Integrins mediate their unconventional, mechanical-stress-induced secretion via RhoA and PINCH in *Drosophila*

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Accepted 22 April 2009
Journal of Cell Science 122, 2662-2672 Published by The Company of Biologists 2009
doi:10.1242/jcs.039347

Summary

During the epithelium remodelling such as the flattening of the *Drosophila* follicular epithelium, the α-integrin subunits are unconventionally secreted through a dGRASP-dependent route that is built de novo. The biogenetic process starts with the upregulation of a small subset of targeted mRNAs, including *dgrasp*. Here, we show that *dgrasp* mRNA upregulation is triggered by the tension of the underlying oocyte and by applied external forces at the basal side of the follicular epithelium. We show that integrins are also involved in *dgrasp* mRNA upregulation and the epithelium remodelling. Tension leads to the recruitment of RhoA to the plasma membrane, where it participates in its remodelling. The LIM protein PINCH can cycle to the nucleus and is involved in *dgrasp* mRNA upregulation. We propose that integrins are involved in triggering the biogenesis of their own unconventional secretion route that they use to strengthen adhesion and ensure epithelial integrity at the next stages of development, perhaps by acting as mechanosensors of the underlying tension through RhoA and PINCH.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/15/2662/DC1

Key words: Integrins, Epithelium, *Drosophila*, RhoA, PINCH, LIM, dgrasp, RNA upregulation, Targeting, Mechano-sensing

Introduction

In addition to stabilised cell-cell contacts, epithelium integrity requires adhesion of the basal side of epithelial sheets to the extracellular matrix (ECM) components such as laminin, collagens and in mammals, fibronectin. This is mediated by the large extracellular domains of integrins that are integral plasma membrane α/β heterodimers (Hynes, 1992). Mammals display 18 α-subunits and 8 β-subunits, which are assembled into 24 distinct integrins (Hynes, 2002; Hynes and Zhao, 2000). In *Drosophila*, this reduced to five α-subunits and two β-subunits.

Integrins are part of large signalling complexes. Although they do not exhibit enzymatic activities, they recruit proteins and factors at their cytoplasmic tails that mediate signalling events (Zamir and Geiger, 2001; Lo, 2006). Among many others, the integrin intracellular domain associates with the cytoskeleton through talin and tensin. These two proteins integrate signals and exert their effect by orchestrating changes in the cytoskeleton and by inducing gene expression, through small GTPases, such as Rho, Cdc42 and Rac (DeMali et al., 2003). Integrins also interact indirectly with the LIM family of proteins, including zyxin, paxillin and PINCH (particularly interesting new cysteine-histidine-rich protein) (Kadrmas and Berckerle, 2004).

By linking the ECM to the cell cytoskeleton, integrins are bidirectional-signalling molecules that are able to transduce two types of signals. First, inside-out signalling (also called integrin activation) is regulated by intracellular events leading to a change in the integrin conformation and affinity. Second, outside-in signalling induced by ECM binding can trigger a variety of cellular responses, including actin polymerisation and cell spreading, proliferation, induction of gene expression, initiation of differentiation, and suppression of apoptosis (Hynes, 1992; Juliano and Haskill, 1993; Williams et al., 1994; Hughes and Pfaff, 1998; Takada et al., 2007). In both signalling events, once bound to an ECM ligand, integrins often cluster into adhesion complexes, the best characterised being focal adhesions.

Throughout animal development, the construction of the body is achieved through epithelial morphogenesis during which epithelial sheets transform into more complex shapes. The remodelling includes epithelial migration, folding, delineation and changes in cell shape to form virtually all organs. For this to happen, the epithelium remodels its junctions (Lecuit and Wieschaus, 2002), and it modifies its adhesion. The adhesive properties of integrin have to be downregulated. This can be mediated by (de)-phosphorylation of the focal adhesion components (Cohen and Guan, 2005); the integrins themselves can be downregulated by endocytosis, and their synthesis and transport to the plasma membrane might be slowed down.

Once the epithelial sheets have moved and form organs and tissues, new adhesion sites need to be generated. Integrins can recycle from endosomes (Caswell and Norman, 2006), and new integrin molecules can be synthesised and transported to the remodelled plasma membrane, where they trigger the formation of new focal adhesion sites.

Whether integrins themselves are part of the signal that controls their regulated deposition during epithelium remodelling is not known. To test this hypothesis, we chose the follicular epithelium of the *Drosophila* egg chamber as a model tissue in which remodelling can be investigated (Schotman et al., 2008; Wu et al.,

2008; Horne-Badovinac and Bilder, 2005). At mid-oogenesis (stage 10A), the follicle cells are columnar and actively deliver yolk proteins to their apical side, which are then internalised by the oocyte through endocytosis. This uptake leads to the growth of the oocyte, and because there is no division of the epithelial cells to accommodate the oocyte growth, the epithelial sheet flattens by expanding both their basal and apical membrane domains. The membrane required for the expansion of the apical side (facing the oocyte) is probably stored in microvilli (because they seem to disappear from stage 9 to stage 11) (supplementary material Fig. S1), but no membrane reservoir is present at the basal side. Therefore, a portion of the lateral membrane previously engaged in cell-cell interactions, the ZOC (zone of contact) is converted into basal membrane (open ZOC) and now faces the basal ECM (Fig. 8), but it is not equipped for adhesion (Schotman et al., 2008). Both αPS1 and βPS integrin subunits are therefore deposited to this converting membrane during the flattening at stage 10B. There, they recruit focal adhesion components, such as ILK and PINCH, and F-actin is polymerised, leading to a strengthening of adhesion (Schotman et al., 2008).

Surprisingly, αPS1 and βPS take different routes to the open ZOC. βPS follows the typical ER–Golgi–plasma-membrane pathway similarly to most transmembrane proteins. By contrast, transport of αPS1 is seemingly independent of the Golgi and uses an unconventional secretion route that depends on dGRASP, the *Drosophila* homologue of the peripheral Golgi protein GRASP65 and GRASP55, as well as on its mRNA transcript. In a *dgrasp* mutant, αPS1 is retained intracellularly and the follicular epithelium is strongly disorganised (Schotman et al., 2008). In fact, *dgrasp* and αPS1 *Drosophila* mutant (*mew* alleles) share some striking similarities. A similar pathway also operates during wing disc elongation (Schotman et al., 2008). Taken together, this illustrates the existence of a dGRASP-dependent unconventional integrin secretion pathway.

We have proposed that this unconventional route is built up de novo, and one of the first steps in this biogenesis is the targeting of *dgrasp* mRNAs, as well as a small number of mRNAs encoding proteins functioning in the secretory pathway, that we name open-ZOC mRNAs (Schotman et al., 2008). *dgrasp* mRNAs are translationally active and encoded protein is locally translated and anchored at the open ZOC. αPS1 mRNA is then also upregulated and basally localised (Schotman et al., 2008) and dGRASP mediates the deposition of newly synthesised αPS1 protein to the open ZOC.

Here, we have deciphered the nature of the upstream signalling events leading to the upregulation of the open-ZOC mRNAs (taking *dgrasp* as an example) and ultimately to the biogenesis of this unconventional integrin secretion route. We show that the oocyte growth and tension are necessary for the *dgrasp* mRNA upregulation in the follicular epithelium at stage 10A. Similar results were obtained when external pressure was applied to the egg chambers. The integrins present at the basal side of the follicle cells are also required, because this upregulation is not observed in their absence. We show that on one hand, pressure to the follicular epithelium induces the recruitment of RhoA to the closed ZOC, leading to its opening. On the other hand, it leads to the transduction of a signal to the nucleus via PINCH, leading to the upregulation of the open-ZOC mRNAs. These results suggest that integrins could perhaps signal the first step in the biogenesis of the unconventional secretion route in response to the mechanical stress imposed by the growth of the oocyte at stage 10A or by external forces. This route is then used by the α-subunit for its targeted deposition at stage 10B. We propose that integrins are able to prepare the cellular and membrane context necessary for the next stage of development in which they are required, making them the key regulators of their own function in epithelial integrity. In a broader perspective, this sheds light on how developmentally controlled signalling and membrane traffic are integrated in vivo to sustain events as important as tissue dynamics.

Results

dgrasp RNA upregulation and targeting

The targeting of the open-ZOC mRNAs is a striking feature of the events taking place at the onset of the follicle-cell flattening. However, whether this correlates with RNA upregulation, or to a mere relocalisation of transcripts present at other cellular locations needs to be determined. We used fluorescence in situ hybridisation (FISH) and confocal microscopy (see Materials and Methods) to evaluate the level of mRNA expression using two open-ZOC mRNAs, *dgrasp* and *dgos28*, at defined stages of oogenesis (Fig. 1A).

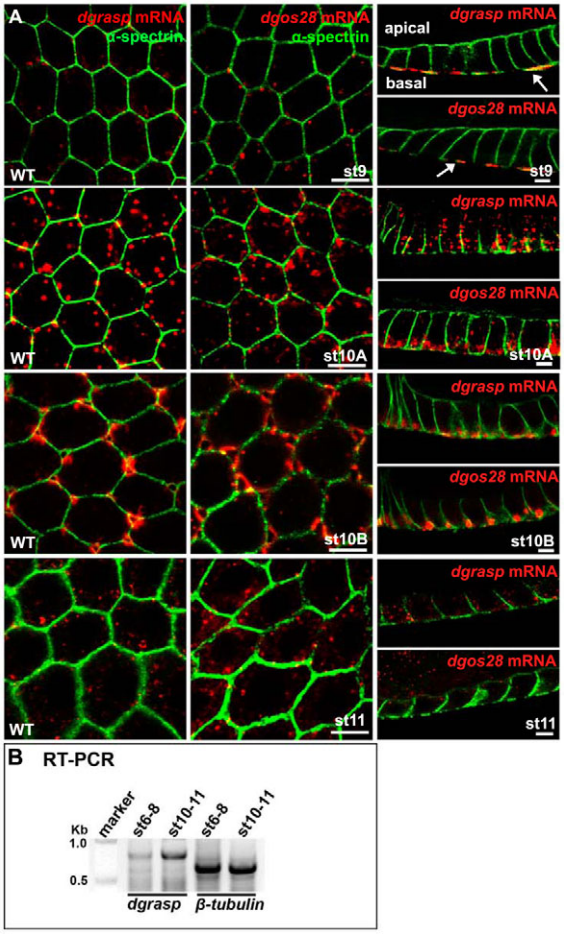

Fig. 1. *dgrasp* mRNA is upregulated at stage 10A. (A) Fluorescence in situ hybridisation (FISH) detection of *dgrasp* and *dgos28* mRNA (red) from stage 9 to 11 follicle cells with α-spectrin labelling the plasma membrane (green). mRNA expression patterns are prominent in stage 10A,B follicle cells. Panels in the two left columns show cells viewed from the basal side, and panels in the right show an apical-basal orientation. The white arrows indicate background labelling in the muscle layer tightly associated with the stage 9 follicular epithelium. (B) RT-PCR of the *dgrasp* (820 bp) and *β-tubulin* mRNA transcripts (703 bp) on egg chambers at stage 6-8 and stage 10-11. Note that *dgrasp* mRNA expression is increased threefold in the latter stages. Scale bars: 5 μm.

At stage 9, both mRNAs were hardly detectable in the cytoplasm. At stage 10A, the number of the cytoplasmic mRNA spots increased significantly both apically and basally, although they appeared to be more concentrated at the basal side (Fig. 1A, see side view stage 10A). At the onset of cell flattening (stage 10B), both mRNAs were found in the typical open-ZOC pattern, that is, in the form of a triangle when viewed from the top basal side (Schotman et al., 2008). At stage 11, when the cells have flattened, the mRNAs were downregulated. These results show that the mRNAs are upregulated from stage 9 to 10A followed by their targeting to the basal side and their concentration around the open ZOC, where they form this unique pattern at stage 10B.

To confirm the upregulation of *dgrasp* mRNA, we performed RT-PCR on egg chambers at different stages of development (Fig. 1B). Using nested primers, we showed that *dgrasp* mRNA expression increases at least threefold in stages 10A-B egg chambers compared with those at stage 7-8, for comparable levels of mRNA encoding β-tubulin. Taken together, these results suggest that *dgrasp* and *gos28* mRNAs are upregulated at stage 10A and maintain a high expression until stage 11. In the subsequent experiments, we therefore used the upregulation of the *dgrasp* transcript as a read-out for the biogenesis of the unconventional secretion route.

Impaired yolk uptake reduces the open-ZOC mRNA expression

Open-ZOC mRNA upregulation is concomitant with epithelium re-arrangement, whether for the follicle cells or the elongating wing disc cells (Schotman et al., 2008). In the follicular epithelium, the open-ZOC mRNA upregulation at stage 10A coincides with the growth of the oocyte that has started at stage 8. At stage 10A, the oocyte has already tripled its size. We hypothesised that the increase of the oocyte size imposes a tension to the follicular epithelium. In turn, the follicle cells could transform this mechanical cue into a chemical signal sent to the nucleus to initiate mRNA expression.

To test this, we used a *yolkless* (*yl*^{15}) mutant (DiMario and Mahowald, 1987; Schonbaum et al., 2000). The receptor Yolkless mediates the uptake of yolk into the oocyte, and, in the *yl*^{15} mutant, the yolk occupies only 10% of the cytoplasm versus 35% in the wild type (Fig. 2A) (Vanzo et al., 2007). The oocytes are slightly smaller but they are deflated. Using FISH to detect *dgrasp* mRNA, we found that it was hardly upregulated in *yl*^{15} egg chambers when compared with the wild type at stage 10A (Fig. 2B), suggesting that the oocyte growth and tension through the yolk uptake is a major trigger initiating the open-ZOC mRNA upregulation.

In addition, we noticed that the follicular epithelium was disrupted in *yl*^{15} mutants (Fig. 2C), as it is in the *dgrasp* mutant. This suggests that mechanical tension imposed by the oocyte to the follicular epithelium might lead to proper adhesion and follicular integrity at later stages of development.

To rule out the idea that Yolkless itself has a role in mRNA upregulation, we repeated a similar experiment using egg chambers of another genetic background that also leads to a less-efficient endocytosis of yolk. We used egg chambers in which actin-GFP (http://flybase.org/reports/FBrf0188667.html) is overexpressed under the control of a triple nanos Gal4 driver (Van Doren et al., 1998) (Fig. 2A). The yolk content of these oocytes, was reduced by at least half, although variability was observed, and clathrin-mediated yolk endocytosis was impaired (C.R., unpublished results). In the wild-type follicular epithelium surrounding these mutant oocytes, upregulation of *dgrasp* mRNA was also reduced (Fig. 2B),

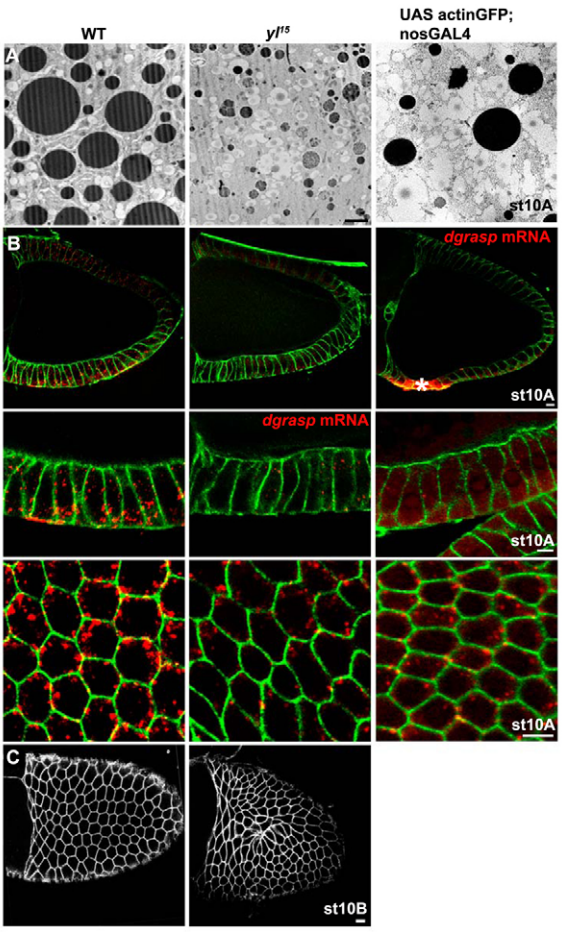

Fig. 2. Oocyte tension induces *dgrasp* mRNA upregulation. (A) Visualisation by electron microscopy of the yolk granules (as electron-dense spheres) in the cytoplasm of a wild-type and *yl*^{15} mutant oocyte and in an oocyte overexpressing UAS actin-GFP under the control of nanos GAL4. Note that in the wild type, the yolk occupies 35% of the cytoplasm and this is reduced to 10% in the *yolkless* mutant and 15% in the UAS actin-GFP;nosGAL4. (B) FISH detection of *dgrasp* mRNA (red) in a stage 10A follicular epithelium surrounding the oocytes described in A. The plasma membrane is labelled with α-spectrin (green). The cells are viewed from the basal side and with an apical-basal orientation. In stage 10A-B mutant follicular epithelium, the *dgrasp* mRNA expression is not upregulated. White asterisks indicate contamination by the surrounding muscle layer. (C) Stage 11 wild-type and *yl*^{15} follicular epithelium. The plasma membrane is labelled with α-spectrin. Scale bars: 200 nm (A), 5 μm (B,C).

confirming that apical pressure and/or tension contributes to the upregulation of *dgrasp* mRNA.

We then tested whether external pressure applied to the basal side was also efficient in upregulating *dgrasp* mRNA. For this, we designed pressure chambers in which the egg chambers were exposed to a hydrostatic pressure of up to 4 bars. The pressure was applied for 10 minutes followed by 15 minutes of recovery. This treatment resulted in the premature upregulation of *dgrasp* mRNA at stage 9 (compare Fig. 3A and 3C), and in its localisation to the closed-ZOC at stage 10A (compare Fig. 3A′ and 3C′). However, a reduced pressure (2.5 bars) did not change the *dgrasp* mRNA expression pattern (Fig. 3B,B′).

We then investigated whether applying external pressure could rescue the low *dgrasp* mRNA expression observed in *yolkless yl*^{15}

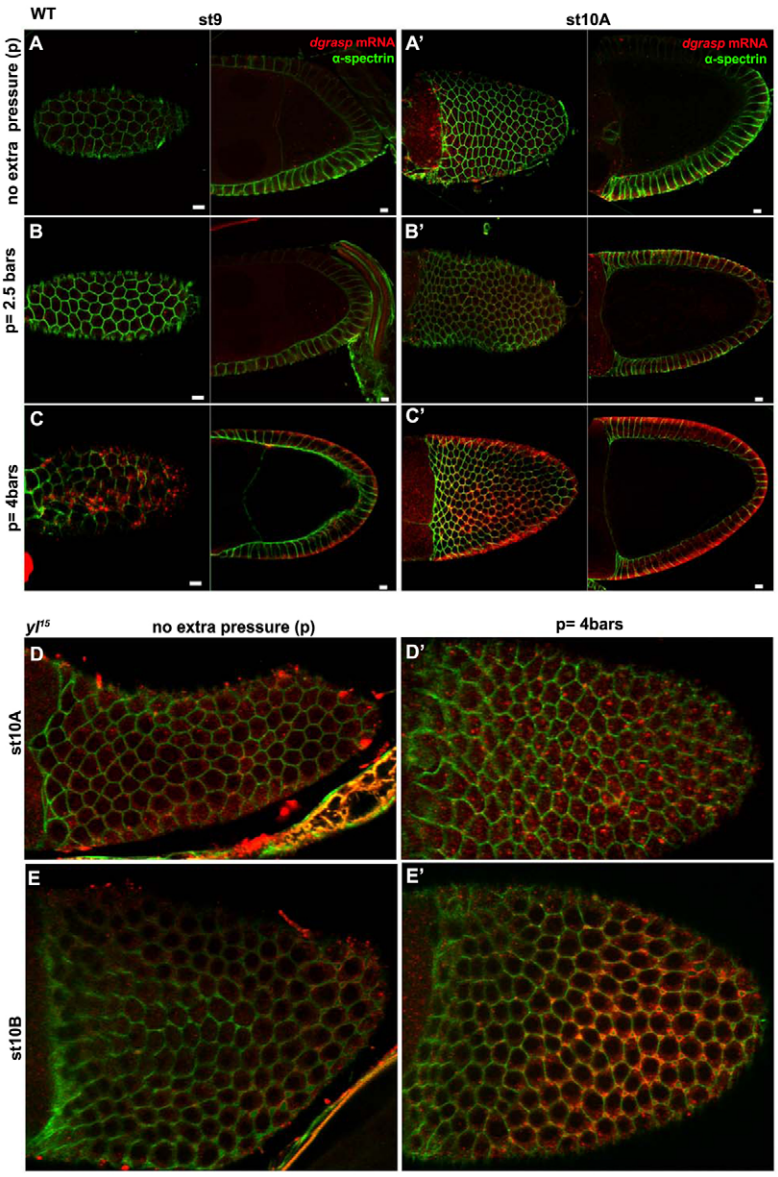

Fig. 3. External basal mechanical stress leads to *dgrasp* mRNA upregulation. (A-C′) FISH detection of *dgrasp* mRNA (red) in a stage 9 (A,B,C) and 10A (A′,B′,C′) wild-type follicular epithelium subjected to external hydrostatic pressure of 0 bar (A,A′), 2.5 bars (B,B′) and 4 bars (C,C′). The plasma membrane is labelled with α-spectrin (green). Note that a pressure of 4 bars elicits a significant upregulation at stage 9 and 10A. (D-E′) FISH detection of *dgrasp* mRNA (red) in a stage 10A (D,D′) and 10B (E,E′) yl^{15} mutant follicular epithelium subjected to an external hydrostatic pressure of 0 bar (D,E) and 4 bars (D′,E′). The plasma membrane is labelled with α-spectrin (green). Note that mRNA expression is rescued by the pressure treatment. Scale bars: 5 µm.

mutants. To do so, we subjected *yolkless* egg chambers to 4 bars of pressure and strikingly, the *dgrasp* RNA expression was upregulated at stage 10A (Fig. 3D,D′) and the transcript was localised at stage 10B (Fig. 3E,E′) as in the wild type.

This suggests that the internal mechanical pressure due to the oocyte tension and/or a possible external pressure is transduced to the basal side of the follicle cells, where it is sensed to elicit the upregulation of the open-ZOC mRNAs.

Integrins are required for *dgrasp* mRNA upregulation

To trigger a signalling cascade leading to mRNA upregulation, the initial mechanical stress induced by the oocyte growth needs to be sensed and transduced. Because integrins link the ECM to the actin cytoskeleton, they are in an optimal position to transform a mechanical signal into a chemical one. On the basis of an elegant experiment that described how stretching of mammalian plasma membrane induces mRNA upregulation near the site of stretching (Chicurel et al., 1998), we hypothesised that the integrins that are deposited at the plasma membrane at stage 10A could be involved in the mRNA upregulation, perhaps by sensing the mechanical stress imposed by the oocyte growth.

To test this, we first showed that αPS1 and βPS are deposited at the basal side of the follicle cells at stage 9-10A (Fig. 4A,A′; supplementary material Fig. S2). Then, we investigated whether *dgrasp* upregulation was observed in the absence of integrins at stage 9-10A. We used hypomorphic *mew* alleles (encoding αPS1) (Wilcox et al., 1981; Brower et al., 1984) (not shown), and induced *mew*-null clones in a stage 9-10A follicular epithelium (Fig. 4B). Using FISH, we found that *dgrasp* mRNA is not upregulated in stage 10A in the absence of αPS1 when compared with levels in the surrounding heterozygous tissue (Fig. 4C). Accordingly, *dgrasp* mRNA was also not targeted at stage 10B (Fig. 4D,D′).

Since βPS is also absent in the *mew* clones (supplementary material Fig. S3A), these results strongly suggest that the αPS1-βPS heterodimer is crucial for the upregulation of the *dgrasp* mRNA, perhaps by sensing the mechanical stress and transducing it chemically to the cell nucleus where transcription is activated, as it is proposed by the tensegrity model (see Discussion).

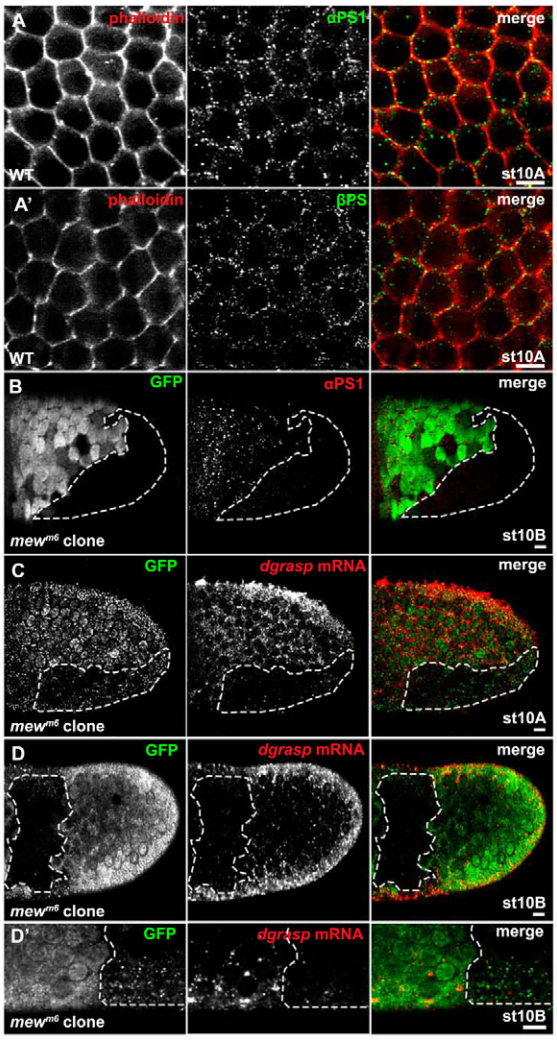

Fig. 4. *dgrasp* mRNA is not upregulated in the absence of integrins. (A,A′) Localisation of integrin subunits αPS1 (A) and βPS (A′) (green) in stage 10A wild-type follicle cells. The plasma membrane is outlined by phalloidin staining of the actin cytoskeleton (red). Cells are viewed from the basal side. (B) Localisation of αPS1 (red) in a stage 10B mosaic follicular epithelium comprising a *mew*^{m6} homozygous clone (outlined with white dashed lines and characterised by the absence of nuclear GFP). Note that αPS1 is absent in the *mew*^{m6} homozygous clone. (C) FISH detection of *dgrasp* mRNA (red) in a stage 10A mosaic follicular epithelium comprising a *mew*^{m6} homozygous clone labelled as above. *dgrasp* mRNA expression is markedly reduced in the *mew*^{m6} homozygous clone compared with the surrounding heterozygous tissue. Note that the GFP is not always neatly visualised in the nucleus when combined with FISH (see Materials and Methods). (D,D′) FISH detection of *dgrasp* mRNA (red) in a stage 10B mosaic follicular epithelium comprising a *mew*^{m6} homozygous clone. *dgrasp* mRNA is not targeted to the open ZOC in the *mew*^{m6} homozygous clone. Scale bars: 5 μm.

RhoA is involved in ZOC opening, but not in *dgrasp* mRNA upregulation

Whether they act as mechanosensors or not, integrins are involved in the upregulation of *dgrasp* mRNA. However, they have no enzymatic activity and they interact directly or indirectly with a number of proteins that activate signalling cascades. Many of the outside-in integrin-mediated signalling events involve actin dynamics that are mediated by the small GTPases RhoA (also named Rho1), Cdc42 and Rac. These are key signalling components that promote cytoskeletal rearrangements, leading to the formation of actin stress fibres, filopodia and lamellipodia, respectively (Schoenwaelder and Burridge, 1999), all in response to integrin activation and signalling.

Using immunofluorescence, we first investigated RhoA localisation in the follicular epithelium at different stages, and found that its localisation was dynamic (Fig. 5A). In particular, RhoA was strongly concentrated to the closed ZOC at stage 10A (arrows in Fig. 5A), but at a much reduced level at the open ZOC at stage 10B (arrowheads in Fig. 5A). To elicit the cascade of events leading to actin polymerisation, RhoA needs to be activated, and we therefore investigated the localisation of Pebble-GFP. Pebble is a RhoGEF that has been shown to activate Rho1, at least during cytokinesis (O'Keefe et al., 2001; Prokopenko et al., 1999). Expression of Pebble-GFP in the entire follicular epithelium resulted in the lack of female progeny. Therefore a driver that promoted expression in the anterior part of the tissue was used. Although mostly nuclear in interphase cells, Pebble-GFP was clearly visible at the basal plasma membrane at stage 9 and 10 A (supplementary material Fig. S4A) and disappeared from the membrane at stage 10B (supplementary material Fig. S4B), consistent with the localisation of RhoA in the corresponding stages.

To examine whether the dynamics of RhoA is relevant to the building of the unconventional secretion pathway, in particular with regard to the integrin signalling, we assessed RhoA dynamics in the *mew* clones. We found that in the absence of integrins, RhoA no longer concentrated at the closed ZOC at stage 10A (Fig. 5B,C). To rule out a global focal adhesion destabilisation because of the absence of integrins, we repeated this experiment in the *yolkless* mutant *yl*^{15}. Indeed, integrins were properly localised in this mutant (supplementary material Fig. S5), yet RhoA was not localised to the closed ZOC in this mutant (Fig. 5D, st10A, and compare with Fig. 5A, st10A, arrows), similarly to the *mew* clones, suggesting that dynamic distribution of RhoA to the ZOC is triggered by the integrin signalling.

We next addressed the function of RhoA at the ZOC. We first used the specific RhoA-associated kinase (ROK) inhibitor Y-27632 and the myosin II inhibitor blebbistatin. We found that egg chambers cultured in the presence of these inhibitors did not exhibit an open ZOC at stage 10B (compare Fig. 6A′,B′ with Fig. 6C′, arrowheads). To test this further in vivo, RhoA was depleted from the follicle epithelium by expressing UAS RhoA dsRNA (#12734 VDRC) under the control of the follicle cell GAL4 driver C355. However, the cross was female sterile at 25°C and 18°C. Using the weaker anterior follicle cell driver 55B, we got few females, which did not display open ZOC at stage 10B in the first anterior third of the follicular epithelium (compare Fig. 6D,D′ with Fig. 6E,E′) where RhoA is depleted (supplementary material Fig. S6). Surprisingly, the ZOC did not open as much in the posterior part of the mutant epithelium as it did in the wild type.

In agreement with these results, we found that when RhoA did not localise to the ZOC, such as in the *yolkless* mutant at stage 10B (Fig. 5D, compare with Fig. 5A, arrowheads) or in the *mew* clones (Fig. 5B,C), the ZOCs did not open as much as those in the wild type. Taken together, these results show that the localisation of RhoA to the closed ZOC at stage 10A is instrumental to their opening at stage 10B. This suggests that, probably in response to integrin signalling, RhoA mediates the initial stretching of the epithelium into the opening of the ZOC. Once the ZOC has opened, at stage 10B, RhoA is no longer localised.

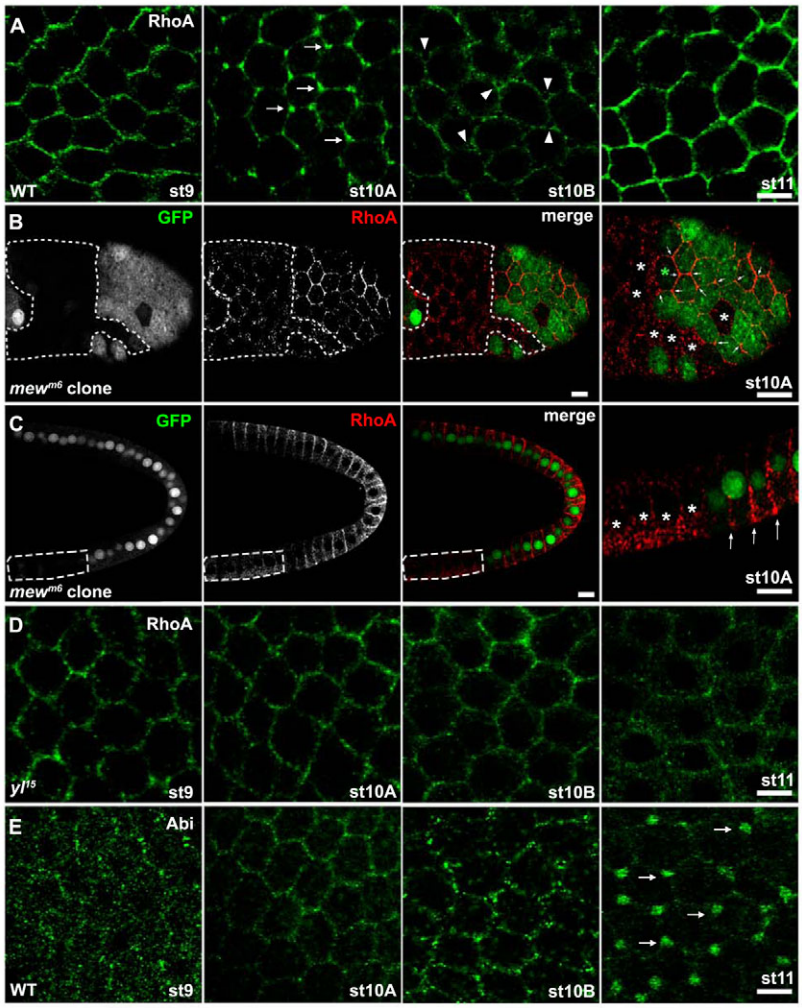

Fig. 5. Integrins induce RhoA localisation to the closed ZOC. (A) Localisation of RhoA (green) in wild-type follicle cells at different stages of egg-chamber development. Note that in a stage 10A follicular epithelium, RhoA concentrates at the ZOC (arrows) whereas it is absent at stage 10B (arrowheads). (B,C) Localisation of RhoA (red) in a stage 10A mosaic follicular epithelium comprising a *mew*^{m6} homozygous clone (outlined with white dashed lines and characterised by the absence of nuclear GFP). Note that RhoA does not localise to the cortex and is not concentrated at the ZOC in the *mew*^{m6} homozygous clone. The follicular epithelium is viewed from the basal side (B) and with an apical-basal orientation (C). (D) Localisation of RhoA (green) in *ylk*^{15} follicle cells at different stages of egg-chamber development. At stage 10A, RhoA does not concentrate at the ZOC. (E) Localisation of Abi (green) in wild-type follicle cells at different stages of egg-chamber development. Abi is enriched at the open ZOC at stage 10B and 11, in agreement with lamellipodia formation, but not on the ZOC at stage 10A. Scale bars: 5 μm.

To assess the specificity of the role for RhoA in the ZOC opening, we investigated the localisation of one of the Rac effectors, Abi (Fig. 5E). Abi and Scar form a complex with three other partners to mediate the recruitment of Arp2/3, thus leading to the polymerisation of F-actin (Stradal et al., 2004). The Abi localisation pattern was very different from that of RhoA. In particular, it was not enriched at the ZOC at stage 10A. Rather, it became gradually enriched to the open ZOC at stage 10B and was found to be concentrated at stage 11 (Fig. 5E), in agreement with the formation of lamellipodia we previously reported to happen at that stage (Schotman et al., 2008). This suggests that at stage 10A, integrins crosstalk preferentially to RhoA to trigger the ZOC opening, though they can also signal to other Rac effectors.

To test whether in addition to basolateral membrane remodelling, RhoA was also involved in the upregulation of the open-ZOC mRNAs, we monitored *dgrasp* mRNA expression in the presence of Rho pharmacological inhibitors. We found that in the presence of Y27632, *dgrasp* mRNA was normally upregulated at stage 10A (Fig. 6A″,B″) compared with the wild type (Fig. 6C,C″), and to some extent targeted to the ZOC at stage 10B, even though it did not open (Fig. 6A,A″). However, in the presence of blebbistatin, no significant targeting was observed, suggesting a direct role for myosin II in this process (Fig. 6B,B″). These results support the existence of a parallel pathway, which is independent of RhoA that is involved in the upregulation of the open-ZOC mRNAs.

The LIM protein PINCH mediates *dgrasp* mRNA upregulation

In theory, this parallel pathway should involve integrins, perhaps through their signalling at focal adhesion sites, and should result in the activated transcription of the open-ZOC mRNAs. Interestingly, the LIM proteins are cytoskeleton-associated focal adhesion proteins that are known to shuttle from focal adhesion sites to the nucleus to modulate gene expression (Cattaruzza et al., 2004; Muller et al., 2002; Kadrmas and Beckerle, 2004). LIM proteins have a double zinc-finger structure (LIM domain), mediating protein-protein interactions and some can bind to DNA in vitro (Nishiya et al., 1998). In this regard, we investigated the role of *Drosophila* LIM protein PINCH.

Drosophila PINCH is involved in integrin-mediated cell adhesion. Furthermore, its focal adhesion localisation depends on integrins (Clark et al., 2003). Accordingly, PINCH is not localised to focal adhesion in *mew* clones (supplementary material Fig. S3B). PINCH contains five LIM domains and interacts with ILK (Tu et al., 1999; Clark et al., 2003). Its *C. elegans* homologue unc-97 functions in mechanosensing and has also been observed in the nucleus (Hobert et al., 1999), although the mechanism that regulates its cycling between the focal adhesion sites and the nucleus is not understood.

To be part of the pathway upregulating the open-ZOC mRNAs during the flattening of the follicular epithelium, PINCH needs to respond to three criteria. First, it should be found at the basal side

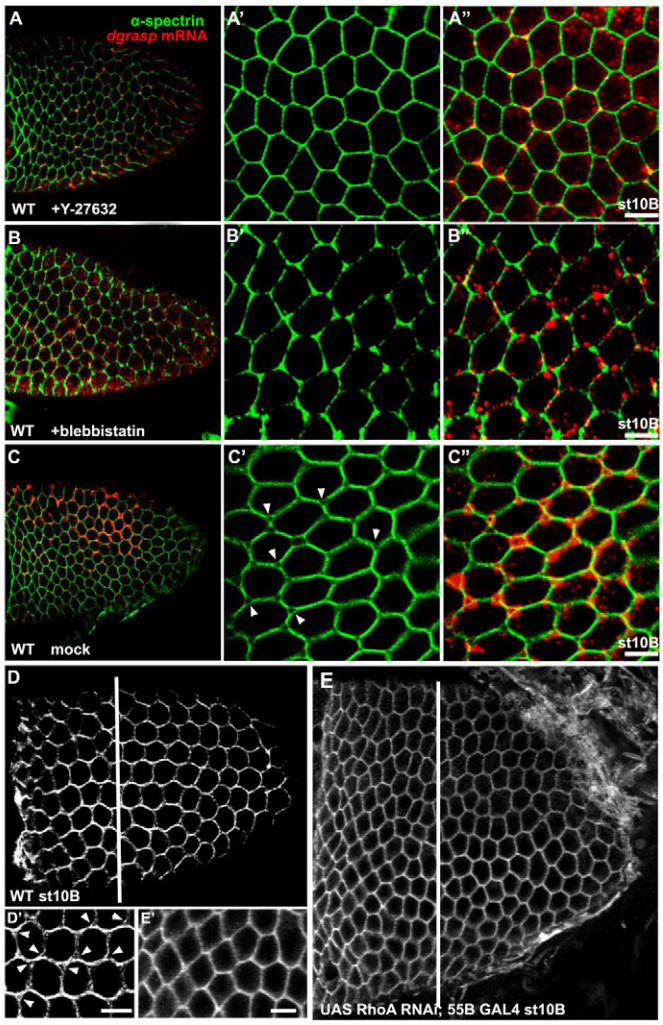

Fig. 6. Inhibition of Rho, ROK and myosin II prevents open ZOC formation. (A-C) FISH detection of *dgrasp* mRNA (red) in stage 10B follicle cells treated with the Rho kinase inhibitor Y-27632 (A), or blebbistatin (B) in a double labelling with the α-spectrin (green) outlining the plasma membrane. Treatment with Y-27632 or blebbistatin prevents the opening of the ZOC (A′,B′), but does not interfere with *dgrasp* mRNA upregulation (A″,B″). In mock-treated follicle cells, the ZOC opening (arrowheads, C′) and the *dgrasp* mRNA upregulation (and targeting) (C″) is not altered. (D-E′) IF localisation of spectrin in wild-type (D,D′) and UAS RhoA dsRNA; 55B GAL4 follicular epithelium (where RhoA is depleted roughly in the anterior third of the tissue, marked by the white line) (E,E′). The anterior third of the follicular epithelium clearly displays the open ZOC in the wild type (D′, arrowhead) and their absence in the depleted tissue (E′). Scale bars: 5 μm.

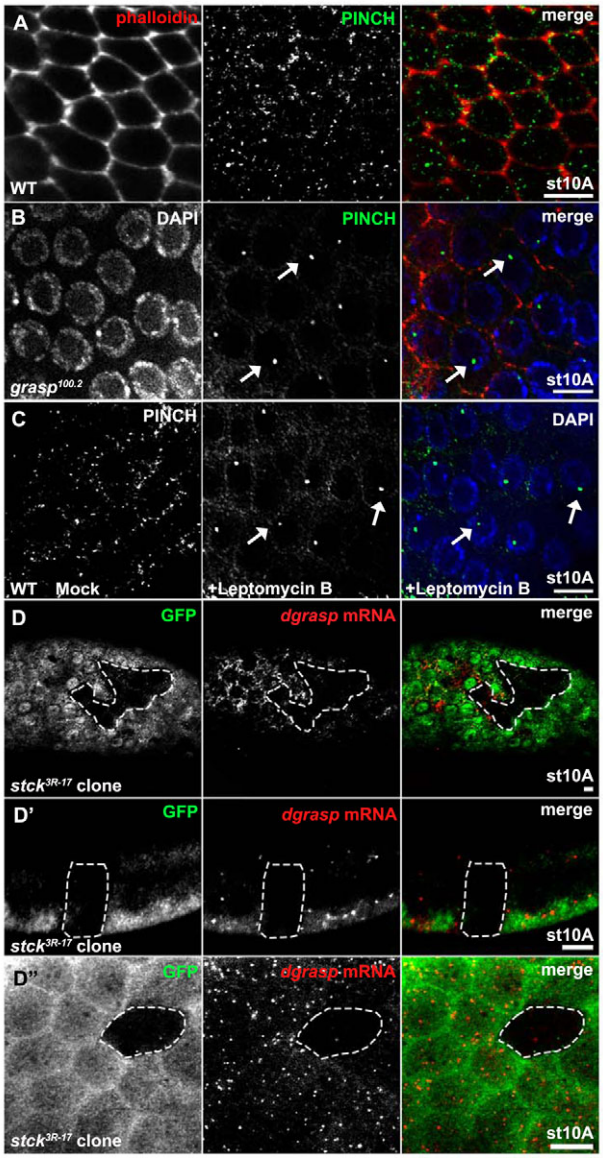

Fig. 7. *dgrasp* mRNA upregulation requires PINCH. (A) Localisation of PINCH (green) in wild-type follicle cells at stage 10A. The plasma membrane was stained with phalloidin. Note that PINCH localises with a similar pattern to αPS1 and βPS in stage 10A follicle cells (compare with Fig. 3A,A′). Cells are viewed from the basal side. (B) Localisation of PINCH (green) in stage 10A *dgrasp*$^{100.2}$ mutant follicle cells in a triple labelling with phalloidin (red) and DAPI (blue). Note that PINCH localises in a single nuclear spot (arrows). (C) Localisation of PINCH in stage 10A wild-type follicle cells non-treated (Mock) and treated with leptomycin B. The last panel in the row shows the nuclear DAPI staining (blue) and PINCH (green). Note that PINCH is trapped in the single nuclear spot under leptomycin (arrows). (D-D″) FISH detection of *dgrasp* mRNA (red) in a stage 10A mosaic follicular epithelium comprising a *stck*$^{3R-17}$ (*pinch*) homozygous clone (outlined with white dashed lines and characterised by the absence of nuclear GFP). Note that *dgrasp* mRNA expression is almost absent in the absence of PINCH. The cells are viewed from the basal side (C,C″) and with an apical-basal orientation (C′). Scale bars: 5 μm.

of the follicle cells at stage 9-10A. Immunofluorescence examination revealed that it is (Fig. 7A). Second, it needs to cycle in and out the nucleus of the follicle cells. To test this, we treated wild-type egg chambers with leptomycin B, a specific inhibitor of the nuclear export factor CRM1 (exportin1) and visualised PINCH by immunofluorescence. In leptomycin-B-treated cells, PINCH was found in one, or sometimes two, dots in the nucleus of the follicle cells (Fig. 7C; supplementary material Fig. S7), as well as in the nurse cells (not shown). The dots did not correspond to the nucleolus, as assessed by DAPI staining. Furthermore, although we do not fully understand the biology of this event, we found that PINCH adopted an identical nuclear localisation in the non-treated *dgrasp*$^{100.2}$ mutant lacking dGRASP (Schotman et al., 2008) (Fig. 7B), strengthening the evidence for the cycling property of this protein. These results indicate that PINCH can cycle in and out the nucleus.

Third, in the absence of PINCH, *dgrasp* mRNA should not be upregulated. To investigate this, we generated PINCH mutant clones

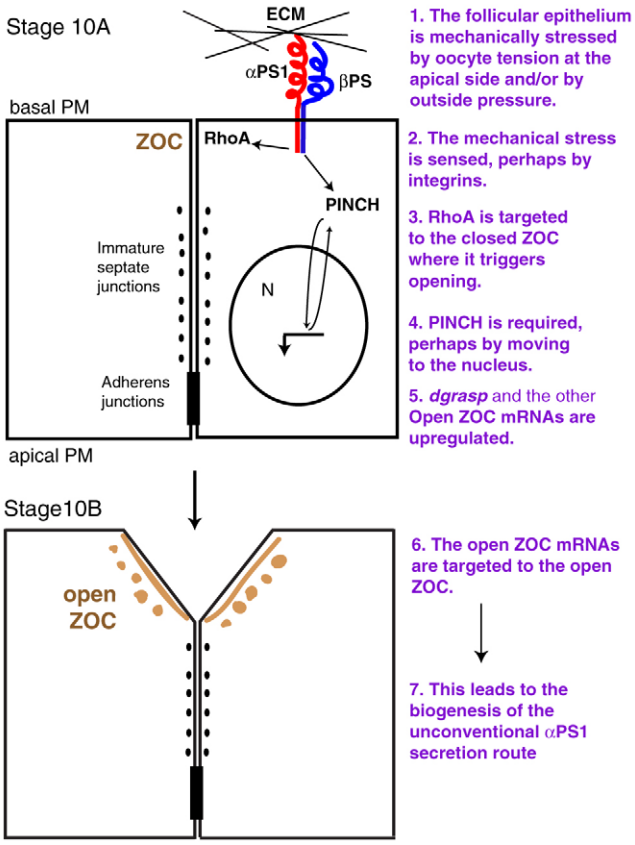

Fig. 8. Upregulation of the open-ZOC mRNAs is mediated by mechanical stress, integrins, RhoA and PINCH, and leads to the biogenesis of the dGRASP-dependent unconventional αPS1 secretion pathway. The oocyte tension or the external pressure exerted to the follicular epithelium (1) is sensed at stage 9-10A at the basal side of the follicular epithelium, perhaps by the integrins (2). As a result, RhoA is recruited to the ZOC and leads to its opening (3). It also triggers PINCH-dependent upregulation of the open-ZOC mRNAs (4-5), thus positively contributing to the biogenesis of the dGRASP-dependent unconventional integrin secretion route at stage 10B (6-7). ECM, extracellular matrix; N, nucleus; PM, plasma membrane.

in the follicular epithelium and assessed *dgrasp* mRNA expression by FISH. In the absence of PINCH, *dgrasp* mRNA was not upregulated at stage 10A (Fig. 7D-D″) and was not targeted at stage 10B. This suggests that PINCH is part of the cascade of events leading to mRNA transcription and that its movement to the nucleus could be triggered by integrin signalling.

Discussion

Our results suggest that there is a series of events leading to the upregulation of the open-ZOC mRNAs at stage 10A, the first step in the biogenesis of the unconventional integrin secretion route (Fig. 8). The signalling events we reported here are endogenous, physiological and developmentally controlled. We propose that the upregulation of open-ZOC mRNAs is triggered by the stretching of the follicular epithelium that is induced by the growth and the tension of the underlying oocyte. This mechanical stress is then transformed into two chemical signals. The first is the relocalisation of the small GTPase RhoA to the ZOC at stage 10A, leading to the active opening of the ZOC. The second is the transduction of a message to the nucleus to activate transcription, and we show here that this is mediated either directly or indirectly by the LIM protein PINCH. Integrins themselves are also involved in the upregulation of the open-ZOC mRNAs, perhaps as mechano-transducers. Taken together, this constitutes one of the first examples of the integration of signalling and secretion where the primary signalling molecule integrin αPS1 is also the substrate of this secretion pathway regulated in time and space.

Mechanical stress induces open-ZOC mRNA upregulation.

During mid-oogenesis, open-ZOC mRNA expression in follicle cells is dynamic and tightly controlled. In particular, the upregulation is very clearly visible at stage 10A in response to mechanical stress. This could be due to the tension imposed by the growing oocyte towards the follicular epithelium. Alternatively, an external force could be directly applied to the basal side of the follicular epithelium, such as the one described here using the pressure chamber. This perhaps mimics the pressure exerted by the muscle layer surrounding each ovariole, acting along the long axis of each developing egg chamber. Interestingly, it is the contraction of the muscle layer that allows the mature egg to be extruded for fertilisation. The contractions could perhaps serve another function such as triggering open-ZOC mRNA upregulation.

Our results are comparable to the unaxial exogenous compression of *Drosophila* embryos that induces the ectopic expression of the *twist* transcript (Farge, 2003). More generally, they expand the list of environmental stress conditions known to induce mRNA upregulation (Anderson and Kedersha, 2006). Whether the mRNA structures observed in the follicle cells share properties with stress granules or P-bodies remains to be investigated.

As mentioned in the Results, the growth of the oocyte starts at stage 8 but this does not trigger mRNA upregulation. This is probably due to the fact that the follicle cells are migrating at that stage and that their integrin-mediated adhesion is downregulated. The stress imposed by the oocyte tension or the muscle layer might be below a threshold. The measuring of the pressure conditions necessary to initiate the mRNA upregulation remains to be investigated.

Interestingly, in agreement with the role we propose for mechanical stress in inducing the biogenesis of the unconventional integrin secretion route (thus ensuring epithelium integrity), we found that the *yolkless* follicular epithelium is as disorganised as observed in *mew* or *dgrasp*. On one hand, this strengthens the prominent role of the mechano-transduction in maintaining epithelial integrity. Along the same line of reasoning, a recent study has shown that mammary epithelial cells grown in 3D matrix switch from cysts to tumour masses when the matrix tension increases (Paszek et al., 2005). On the other hand, this is surprising because without oocyte growth, the follicle cells would not need to flatten and therefore open their ZOC and deposit integrins to maintain the epithelium integrity. This disorganisation might be due to later events, such as the cytoplasmic dumping that occurs at the end of stage 10B (Wheatley et al., 1995), massively increasing the volume of the oocyte.

Finally, we have shown that the GRASP-mediated unconventional integrin secretion also occurs in elongating wing imaginal discs (Schotman et al., 2008) and we also observed *dgrasp* mRNA upregulation (not shown), suggesting that the mechanical forces exerted on the cells during their rearrangement might trigger a common signalling pathway.

Are integrins mechanosensors?

Since the tension coming from the oocyte has similar effects as pressure applied from outside, we propose that it directly imposes a

mechanical stress to the basal ECM overlaying the follicular epithelium where it needs to be sensed. Integrins have the ability to bind both the ECM and the actin cytoskeleton and they have been shown to act as mechanosensors. In this position, they can sense distortion of the ECM, or an increase in its rigidity, a concept that is encompassed in 'tensegrity' (Ingber, 2006), and transform this mechanical signal into a chemical signal that is sent to the nucleus to initiate mRNA upregulation, as part of the outside-in signalling described in the introduction. We hypothesise that this is the sequence of events in the follicular epithelium as, in the absence of integrins, the open-ZOC mRNAs are not upregulated. Whether integrins indeed play a role in mechano-transduction in the follicular epithelium remains to be investigated further as well as the exact mechanism of this action. The unravelling of the nature and role of intermediates such as P130cas (Sawada et al., 2006), Focal adhesion kinase, or ILK might help shed light on this aspect. It is also possible that the transmembrane proteins of the sub-apical region, the adherens junctions (E-cadherins) or those, such as Cad99C, that are located in the microvilli at the apical side (Schlichting et al., 2006) facing the oocyte, play a role in the transmission of this initial stretching.

In any case, the role of integrins at stage 10A in upregulating the open-ZOC mRNAs constitutes an intriguing case of signalling and secretion integration during tissue dynamics. We propose a feedback mechanism in which integrins sense a change in their environment (here in the epithelium) and signal to initiate the transcription of a number of mRNAs that will elicit the biogenesis of an unconventional secretion route. The route then serves to deposit αPS1 to the plasma membrane, to a locus destined to become a focal adhesion at the next stage of development, thus ultimately strengthening adhesion. Once this is achieved (at stage 11), integrins stop signalling the maintenance of this unconventional pathway. In other words, through their signalling properties, integrins at stage 10A prepare their deposition at stage 10B where they have a crucial role.

RhoA relocalises to the closed ZOCs and leads to their opening.

The outside-in signalling through integrins often leads to actin cytoskeleton rearrangements. Here, we have evidence suggesting that RhoA is one of the small GTPases involved. First, RhoA localises to the closed ZOCs in wild-type stage 10A follicular epithelium but not in *yolkless* mutants nor in *mew* clones, suggesting that this localisation is a response to the sensing of mechanical stress, perhaps through integrin signalling. Second, when the RhoA-ROK-myosin II cascade is inhibited by pharmacological means, or when RhoA is depleted in vivo, the ZOCs do not open, suggesting that RhoA is involved in the epithelium rearrangement. Interestingly, inhibiting ROCK allows mouse mammary epithelial cells to avoid the transition from cysts to tumour masses upon increasing matrix tension, thus avoiding epithelium remodelling (Paszek et al., 2005).

One explanation fitting with our results is that the passive stretching of epithelium due to the oocyte growth is transformed into an active opening of the ZOC. Indeed, RhoA targets molecules, such as ROK that in turn stimulate myosin-II-driven contractility by phosphorylating, and thereby inactivating, the myosin light chain phosphatase (Essler et al., 1998). Here, the concentrated RhoA localisation at stage 10A at the ZOC could lead to the formation of contractile/stress actin fibres (Roeper et al., 2005) that would pull the membrane and open the ZOC. Alternatively, RhoA activity to the closed ZOC could release the mechanism that can keep the membrane in close contact. A last possibility is that the actin polymerisation creates a site for targeted secretion as it is the case in yeast at the bud neck (Guo et al., 2000). This could lead to the specific deposition of membrane to the ZOC leading to their opening. At present, we cannot distinguish between these three possibilities, though inhibiting membrane transport from the ER does not prevent ZOC opening (Schotman et al., 2008).

We can further speculate that the presence of RhoA is instrumental in the formation of focal adhesion site at the open ZOC (Schotman et al., 2008). Indeed, myosin II-driven cell contractility via the formation of stress fibres is necessary for focal adhesion formation (Bershadsky et al., 2006; Chrzanowska-Wodnicka and Burridge, 1996; Volberg et al., 1994; Helfman et al., 1999). This suggests that RhoA localisation to the ZOC could have a role not only in the ZOC opening but also in the recruitment of the focal adhesion components at stage 10B. Furthermore, RhoA has recently been shown to be active in the breakdown of the basement membrane during epithelial-mesenchyme transition, thus allowing the remodelling of the basal plasma membrane. Whether it has a similar role during the follicular epithelium remodelling remains to be investigated (Nakaya et al., 2008).

PINCH is involved in the upregulation of the open-ZOC mRNAs

The RhoA pathway, however, is not involved in the upregulation of the open-ZOC mRNAs. Signalling to the nucleus could therefore involve kinases that integrins interact with but this is not likely to be the case. FAK is involved in mechanosensing and is an ideal candidate to transduce integrin signalling cascade in response to mechanical stress. However, flies lacking the only Fak gene in *Drosophila*, Fak56, are viable and fertile, suggesting that Fak is not essential for integrin function or upregulation in adhesion, migration, or signalling (Grabbe et al., 2004).

Here, we provide evidence that a second pathway that involves PINCH is implicated in the upregulation of the ZOC mRNA. In the absence of PINCH, the open-ZOC mRNAs fail to be upregulated. PINCH is part of the LIM family of proteins that are capable of shuttling between focal adhesion sites and the nucleus (Tu et al., 1999; Clark et al., 2003). PINCH also shares these properties (Hobert et al., 1999) (and this study), and upon entry to the nucleus, it could mediate the open-ZOC mRNA upregulation. We speculate that PINCH derived from the focal adhesion is relocalised to the nucleus in response to the mechanical stress. However, our experiments do not address whether PINCH cycling is instrumental to this upregulation, only that PINCH is required. Thus, we do not rule out that PINCH cycling to nucleus might represent a different pool from the PINCH found in the ZOC, and future work will tackle this possibility. For instance, localisation of ILK and Pinch to integrin adhesion sites and their stabilisation depends on their interaction (Wu, 2005). Therefore, the translocation of Pinch into the nucleus should be associated with a decrease of ILK at the same site.

Also whether the open-ZOC mRNA upregulation is directly mediated by PINCH remains to be investigated. PINCH has not been formally shown to bind DNA in vivo or to have transcription activity, although in vitro studies have shown that LIM domains can bind to DNA (Nishiya et al., 1998), as it is the case for LHX (Shirasaki and Pfaff, 2002), Hic-5 (Nishiya et al., 1998). Other LIM proteins, such as the FHL, have been shown to interact with transcription factors, such as the cyclic-AMP-response-element-binding protein (CREB) (Fimia et al., 2000).

PINCH could also bind a transcription factor and bring it to the nucleus. We favour this hypothesis because PINCH shuttling to the

nucleus is not specific for stage 10A of oogenesis. The specificity would therefore be implemented through the binding of a transcription factor upregulated at this stage. The nature of this factor remains to be elucidated but it does not seem to be Armadillo (β-catenin) as during embryo compression (Farge, 2003), or MAL-D/SRF as during border cell migration (Somogyi and Rorth, 2004).

Interestingly, PINCH localisation to focal adhesions depend on integrins (Clark et al., 2003) (and this study) and in turn, we show here that PINCH is instrumental in the deposition of integrins to newly formed focal adhesion sites at the next stage of development. This shows that in addition to the integrins themselves, RhoA and PINCH also contribute to the preparation of the next stage of development.

Taken together, we propose the model according to which integrins act as mechanosensors, initiating at least two signalling pathways leading to the RhoA induced ZOC opening and to the PINCH dependent mRNA upregulation. Both positively contribute to the biogenesis of the dGRASP-dependent unconventional integrin secretion route (Schotman et al., 2008) (Fig. 8), and to the formation of focal adhesion sites critical for epithelial integrity and necessary during remodelling and morphogenesis. We propose that integrins foresee their own requirement and control their own deposition in time and space at the next step of development.

Materials and Methods

Drosophila stocks and crosses

The *Drosophila melanogaster* stocks come from the Bloomington *Drosophila* stock Center (http://flystocks.bio.indiana.edu/) and the Vienna *Drosophila* RNAi Center (http://www.vdrc.at/stock-screening-center/). The wild-type strain was Oregon R (OreR). The d*grasp*$^{100.2}$ mutant was previously described (Schotman et al., 2008) and UAS-Pebble-GFP was a gift from Robert Saint (Australian National University, Canberra, Australia). The flies were all raised on standard cornmeal-agar medium at 25°C.

To express UAS-Pebble-GFP and UAS-RhoRNAi/CyO, males were crossed with virgins homozygous for the follicle cell driver lines *P{w[+mW.hs]=GawB}c355*, *w[1118]* or *w[*]; P{w[+mW.hs]=GawB}55B* that allow the expression of UAS transgenes in the entire follicular epithelium or in the anterior third of it, respectively. Females carrying the UAS gene were selected.

Expression of UAS-actin-GFP was driven by the nosGal4 driver *y*^{1} *w*; P{GAL4-nos.NGT}40*. To induce *multiple edematous wings* (*mew*) mutant clones, females (y, *mew*^{m6}, *p{neoFRT18A}/FM7*), carrying the mutation (X chromosome) were crossed with males carrying a GFP cell marker (X chromosome), and a flippase under control of a heatshock promoter *w, p{ubiGFP(S65T)nls}, p{neoFRT18A}/Y*; *pr, pwn,p{hsFLP}/+* (which was collected from the cross *w*^{1118} *P{Ubi-GFP(S65T)nls}X P{neoFRT}18A* × *pr*^{1} *pwn*^{1} *P{hsFLP}38/CyO; Ki*^{1} *kar*^{1} *ry*^{506}). The F1 virgin females (*y, mew*^{m6}, *p{neoFRT18A}/ w, p{ubiGFP(S65T)nls}; pr, pwn,p {hsFLP}/+*), were selected and heat-shocked after hatching for 60 minutes at 37°C. The clones are characterised by the absence of nuclear GFP.

The *steamer duck* (*pinch*) mutant clones were induced by crossing females (*y,w; p{neoFRT82B} stck*$^{3R-17}$*/TM6B,Tb*) with males (*pr, pwn, p{hsFLP}/CyO; Ki,kar,ry*). In parallel with this cross, females (*y,w; p{neoFRT82B}, p{ubiGFP(S65T)nls}*) were crossed with males (*pr, pwn, p{hsFLP}/CyO; Ki,kar,ry*). Subsequently, females (*pr, pwn, p{hsFLP}/+; p{neoFRT82B} stck*$^{3R-17}$*/Ki,kar,ry*) were crossed with males (*pr, pwn, p{hsFLP}/+; p{neoFRT82B},p{ubiGFP(S65T)nls/Ki,kar,ry*). The clones were generated by heat-shocking virgin females (*pr, pwn, p{hsFLP}/+; p{neoFRT82B} stck*$^{3R-17}$*/ p{neoFRT82B},p{ubiGFP(S65T)nls*) after hatching for 60 minutes at 37°C, and were characterised by the absence of nuclear GFP.

Note that clones are characterised by the absence of their nuclear GFP. However, the GFP fluorescence disappears when egg chambers are processed for FISH. The GFP is therefore detected by antibodies (Molecular Probes, Eugene, OR, 1:200 or Roche monoclonal 1:100) that give an acceptable nuclear pattern but also a fair amount of background.

Antisense probes, antibodies and reagents

Antisense probes to detect mRNAs were made with the DIG RNA Labeling Kit (SP6/T7, Roche) using the following cDNA clones. For *dgrasp*, a partial cDNA was used as described (Kondylis et al., 2005); the clone RE64493 was used for *dgos28* (Ordway et al., 1994; Kondylis et al., 2001).

The following antibodies were used: mouse monoclonal antibodies against integrin αPS1 (DK 1A4, 1:100), βPS (CF.6G11), RhoA (p1D9, 1:50) and α-spectrin (3A9) (Developmental Studies Hybridoma Bank, DSHB, IA); rat polyclonal anti-DE-cadherin (DSHB, DCAD2, 1:200); rabbit polyclonal affinity-purified serum anti-PINCH (B83/245, 1:500) (Clark et al., 2003); the anti-Abi antibody (Bogdan et al., 2005); the anti-Cad99C (Schlichting et al., 2006). Secondary antibodies conjugated to Alexa Fluor 488 and 568 (Invitrogen, Molecular Probes) were used at 1:500. Phalloidin-TRITC was used at 1:10,000 to label the cortex and 1:1000 to show enrichment at the open ZOC.

Rho-kinase inhibitor Y-27632, blebbistatin and leptomycin B treatment

Ovaries were dissected and placed in Schneider's insect medium supplemented with 10 μM Y-27632 or 20 μM blebbistatin for 60 minutes at 27°C, or 50 nM leptomycin B for 25 minutes at 27°C and with solvent (ethanol) at the appropriate concentration as negative controls. After drug treatment, the ovaries were fixed for 10 minutes in 4% paraformaldehyde (PFA) in PBS. Detection of *dgrasp* mRNA was performed by standard FISH protocol.

Immunofluorescence and RNA fluorescence in situ hybridisation (FISH)

Ovaries were fixed in 4% paraformaldehyde (Polysciences) in PBS for 15 minutes, followed by several washes with PBT (PBS + 0.3% Triton X-100) for IF or PTW (PBS + 0.1% Tween-20) for FISH. They were processed for immunofluorescence (Herpers and Rabouille, 2004) and FISH (Dunne et al., 2002) as described, using the tyramide detection TSA Cyanine 3 (PerkinElmer). After mounting in Vectashield containing DAPI (Vector), imaging was performed on a Leica TCS-NT or Zeiss LSM-510 confocal microscope using a 40× or 63× oil-immersion lens.

Electron microscopy

Wild-type egg chambers were fixed (Vanzo et al., 2007), processed and sectioned along the long axis. Images were captured on a Jeol EX1200 electron microscope. The method for estimation of the volume of the cytoplasm occupied by yolk granules was as described elsewhere (Vanzo et al., 2007).

RT-PCR

RT-PCR of total mRNA extracted from isolated stage-specific wild-type egg chambers (using RNeasy kit from Qiagen). The RT reaction was performed using random primers (random hexamer primers from Promega and with the Superscript III reverse transcriptase kit from Invitrogen) on 400 ng total RNA. The subsequent PCR was performed using the primer pair: (GACCACGTAAACGCAAACCT and GCTCGTGCGTTTCGATCAG) for *dgrasp* and primer pair: (GGCCGGT-CAATGCGGTAACC and GTAGGTGGGCGTGGTCAGC) for β-tubulin 85D.

Pressure treatment of *Drosophila* ovaries

Wild-type and *yolkless* ovaries were dissected and placed in a screw-cap tube with 300 μl Schneider's insect medium supplemented with 8% serum. A needle was perforated through a screw cap and sealed in place. The tube harbouring ovaries was attached to the cap connected to an air supply, and a pressure of 2.5 or 4 bar was applied for 10 minutes. After releasing the pressure, the ovaries were allowed to recover for 15 minutes before fixation in 4% PFA in PBS for 15 minutes at room temperature. Detection of *dgrasp* mRNA and α-spectrin was performed by standard protocols.

We thank the Rabouille group for helpful discussions, and Sandrine Pizette and Madelon Maurice for critically reading the manuscript. We thank Mary Beckerle for the anti-dPINCH, Christian Dahmann for the anti-dCad99C, Christian Klambt for the anti-Abi, Jan Soetaerd for preliminary injection experiments, Robert Saint for the UAS-PebbleGFP fly stocks, Ilan Davis for the nosGAL4 fly stock, the Vienna Stock Center for the UAS RhoA dsRNA fly stock (http://www.vdrc.at/stock-screening-center/), and the Bloomington Stock Center for other fly stocks (https://dgrc.cgb.indiana.edu/). We acknowledge the use of flybase (http://flybase.bio.indiana.edu/). H.S. is supported by a ZonMw grant (NWO 912-04-009) and L.K. by a fellowship from the Academy of Finland.

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