milder, than those of *crb¹¹⁴²²* 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^{S010409}* tracheal cells internalised in a disorganised way, and often a cavity broader than in the wild-type embryo formed (27% of placodes, $n=77$; Fig. 3H). Finally, by the

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. 4B), DEcad remained apically accumulated, although it did not form a continuous belt [probably reflecting that the zonula adherens (ZA) was not properly assembled (Tepass, 1996)] (Fig. 4D), whereas the basolateral marker discs large 1 tumor suppressor protein (Dlg) appeared normally localised (Fig. 4D). These results

Fig. 3. The Crb protein is required for proper tracheal invagination. (A–I) Confocal sections showing a single tracheal metamer of embryos of the indicated genotypes stained to visualise the indicated proteins. For each placode scanned in the *X–Y* axis, we show below a reconstruction of a *Z*-section (indicated by the dotted line). To the side, we show a schematic representation of the invagination phenotypes. Schemes for A,D,G are *X–Y* representations, whereas the rest are *Z* representations. Note that an apical constriction (in red) is absent in *crb*-null mutants (D) and irregular in hypomorphic *crb* alleles (G). Later, the internalisation of tracheal cells and the formation of the initial tube is compromised in *crb* loss-of-function conditions (E,F,H,I). (J) Frequency of invagination phenotypes at the end-invagination step shown by embryos of the indicated genotypes. *n* indicates the number of placodes analysed. Scale bar: 10 μ m.

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*

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,J,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 μ m.

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-Crb^{WT}*) or the cytoplasmic domain of Crb (*UAS-Crb^{CT}*), 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

(for reviews, see Fehon et al., 2010; Miller, 2003; Polesello and Payre, 2004) 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

UAS-Crb^{WT} or *UAS-Crb^{iY10A}* in *crb*-null mutants was able to restore apicobasal polarity, as judged by Crb (Fig. 11,I', Fig. 5J) (Klebes and Knust, 2000; Wodarz et al., 1995), Sdt and PATJ (Fig. 5D; supplementary material Fig. S2B) apical accumulation and a continuous belt of AJ markers (Fig. 5G; supplementary material Fig. S2C). It also rescued the formation of the ring of pMoe (Fig. 5A; supplementary material Fig. S2A) and the strong invagination phenotype of *crb* mutants (Fig. 3J, Fig. 5J; supplementary material Fig. S2D). We next tested the activity of a Crb construct that should be defective in recruiting Moe (*UAS-Crb^{iY10A}*) 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. Finally, we also tested a mutant form of Crb protein that lacks the carboxyl-terminal ERLI domain, encoded by *UAS-Crb^{iAERLI}* (Klebes and Knust, 2000). The ERLI domain of Crb is required for the interaction with Sdt, which plays a key role in maintaining apicobasal cell polarity (Bachmann et al., 2001; Grawe et al., 1996; Hong et al., 2001). As expected (Klebes and Knust, 2000), we observed that this construct could restore neither the accumulation of apical polarity 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-Crb^{iAERLI}* 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 *lgl⁴; 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 *engrailed* (*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,

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 metamer (marked with trh or outlined by dotted lines) of stage 11 *crb^{11A22}* 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 Crb (J), Sdt (D), AJ organisation (G) and pMoe recruitment (A). A Crb construct lacking the ERLI domain is also able to rescue the invagination defects (L) and pMoe accumulation (C,L) of *crb* mutants but not apicobasal polarity and AJ assembly (F,I). By contrast, a Crb construct defective in its FERM-binding domain partially restores apicobasal polarity and adhesion (E,H) of *crb* mutants, but neither is pMoe recruited to the invagination site (B,K) nor is invagination rescue observed (K). Arrows point to the invagination site. (M,N) Invagination phenotypes of *lgl⁴; crb^{11A22}* double-mutants stained for pTyr. In double-mutant embryos, apical constriction is not observed at early stage 11 (X–Y section in M), and the initial tube show a strong invagination phenotype (Z-section in N). Scale bar: 25 μm.

Fig. 6. *crb* acts independently of *Egfr* in invagination and can promote internalisation. (A–F) Confocal sections showing two tracheal metameres of late stage 11 embryos overexpressing GFP alone or *crb* and GFP in the pattern of *en* (in green) stained to visualise the indicated proteins. The overexpression of Crb can lead to increased levels of pMoe and actin (compare B,B' with A,A', and C,C' with D). The tracheal pits are usually enlarged (compare arrows in D,E). Additionally, precocious and/or ectopic ectodermal depressions are observed (arrows in F). The lower part of panel F displays a cross-section to show the depression, marked with an arrow. (G–I') Confocal sections showing two tracheal metameres of early stage 11 embryos of the indicated genotypes stained to visualise the indicated proteins. The pattern of dpERK is not modified by Crb activity. The arrow in I' points to cells overexpressing Crb. (J–L) Confocal sections showing a single tracheal metamere at mid (J,K) or late (L) stage 11 stained to visualise trh and Crb (J,K) or pMoe (L). Crb is enhanced in the dorsal part of the tracheal placode in wild-type and *Egfr* mutants (J). In *Egfr* mutants, pMoe accumulates to high levels during invagination, as in the wild-type embryo (compare with Fig. 4K). Z-sections are shown below. Scale bars: 25 μ m (A,G); 10 μ m (J).

when Crb^{WT} 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 (Brodu 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*^{f2} 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,I').

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 (Brodu 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*^{11A22} mutants, *Egfr*^{f2} 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 autocellular junctions sealing a lumen encircled by a single tracheal cell; these autocellular 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 (*crb*^{16AT9A}), which is non-phosphorylatable in vitro by aPKC and

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, *crbi*^{T6AT9A} 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).

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 *crbi*^{T6AT9A} in the trachea (Fig. 7E), indicating that *crb* might not be directly regulating this circuit. Consistent with the phenotype of *crbi*^{T6AT9A}, *crbi*^{S010409} and *crbi*^{11A22} mutants also displayed strong defects in tracheal intercalation (Fig. 7F–G'). As previously described (Tepass et al., 1990), *crbi*^{11A22} 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-Crb*^{WT-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,

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 (Izaddoust 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 proposed for ERM proteins in the nematode *Caenorhabditis elegans* and mouse (for a review, see Hughes and Fehon, 2007). Although direct or indirect links between *crb* or other SAR components and the actin cytoskeleton have been proposed earlier (for reviews, see Bazellieres et al., 2009; Bulgakova and Knust, 2009), this work represents one of the first examples of this interaction during organogenesis.

We show that Crb appears to act in parallel to the Egfr signal, which is also required for apical constriction and tracheal invagination (Brodu and Casanova, 2006; Nishimura et al., 2007). Egfr controls the activity of the Rho GTPase Rho1 during tracheal invagination by regulating the RhoGAP cv-c (Brodu and Casanova, 2006). By contrast, Moe antagonises the activity of Rho1 in epithelial cells (Speck et al., 2003). Therefore, the Crb and Egfr signals could both impinge on Rho1 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 trachea 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 *y¹w¹¹⁸* was used as the wild-type strain. Mutant strains used for the study were: *crb^{1A22}*, *crb⁸⁰¹⁰⁴⁰⁹*, *trh¹⁰⁵¹²*, *vvf^{G43}*, *Egfr^{Δ2}*, *Df(1)os1A2*, *Igf^Δ*. To select the homozygous mutant embryos, we used appropriate blue- or GFP-marked balancer chromosomes.

The transgenic lines used for overexpression experiments were: *UAS-Crb^{AERL1}*, *UAS-Crb^{Y10A}*, *UAS-Crb^{WT}* and *UAS-Crb^{WT-Weak}* (*UAS-Crb^{WT-11d}*), gifts from E. Knust, and *UAS-Crb^{T6AT9A}*, *UAS-Crb^{WT}* and *UAS-ezrin^{DN}GFP*. These stocks are described in Flybase (<http://flybase.bio.indiana.edu>). *Drosophila* stocks and crosses were kept under standard conditions at 25°C.

The following Gal4 drivers were used: *btGal4* to drive expression in all tracheal cells from invagination onwards, *armGal4* and *69B* to drive ubiquitous expression and *enGal4UAS-GFP* to drive expression in ectodermal stripes. Overexpression experiments were conducted at 29°C. Rescue or overexpression experiments using *UAS-Crb^{WT}* were conducted at 18°C.

Immunohistochemistry and in situ hybridisation

Embryos were staged as described previously (Campos-Ortega and Hartenstein, 1985) and stained following standard protocols. Immunostainings were performed on embryos fixed in 4% formaldehyde for 20–30 minutes, except for DCAD2 stainings, in which we fixed for 10 minutes, and in stainings for Arm, in which we fixed by heat treatment. The following primary antibodies were used: anti-Trh 1:600 (made by N. Martin in the laboratory of J. Casanova), anti-DEcad (DCAD2) 1:100, anti-Arm (N2 7A1) 1:100, anti-Crb (Cq4) 1:5 and anti-Dlg (4F3) 1:500 from Developmental Studies Hybridoma Bank, anti-actin 1:1000 (MP Biomedicals), anti-aPKC ζ C-20 1:500 (Santa Cruz Biotechnology), anti-PATJ 1:1000 (H. Bellen), anti-Sdt 1:500 (E. Knust), anti-DmPar-6 1:500 (J. A. Knoblich), anti-pTyr 1:1000

(Upstate), anti-pMoe 1:500 (S. Karagigious and Cell Signaling), anti-dpERK 1:20 (Cell Signaling), anti-Kni (developed by J. Reivitz and provided by M. Ruiz-Gomez) 1:1000, anti-GFP 1:600 (Molecular Probes and Roche) and anti-βGal 1:600 (Cappel and Promega). Biotinylated or Cy3-, Cy2- and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:300 in PBT plus 0.5% BSA. Chitin was visualised with Chitin-binding protein CBP (NEB) at 1:500.

In situ hybridisation was performed according to standard protocols; *crb* and *aPKC* riboprobes were marked using a DIG RNA Labeling Kit (Roche). Confocal images were obtained with a Leica TCS-SPE or TCS-SP5 system. Images were imported into Adobe Photoshop and assembled into figures using the Adobe Illustrator software.

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