

# Scarface, a secreted serine protease-like protein, regulates polarized localization of laminin A at the basement membrane of the *Drosophila* embryo

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**Cell–matrix interactions brought about by the activity of integrins and laminins maintain the polarized architecture of epithelia and mediate morphogenetic interactions between apposing tissues. Although the polarized localization of laminins at the basement membrane is a crucial step in these processes, little is known about how this polarized distribution is achieved. Here, in *Drosophila*, we analyse the role of the secreted serine protease-like protein Scarface in germ-band retraction and dorsal closure—morphogenetic processes that rely on the activity of integrins and laminins. We present evidence that *scarface* is regulated by c-Jun amino-terminal kinase and that *scarface* mutant embryos show defects in these morphogenetic processes. Anomalous accumulation of laminin A on the apical surface of epithelial cells was observed in these embryos before a loss of epithelial polarity was induced. We propose that Scarface has a key role in regulating the polarized localization of laminin A in this developmental context.**

Keywords: JNK; morphogenesis; integrin; basement membrane  
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## INTRODUCTION

Integrins, which are heterodimeric transmembrane receptors, interact with laminins, which are extracellular matrix molecules, to mediate signalling, stable adhesion of differentiated epithelia to the basement membrane (BM) and transient adhesion during cell spreading or migration (Bokel & Brown, 2002). Considerable progress has been made in our understanding of the mechanisms involved in the polarized transport of integrins and other basolateral membrane proteins, but little is known about how laminins and other basally secreted proteins are sorted and delivered specifically to the BM (Rodríguez-Boulan *et al*, 2005). Although a pathway involved in the polarized transport of BM proteins was proposed many years ago (Caplan *et al*, 1987; Boll

*et al*, 1991), the first protein regulating this process has only recently been characterized (Denef *et al*, 2008). Crag, a protein with domains involved in membrane transport and localized in early and recycling endosomes, has been shown to have a role in regulating the polarized secretion of BM proteins in *Drosophila* epithelial cells.

Here, in *Drosophila*, we characterize the embryonic role of *scarface* (*scarf*; Fig 1), a gene encoding for a putative secreted serine protease-like molecule without catalytic activity (Bonin & Mann, 2004). We present evidence that *scarf* expression is regulated by c-Jun amino-terminal kinase (JNK) signalling and *scarf* mutant embryos show defects in germ-band retraction (GBR) and dorsal closure (DC)—morphogenetic processes that rely on the activity of integrins and laminins (Leptin *et al*, 1989; Schock & Perrimon, 2003; Narasimha & Brown, 2004; Reed *et al*, 2004; Homsy *et al*, 2006). Our data indicate that Scarf has a key role in regulating the polarized localization of laminin A (LanA) in this developmental context.

## RESULTS AND DISCUSSION

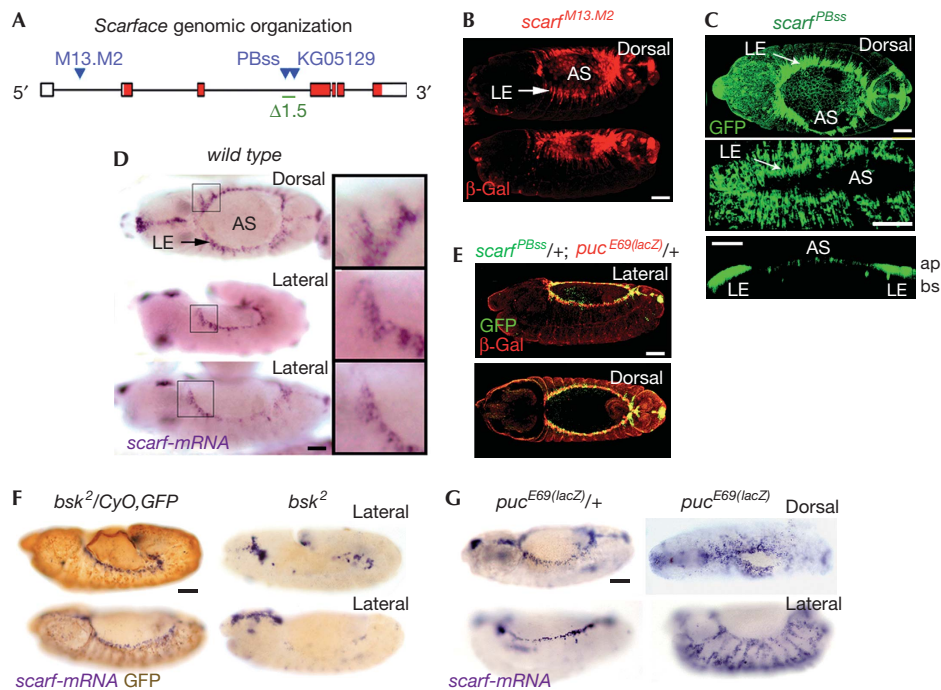
During GBR, the tail end of the germ band, or embryo proper, interacts with the amnioserosa (AS), an epithelium of large flat cells that does not contribute to the larva, and moves to its final posterior position (Fig 2E; Schock & Perrimon, 2002). After GBR, the ectoderm has a gap on its dorsal side that is occupied by the AS. The dorsal-most cells of the ectoderm, on both sides of the embryo, are in contact with the AS and are called leading edge (LE) cells. During DC, LE cells direct the movement of the epidermal sheets migrating dorsally over the apical side of the AS until they meet and fuse at the dorsal midline (Fig 2E; Jacinto *et al*, 2002). Our attention was drawn to the function of *scarf* on the basis of its embryonic expression pattern. Two *piggyBac* insertions, *scarf*<sup>PBss</sup>(GFP) and *scarf*<sup>M13.M2</sup>(lacZ; Fig 1A), were expressed at a high level in LE cells during GBR and DC and at a low level in AS cells (Fig 1B,C). The expression of *scarf* was confirmed by *in situ* hybridization (Fig 1D). It was also expressed in the head and tail regions, and in the ventral ectoderm in a segmental pattern (Fig 1D and data not shown).

The JNK pathway is activated in LE cells and leads to the expression of the signalling molecule Dpp and the phosphatase

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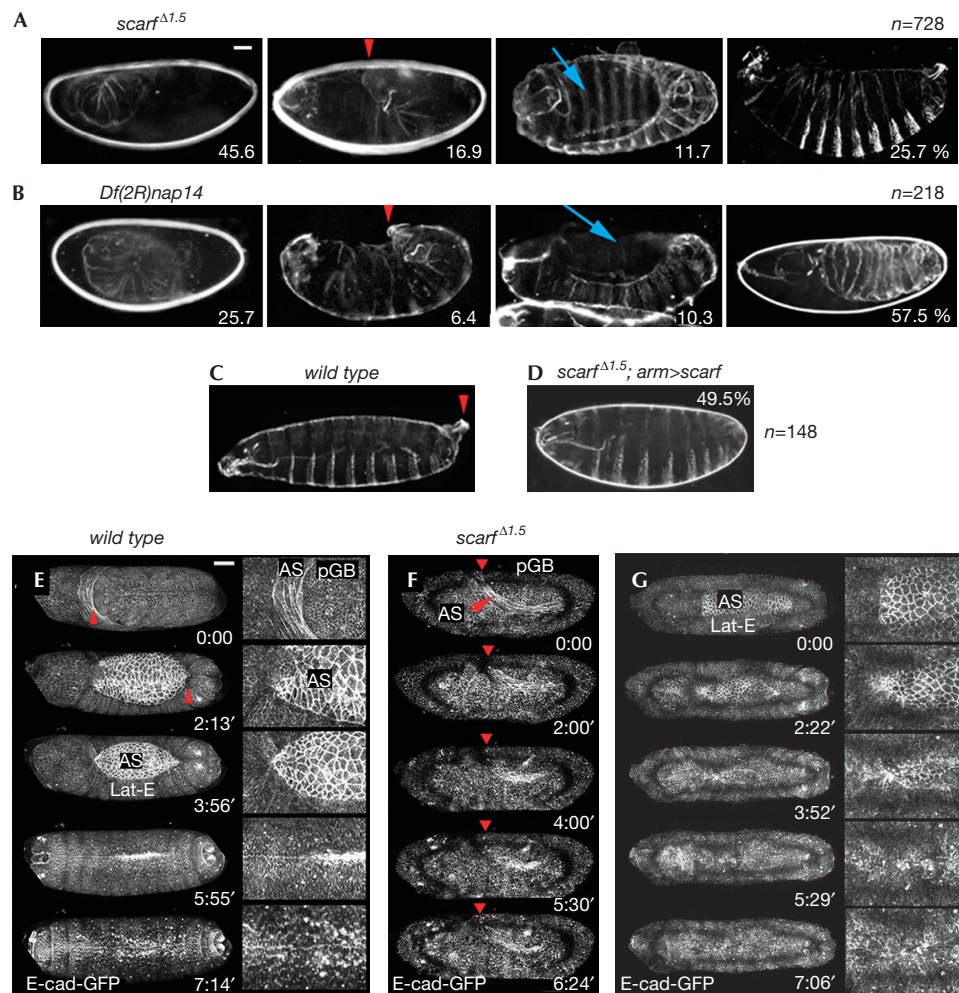
**Fig 1** | *scarf* is regulated by JNK in leading edge cells. (A) Genomic organization of the *scarf* locus. P element (*KG05129*) and *piggyBac* (*PBss* and *M13.M2*) insertions (blue triangles), the *scarf* <sup>$\Delta 1.5$</sup>  deletion (green), exons (white boxes) and the open reading frame (red boxes) are shown. (B–G) Embryos of stages 12–14 labelled to visualize  $\beta$ -gal, GFP or *scarf* mRNA expression. (C) The bottom panel shows a cross-sectional view. (D) Magnifications of the boxed tissues are shown. Scale bars, 25  $\mu$ m. ap, apical; AS, aminoserosa; bs, basal; GFP, green fluorescent protein; JNK, c-Jun amino-terminal kinase; LE; leading edge; mRNA, messenger RNA.

puckered (*Puc*). The *scarf* gene was expressed in the same cells as *puc* and *dpp* at the LE (Fig 1E; supplementary Fig S1 online). Reduced JNK—basket (*Bsk*) in *Drosophila*—or expression of a dominant-negative version of *Bsk* or *Puc*, which mediates a feedback loop repressing JNK activity (Martin-Blanco et al, 1998), led to the loss of *scarf* expression in LE cells (Fig 1F; supplementary Fig S1 online). Loss of *puc* activity, which leads to increased levels of JNK activity, or expression of an activated version of JNK-activating kinase—Hemipterous in *Drosophila*—led to the expansion of the *scarf* expression domain throughout the lateral ectoderm (Fig 1G; supplementary Fig S1 online). Dorsal cells had a stronger response to increased JNK, suggesting that JNK requires the activity of another factor expressed in this region to induce *scarf*. As stated, the JNK cascade drives the expression of the secreted molecule *Dpp*. In embryos mutant for the *Dpp* receptor *Thickveins*, *scarf* expression at the LE was not lost and ectopic activation of the *Dpp* pathway did not induce the ectopic expression of *scarf* (supplementary Fig S1 online). These results indicate that *scarf* expression is induced by JNK in LE cells.

Homozygosis or *trans*-heterozygosis for the *piggyBac* insertions *scarf*<sup>*PBss*</sup> and *scarf*<sup>*M13.M2*</sup> caused semi-lethality and survivors had scars on the head (data not shown; Bonin & Mann, 2004), phenotypes that were reverted by precise excision of the transposon elements (supplementary information online). As embryos showed no obvious cuticle phenotype (data not shown), we generated stronger alleles of *scarf* by imprecise excision of a P element located in the third intron of *scarf* (*scarf*<sup>*KG05129*</sup>; Fig 1A). We isolated one allele (*scarf* <sup>$\Delta 1.5$</sup> ) that led to the loss of *scarf*

messenger RNA expression (supplementary Fig S2 online). This is an embryonic lethal allele and *trans*-heterozygous combinations of *scarf* <sup>$\Delta 1.5$</sup>  or *Df(2R)nap*<sup>*14*</sup>, a deficiency that uncovers the *scarf* locus, with *scarf*<sup>*PBss*</sup> or *scarf*<sup>*M13.M2*</sup> were semi-lethal and resulted in scars on the head (data not shown). Homozygous animals for *scarf* <sup>$\Delta 1.5$</sup>  or *Df(2R)nap*<sup>*14*</sup> showed similar phenotypes (Fig 2A,B; supplementary Table S1 online). Their larval cuticles were either dorsally wrinkled or presented a dorsal hole or a U-shaped phenotype (Fig 2A–C), which is suggestive of failures in GBR and DC. A high proportion of embryos showed undifferentiated cuticles. Ubiquitous expression of *scarf* largely rescued the *scarf* <sup>$\Delta 1.5$</sup>  phenotype (Fig 2D; supplementary Table S1 online).

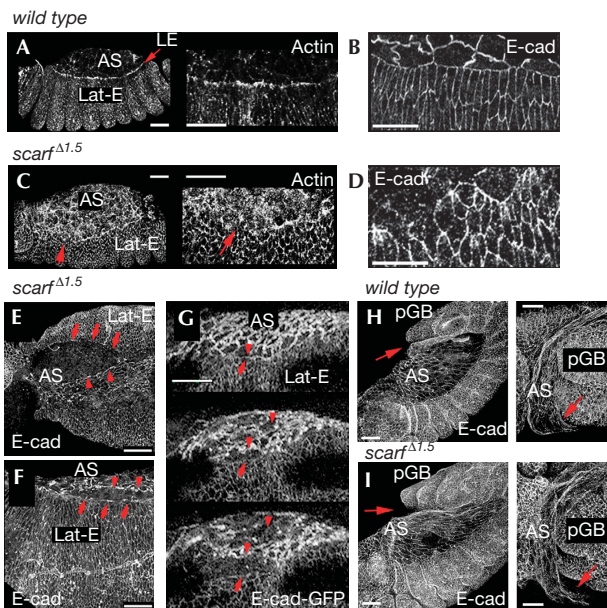
Time-lapse recordings indicated that these embryos were delayed in development (supplementary Table S2 online) and showed a failure in GBR (Fig 2E,F; supplementary Fig S3 online; supplementary Movies S1–S3 online) and DC (Fig 2E,G; supplementary Movies S4,S5 online). Frequently, the attachment of the AS to the tail end of the germ band was compromised (Fig 3H,I) and the germ band did not retract (Fig 2F; supplementary Movie S2 online) or retracted only partly (supplementary Fig S3 online; supplementary Movie S3 online). In embryos that were able to retract (Fig 2G), DC started and LE cells began to direct the movement of the epidermal sheets migrating dorsally over the apical side of the AS. However, either the AS detached from the neighbouring epidermal sheet (Fig 3E–G; supplementary Movie S5 online) or the epidermal sheets met and fused at the dorsal midline but the dorsal epidermis reopened (Fig 2G; supplementary Movie S4 online). We analysed the requirement



**Fig 2** | Germ-band retraction and dorsal closure defects in the absence of *scarf*. (A–D) Cuticle preparations of the indicated embryos. Red arrowheads point to the posterior tail and blue arrows to a hole in the dorsal part of the cuticle. Percentages of embryos with the corresponding phenotypes and the number of embryos scored (*n*) are shown. (E–G) Live image sequences of developing *ubi-E-cad-GFP* (E) and *ubi-E-cad-GFP scarf*<sup>Δ1.5</sup> (F,G) embryos during GBR and DC. E-cad-GFP is shown in white. Red arrowheads point to the pGB. (E) In wild-type embryos, GBR and DC last around 3 h each. (F,G) Defects in GBR and DC are observed in the *scarf*<sup>Δ1.5</sup> embryos. Movies are shown as supplementary information online. Scale bars, 25 μm. AS, aminoserosa; DC, dorsal closure; GBR, germ-band retraction; GFP, green fluorescent protein; pGB, posterior end of the germ band.

of *scarf* in DC in greater detail in fixed staged embryos. During the initial steps of DC, lateral ectodermal cells start to elongate dorsally and an actin cable at the LE is formed, which helps to generate a linear fence at the interface between LE and AS cells (Fig 3A,B; Jacinto *et al*, 2002). In *scarf*<sup>Δ1.5</sup> embryos, elongation of the lateral ectoderm was compromised frequently, the actin cable was not formed properly, the interface between LE and AS cells became irregular (Fig 3C,D), and eventually rips appeared at the AS–LE interface (Fig 3E–G). The elongation of lateral ectodermal cells and adhesion between AS and LE cells are mediated by the activity of Dpp (Affolter *et al*, 1994; Fernandez *et al*, 2007) and JNK activity controls the polymerization of actin into a cable at the LE (Jacinto *et al*, 2002). In embryos lacking *scarf*, the activity of the Dpp and JNK signalling pathways was not affected (supplementary Fig S4 online). Thus, Scarf has a role in DC without affecting the activity levels of these pathways.

Integrins and laminins have a fundamental role in GBR and DC by mediating interactions between AS cells and neighbouring cell populations. As defects were observed in these processes on *scarf* depletion and a reduction in *scarf* levels increased the frequency of cuticle phenotypes caused by depletion of the β-integrin position-specific (βPS) subunit (supplementary Table S1 online), we examined the contribution of Scarf to the regulation of the expression levels and subcellular localization of these proteins. Cross-sectional views of properly staged *wild-type* and *scarf*<sup>Δ1.5</sup> embryos stained for βPS integrin revealed similar levels and localization of integrins (Fig 4A,B). The JNK signalling regulates the expression of the βPS integrin subunit during DC (Homsy *et al*, 2006). Although ectopic activation of JNK induced increased expression of βPS integrin, ectopic expression of *scarf* did not exert this effect (supplementary Fig S5 online). We then analysed the expression of LanA, one of the two existing fly α-laminins. In *wild-type* embryos, high levels of LanA were localized at the



**Fig 3** | Dorsal closure and aminoserosa attachment defects in the absence of *scarf*. Wild-type (A,B,H) and *scarf*<sup>Δ1.5</sup> (C–G,I) embryos labelled to visualize actin and E-cadherin (E-cad) protein expression or expression of an *E-cad*–GFP transgene. Red arrows in (A,C) point to the LE; those in (E–G) point to Lat-E cells; and those in (H,I) point to the attachment between AS cells and pGB. Red arrowheads in (E–G) point to AS cells. Scale bars, 25 μm. AS, amnioserosa; GFP, green fluorescent protein; Lat-E, lateral ectodermal; LE, leading edge; pGB, posterior end of the germ band.

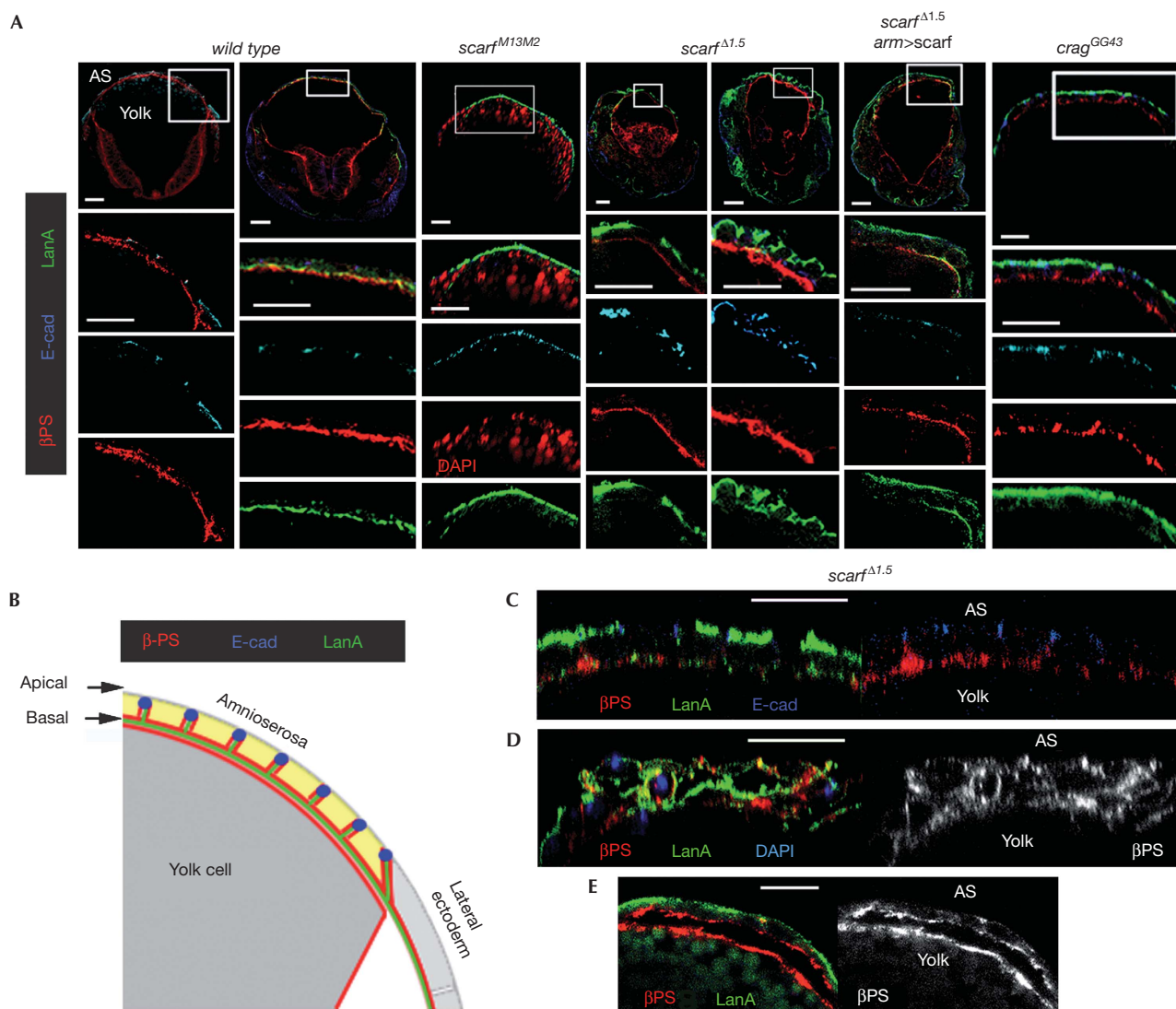
BM of AS cells (Fig 4A,B). Interestingly, *scarf* embryos revealed strong defects in the polarized distribution of LanA. It was localized on the apical side of the AS epithelium and its protein levels were largely reduced in the BM (Fig 4A; supplementary Fig S6 online). Ubiquitous expression of *scarf* largely rescued the levels of LanA at the BM (Fig 4A). We observed similar defects in the polarized distribution of LanA in the lateral ectoderm of *scarf* mutant embryos (supplementary Fig S6 online), and these defects were also rescued largely by the ubiquitous expression of *scarf* (supplementary Fig S6 online). Apico-basal polarity of AS cells, visualized by the basal localization of βPS integrin and the localization of E-cadherin (E-cad) at the adherens junctions, was not affected in hypomorphic *scarf*<sup>PBss</sup> and *scarf*<sup>M13.M2</sup> embryos (Fig 4A; supplementary Fig S6 online) and in some *scarf*<sup>Δ1.5</sup> embryos (Fig 4C) even though LanA was localized on the apical side. However, *scarf* depletion was frequently found to cause defects in the apico-basal polarity of AS cells and this was accompanied by multilayering of the AS epithelium (Fig 4D; supplementary Fig S6 online). These observations suggest that before inducing a loss of epithelial integrity, the absence of Scarf caused aberrant localization of LanA on the apical side of the AS epithelium and reduced LanA in the BM, without affecting the distribution of apical and baso-lateral proteins. As integrins are involved in maintaining epithelial polarity (Schock & Perrimon, 2003; Fernandez-Minan et al, 2008), these results suggest that the observed loss of cell polarity is a consequence of reduced integrin-mediated adhesion to the BM. Consistent with this view, defects in

the attachment of the AS cells to the underlying yolk cell were observed frequently (Fig 4E).

Although the defects observed in *scarf*<sup>Δ1.5</sup> embryos resemble those caused by βPS integrin depletion, only defects in the attachment of the AS with the underlying yolk cells are found in *lanA* mutant embryos (Schock & Perrimon, 2003; Narasimha & Brown, 2004). Mutations in *wing blister* (*wb*), the only other fly α-laminin, cause defects in GBR and in the attachment between AS cells and the posterior germ band (Schock & Perrimon, 2003). These data suggest that LanA and Wb have a redundant function and that Scarf most probably promotes BM localization of not only LanA but also Wb. Consistent with the proposed redundancy, halving the dose of *lanA* or *wb* increased the frequency of the βPS integrin loss-of-function cuticle phenotype (supplementary Table S1 online). We were neither able to monitor the expression of Wb protein in AS cells using the available antibodies nor address the role of Scarf in the polarized distribution of other BM proteins, such as Perlecan or Collagen IV, as these two proteins were not expressed in AS cells (supplementary Fig S7 online).

These results indicate that Scarf depletion causes defects in the BM localization of LanA and in epithelial apico-basal polarity. The defects resemble those observed in follicle cells (that is, epithelial cells covering the fly oocyte) mutant for *crag*, a gene encoding for a protein localized in early and recycling endosomes (Denef et al, 2008) and proposed to regulate protein transport and membrane deposition of BM proteins. Zygotic removal of *crag* induced anomalous localization of LanA on the apical side of AS cells (Fig 4A; supplementary Fig S6 online). Zygotic or both maternal and zygotic removal of *crag* produced cuticles with a weak dorsally wrinkled phenotype (supplementary Table S1 online and data not shown), suggesting that Crag has a redundant function with another protein in the fly embryo and that the low BM levels of LanA observed in these embryos are sufficient to exert their function. Interestingly, halving the dose of *scarf* expression gave rise to a large proportion of *crag* cuticles with phenotypes similar to those caused by *scarf* depletion (supplementary Table S1 online).

We next raised antibodies against Scarf to analyse its subcellular localization. As the antibodies did not work properly at embryonic stages and did not detect endogenous levels of Scarf protein, we used the wing imaginal disc, a monolayered epithelium, to analyse its subcellular localization. Scarf was not detected in wild-type wing cells (Fig 5B). When *scarf* and green fluorescent protein (GFP) were expressed in a restricted domain in the wing disc, Scarf was detected not only in the GFP-labelled *scarf*-producing cells, but also on the apical side of the epithelium at long distances from the source (Fig 5A). When atrial natriuretic factor–GFP—a fusion between the secreted rat atrial natriuretic peptide and GFP—was expressed, the protein was also observed at long distances from the source; however, it did not accumulate on the apical side of the epithelium (Fig 5D). Scarf was found to localize in small punctate structures throughout the cytoplasm of *scarf* non-producing cells (Fig 5F–I). These structures corresponded either to Rab5-positive early endosomes (Fig 5F; 38% of the Scarf vesicles ( $n=185$ ) were labelled by Rab5–GFP), to Rab7-positive late endosomes (Fig 5G,I; 22%;  $n=353$ ), or to Rab11-positive recycling endosomes (Fig 5H; 5%;  $n=140$ ). Together, these results indicate that Scarf is secreted apically and is internalized through endocytosis by non-producing cells. To confirm that Scarf is a secreted protein, *Drosophila* S2 cells were

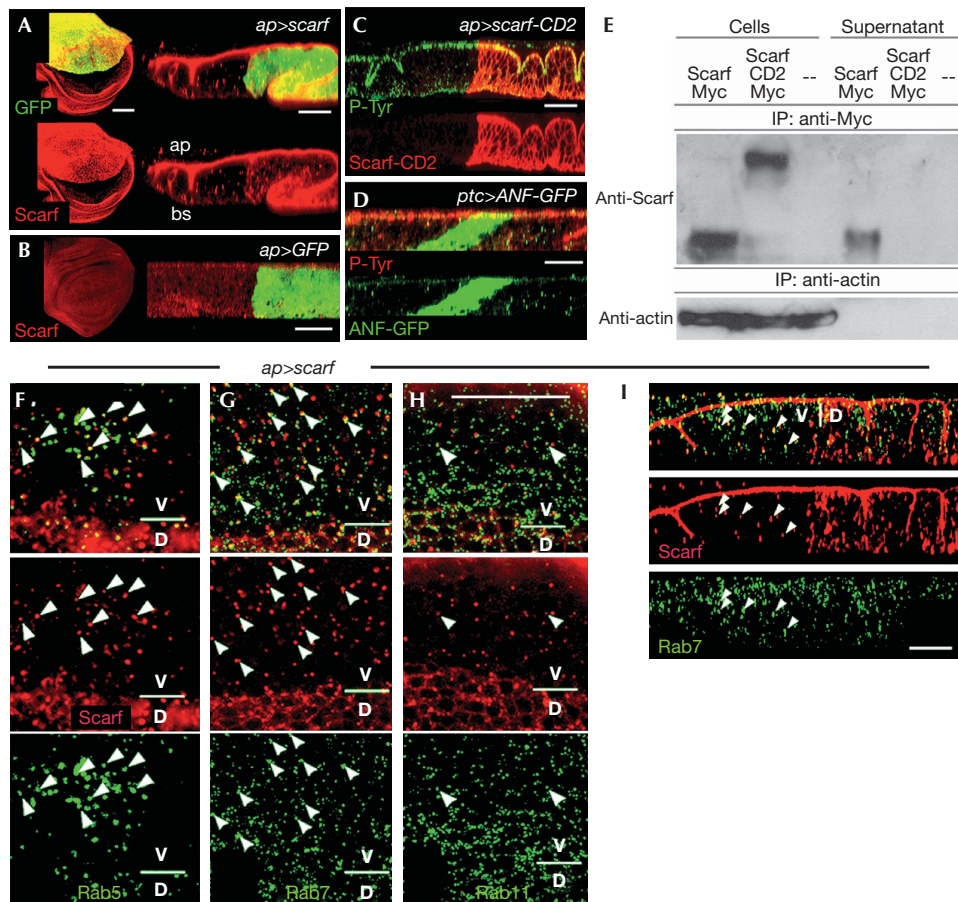


**Fig 4** | Defects in the polarized distribution of laminin A in the absence of *scarf*. (A,C–E) Cross-sectional views of *wild-type* and mutant embryos at stages 12–14 labelled to visualize the subcellular localization of E-Cadherin (E-cad), DAPI or  $\beta$ PS integrin and laminin A (LanA). Lower panels in (A) show magnification of the boxed fields. (B) Schematic representation of a cross-sectional view of the *wild-type* embryo shown in (A). Scale bars, 25  $\mu$ m. AS, amnioserosa; DAPI, 4',6-diamidino-2-phenylindole; PS, position specific.

transfected with either a *scarf*-myc-tagged transgene or a transgene driving the expression of a myc-tagged membrane-tethered form of Scarf (Scarf-CD2; Fig 5C; supplementary information online). Scarf was isolated not only from the protein extract of the *scarf*-myc-expressing cells but also from the supernatant (Fig 5E). Transfected Scarf-CD2 and endogenous actin were not observed in the supernatant (Fig 5E).

Here, we characterize the role of *scarf*, a JNK-regulated gene, in GBR and DC, two morphogenetic processes that rely on cell-matrix interactions between the AS epithelium and neighbouring cell populations. We present evidence that Scarf is a secreted protein expressed in LE cells and involved in promoting the localized accumulation of LanA in the BM of AS cells. Although low levels of Scarf were detected in AS cells (Fig 1B,C), the cuticle

phenotype of *scarf* mutant embryos and the defects observed in BM localization of LanA and epithelial integrity of AS cells were rescued largely by driving expression of a *scarf* transgene only in ectodermal cells (supplementary Table S1 online; Fig 4; supplementary Fig S6 online), indicating that Scarf is acting as a secreted protein. Three alternative hypotheses might explain the role of Scarf and Crag in the polarized localization of LanA to the BM. As Crag and Scarf are localized specifically on the apical side of the epithelium (Fig 5A; Deneff *et al*, 2008), they would have a repulsive role, inhibiting the targeting of LanA-containing vesicles with apical membranes. Alternatively, LanA might be secreted apically and basally and be stabilized preferentially on the basal side or degraded on the apical side. As Scarf encodes for a putative serine protease-like protein without catalytic activity, Scarf would



**Fig 5** | Scarf is a secreted protein. (A–D) Z or XY confocal sections of (A) *ap-Gal4 > UAS-GFP,UAS-scarf* (left panel), (B) *ap-Gal4 > UAS-GFP* (left panel), (C) *ap-Gal4 > UAS-GFP,UAS-scarf-CD2* and (D) *ptc-Gal4 > UAS-ANF-GFP* wing discs labelled to visualize GFP, Scarf and phospho-tyrosine protein expression. (E) Actin (lower immunoblot) and Myc-tagged versions of Scarf and Scarf-CD2 (upper immunoblot) from cell lysates and conditioned medium of S2 cells expressing Scarf-Myc, Scarf-CD2-Myc or an empty vector were immunoprecipitated using actin or Myc antibodies, respectively, and probed using actin and Scarf antibodies. (F–H) Sub-apical XY and (I) Z confocal sections of *ap-Gal4 > UAS-scarf* wing discs labelled to visualize co-localization (white arrowheads) of Scarf protein and (F) Rab5-GFP, (G,I) Rab7-GFP and (H) Rab11-GFP. *ap-Gal4* drives the expression of *scarf* in the dorsal compartment. Scale bars, 25 μm. ap, apical; bs, basal; D, dorsal compartment; GFP, green fluorescent protein; V, ventral compartment.

function, in this scenario, to facilitate the effect of a cascade of serine proteases involved in the degradation of LanA in the apical domain of epithelial cells. Finally, in *scarf* mutant cells, LanA might be transported from the basal to the apical side through transcytosis, a mechanism responsible for the formation of a small apical BM cap over pre-invasive epithelial cells during *Drosophila* oogenesis (Medioni & Noselli, 2005).

Another secreted serine protease-like protein without catalytic activity, Masquerade, regulates cell–matrix interactions at the somatic-muscle attachment in the fly embryo, a process that also depends on integrin and laminin activity (Murugasu-Oei et al, 1995). We speculate that a number of non-functional serine protease-like proteins, such as Masquerade and Scarf, are regulated temporally and spatially to promote the appropriate localization of BM proteins in a context-dependent manner to facilitate cell–matrix interactions and to ensure the maintenance of epithelial integrity. Similarly, among the large class of vertebrate functional

and non-functional serine protease-like proteins involved in remodelling the extracellular matrix (Lopez-Otin & Matrisian, 2007), some of them might exert a similar action.

## METHODS

**Drosophila strains.** *scarf<sup>PBss</sup>* (Bonin & Mann, 2004); *scarf<sup>M13.M2</sup>*, a piggyBac-lacZ insertion, and *scarf<sup>KG05129</sup>*, a P element insertion, are localized in the first and third intron, respectively, of the *scarf* locus. *scarf<sup>A1.5</sup>* is an excision of the *scarf<sup>KG05129</sup>* insertion lacking the 5' end of the P element and containing an intronic deletion of 404 bp of the nearby 5' genomic region (Fig 1A). *Df(2R)nap14* is a deficiency, the breakpoints of which are 41B and 42B1. *UAS-Scarf* and other stocks are described in supplementary information online and in the FlyBase database.

**Antibodies.** Rat E-cad and mouse βPS antibodies were obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, USA), rabbit anti-LanA was provided by S. Baumgartner, other

antibodies are available commercially and antibodies against Scarf are described in supplementary information online. Embryo thick sections were performed as reported by Narasimha & Brown (2004).

**Supplementary information** is available at *EMBO reports* online (<http://www.emboreports.org>).

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#### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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