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Morphogenesis of the follicular epithelium during *Drosophila* oogenesis

Memoria de Tesis Doctoral presentada por Ana Fernández Miñán para optar al grado de Doctor por la Universidad de Granada.

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I. INTRODUCTION

During the development of an organism, complex morphogenetic rearrangements of tissues are required to accomplish the final three-dimensional architecture of an adult organism. These rearrangements are achieved by a number of cell biological processes such as epithelial to mesenchymal transitions (EMT; see review in [2]), apical constriction of epithelial cells, cell intercalation [3-5], cell migration [4, 5], and polarized cell division [6, 7].

Epithelial cells posses a well-defined apicobasal polarity [8] [9]. They can undergo a number of morphogenetic movements and changes to sculpt organs and body plans during development. Although it is clear that epithelial morphogenesis is largely driven by cytoskeletal rearrangements and changes in cell adhesion, still an important aim is to understand how these processes are coordinated to construct complex biological structures from simple sheets of cells.

Despite the differences observed among species in epithelia organisation, there are evidences suggesting that epidermis formation and differentiation might share a large number of homologies between *Drosophila* and vertebrates. This thesis utilises *Drosophila* oogenesis as a model system to identify genes required to control epithelial cell morphogenesis. During *Drosophila* oogenesis, the follicular epithelium of the egg chamber exhibits a diverse range of epithelial rearrangements in a genetically accessible tissue, making it an excellent model system for the study of epithelial morphogenesis. An introduction to oogenesis itself together with an overview of the different molecules required for the morphogenesis of some epithelia in *Drosophila* is presented in order to better understand the experiments here described.

I1. A summary of *Drosophila* oogenesis

The *Drosophila* female produces hundreds of gametes during its lifespan in a process known as oogenesis (Fig.1). This process takes place in the two ovaries that occupy the major part of the adult abdomen (Fig.1A, B). Each ovary is composed of about 15 ovarioles where eggs are formed. Each ovariole contains a line of developing egg chambers that proceed through 14 morphologically distinct stages: the previtellogenic stages (S1-7) and the vitellogenic stages (S8-14) (Fig. 1C) [10, 11]. Ovarioles can be subdivided in two regions: the germarium and the vitellarium. The germarium, where egg chambers or follicles are assembled, is divided in four regions (1, 2a, 2b and 3) (Fig. 1D). Region 1 contains the precursors of the gametes, the **G**ermline **S**tem **C**ells (GSCs) [12], ultimately responsible for the production of gametes through the female life span. Oogenesis begins in this region when a germline stem cell divides asymmetrically. One of the daughter cells remains as a stem cell while the sibling cell becomes a cystoblast. The cystoblast (CB) undergoes 4 synchronous mitoses with incomplete cytokinesis giving rise to a cyst of 16 cells called cystocytes interconnected by intercellular bridges called ring canals. In region 2a of the germarium, one of the cells is selected as the oocyte and is placed posterior to the remaining 15 cystocytes, which become polyploid nurse cells [13-15]. At the boundary between region 2a/2b there is another type of somatic cells, the **F**ollicle **S**tem **C**ells (FSCs) [16]. These stem cells give rise to three types of somatic cells: i) a pair of polar cells at each pole of the follicle, ii) 6-8 stalk cells that form a bridge, the interfollicular stalk, connecting two consecutive egg chambers and iii) the follicle cells.

In region 2b, follicle cells migrate to encapsulate the 16-cell germline cyst [13] (Fig. 1D). Later on, in region 3 (also known as stage

1), both the germline cyst and the adjoining follicle cells adopt a round shape and form a complete follicle or egg chamber. During the transition from region 2b to region 3, contact between the germline and the follicle cells is required for the proper positioning of the oocyte at the posterior of the egg chamber, a critical event necessary for the subsequent anterior-posterior patterning of the follicle [13, 14]. Follicles bud off from the germarium and enter the vitellarium as S2 egg chambers. As they move posteriorly along the ovariole, they mature through different stages and increase dramatically in size to produce a S14 mature egg.

There is an interesting and very dynamic pattern of cell proliferation and differentiation during oogenesis. In the case of the somatic cells, while polar cells and stalk cells stop dividing and are already differentiated cells when the egg chamber leaves the germarium (see section on the Patterning of the follicular epithelium), follicle cells continue proliferating until the end of S6 (Fig. 1C). They undergo 5-6 rounds of division reaching a number of approximately 650 cells and form a simple, monostratified epithelium [16-19]. In dividing cells, the orientation of the mitotic spindle determines the position of the two daughter cells after division. Follicle cells fix their spindle parallel to the surface of the germline cyst so that both daughter cells remain within the monolayer and in contact with the germline cells. During these stages, follicle cells not only proliferate but they also interact with the germline cells to pattern the follicular epithelium along the anterior-posterior axis.

From S7-10, after proliferation ceases, follicle cells become polyploid through three rounds of endoreduplication [20]. In addition, from S9 onwards, follicle cells will undergo dramatic rearrangements consisting in changes in cell shape and migration processes that will end up with most of the follicle cells covering the oocyte by the end of S9 (see

section on Rearrangements of the follicular epithelium). Concomitantly, interactions between the follicle cells covering the oocyte and the oocyte will establish the dorsal-ventral axis [21]. At later stages of oogenesis, the follicle cells covering the oocyte synthesise yolk proteins and secrete the eggshell components characteristics of a mature egg.

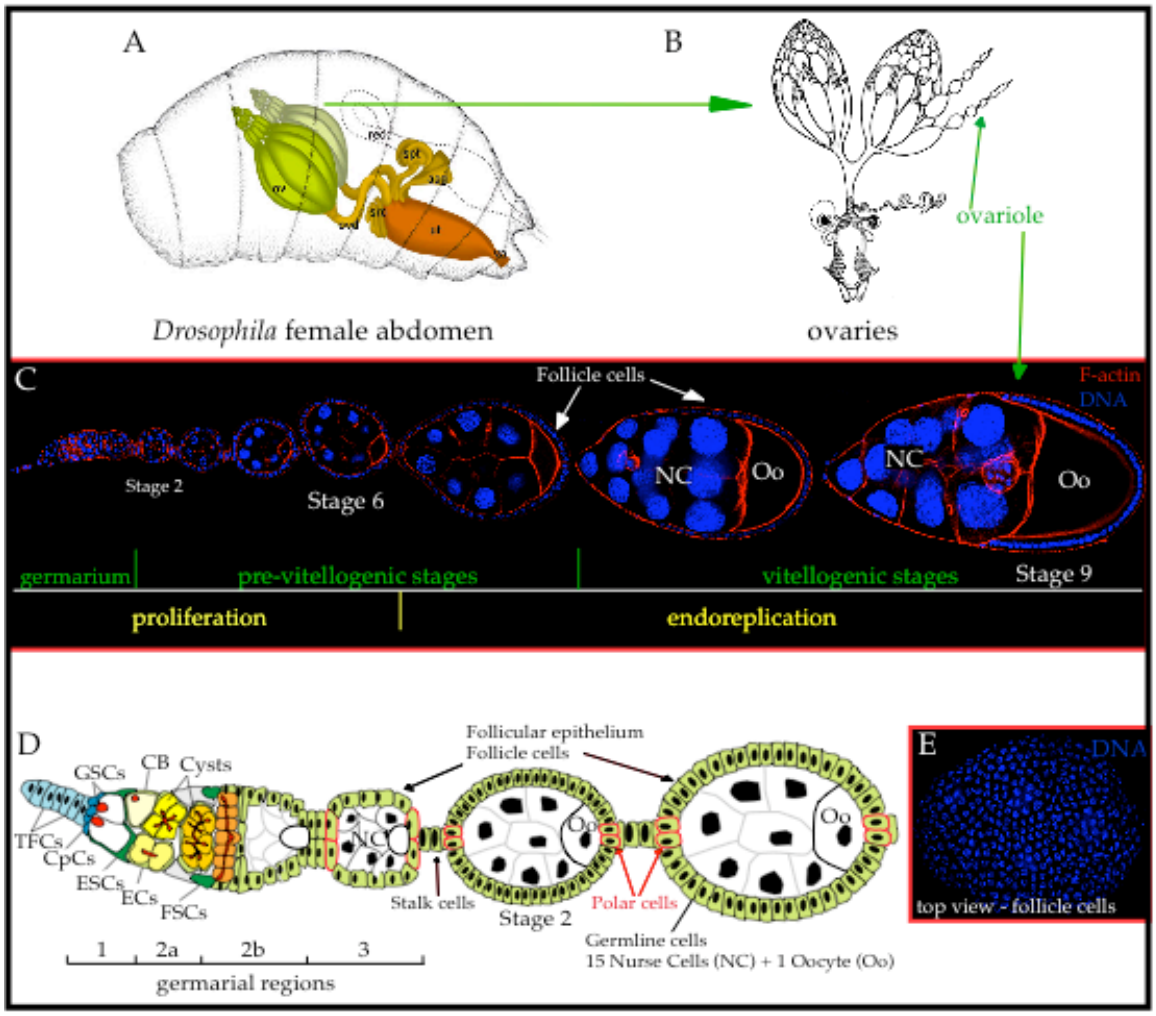


Figure 1. *Drosophila* oogenesis

(A, B) Schematic representation of the *Drosophila* female abdomen and ovaries, respectively. (C) A *Drosophila* ovariole stained against □-Filamentous actin (red) and TOPRO-3 (blue) to label DNA to show egg chambers at different stages of development. (D) Schematic representation of the germarium and S2-3 egg chambers to show the different cell types present in the *Drosophila* ovary. **G**ermline **S**tem **C**ells (GSCs), Cystoblast (CB), **C**ap **C**ells (CpCs), **T**erminal **F**ilament **C**ells (TFCs), **E**scort **C**ells (ECs), **E**scort **S**tem **C**ells (ESCs), **F**ollicle **S**tem **C**ells (FSCs). (E) Top view of an egg chamber labelled with TOPRO-3 to visualize the follicle epithelium monolayer. Unless otherwise noted, anterior is to the left in all figures.

I2. The follicular epithelium

I2.i. Follicle cells display an apical-basal polarity and a planar polarity (Fig. 2).

The follicular epithelium displays features from both primary and secondary epithelia. It is formed through a mesenchymal to epithelial transition, as do secondary epithelia. During this transition, follicle cells are in contact with the basement membrane and the basal domain of these cells is established. Posterior contact with germline cells, in region 2a of the germarium, leads to the initation of the establishment of apical and lateral domains within the follicle cells membrane. As a consequence, *adherens junctions* can be detected as early as S2. By S6, incipient *septate adherens*, where Discs large (Dlg) is found, appear and by S10 they are completely formed [22]. As primary epithelia, the follicular epithelium presents a *zonula adherens* where *D*E-cadherin, *D*β-catenin and armadillo (β-catenin) are localised [13, 23, 24]. Apically to the *zonula adherens*, in the marginal zone, are found proteins such as Crumbs-*D*Patj-Stardust, Bazooka-atypical Protein Kinase C (aPKC)-*D*mPar6 complexes [25-29]. The lateral and basal domains of the follicle cells are in contact with a laminin-rich extracellular matrix. The integrin receptors are expressed at the basolateral domain of these cells in all stages of oogenesis [30].

In addition to the cell membrane polarity, the microtubule cytoskeleton of the follicle cells also display polarity features. The microtubule lattice is polarised with the minus ends of the microtubules at the apical domain [31]. In addition, some of the components of the spectrin network show a differential localisation. Thus, while the β-spectrin subunit is distributed uniformly, the βH-spectrin subunit is found in the apical domain at the *zonula adherens* and the α-spectrin subunit is localised laterally [32, 33].

It has been previously shown that some of these polarity markers are involved in the formation of the follicular epithelium and its subsequent maintenance. For instance, *septate adherens* are essential to keep the follicular epithelium monolayer. Loss of any component of the Discs large-Scribble-Lethal giant larvae complex results in a massive overproliferation and in an invasive behaviour of the Follicle cells into the germline cluster [34-36]. This role of septate jnction components is in contrast with the requirement of the adherens junctions in the maintenance of the structure of the follicular epithelium. The cadherin-catenin complex is required for cell shape maintenance and contact between follicle cells. In fact, loss of *armadillo* (β-catenin) in follicle cells results in loss of cell shape and cell contacts [37]. However, loss of *D*E-cadherin does not have a detectable consequence on the overall structure of the follicular epithelium. This is most likely due to the redundant presence of *D*N-cadherin another typical cadherin [38].

Similar to *adherens junctions* and *septate junctions* components, apical components are also required to maintain the follicular epithelium monolayer. For instance, follicle cells mutant for Crumbs and *D*Patj, which localise to the marginal zone, display defects in polarity and give rise to a multilayered epithelium [37, 39].

In addition to the above mentioned polarity markers, components of the spectrin skeleton are also involved in the maintenance of the simple structure of the follicular epithelium. *βH-spectrin* mutant shows defects in cell shape and in the *zonula adherens* structure [40]. In contrast, *β-spectrin* mutants display more severe defects, the *zonula adherens* is disrupted, apical-basal polarity is lost and the epithelium becomes multilayered [41]. These additional defects are due to the fact that β-spectrin is required for the proper localization of the two β-spectrin subunits.

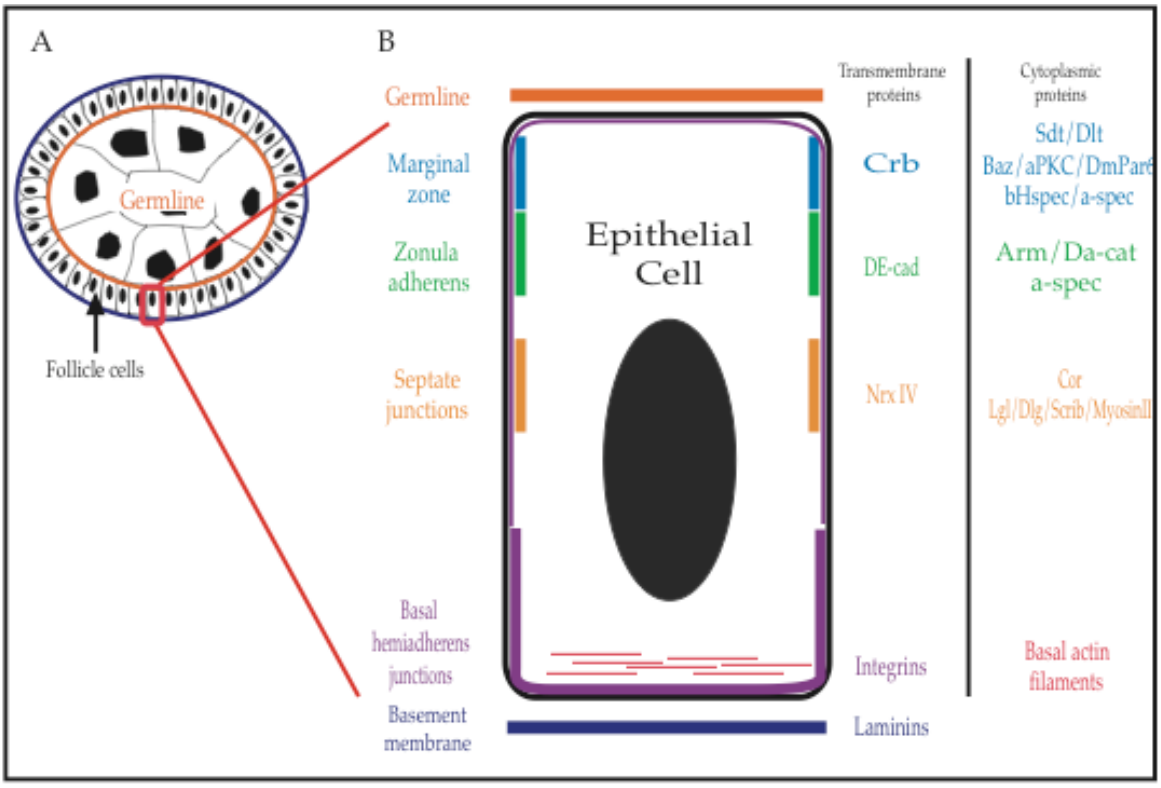


Figure 2. Follicle cells are polarised

(A) Scheme of an egg chamber. The monolayer of follicle cells is surrounding the germline cells. (B) Schematic magnification of a follicle cell after contact with the germline is established. The apical membrane of the follicle cell is facing the germline. Some of the transmembrane and cytoplasmic proteins present in the membrane domains are showed.

In summary, follicle cells are highly polarized epithelial cells, a feature that allows them to exert different functions throughout oogenesis. For example, interactions between the apical follicle cell surface and the germline cells will participate in signalling events such as the establishment of the anterior-posterior axis of the egg (see section on Patterning of the follicular epithelium). In addition, follicle cells secrete the eggshell material from their apical membrane domain at later stages of oogenesis.

In addition to the apical-basal polarity, follicle cells also display a polarity within the plane of the epithelium, known as planar polarity. Follicle cells show a basal array of actin bundles perpendicular to the anterior-posterior axis of the egg chamber [42]. This pattern is first establish during S5-6 around the Polar cells at the follicle poles and then spreads to the centre. At S7 all follicle cells display this stereotyped basal actin organization which is maintained until S14 [42, 43]. Interestingly, the major component of the basal extracelular matrix that surrounds the follicle cells, Laminin A, is organised into complementary circumferential fibres [44]. This polarised organization of Actin and Laminin is thought to act as a molecular corset for the generation of an elongated egg. Mutations in proteins that mediate interactions between the actin cytoskeleton and the extracelular matrix, such as Dystroglycan, the receptor-like tyrosine phophatase *D*Lar and the b subunit of the integrins, produce spherical eggs where the actin fibres are correctly formed but lack a planar orientation [30, 43, 45, 46].

I2.ii. Patterning of the follicular epithelium (Fig. 3)

Interactions between follicle cells themselves and between follicle cells and the underlying germline cells pattern the follicular epithelium. Correct patterning of the follicle cell monolayer along the anterior-posterior and the dorsal-ventral axis is essential to obtain a functional mature egg and to establish the polarity of the oocyte and, as a consequence, that of the future embryo.

The first differences among the follicle cells are already evident when the egg chamber exits the germarium. Two different somatic cell types can be distinguish: i) 5-8 stalk cells form the Interfollicular Stalk (IS), which connects two consecutive follicles and ii) the polar cells, two at each pole of the egg chamber. Stalk and polar cells are determined in the germarium and derive from a single precursor cell type specified by a Hedgehog signal originated in the terminal filament [18, 47, 48]. Hedgehog induces the downregulation of the transcription factor Eyes absent, a polar cell fate suppressor [49].

Early during oogenesis, polar cells can be distinguished within the follicular epithelium because they express higher levels of proteins such as Fasciclin *III* and Neuralised [16, 50], [51]. By S6-8, the polar cells become morphologically different; they round up and lose contact with the basal lamina, and also exhibit a distinct subcellular distribution of some proteins such as *D*E-Cadherin and Armadillo [13, 23, 51, 52]. In addition, polar cells have been highlighted as organizing centres that pattern the follicular epithelium. Indeed, the polar cells are responsible for the patterning of Terminal Domains (TDs) at each pole of the egg chamber in a Notch-dependent manner [13, 18, 19, 53]. The follicle cells found between these terminal domains constitute the Main Body Domain (MBD) (Fig. 3A, B). Thus, the egg chamber is symmetrically patterned with the TDs at the poles and the MBD in between them.

By S6, symmetry is lost when the oocyte, located at the posterior pole of the follicle, signals to the adjacent follicle cells and induces them to adopt a posterior fate [19, 21, 54]. The signal coming from the oocyte is Gurken, a **T**ransforming **G**rowth **F**actor (TGF) □-like ligand of the **E**pidermal **G**rowth **F**actor **R**eceptor (EGFR) pathway. Therefore, the activity of the EGFR pathway is responsible for the establishment of the anterior-posterior axis within the egg chamber.

Follicle cells at the anterior pole do not receive the signal from the oocyte and adopt anterior fates. Depending on the distance from the anterior Polar cells, the subdivision of the follicular epithelium results in the following anterior follicle cell types: anterior polar cells, border cells, stretched cells and centripetal cells (Fig. 3D).

Another pathway involved in the patterning of the follicular epithelium is the **J**anus **K**inase - **S**ignal **T**ransducer and **A**ctivator of **T**ranscription (JAK-STAT) pathway. One of the ligands of the pathway, Unpaired (Upd), is secreted by the polar cells and has been shown to form a gradient required for the specification of both, posterior terminal cell fates within the posterior domain and border cell fate in the anterior one [55-57].

Once posterior follicle cells are determined, they signal back to the oocyte during S6-9 triggering the reorganization of this cell´s microtubule cytoskeleton. As a consequence, the oocyte nucleus and *Gurken* are localised to the anterior/dorsal corner of the oocyte. Meanwhile, and following a series of cell shape changes and cell migration events that take place in the follicular epithelium, most of the follicle cells cover the oocyte by the end of S9. Thus, localised Gurken signals a second time to induce the adjacent follicle cells to adopt a dorsal rather than a ventral fate, establishing the dorsal-ventral axis (Fig. 3F). In addition to the EGFR pathway, Decapentaplegic (Dpp - the *Drosophila* homolog of the human bone morphogenetic proteins BMP2

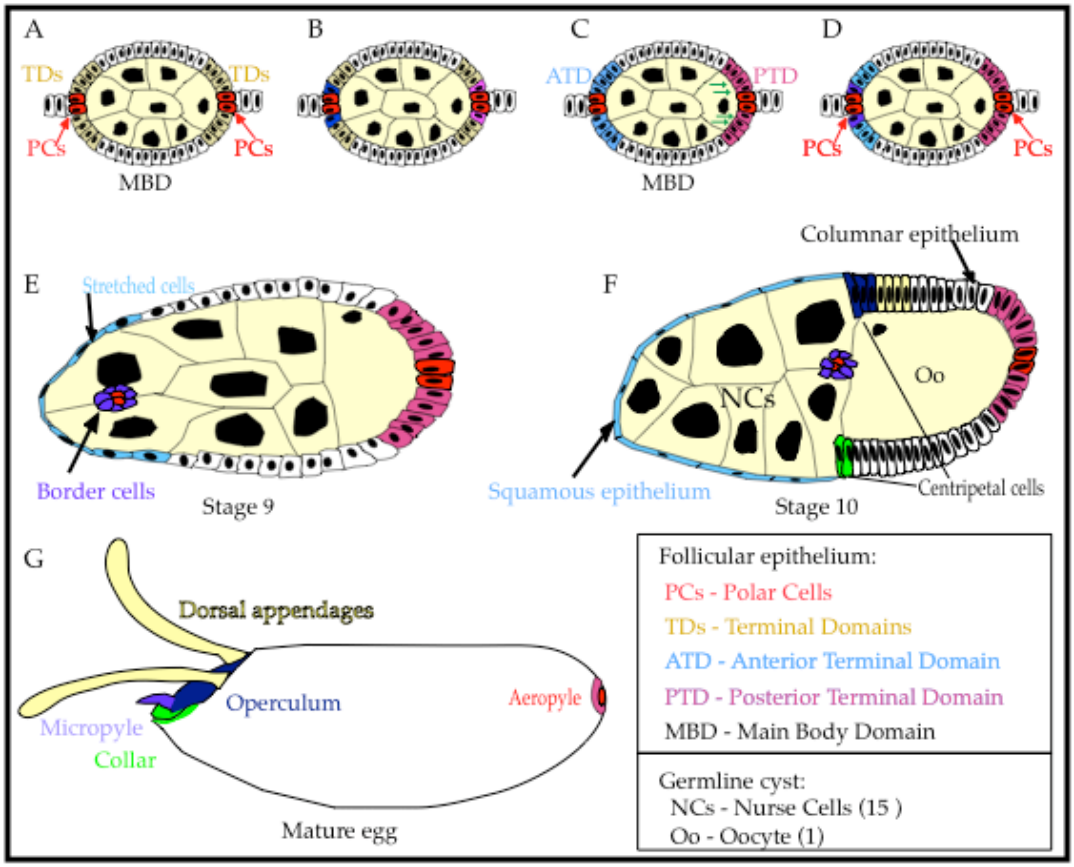


Figure 3. Patterning and rearrangements of the follicular epithelium

(A-D) Egg chamber schemes. (A) Polar cells (red) at both poles of the egg chamber pattern both TDs versus the central MBD. (B) Follicle cells within the TDs are subdivided depending on their distance to the polar cells. (C, D) Symmetry is lost when the oocyte signals to the adjacent follicle cells and induces them to adopt a posterior fate. Follicle cells within the anterior TD are subdivided in anterior polar cells, border cells, squamous cells and centripetal cells. (E, F) At S9 follicle cell rearrangements start: border cells delaminate from the follicular epithelium and migrate posteriorly towards the oocyte. At the same time, main body follicle cells migrate posteriorly to form the columnar epithelium over the oocyte while the stretched cells form the squamous epithelium over the nurse cells. (G) Mature egg chamber showing the diverse eggshell structures formed by the follicle cells. The colour code (F-G) indicates which cell type gives rise to a certain eggshell structure.

and BMP4) signalling is also involved in the patterning of anterior-dorsal eggshell structures such as the operculum and the dorsal appendages [17, 58] (Fig. 3G).

I2.iii. Rearrangements of the follicular epithelium

The follicular epithelium is an excellent model system where to study the processes required for tissue morphogenesis. Initially, the follicle cells within the follicular epithelium acquire a cuboidal shape. As the egg chamber matures, follicle cells undergo a number of morphological changes that include changes in cell shape and in cell motility. During egg chamber maturation three cell migration events can be observed (Fig. 3E, F):

a) Starting at S9, the cuboidal follicle cells change shape and begin to migrate posteriorly, so that approximately 95% of the main body follicle cells form a columnar epithelium over the oocyte. The remaining 5% flatten to form a squamous epithelium over the nurse cells.

b) Simultaneously, a group of 6 to 8 follicle cells, known as border cells, undergo a partial epithelial-mesenchymal transition and delaminate from the anterior pole of the follicular epithelium. Border cells migrate posteriorly following the most direct route between the nurse cells until they reach the anterior membrane of the oocyte by S10. Border cells then migrate dorsally along the nurse cells-oocyte boundary.

c) Migration of a group of follicle cells - the centripetal cells - at S10a between the nurse and the oocyte to cover the anterior part of the oocyte.

At later stages of oogenesis, the different types of follicle cells will form the diverse eggshell structures. The columnar follicle cells over the oocyte synthesize and secrete the vitelline membrane and the chorion. Border cells are necessary for the proper formation of the micropyle pore, an opening in the egg´s outer layers through which the sperm will enter to fertilize the female gamete. Finally, the centripetal cells undergo further morphogenetic processes that result in the formation of bot, the operculum, which will provide an exit for the future larva, and the two dorsal respiratory appendages. At the posterior pole, the polar cells and the adjacent cells will form the aeropyle (Fig. 3G).

I2.iv. Migration of the border cells (Fig. 4)

Border cells execute a stereotyped migration from the anterior pole of the egg chamber to the anterior-dorsal corner of the oocyte where they help to the formation of a functional micropyle [59, 60]. Border cells also play a role in embryonic development, as they express the gene *torso-like* (*tsl*), a secreted protein essential for the correct development of the terminal region of the embryo [61].

Border cell migration is highly regulated, both spatially and temporally. Border cells migrate around 150 microns in approximately 6 hours following the most direct route from the anterior pole of the egg chamber towards the oocyte membrane. Therefore, border cell migration represents an excellent model system in which to study the regulation of cell migration.

The border cell cluster is formed by a group of about 8 follicle cells - 6 outer border cells and the 2 anterior polar cells, which occupy a central position. At stage 8, 4-8 follicle cells adjacent to the anterior polar cells are recruited to form the border cell cluster [62]. Before

migration, border cells are part of the follicular epithelium and adhere to the neighbouring follicle cells.

A number of molecules have been reported to participate in border cell migration. For instance, and much like the polar cells, the outer border cells also upregulate *D*E-Cadherin expression during migration [63]. *D*E-Cadherin is required in both, border cells and nurse cells for border cell migration. This homophilic cell-cell adhesion molecule seems to provide the traction necessary for border cells to travel between the nurse cells towards the oocyte [63]. Interestingly, Myosin VI which is also upregulated in border cells and its loss of function results in a failure of border cell migration, has been shown to be part of a complex with *D*E-Cadherin and Armadillo where it acts to stabilize the components of this complex [64].

In addition to *D*E-Cadherin, another adhesion molecule has been shown to play a role in border cell migration. Fasciclin II, a transmembrane cell-adhesion molecule, controls the timing of border cell cluster motility [36]. Fasciclin II expression is lost from all the anterior follicle cells except from the polar cells at the time of border cell differentiation. Fasciclin II in the polar cells regulates the localization of the tumour supressor proteins Discs-large and Lethal-giant-larvae in border cells. These two genes inhibit the rate of movement of the border cell cluster [34, 65]. At the time of border cell differentiation, Fasciclin II expression is lost from all the anterior follicle cells except from the polar cells [36]. Fasciclin II in turns regulates the localization of Discs-large and Lethal-giant-larvae in border cells [34, 65] to the apical membrane of the cell, facing the germline [36]. Partial loss of FasciclinII, Discs-large or Lethal-giant-larvae leads to a precocious delamination and faster migration of the border cell cluster. However, completely loss of these three proteins causes loss of polar cell polarity

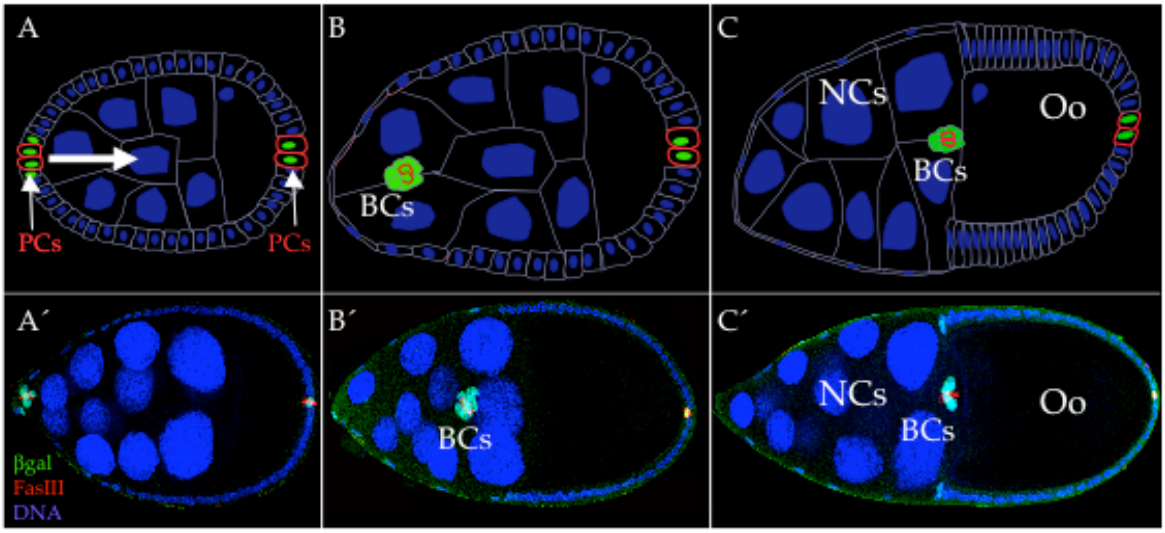


Figure 4. Migration of the border cell cluster

(A, B, C) Schematic diagrams of S8, S9 and S10a egg chambers respectively. PC stands for Polar Cells and BC for Border Cells. (A´, B´, C´) Egg chamber stained against anti-βgal (green) to label the border cells, anti-FasIII (red) to label the polar cells and TOPRO-3 (blue) to label the DNA. (A, A´) At S8, polar cells recruit adjacent follicle cells to form the border cell cluster. (B, B´) At S9 the border cell cluster, with the polar cells in a central position, migrate posteriorly between the nurse cells until they contact the anterior membrane of the oocyte by S10 (C, C´).

resulting in a delayed delamination without affecting the rate of migration.

As a consequence of the acquisition of "motile" polarity, border cells undergo a partial epithelial-mesenchymal transition and delaminate from the follicular epithelium. After losing contact with the neighbouring cells, border cells extend filamentous-actin-filled cytoplasmic protrusions and finer filopodia between the nurse cells and acquire an irregular fibroblast-like morphology [66]. Concomitantly, apical localization of Crumbs and *D*E-Cadherin in border cells is lost but still these cells retain some epithelial characteristics, as they remain attached to each other and to the central polar cells via *D*E-Cadherin-containing adhesive junctions. *D*E-Cadherin is also highly expressed in the cytoplasmic protusions and in fact their formation is abolished when both border cells and the gemline lack *D*E-Cadherin function indicating that formation or stabilization of the border cell cellular extension requires specific substrate adhesion [66]. Polar cells also retain some aspects of epithelial polarity since Crumbs is found at the apical domain of these cells. Polar cells are situated in the centre of the cluster and do not undergo an active migration; rather, they become passive cargoes carried by the outer border cells [67].

I2.v. Genes involved in border cell migration

The transition from non-motile epithelial cells to migratory border cells requires changes in gene expression, cell adhesion and cytoskeleton organization. To date, four different pathways have been described to be involved in border cell determination and migration.

The **Janus Kinase - Signal Transducer and Activator of Transcription** (**JAK-STAT**) **pathway** is involved in the recruitment of the outer border cells and in the transition from stationary to motile cells [56]. This pathway consists of a highly localized cytokine signal that activates initially the Janus kinase and, as a consequence, leads to the activation of the transcription factor STAT that in turn regulates target gene expression [68]. One of the ligands of the pathway, Unpaired, is expressed in the polar cells prior and during migration and it signals to the surrounding cells, which express the Upd receptor Domeless [57, 69]. Mutations in different components of the pathway result in fewer border cells and in a failure to initiate migration. In addition, ectopic expression of the pathway in non-motile cells makes them migratory. Thus, activation of the JAK-STAT pathway is necessary and sufficient to induce migration. Activation of the JAK-STAT pathway leads to the expression of a border cell marker, Slow Border Cells (Slbo), the *Drosophila* C/EBP transcription factor, a basic region- leucine zipper transcriptional activator [57, 59]. Slbo is expressed in border cells before and during migration, and its expression has been shown to be regulated by ubiquitination and proteosome-dependent degradation [70]. Recent genomic analysis of the gene expression changes due to the transcriptional switch promoted by Slbo function have revealed a large number of Slbo targets. Highly represented among these targets are regulators of the actin and microtubule cytoskeleton dynamics, as well as genes involved in the secretory and endocytic pathways and

muscle-specific genes [71, 72]. In addition, *D*E-Cadherin and Myosin VI have been found to respond to Slbo regulation [63, 64]. This relationship has biological significance, since *D*E-Cadherin upregulation fails to occur in slbo mutants border cells [63].

Slbo is essential for border cell motility but not sufficient, since earlier expression of this gene in border cells does not cause a precocious migration [70]. Conversion of the border cells from a stationary group of epithelial cells to invasive cells and the definition of the timing of migration requires the integration of the activity of Slbo and that of the **Taiman pathway**. Taiman is a steroid hormone ecdysone coactivator [73]. Ecdysone levels are highest during the time of border cell migration, indicating a coordination between egg production and an adequate nutrition. Taiman functions independently of Slbo. In *taiman* mutant border cells, *D*E-cadherin is still present but its distribution is altered, as there is a higher accumulation of *D*E-Cadherin at the interfaces between border cells and nurse cells. Thus, Slbo is required for *D*E-Cadherin, while Taiman is required for DE-Cadherin proper localization via the stimulation of adhesion complexes turnover [73].

Two **R**eceptor **T**yrosine **K**inases (RTK) pathways, the **Epidermal Growth Factor Receptor (EGFR) pathway** and the **Platelet derived growth factor and Vascular growth factor Receptor (PVR) pathway**, cooperate in a redundant manner to guide border cells until their final destination. As mentioned above, border cell migration takes place in two steps: a posterior directed migration, in which both PVR and EGFR participate, and a dorsally directed migration that depends solely on the EGFR pathway [74, 75]. During the posterior migration, the ligands of both pathways are expressed in the germline before and during the migration process, and their receptors are expressed in the border cells. Independent overexpression of gain of function and

dominant negative forms of components of either of the two pathways give similar results, as border cell migration is affected but not fully abolished. However, when both pathways are blocked at the same time there is a dramatic effect on Border Cell migration, indicating a redundant function of the two pathways [76]. During the dorsally-directed migration of border cells, only ligands of the EGFR pathways are expressed differentially on the dorsal side [75]. At the cellular level, PVR and EGFR seem to regulate F-actin cellular extensions arising from the border cells at the initiation of their migration. This is based on the fact that expression of a dominant negative (DN) form of PVR and, to a lesser extent, that of a DN-EGFR construct, reduce the formation of long cellular extensions. Moreover, the two pathways act synergistically because over-expression of both DN forms abolishes this cellular extension. Experiments involving the misexpression of the PVR and EGFR ligands, PVF1 and Vein, respectively, and the inhibition of one of the pathways while over-activating the other, have shown that the formation of this cellular extension is not simply triggered by PVR or EGFR signalling. Rather it is a consequence of active guidance [66].

I3. Molecules controlling epithelial morphogenesis in *Drosophila*

I3.i. *D*JNK pathway in *Drosophila* (Fig. 5)

The ***Drosophila* c-Jun N-terminal Kinase** (***D*JNK**) pathway is a **M**itogen-**A**ctivated **P**rotein **K**inase (MAPK) module. All MAPK modules consist of three sequentially acting protein kinases: a MAPKKK (MAP3K), a MAPKK (MAP2K) and a MAPK (Herskowitz 95). JNK activation has been implicated in cytoskeleton rearrangements, immune and cellular stress responses, growth arrest, and the regulation of apoptosis, leading to the surprising conclusion that a single signalling cascade may control both specific developmental events and general physiological responses to numerous extracellular signals and intracellular signalling molecules. A primary consequence of JNK activation is the regulation of gene expression by target transcription factors such as the **A**ctivator **P**rotein-**1** (AP-1) transcription factor. Active AP-1 complexes are dimmers of basic-region leucine zipper (bZip) transcription factors, formed predominantly by members of the Jun and Fos gene families. JNKs are so called because they activate the transcription factor c-Jun by phosphorylating specific residues in its N-terminal domain. c-Jun homodimers or heterodimers between c-Jun and c-Fos proteins act as transcription factors, which can control the expression of many known mammalian genes by interacting with AP-1 binding sites in gene regulatory sequences.

The triple-kinase module and its activation mechanism are highly conserved from yeast to mammals [77]. In *Drosophila,* homologues of the different components of the JNK pathway have been identified. ***Drosophila*** JNK (*D*JNK) consists of a cascade of three MAPKs: *slipper*, a *D*JNKKK [78], *hemipterous,* a *D*JNKK[79], and *basket,* a *D*JNK [80, 81]. *D*JNK activity results in the phosphorilation of *D*Jun. Like its mammalian

homologue c-Jun, *D*Jun [82-86], forms homodimers and heterodimers with *D*Fos, the *Drosophila* c-Fos homologue encoded by *kayak* [87], and binds to AP-1 DNA binding sites. In contrast to mammalian Fos, *D*Fos also forms homodimers suggesting *D*Jun-independent functions.

Nuclear *D*Jun, together with *D*Fos, leads to distinct downstream gene transcription. One of their targets is *puckered* (*puc*), a Cl-100-like MAP kinase phosphatase [88, 89]. Puckered dephosphorilates Hemipterous and therefore inhibits *D*JNK activation [88]. Thus, the JNK pathway posseses also a negative feedback loop which represents an internal control mechanism allowing a response intensity regulation once the signalling cascade is activated.

The intensive study of the implications of the *D*JNK pathway in the epithelial migration process known as dorsal closure, in which a series of dynamic changes in cell shape, mobility and adhesion has been shown to take place, has highlighted the importance of this pathway as a regulator of cell morphogenesis and motility. Dorsal closure begins during mid-embryogenesis and takes approximately 2 hours (S13-15). During this process, embryonic lateral epithelial cells stretch over an extraembryonic dorsal cell layer, the amnioserosa, and fuse at the dorsal mid-line to cover the dorsal half of the embryo [90, 91]. At least two cell types are involved in dorsal closure. The leading edge cells are the most dorsal epidermal cells and they are marked by the expression of *puckered* and *decapentaplegic* (the *Drosophila* BMP4 homologue) [79, 89] and other specific proteins such as FasIII, PAK, Paxillin, Flamingo, Frizzled and Dishevelled, Filamentous-actin (F-actin), Myosin, □PS2, Coracle, Canoe, and □PS [90, 92-100]. The leading edge cells are the ones that initiate the dorsoventral movement and are followed by the more lateral cells [79, 89, 94].

The onset of dorsal closure is marked by a change in the distribution of cell surface proteins along the dorsal-ventral plane of the leading edge cells [89, 93], that lead to the assembly of actin-nucleating centres [93, 94, 101, 102], at the level of adherens junctions. Accumulation of large amounts of F-actin and Myosin at the actin-nucleating centres results in the polarisation of leading edge cells towards the direction of movement. Maturation of these actin-nucleating centres into an actomyosin cable will provide the leading edge cells with the contractile forces required for these cells to stretch dorsally over the subjacent amnioserosa cells [93, 103].

One of the roles of *D*JNK during dorsal closure is the regulation of the organization of the leading edge. In *D*JNK pathway mutants the initial elongation and polarization of the leading edge cells occurs normally. In contrast, *D*JNK is required for the proper maturation of the actin-nucleating centres and correct actin dynamics [78, 79, 93], similar to its role in vertebrates [79, 94, 101, 104, 105]. In fact, one of the downstream targets regulated by *D*JNK is *chickadee,* which encodes for Profilin, a protein critical for actin polimerization [106].

The *D*JNK pathway has also been reported to regulate actin reorganization in thorax closure, another epithelial migration process [107].

I3.ii. *misshapen* in *Drosophila*

Drosophila Misshapen is a protein kinase (MAP4K) member of the mammalian Germinal Centre Kinase-IV (GCK-IV) subfamily of Ste20 kinases and is homologue to the mammalian Ste20 kinase **N**ck **I**nteracting **K**inase (NIK) [108]. Misshapen has an N-T kinase domain and a C-T regulatory domain with a region in between containing binding sites for Nck and TRAF but not for small GTPases as do other MAP4Ks [109], [110, 111]. NIK has the ability to activate JNK in cell culture experiments [110]. Biochemical studies have shown that, like its mammalian homologue NIK, Misshapen acts upstream of the *D*JNK module and that both, the N-T and C-T domains are required for *D*JNK activation [110, 112, 113]. Similarly, genetic studies place Misshapen upstream of the *D*JNK pathway in dorsal closure in the *Drosophila* embryo [112]. In fact, mutants for *misshapen*, as do mutants for each of the components of the *D*JNK module, present a dorsal open phenotype. Furthermore, Misshapen also regulates *decapentaplegic* expression specifically in the leading edge cells [112].

Similarly to the *D*JNK pathway, some of Misshapen's functions are linked to rearrangement of the cytoskeleton leading to cell-shape changes and cell motility. However, Misshapen does not act uniquely by the activation of the *D*JNK pathway. For instance, during *Drosophila* eye development *misshapen* is required for the correct targeting of photoreceptor axons, a system where *basket* is not required [114, 115]. In this case, Misshapen has been shown to interact with Dreadlocks (Dock), a SH2/SH3 adaptor protein [110, 113, 114, 116]. This shows the ability of Misshapen to interact with distinct classes of adaptor molecules depending on the biological process. Similar to the role of Misshapen in dorsal closure, a direct link between Misshapen and the actin cytoskeleton has been found in the eye. In this context Misshapen

regulates Bifocal, a cytoskeletal regulator that binds F-actin in vitro [117], to control cytoskeletal changes during the targeting of photoreceptor growth cones [115]. In fact, in vitro experiments show that Misshapen binds directly to Bifocal and phosphorilates it. Thus, Bifocal links Misshapen activation with changes in the actin cytoskeleton necessary for the correct targeting of photoreceptor growth cones.

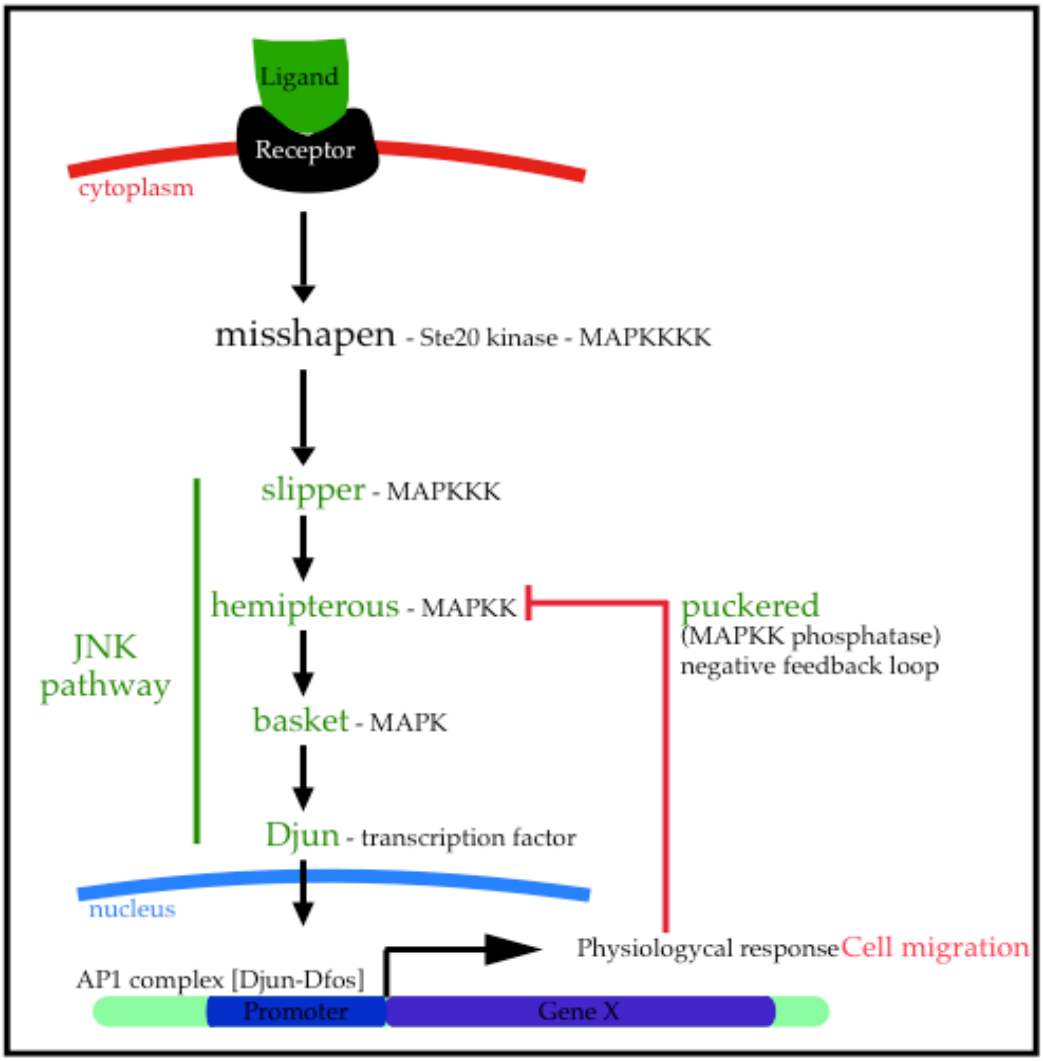


Figure 5. Misshapen and the *D*JNK pathway in *Drosophila*

An unknown signal activates the MAPKKKK Mishapen which in turns activates the DJNK pathway, a MAPK cascade consisting of three MAPKs: Slipper, a *D*JNKKK, Hemipterous, a *D*JNKK, and Basket, a *D*JNK. *D*JNK activation results in the phosphorilation of *D*Jun, a transcription factor. Nuclear *D*Jun, together with *D*Fos, leads to distinct downstream gene transcription to regulate a number of physiological responses, being cell migration one of them. One of their targets is Puckered, a MAP kinase phosphatase that dephosphorilates Hemipterous and thus inhibits *D*JNK activation. Therefore, the pathway presents also a negative feedback loop to regulate the response intensity once the signalling cascade is activated.

I3.iii. Integrins

Integrins are a widely expressed family of cell surface receptors. Most integrins are expressed on a variety of cell types and most cells express several integrins. Integrins are transmembrane receptors that mediate adhesion between the cell and the extracellular matrix (ECM). Integrins are heterodimeric receptors composed of two non-covalently linked class I transmembrane proteins, an α and a β subunit [118] (Fig. 6). Both integrin subunits contain large extracellular domains that interact mainly with proteins of the extracellular matrix but also with transmembrane proteins, transmembrane domains and smaller intracellular domains by which they interact with the actin cytoskeleton and diverse intracellular proteins [119]. They regulate several fundamental processes during epithelial morphogenesis by orchestrating the recruitment of both cytoskeletal and signalling molecules that regulate cell-ECM adhesion, assembly of the ECM, cell migration and cell shape [120, 121]. They are also involved in the regulation of cell proliferation and cell survival, and they direct the differentiation of tissues and organs [120, 121].

Integrins are extremely well conserved throughout evolution [122, 123]. They are present in metazoans ranging from sponges to mammals [124]. In *C. elegans* there are one α subunit and two β subunits. In *Drosophila* there are two β subunits, βPS and βv - and five α subunits, αPS1 to αPS5. In vertebrates there are 8 β subunits and 18 α subunits to form at least 24 different receptors [121]. Despite the larger number of integrins in vertebrates, both vertebrate and invertebrate integrins show conserved structural and functional features. Based on the specificity for the extracellular matrix ligands, integrins are classified in three types: 1) RGD receptors that bind to extracellular matrix components containing the Arg-Gly-Asp motif such as fibronectin and vitronectin, 2) laminin receptors and 3) collagen receptors (Fig. 7)

[1]. Phylogenetic studies have shown that two of these subfamilies of integrins arose in early during metazoan evolution: one laminin-specific and one recognizing RGD sequences. *Drosophila* αPS1, mammalian α3, 6 and 7 and *C. elegans* αG8.3 bind to laminin, while *Drosophila* αPS2, mammalian α5,8 and v and *C. elegans* αF2.1 bind to RGD-containing proteins. *Drosophila* αPS3, 4 and 5 are closely related and form a separate evolutionary group with no orthologues in other species. Vertebrates have evolved many more α subunits, half of them include an extra I domain (which shares homology with the von Willebrand A domains) that is not found in fly or worm integrin α subunits. I domains are found in integrins that bind to collagens and in leukocyte integrins [122]. Apart from the extracellular matrix components, some integrins such as the b2 subgroup mediate cell-cell adhesion by binding to counter receptors on other cells. Many integrins also bind to soluble proteolytic fragments of vascular basement membranes such as endostatin [125], and a number of pathogens make use of integrins as receptors to enter into the cell [126].

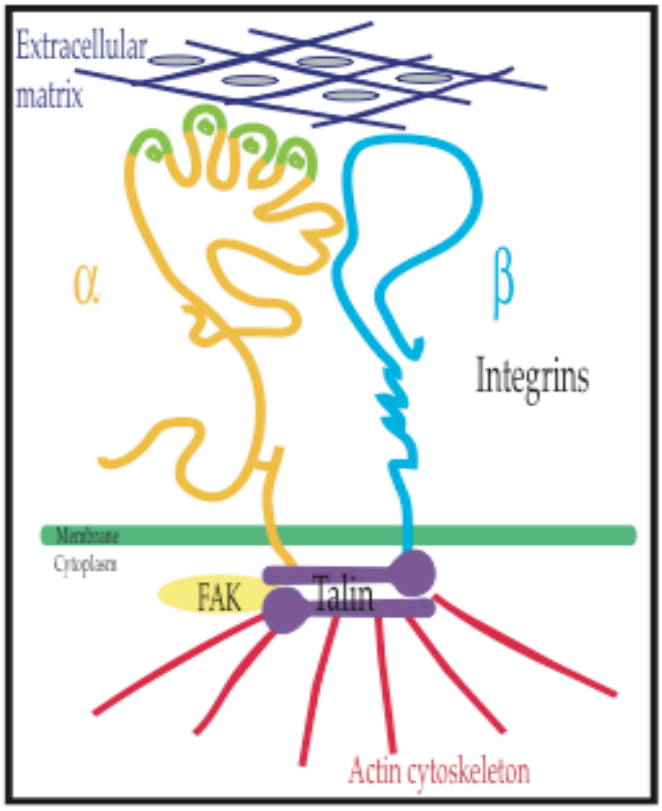


Figure 6. Integrins

Integrins are heterodimeric transmembrane proteins formed by an α and a β subunit. The large extracelular domains interact with proteins of the extracellular matrix and other transmembrane proteins. The smaller intracellular domains interact with the actin cytoskeleton and a number of intracellular proteins, such as the cytoplasmic actin-binding protein Talin, a core component of the integrin pathway

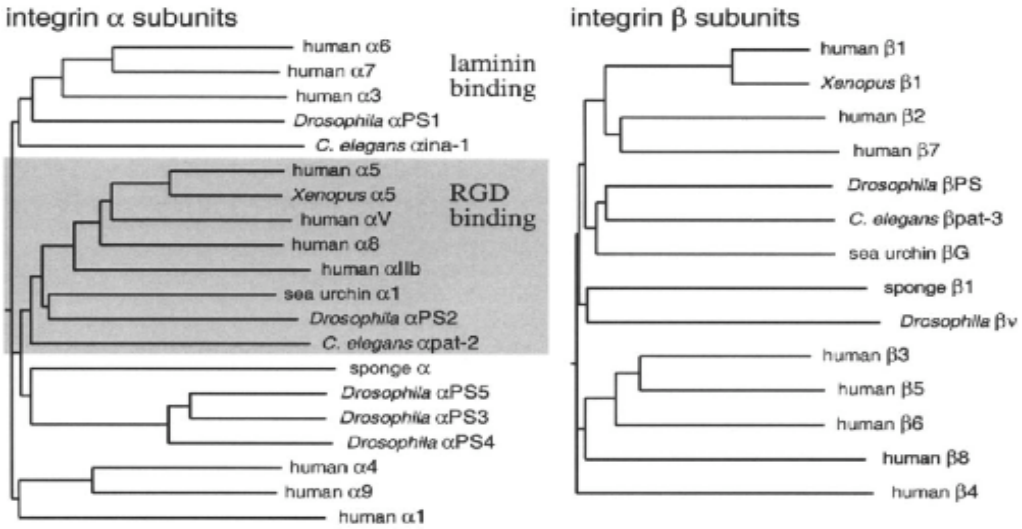


Figure 7. Phylogenetic Tree of the Integrin α and β Subunits (from [1]).

Comparisons of vertebrate and invertebrate integrin α subunits at the left and β subunits at the right. Distinct functional sub-divisions can be made on the basis of ligand specificity finding three types of integrins: RGD, laminin and collagen receptors.

I3.iii.1. Integrins in *Drosophila*

In *Drosophila* the genes encoding the different subunits have been identified and mutants of these genes have been isolated. All *Drosophila* 5 □ subunits (encoded by *multiple edematous wing* (*mew* - □PS1), *inflated* (*if* - □PS2), and (*scab* - □PS3-4)) likely form heterodimers with the □PS subunit (encoded by *myspheroid*). This has been demonstrated for the □PS1, □PS2 and □PS3 subunits, which have been purified biochemically [127-131]. □□ is less conserved in its sequence than other □ subunits. □□ is the only adhesion molecule in *Drosophila* with a von Willebrand A homologous domain. So far, the only known partner of □□ is the □PS3 subunit [132]. Because of the high sequence similarity between □PS3 and □PS4 and □PS5, it is possible that □□ forms heterodimers with these subunits as well. *Drosophila* □□ is most strongly expressed in the endodermal cells of the developing midgut of the embryo and this expression is maintained in the larva and pupa [133]. □□ is not essential for viability or fertility. This subunit is only required in the midgut where it can partially substitute □PS function, but no other roles have been detected for this subunit during embryogenesis, imaginal disc development or oogenesis [132].

I3.iii.2. Integrin functions

As mentioned above, integrins have been shown to play different roles during the morphogenesis of distinct types of epithelia during development. To follow I introduce a number of these roles:

- Cell-extracellular matrix adhesion

Integrins mediate stable adhesion between cells and their substrate by linking the extracellular matrix to the actin cytoskeleton [118].

Integrin-mediated adhesion to the extracellular matrix requires the extracellular domains of both subunits since they contribute to the binding site for ligands present in the extracellular matrix [134]. Since integrins are localised at the basal domain of the cell membrane in most cell types, the connections to the actin cytoskeleton at the hemiadherens junctions and to the intermediate filaments at the hemidesmosomes are mediated at the basal sides. The β cytoplasmic domain is the primary responsible for the binding of intracellular proteins required for integrin function in signalling and adhesion [121]. The short cytoplasmic tails of the α and β subunits have no catalytic function. Rather, they transduce signals by binding to a variety of effector proteins that may be either directly or indirectly associated with the actin cytoskeleton. The intracellular, cytoskeletal connections of integrins have been studied extensively in vertebrates and they appear to be largely conserved in *Drosophila.* The typical integrin-actin microfilament linkers such as Talin, α-Actinin, Vinculin, Paxilin, and Tensin are present in a single copy in *Drosophila* and in multiple copies in vertebrates [122]. Integrins and their cytoplasmic binding partners are organized into large, highly dynamic multiprotein complexes termed focal contacts.

Integrin-mediated adhesion is required for the development of the muscles in *Drosophila* and *C. elegans* [1, 135]. In fact, the gene encoding the *Drosophila* βPS subunit, *myspheroid*, was given its name because its mutant phenotype results in the rounding up of detached embryonic muscles [135]. In *Drosophila*, the integrin adhesive function has been extensively studied in the wing epidermis [96, 136]. The *Drosophila* adult wings are composed of two monolayers of cells that are held together through a thin extracellular matrix at their basal sides. Since loss of integrins results in wing blisters, integrin-based

connections among wing layers and the extracellular matrix deposited between them are required to maintain both layers together [96, 137] [138]. In addition, experiments of tissue-specific knockout mice and the characterization of certain human heritable skin disorders have highlighted the importance and the multiple roles of integrins in the epidermis. The mammalian epidermis is a stratified epithelium over a thick extracellular matrix consisting of a basement membrane connected by anchoring fibrils to the collagen-rich mesenchymal dermis. Patients with epidermolysis bullosa, in which there is a loss of integrin-mediated adhesion, present skin blistering caused by the detachment of the stratified epidermal epithelium from the basement membrane, gastrointestinal scarring, muscular dystrophy and motoneuron disease [139] [140]. In the mouse, mutations of components of the hemidesmosomes, a specialized integrin junction, also result in skin blistering [141].

While similar integrin functions in adhesion have been documented in *C. elegans*, *Drosophila*, mice and humans [120] [142], other Integrin activities are more variable between model organisms.

- Assembly of the extracellular matrix

The extracellular matrix is composed of a mixture of glycoproteins that form a meshwork in the extracellular space between layers. The components of the extracelular matrix have been also highly conserved during evolution [122]. The basic components are laminin, type IV collagen, nidogen/entactin and proteoglycans of the perlecan type. *Drosophila* laminin comprise 3 subunits (□, □ and □) and is related to vertebrate and to *C. elegans* laminins (which has: 2□, 1□ and 1□). In *Drosophila* and *C. elegans* there is one pair of type IV collagen genes and in mammals there are three such pairs. Both the nidogen/entactin

and the proteoglycan perlecan have also homologues in *C. elegans*, *Drosophila* and vertebrates.

These extracellular matrix components are organized by interactions with cell-surface receptors into diverse structures. These range from basic basement membranes formed by amorphous extracellular material underlying epithelial cells to more specialized structures such as the tendons. Tendons are composed of collagen fibres arranged in a parallel fashion forming structures which connect the muscles to the bone [143].

The extracellular matrix is not a passive structure, it can also be an important source of signals that promote proliferation and differentiation. Therefore, the proper modulation of the structure and activity of the extracellular matrix has profound effects on its function and the consequent behaviour of cells residing on or within it.

The organisation of tissues during development is linked to the assembly of the extracellular matrix. Integrins are involved in the assembly of the extracellular matrix [144]. For instance, in mice, the $\alpha 3\beta 1$ integrin is required for the basement assembly at the basal surface of the epidermis [145, 146]. During amphibian gastrulation, integrins are involved in the deposition of the fibronectin-containing extracellular matrix. In *Drosophila*, during embryogenesis, integrins are also required for the assembly of the extracellular matrix [147, 148].

- Cell migration

Integrins can affect the actin cytoskeleton organization directly, by regulating the activity of the Rho family of small GTPases [149]. Both, in flies and vertebrates, this integrin function contributes to cell rearrangements and cell migration. Integrins participate in cell migration events essential for embryonic development and adult life [120]. Cell migration requires an interaction between the cells

themselves and their substrate, and depends on the recognition of this substrate by the cell´s integrin complement [150]. The first process that requires integrins takes place early during gastrulation. Mice embryos lacking the β1 integrins are arrested early in development, as cells from the inner cell mass fail to migrate or differentiate and as a consequence they do not generate primitive ectoderm and ectoderm layers [151, 152]. During amphibian gastrulation, integrins are required in migrating mesodermal cells and in epidermal cells of the blastocoel roof for radial intercalation and thinning of the blastocoel roof during epiboly [153-155]. In the *Drosophila* embryo, lack of integrins results in a defective migration of the primordial midgut cells and in a defective migration of cells forming the branches of the tracheal system [156-158]. In vertebrate embryos, integrins are required for neural crest migration [159, 160]. In *C. elegans*, integrins are required in the migration of the gonadal distal tip cells [161] .

In the central nervous system, integrins are required for the migration ofneural precursors in mammals, for the migration of neuronal cell bodies in the mouse olfactory interneuron precursors and for guidance of the growth cone in *Drosophila*. Integrins are also necessary for leukocyte migration and adhesion [162, 163].

Integrins also play a role in cancer. Dysfunctional integrin-mediated signalling can contribute to cancer progression by promoting invasion and metastasis of neoplastic cells, and tumor angiogenesis {Goel, 2004 #399}.

I3.iii.3. Other functions of integrins

- Integrins in intracellular signalling

It still remains unclear to what extent signalling may contribute to the function of integrins during morphogenesis. One of the main problems is how to distinguish between direct integrin signalling and the indirect effects caused by the loss of integrin adhesion.

Integrins are bidirectional signalling receptors that can be activated both by extracellular ligands and by intracellular proteins. When activated by extracellular ligands, the process is known as outside-in integrin activation. The outside-in activation leads to the clustering of integrins at the integrin-containing structures known as focal contacts. Since both subunits contribute to the ligand-binding site, ligand specifity is therefore determined by the integrin heterodimer composition and also by the cellular context. After integrin binding to the extracellular matrix, there is a conformational change of the heterodimer that induces the displacement of the α subunit tail and that exposes sites in the β tail by which it is then free to interact directly or indirectly with about 60 cytoplasmic proteins [164]. The fact that the cytoplasmic tail of integrin β subunit is only 30 aminoacids long suggests that the interactions vary depending on the cellular context. Similar to the integrin-actin microfilament linkers mentioned before, the integrin-linked signal transduction molecules such as Focal Adhesion Kinase (FAK), Integrin Linked Kinase (ILK), p95PKL and p130CAS are also conserved between *Drosophila* and vertebrates [122].

In addition to the outside-in activation, integrins can also be activated from inside the cell in response to pathways acting downstream of other cell surface receptors. This process is known as inside-out integrin activation. A core component of the integrin pathway, Talin, plays a key role in the inside-out activation, since

different pathways converge in Talin in order to activate integrins. Talin binding to the cytoplasmic tails of integrins induces conformational changes in the integrin subunits triggering the separation of integrin tails and producing a transition to the high affinity state of integrins for extracellular matrix ligands [165-170] [171].

- Integrins in gene expression

The study of integrin-mediated signalling in cells inculture or in whole organisms render different results. For instance, integrins are essential for muscle differentiation in culture plates. In the absence of integrins the expression of muscle-specific proteins is abnormal [172]. However, *C. elegans*, *Drosophila* or mouse embryos do not show this phenotype [173] [174] [175] [176].

In *Drosophila* the only evidence of integrin signalling occurs in the gut, where several genes that require integrin for their activation or repression have been reported [177]. In order to discriminate between the two functions of integrins, adhesion and signalling, chimeras containing the intracellular domain of the subunit fused to different, labelled extracellular domains of a variety of proteins have been generated. The use of these chimeras in cell culture studies has shown that they are able to cluster at the focal contact sites and that are sufficient to induce the phosphorilation of Focal Adhesion Kinase (FAK) [178, 179]. In *Drosophila*, the Tor^{D}/β_{cyt} (dibeta) chimera contains the cytoplasmic tail of the integrin subunit fused to the extracellular and transmembrane domains of a dominant gain-of-function mutant allele of the Torso receptor tyrosine kinase that dimerizases in absence of ligand bindin. Because integrins can be activated by clustering, this chimera is a constitutively active integrin independently of ligand binding (Fig. 8). dibeta, like the endogenous integrins, accumulates at the focal contact

sites and competes with the endogenous integrin in the recruitment of intracellular components of the integrin signalling pathway and therefore acts as a dominant negative (DN) form. This fact makes the use of this chimera an excellent tool to distinguish between adhesion and signalling. When overexpressed in an otherwise wild-type cell, the Tor^{D}/β_{cyt} fusion protein act as a dominant-negative form sequestering the integrin cytoplasmic partners and therefore blocking the integrin-mediated adhesion function of integrins. In addition, in the absence of endogenous integrins, Tor^{D}/β_{cyt} is able to rescue integrin-dependent signalling but not adhesion since it lacks the integrin extracellular domains to interact with the extracellular matrix ligands [177].

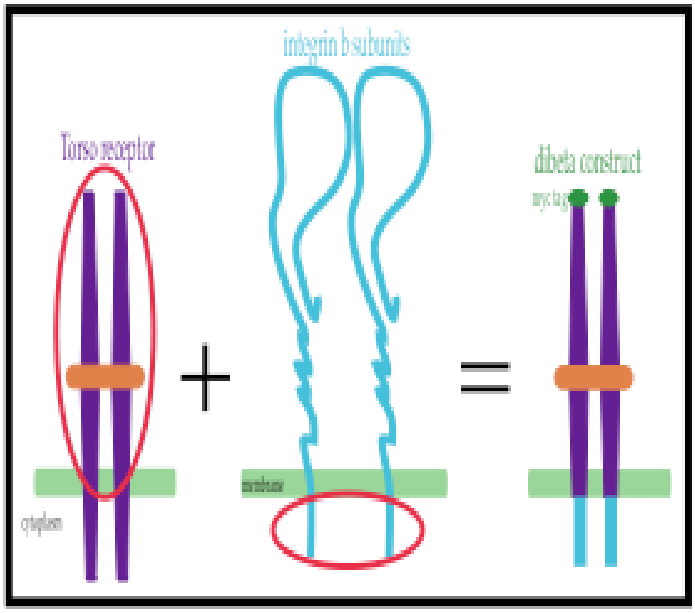


Figure 8. TorD/βcyt (dibeta) construct

Fusion of the extracellular and transmembrane domains of a dominant gain-of-function mutant allele of the Torso receptor tyrosine kinase to the intracellular domain of the integrin β subunit results in a chimera consitutively active independently of ligand binding.

- Integrins in cell proliferation and in the establishment of cell polarity

Integrins have been also implicated in regulating cell proliferation and establishment of polarity in many different vertebrates tissues [180]. For example, in culture experiments with mammalian cells have shown that Integrins are required to establish cell polarity and proved anchorage-dependent cell proliferation [181, 182]. Moreover, in vivo experiments show that integrins are important for cell proliferation during the development of a number of tissues including skin, bone and embryonic ectodermal ridge cells [146, 183, 184]. There is also evidence for integrins playing a role in epithelial architecture and polarity in the pulmonary epithelia in vivo and in vitro [182, 185].

However no similar functions for integrins have been found in *Drosophila* or *C. elegans*. In *Drosophila* embryos, integrins are not required for the establishment of cell polarity, but for their maintenance. Studies in the *Drosophila* wing pouch and midgut epithelia have reported that integrins are neither required for the organization or proliferation of disc epithelial cells nor for the initial apical-basal polarization of midgut endodermal cells [132] [186].

- Integrins in cell division

Different steps during cell cycle are accompanied by changes in the adhesion to the extracellular matrix (Glotzer, 2001). Recent data from cell culture experiments have also involved integrin in the regulation of centrosome function, the assembly of the mitotic spindle, and cytokinesis [187]. A mutation in the integrin □ subunit cytoplasmic domain that suppresses integrin activation allows entry in mitosis but inhibits the assembly of microtubules from the centrosome and disrupts

cytokinesis by preventing the formation of a normal bipolar spindle [187].

I3.iii.4. Integrins and other signalling pathways

Integrins can act as signalling-transducing molecules. The extracellular signals transmited via integrins can be interpreted in the receiving cell as growth, differentiation or survival signals since integrins can regulate transcription through components shared with other pathways (JAK/STAT or MAPK signalling) [180] [188]. However, it is not clear how integrins regulate transcription. Instead of transmitting a signal to the nucleus, integrins seem to modulate other signalling pathways mostly at the membrane level acting as a clustering centre that enhances the efficiency of these pathways. This correlates with the large number of molecules that have been described to interact with integrins [164, 189]. In other cases, the interacting pathway can be spatially separated. During frog gastrulation, Dishevelled, a member of the Wnt pathway that localizes apically, is recruited to the plasma membrane in response to basal integrin-mediated adhesion to the extracellular matrix [155].

I3.iii.5. Molecules downstream of integrins

- Talin

Talin is an important component of the focal adhesion complexes. Talin is a cytoplasmic actin-binding protein that also binds to Integrins [190] linking them, either directly or indirectly to the actin cytoskeleton. Talin self-associates via the rod-shaped carboxi-terminal domain to form a homodimer [191]. The globular head of talin contains a **F**our-point-one, **E**zrin, **R**adixin, **M**oesin (FERM) domain. The FERM domain binds with

high affinity to a conserved NpxY motif in the cytoplasmic tail of Integrin b1, b2, b3 and b5[166, 168]. In addition to integrins, Talin binds to multiple adhesion molecules. The head region also contains binding sites for Focal Adhesion Kinase (FAK) phosphatidylinositol-4, 5-biphosphate (PIP2), and phosphatidylinosito-4-phosphate 5-kinase type Ig (PIPKIg) [192, 193]. The tail region contains an additional low affinity binding-site for □-integrin tails and two actin and vinculin binding sites [194-196].

Talin is not required for integrin localization. Rather it participates in an early step in the formation of integrin-mediated extracellular matrix-actin connections. The coordinated recruitment of Talin, PIPKIg and Vinculin to activated integrins contributes to the formation of focal complexes, to link them to the cytoskeleton and promote their maturation into focal adhesions [169, 197, 198]. Therefore, in addition to its structural role, Talin plays a central role in Integrin activation [165-171].

In *Drosophila*, *C. elegans* and mice, the mutant phenotype of *talin* closely mimics that of □PS, suggesting that Talin is required for most of integrin functions [198-200]. These studies show that Talin is essential for the stable linkage between integrins and the actin cytoskeleton, for the organization of actin-based lattice and for integrin-mediated signalling.

- Focal Adhesion Kinase

Focal Adhesion **K**inase (FAK) is a non-receptor protein tyrosine kinase that plays a central role in signalling through integrins [201]. FAK contains a N-Terminal FERM (**F**our-point-one, **E**zrin, **R**adixin, **M**oesin) domain that interacts with the cytoplasmic tail of the □ subunit [202]. FAK is not involved in the assembly of integrin adhesive complexes but

is involved in their remodelling [203]. In mice, lack of FAK results in embryonic lethality, however FAK is not required for integrin function or viability in *Drosophila* [204, 205]. Nevertheless, when overexpressed, FAK has been revealed as a potent inhibitor of integrin binding to the extracellular matrix [205, 206].

II. OBJECTIVES

As mentioned in the Introduction section, the follicular epithelium of the *Drosophila* ovary is formed by a monolayer of follicle cells. These follicle cells display features of both apical-basal polarity and planar polarity. In addition, after several rounds of division, follicle cells undergo a number of rearrangements involving changes in cell shape and migration events. One of such migration processes is performed by the border cells. Therefore, the follicular epithelium of the *Drosophila* ovary represents an excellent model system where to study cell migration, epithelial morphogenesis and cell polarity.

This project pretends to study, at the genetic and molecular levels, border cell migration and the maintenance of the follicular epithelium monolayer during *Drosophila* oogenesis.

II.1.- The role of integrins during the morphogenesis of the follicular epithelium

Integrins are expressed in most epithelia characterized to date in vertebrates and invertebrates, such as the epidermis and mammary gland epithelia in vertebrates, and in the wing and gut epithelia of *D. melanogaster*, where they play important roles [132, 147, 207-209]. For instance, integrins are required to keep the integrity of the mammalian epidermis, a multilayered epithelium made up of keratinocytes at various stages of differentiation. Diverse integrins are expressed in these cells and exert multiple roles in epidermal homeostasis through the regulation of cell proliferation, migration and differentiation [210].

Recent findings have unveiled some of the mechanisms by which integrins regulate epithelial organization. Integrins participate in the establishment of apical-basal polarity in epidermal cells in mice and are

essential for the homeostasis of the stratified epithelium of the skin [211]. In addition, integrins are necessary for the establishment of membrane asymmetry and for organizing the apical-basal polarity in epithelial cells [182, 185, 212]. Finally, integrins have been described to participate in epithelial morphogenesis and differentiation, as shown for the dorsal epidermis of the *Drosophila* embryo and in the mammary glandular epithelium in mice [148, 213, 214].

In order to analyse the role of integrins in the follicular epithelium I intend to investigate the following points:

a.- The pattern of integrin expression in the follicular epithelium.
b.- The analysis of the phenotypic consequences of removing integrin function in the follicle cells.
c.- The role of integrins in the establishment and/or maintenance of apical-basal polarity of the follicle cells.
d.- The role of integrins in the control of follicle cell proliferation.
e.- To address which of the two functions of integrins, adhesion or signalling, is responsible for integrin function during oogenesis.
f.- To elucidate the molecular mechanisms by which integrins are exerting their function in follicle cells.

II.2.- The role of Misshapen during border cell migration

Cell migration plays a key role in a wide variety of biological phenomena that take place during both embryogenesis and in the adult organism. Therefore, the progress in the knowledge of the mechanisms that regulate cell migration is essential to understand human pathologies, such as metastasis, as well as normal physiological conditions such as the migration of fibroblasts during wound healing.

Previous studies have reported *misshapen* as being involved in dorsal closure, a well characterized cell migration process that takes place in the *Drosophila* embryo [78-80, 85, 88, 112]. In addition, *misshapen* is also required for normal photoreceptor cell shape and orientation and for growth cone motility in the eye and for hair development in the wing [109, 114, 215]. To gain insight in the mechanisms by which *misshapen* controls cell migration, I set out to study the role of *misshapen* in border cell migration.

To address the role of *misshapen* during border cell migration I have analysed:

a.- The expression pattern of Misshapen during border cell migration

b.- The phenotype of the loss of Misshapen function in the process of border cell migration

c.- The consequences at the cellular level of removing *misshapen* in the border cells

d.- The interactions of Misshapen with other pathways involved in border cell migration

III. THE ROLE OF INTEGRINS DURING THE MORPHOGENESIS OF THE FOLLICULAR EPITHELIUM

III.1. RESULTS

III.1.i. Integrins are expressed in the basal, lateral and apical domains of epithelial follicle cells

The *Drosophila* genome encodes two integrin subunits: the widely expressed PS subunit encoded by the *myospheroid* gene and the chain, detected only in the embryonic midgut endoderm [133] [1]. The PS subunit is likely to form functional heterodimers with all five subunits reported in the *Drosophila* genome [1]. Since the only chain present in the ovary is the PS subunit [132, 136], the pattern of PS expression should reflect the distribution of all functional integrin complexes in the adult ovary. Using an antibody specific to PS, we observed that this subunit is expressed in the somatic cells of the germarium, in the follicular epithelium and at higher levels in the interfollicular stalks that connect adjacent follicles. In addition, PS is found in the germline until stage 3-4 (Fig.9A-C). Similar to other epithelial cells where PS expression is restricted to their basal side {Brown, 2002 #392}, integrins have been described to localize in a punctuate pattern at the basal side of follicle cells [30]. In our experimental conditions, however, PS was also localized along the lateral and apical domains (Fig. 9D, E), a localization that was not a consequence of PS accumulation in germline cells, as *myspheroid* mutant germline clones presented a similar pattern of expression (data not shown). This distribution resembles that of other integrin members that are expressed in the basal cells of the mouse epidermis, where they accumulate along the entire cell periphery [207] and suggests that

integrins might interact not only with basement membrane components in the *Drosophila* ovary, but also with the extracellular matrix deposited around follicle cells (ref).

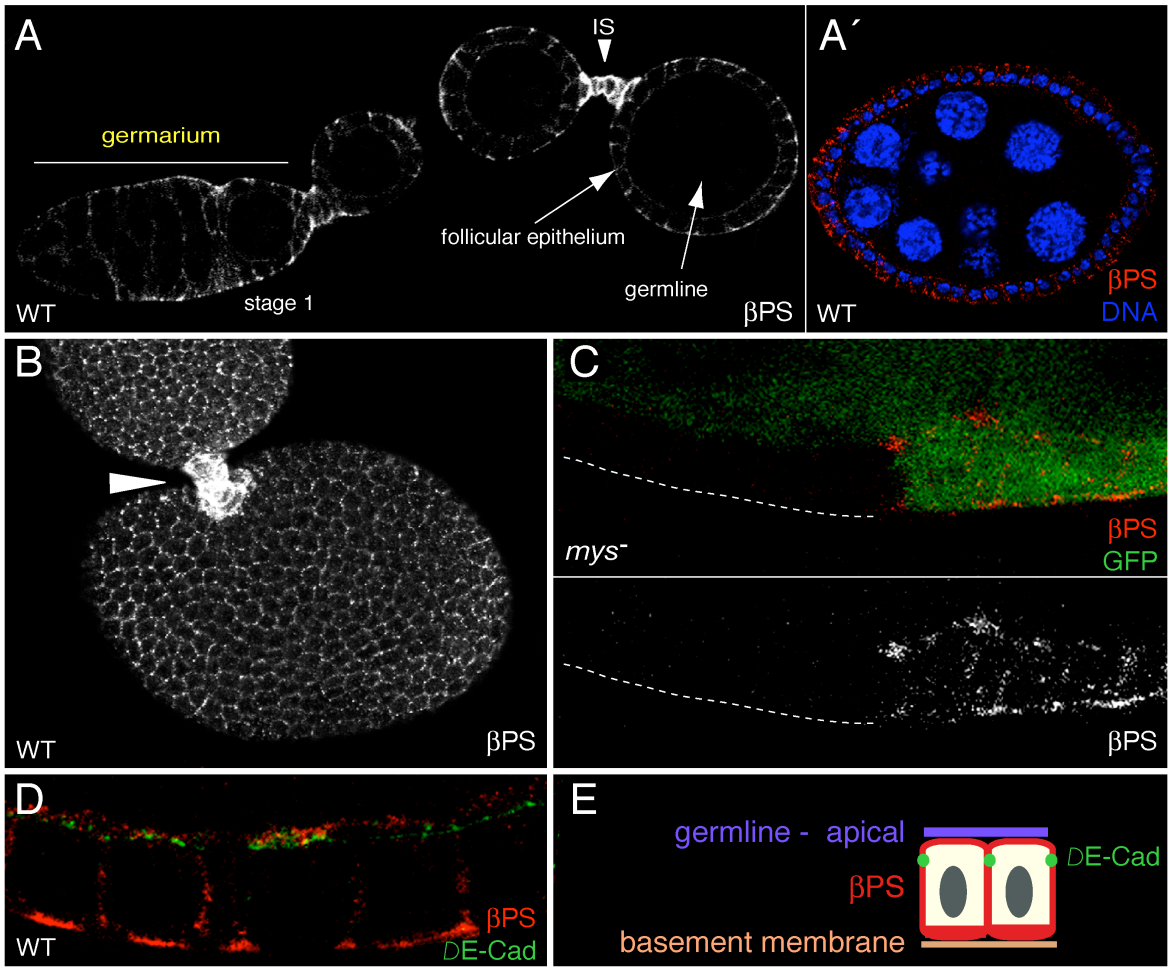


Figure 9. βPS is expressed in the apical and basolateral sides of follicle cells

(A) The βPS subunit is detected in germline and somatic cells in the germarium. Later, its expression is restricted to somatic cells. The cells forming the interfollicular stalk (IS) possess higher expression levels (arrowheads in A and B). (A') Wild-type egg chamber stained with anti-βPS and the DNA dye TO-PRO-3 to show the follicular epithelium monolayer. (B) Top view of a S5 egg chamber to label the expression of βPS in the basal side of follicle cells. (C) Mosaic follicular epithelium stained for βPS (red) and GFP

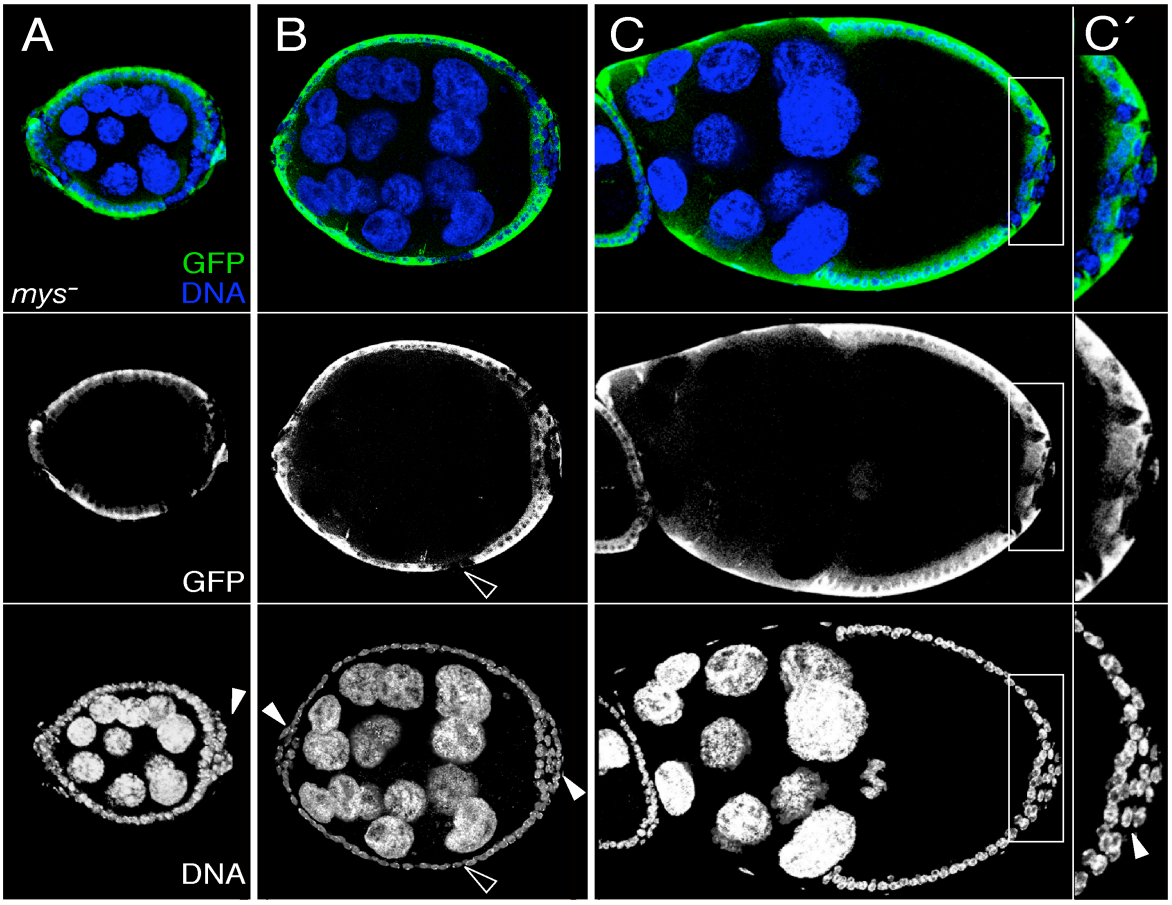
(green) to demonstrate the specificity of the anti-βPS antibody. The mutant cells (GFP-negative; dashed line) are homozygous for the protein-null allele *mys*^{XG13} and show no signal with the anti-βPS serum. (D) βPS is localized basolaterally in follicle cells. It is also found at the apical side, in contact with the germ line, as shown by the localization of βPS (red) apical to the zonula adherens component *D*E-cadherin (green). (E) Schematic representation of βPS expression in follicle cells.

Unless otherwise noted, cells expressing GFP are wild type whereas non-GFP cells are mutant.

III.1.ii. Integrins are required to maintain the simple structure of the follicular epithelium

In order to analyze the phenotypic consequences of removing integrin function in the follicle cells, we used the null allele *mys*^{11} to generate mutant clones [216, 217]. Egg chambers with mosaic epithelia containing *myspheroid* mutant follicle cells very often lost their monolayer structure and grew between one and four extra cell layers. These ectopic layers were composed of both mutant and wild-type cells, suggesting a non-autonomous effect of the lack of integrin function (Fig. 10A-C). This result strongly suggests that integrin function is required to preserve the simple structure of the follicular epithelium. This conclusion is further supported by the fact that removal of Talin, a core component of the integrin complex encoded by *rhea* in *Drosophila* [198], or the use of a second null allele of *myspheroid*, *mys*^{10}, gave rise to phenotypes identical to *mys*^{11} (Fig. 10F and data not shown). Finally, we determined that the main integrin responsible for the maintenance of the monolayer was the heterodimer βPS1αPS and that βPS2αPS did not play a significant role in this process (Fig. 11).

Interestingly, the abnormal morphology of *myspheroid* mutant mosaic follicular epithelia was only caused by mutant cells positioned at either end of the egg chamber, but not by mutant clones within the central domain of the follicle (Fig 10B). Positional mapping of the clones that caused the multilayer phenotypes revealed that only loss of integrin function from cells within ~10-cell diameters from the polar cells resulted in formation of stratified epithelia. It has previously been proposed that the follicular epithelium can be subdivided, based on their different developmental competence, into terminal domains at both poles and a single intervening main body domain [17, 19, 55]. Interestingly, the area where loss of integrin function induced epithelial stratification corresponds to both terminal domains, providing additional support for the subdivision of the follicular epithelium into areas of competence.



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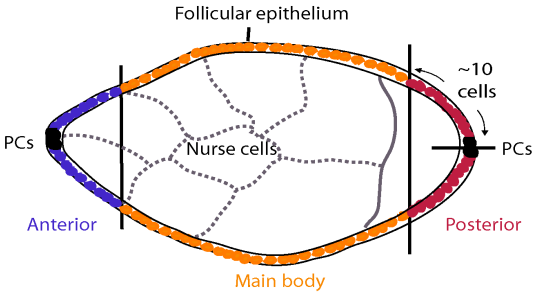

E

FE domains	% of mutant clones showing a multilayered phenotype			
	Total	S 3-6	S 7-8	S 9-10
Anterior	73.4% n=79	77% n=60	63% n=19	nd
Main Body	3% n=126	5% n=82	0% n=23	0% n=21
Posterior	98.8% n=169	98% n=102	100% n=28	100% n=39

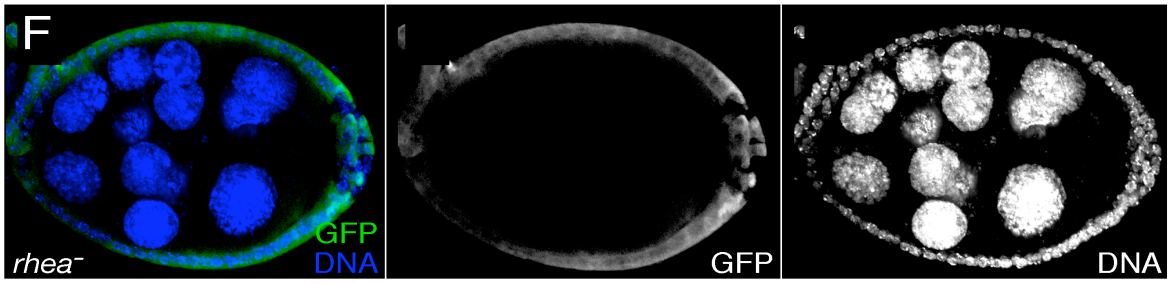

Figure 10. Loss of integrin function in follicle cells results in a multilayer epithelium

Follicles containing *myspheroid* mutant follicle cell clones stained with anti-GFP and TO-PRO-3. (A-C) S5, 7 and 10a egg chambers, respectively. (C´) Magnification of the white box in C. Absence of βPS activity in the follicle cells causes a stratification of the follicular epithelium only when the mutant clones are located at either pole of the developing egg chamber (arrowheads). Mutant clones falling within the main body domain do not give rise to a multilayer epithelium (empty arrowhead). (D) Schematic representation of a S7 egg chamber showing the anterior, main body and posterior domains of the follicular epithelium [19]. The regions susceptible to form a multilayer in the absence of integrins are restricted to about 10-cell diameters from the anterior and posterior polar cells (PCs; in black). (E) Table correlating the localization of mutant clones and their ability to give rise to a multilayer phenotype. Mosaic egg chambers were grouped into three developmental stages. (n= number of clones analyzed; nd = not determined). (F) Mosaic egg chamber containing *rhea* mutant follicle cells stained with anti-GFP and TO-PRO-3.

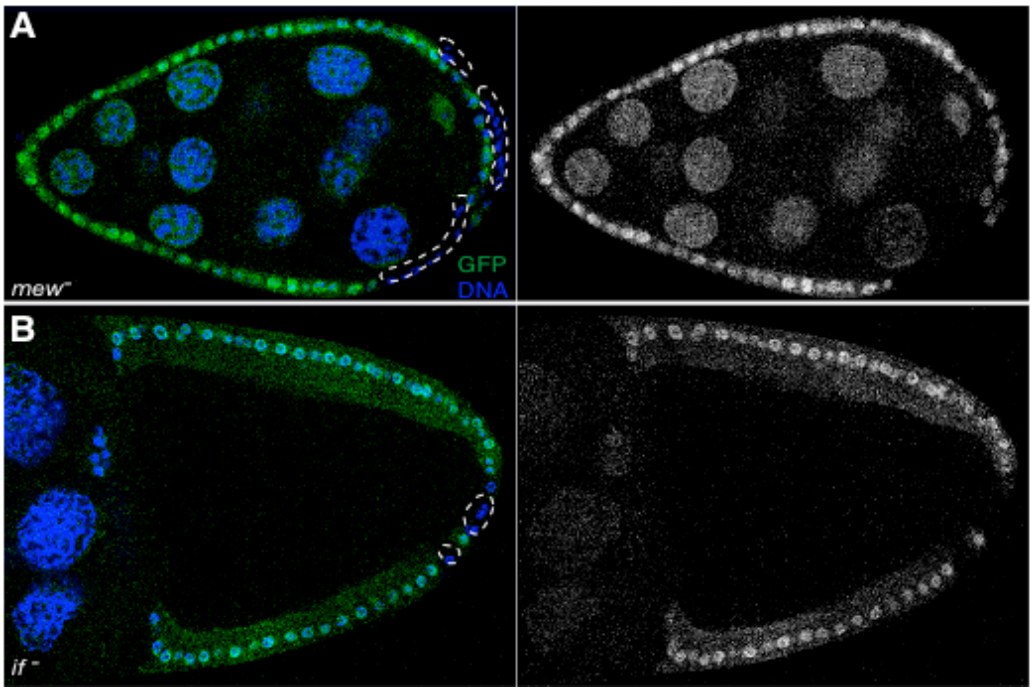

Figure 11. Loss of *mew* in follicle cells, but not *if*, results in a multilayer epithelium

Follicles containing *mew* (A) or *if* (B) mutant follicle cell clones stained with anti-GFP (green) and TO-PRO-3 (blue). (A) Absence of *mew* activity in the follicle cells causes a stratification of the follicular epithelium in mutant clones located at either pole of the developing egg chamber. (B) In contrast, *if* is not required to maintain the monolayer structure of the follicular epithelium. Dashed lines indicate the position of mutant cells.

III.1.iii. Proliferation is not affected in *myspheroid* mutant follicle cells

Integrins have been involved in the control of cell proliferation and tumour growth [218][Bottazzi, 1997 #436. In our system, however, the similar size of wild-type twin clones and mutant clones suggests that, like in the case of the wing disc epithelium [96, 132], integrins are not required for epithelial cell proliferation during oogenesis (data not shown). To confirm this observation, we stained wild-type and experimental egg chambers displaying the multilayer phenotype with anti-phosphohistone H3, a mitotic marker, and counted the number of mitotic figures in the follicular epithelium (Fig. 12A, C). First, we determined that mutant cells ceased division at S6, like their wild-type neighbours (data not shown). This indicates that the ectopic layers in mosaic egg chambers are not due to an extension of the mitotically active period in the ovary beyond S6. Next, we assessed mutant and wild-type cells within additional layers of follicle cell epithelia to determine if such cells divide more frequently than cells in contact with the germ line. We subdivided the follicular epithelia of S3-4 and S5-6 egg chambers into three arbitrary areas into which we scored mitotic cells. Our analysis revealed no significant difference in the frequency of cells in mitosis between control and mosaic egg chambers containing extra layers at one or both poles (Fig. 12B, D). Thus, the growth of ectopic cell layers in mosaic egg chambers does not appear to be a consequence of excess proliferation of either *myspheroid* mutant cells or wild-type cells that have detached from the germ line. This conclusion is further supported by our observation that prior to S3 proliferation of mutant cells is comparable to wild-type siblings, excluding the possibility that stratification is the consequence of abnormal cell proliferation.

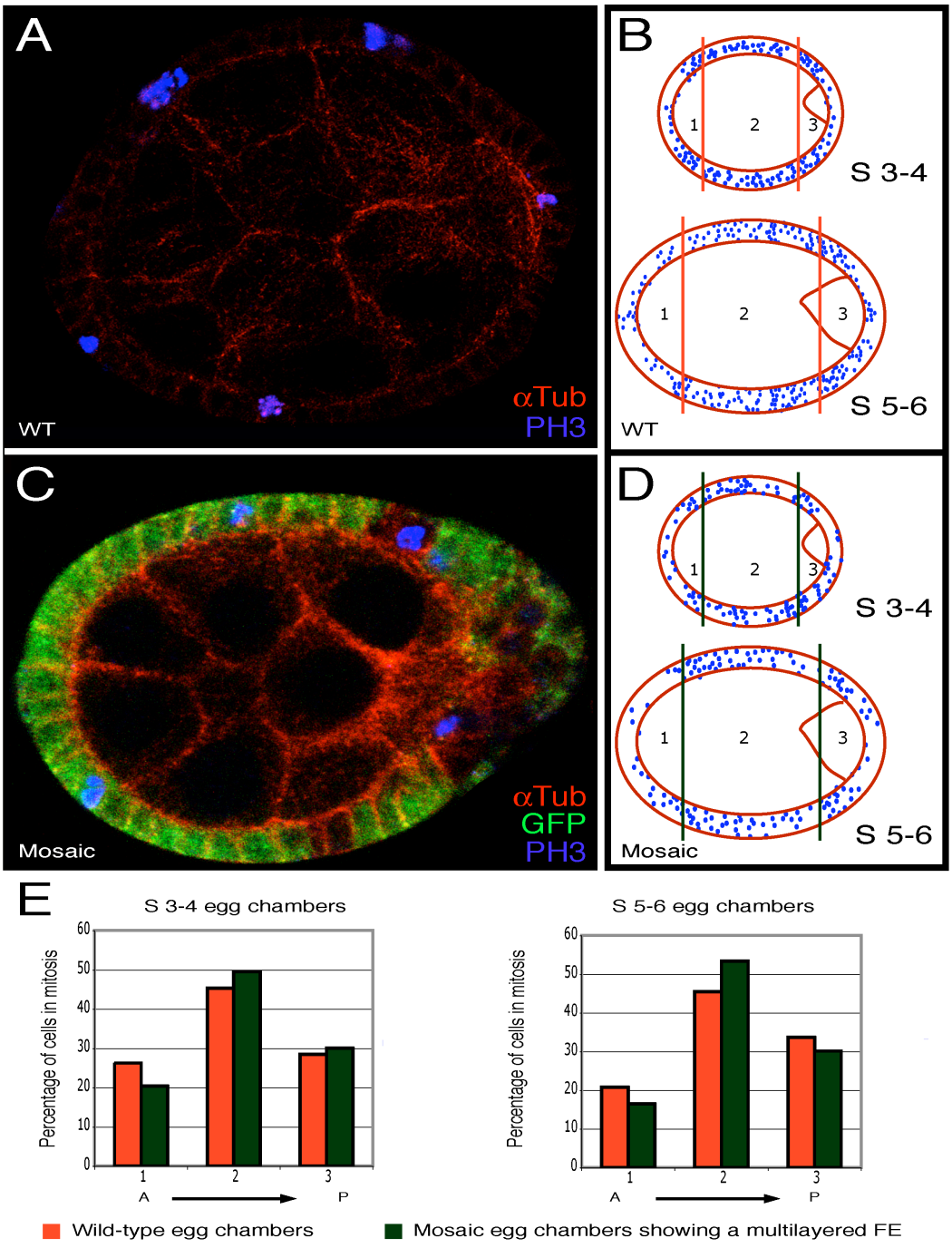

Figure 12. The proliferation rate is not increased in *myspheroid* mutant cells

(A) S5 wild type egg chamber labelled with anti-□-tubulin (red) and anti-PH3 (blue) to mark cells in mitosis. (B) The distribution of the mitotic cells found in a sample of wild-type S3-4 and S5-6 egg chambers is shown. Blue dots represent mitotic figures (n=136/22 for the S3-4 follicles and n=237/26 for the

S5-6 egg chambers). (C) Mosaic egg chamber carrying several *myspheroid* mutant clones and a multilayer posterior pole. (D) S3-4 and S5-6 egg chambers showing a stratified follicular epithelium were scored for mitotic figures (n=116/15 and n=134/13, respectively) and their distribution plotted. (E) Graphic visualization of the data shown in C and D. The follicular epithelium was arbitrarily subdivided into 3 regions along the anterior-posterior axis and the percentage of the total number of mitotic figures *per* area was represented. The distribution of cells in M-phase in control and experimental epithelia showed that the rate of follicle cell proliferation of S3-6 egg chambers is relatively homogeneous along the AP axis (Fig. 3E). n=total number of mitotic figures scored/total number of egg chambers analyzed.

III.1.iv. *myspheroid* mutant cells in contact with the germ line possess normal apical-basal polarity

Epithelial cells polarize into apical and basal-lateral domains in response to cell-cell adhesion and to cell-matrix interactions [182, 212]. Since the loss of function of genes involved in the establishment and maintenance of epithelial polarity such as *crumbs*, *bazooka (baz), atypical Protein Kinase C (aPKC)* and *discs large (dlg)* give rise to multilayer phenotypes [25, 29, 34, 37, 219], and since integrins have been implicated in the polarization of epithelial cells in culture and in the epidermis [211, 220], it is possible that the ectopic layers developed in mosaic epithelia arise because the apical-basal polarity of mutant cells is compromised in absence of integrins. To test this hypothesis, we analyzed the distribution of the apical markers Baz, Patj, βHeavy-spectrin (β_H-Spec), aPKC, *D*E-Cadherin (*D*E-Cad) and Armadillo (Arm), and that of the lateral markers Dlg and β-Spectrin (β-Spec) in *myspheroid* mutant cells [22, 25, 32, 33, 221-224]. We found that all these markers localized correctly in mutant cells directly adjoining the germ line, irrespective of whether they were at the terminal or main

body domains, strongly suggesting that integrin-mediated cell-matrix interaction is not required to polarize the follicle cells that make contact with the germ line (data not shown). This observation is in agreement with a previous report that showed that *D*E-Cadherin is localized apically in follicle cells lacking integrin function [132].

Cell-extracellular matrix interactions are important for establishing spatial asymmetry in epithelial cells [225]. As integrins are also required for the extracellular modelling [226], it is possible that integrins expressed in the germ line are sufficient to organize the matrix between the germ line and the follicular epithelium, which could in turn induce apical-basal polarization of *myspheroid* mutant follicle cells. To test this possibility, we examined the polarity of *myspheroid* mutant follicle cells in contact with *myspheroid* mutant germ line. We found that the membrane polarity of these mutant cells was not affected, as assayed by the localisation of the polarity markers Baz and Dlg, which were distributed normally in *myspheroid* mutant clones (Fig. 13). This finding provides further evidence towards an integrin-independent mechanism for the establishment (or maintenance) of polarity in the follicular epithelium monolayer.

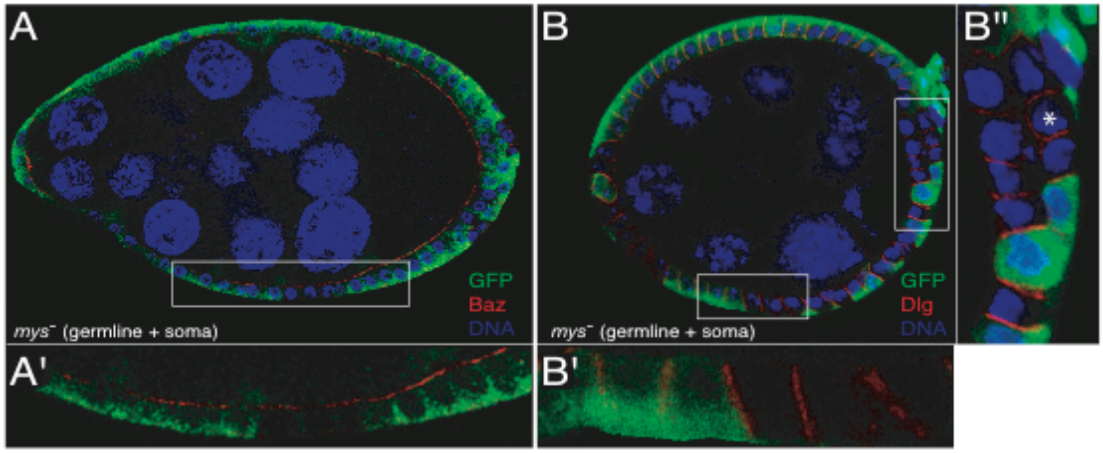

Figure 13. Integrins are not required to maintain the apical-basal polarity of follicle cells in contact with the germ line

Mosaic egg chambers carrying *myspheroid* germline clones and *myspheroid* mutant follicle cell clones labelled with anti-GFP (green), TO-PRO-3 (blue) and with anti-Bazooka (A) or anti-Discs large (B) in red. (A, B) The apical distribution of Baz and the lateral one of Dlg are not visibly affected in mutant main body or terminal follicle cells adjoining *myspheroid* mutant germ line. (A', B', B'') Magnifications of the boxes in (A) and (B), respectively. Asterisk: mutant cell not in contact with the germ line showing an aberrant distribution of Dlg (see legend to Fig. 14 for details).

III.1.v. A role for the germ line and the basement membrane in follicle cell polarity

In contrast to follicle cells that maintain contact with the germ line, follicle cells detaching from the main epithelium at the termini lose their cuboidal shape and display an abnormal distribution of apical and lateral markers. The precise phenotype depends on both, the cell's genotype and its position within the extra-layers. For example, wild-type cells contained within the most external layer exhibit a normal distribution of Baz, *D*E-Cad and Dlg (Fig. 14A and data not shown). Interestingly, although wild-type cells in the inner layers still localized apical markers such as Baz and *D*E-Cad asymmetrically, the place of accumulation was not always orientated with respect to the germ line as these cells could localize apical markers facing the basement membrane (Fig. 14A). In contrast, mutant cells in the ectopic layers showed a distribution of Baz and Dlg that was no longer restricted to the apical and lateral domains, respectively, as defined by the position of the germ line. Instead, they often accumulated over a large fraction of the cell membrane (Fig. 13B''; Fig. 14A, B). Similar, abnormal distribution patterns were obtained for Patj, *D*E-Cad, Arm, β_H-Spec, aPKC and β-Spec (data not shown). Nevertheless, mutant cells in the extra layers seem to maintain the demarcation between apical and basal-lateral membranes, as apical and lateral markers such as aPKC and Dlg do not co-localize (Fig. 14C). Considering that the primary cue for the polarization of the follicular epithelium is contact with the germ line [37], the above observations suggest that integrins — presumably via interactions with the basement membrane — play a reinforcing role in follicle cell apical-basal polarisation, as they are required to establish or maintain follicle cell membrane asymmetry only when contact with the germ line is lost. This hypothesis is supported by the distribution of the laminin-rich

basement membrane in stratified epithelia, which contacts only the most external layer (Fig. 14D). Cells in the intermediate layers fail to contact either the germline or the basement membrane and thus show aberrant polarisation.

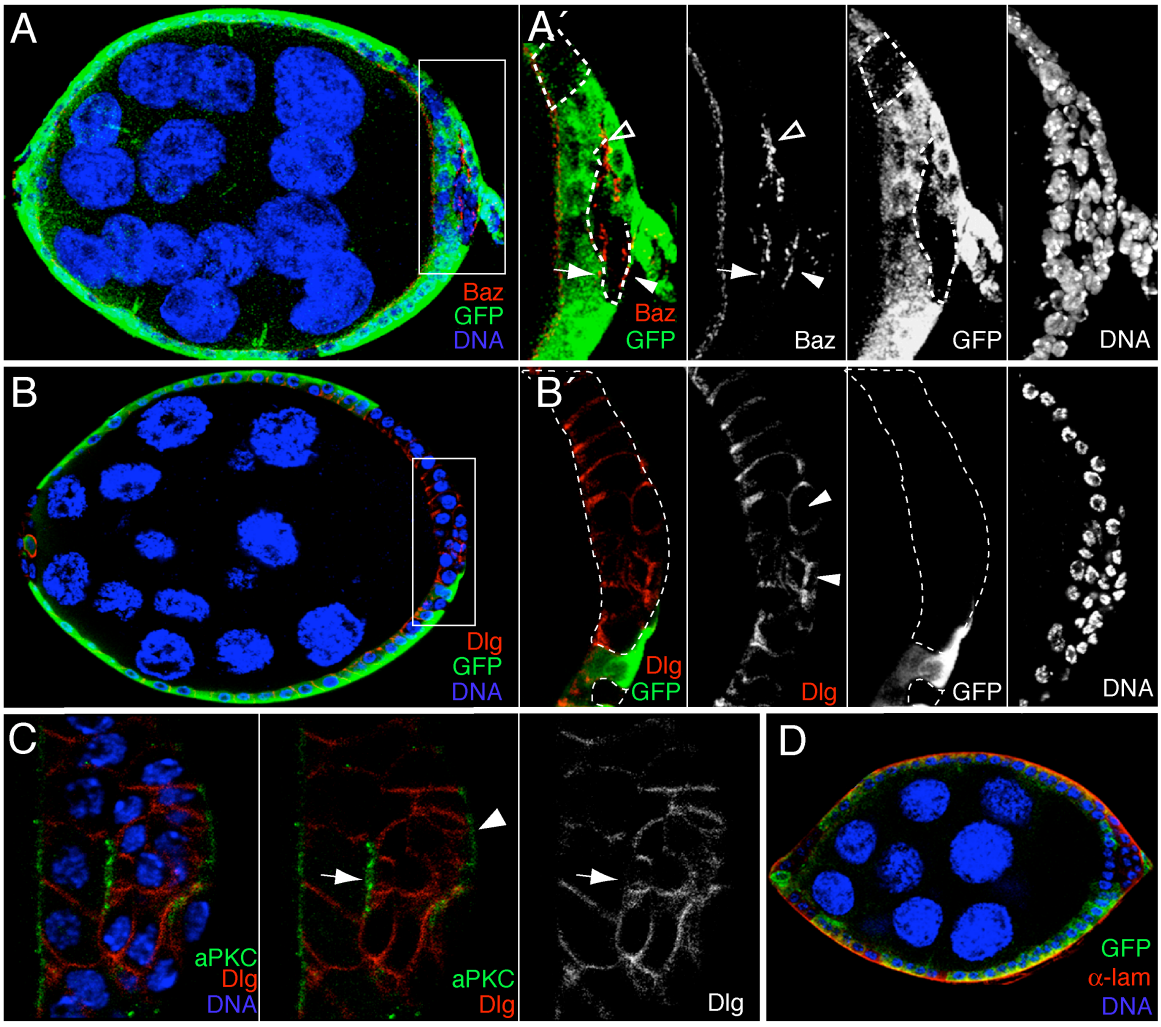

Figure 14. Mutant follicle cells detached from the germ line show an abnormal polarity

(A, B) Mosaic egg chambers harbouring *myspheroid* mutant clones stained with anti-GFP (green), TO-PRO-3 (blue) and anti-Bazooka (A) or anti-Discs large (B). (A) Baz localizes apically in wild-type and mutant posterior follicle cells in contact with the germ line. In contrast, mutant cells in ectopic layers show mislocalized Baz staining (arrowhead; see arrowhead in C for another example

of a mislocalized apical marker in mutant cells in the most external layer). Wild-type cells that form the most external layer display normal localization of Baz (empty arrowhead), while wild-type cells in intermediate layers accumulate Baz distal to the oocyte (arrow). (B) Dlg accumulates laterally in wild-type and mutant cells in contact with the germ line. This distribution is lost in mutant cells forming the additional layers (arrowheads). (C) Posterior pole of a mosaic egg chamber containing *myspheroid* mutant cells. The localization of the apical marker protein aPKC (green), lateral Dlg (red) and the DNA dye TO-PRO-3 (blue) is shown. In spite of the abnormal polarity of mutant cells within the ectopic layers, there is no co-localization of aPKC and Dlg (arrow). (D) Mosaic egg chamber displaying a stratified epithelium at the posterior pole stained with the ECM component Laminin A (red), anti-GFP (green) and TO-PRO3 (blue). The basement membrane, defined by the major concentration of Laminin A, is found only at the basal side of the most external follicle cell layers. (A', B') Magnifications of the boxes in (A) and (B), respectively.

III.1.vi. Integrin function is required to orientate the mitotic spindle of follicle cells

Cell proliferation and cell polarity are normal in myspheroid mutant follicle cells adjoining the germ line and thus cannot be the cause of epithelial stratification. Considering that the orientation of the mitotic spindle determines the position of the two daughter cells after division, we decided to investigate whether a defect in the orientation of the mitotic spindle is responsible for the phenotype observed in mosaic egg chambers. Consistent with this assumption, we found that wild-type follicle cells always align their mitotic spindle parallel to the surface of the germline cells so that both daughter cells remain in contact with the germ line and within the monolayer (Fig. 15A, B). This spindle orientation pattern was also always observed in myspheroid mutant cells

in the main body domain (Fig. 15C). In contrast, *myspheroid* mutant follicle cells within the terminal domains, whether in contact with the germline or in the ectopic layers, aligned their spindles randomly. In fact, 30% of observed spindles (n=20; Fig. 15D) were found completely perpendicular with respect to the germ line. It is important to note that this percentage refers only to spindles positioned strictly perpendicular to the germ line and thus represents an underestimation of the total number of misaligned spindles. In the case of mutant cells in contact with the germ line, this misalignment would most probably result in one of the daughter cells being excluded from the epithelial monolayer.

Our results show that the ectopic layers detected in mosaic epithelia are composed of both wild-type and *myspheroid* mutant cells, implicating a non-autonomous effect of the lack of integrin function in epithelial overgrowth. In order to test if the lack of integrin activity affects the divisions of adjacent wild-type cells, we studied spindle orientation of wild-type follicle cells abutting *myspheroid* mutant cells. Consistent with our previous findings, we did not detect any defects in the orientation of the mitotic apparatus in either mutant or wild-type main body domain follicle cells. However, wild-type cells in direct contact with *myspheroid* mutant cells within the terminal domains exhibited mitotic spindles aligned strictly perpendicular to the germ line in 17% of observed cases (n=18; Fig. 15E, F). Taken together, our observations demonstrate that integrin function is required for proper positioning of the mitotic spindle in follicle cells in a limited non-autonomous fashion. We propose that integrins are necessary to preserve the simple monolayer organization of the follicular epithelium by controlling mitotic spindle alignment.

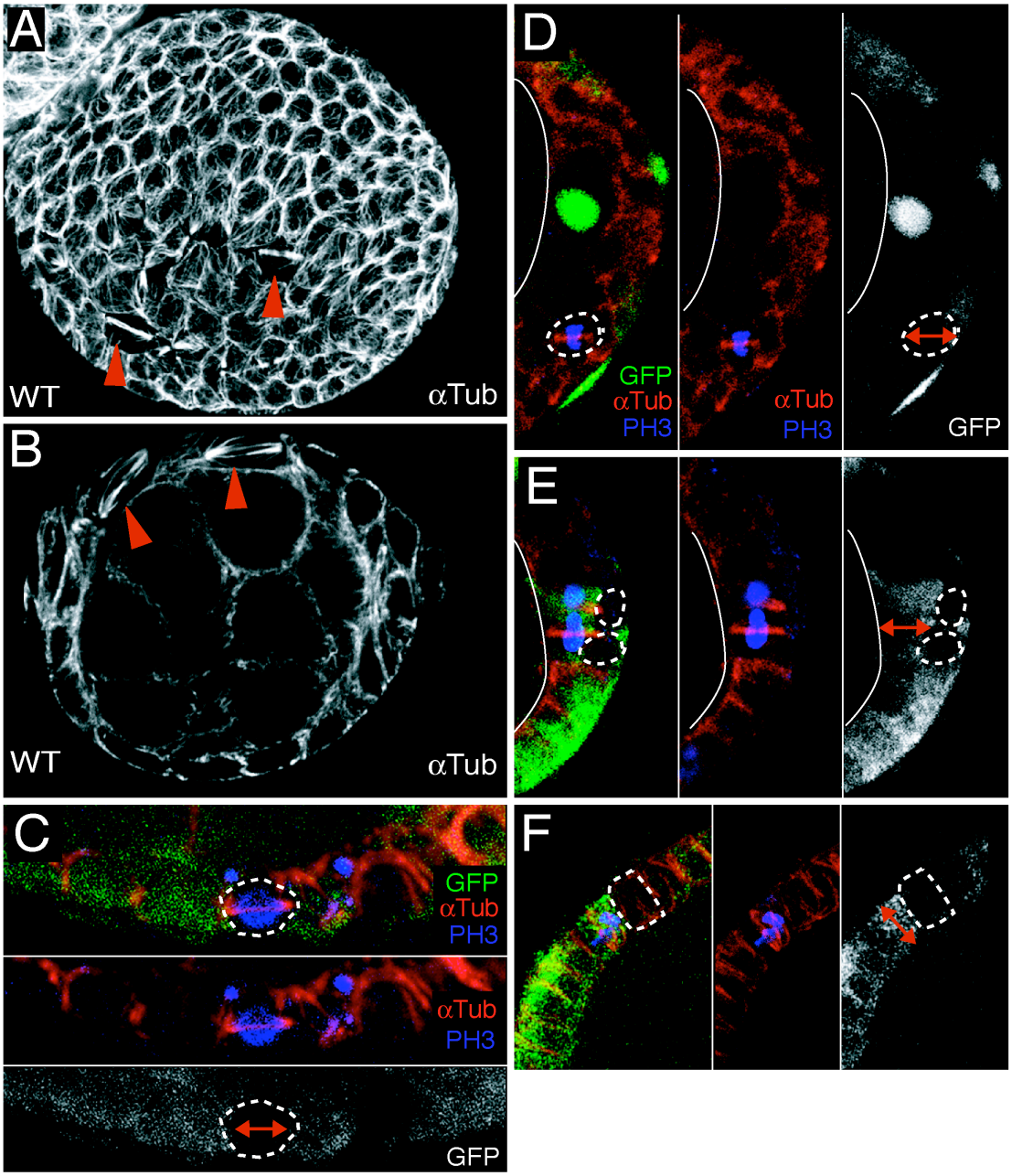

Figure 15. Integrins are required to orientate the mitotic spindle of epithelial follicle cells

(A) Top and (B) lateral views of wild-type egg chambers labelled with anti-□-tubulin to visualize microtubules. Mitotic spindles are found parallel to the surface of the germline cells (arrowheads). (C-F) Mosaic epithelia labelled with anti-GFP (green), anti-□-tubulin (red) and anti-PH3 (blue) to show the chromatin and the orientation of the spindle in mitotic cells. (C) Wild-type and

mutant cells located in the main body domain always position their mitotic spindles parallel to the germ line. (D) In contrast, the mitotic spindle in cells lacking *myspheroid* function in the terminal domains can adopt a random orientation with respect to the germ line. This aberrant orientation of the spindle is non-autonomous, as terminal wild-type cells in contact with *myspheroid* mutant cells can align their spindle perpendicular to the germ line (E, F). Double-arrows indicate the orientation of the mitotic spindles. Dashed lines indicate mutant cells.

III.1.vii. The orientation of the mitotic spindle is dependent on integrin-mediated signalling

Integrins have been described to play important roles in cell-matrix adhesion and in signalling events during cell differentiation [227]. In order to elucidate which of these two integrin-mediated processes is responsible for the stratification phenotype, we utilized two different experimental conditions to uncouple integrin-linked signalling and adhesion. The *Drosophila* homolog of the mammalian Focal Adhesion Kinase (FAK) family of non-receptor protein-tyrosine kinases, Fak56, is dispensable for integrin functions in adhesion, migration or signalling during development. However, overexpression of Fak56 or Fak56:GFP in embryonic muscle or adult wing results in the dissociation of the integrins from the extracellular matrix, most probably by negatively regulating integrin ligand binding affinity [205]. Thus, the ectopic induction of Fak56 in the follicle cells should impair the ability of integrins to bind to the extracellular matrix with high affinity. As shown in Fig 16A, the ectopic expression of Fak56:GFP in large clones of terminal domain follicle cells did not result in epithelial stratification, suggesting that the adhesion of follicle cells to the extracellular matrix might not be essential for the orientation of their mitotic spindle. Second, to corroborate our finding we used the $Tor^{D}/\square_{cyt}$ chimera, which

consists of the cytoplasmic tail of the βPS subunit fused to the extracellular and transmembrane domains of a dominant gain-of-function mutant allele of the Torso receptor tyrosine kinase (Fig. 8). This fusion protein can substitute the endogenous integrin and activate signalling to regulate gene expression [177]. In addition, Tor^{D}/β_{cyt} behaves as a dominant negative and, when overexpressed, blocks adhesion mediated by the endogenous integrins to the extracellular matrix, as shown in embryonic and adult tissues [148, 228]. When ectopically expressed in the follicular epithelium of otherwise wild-type egg chambers, Tor^{D}/β_{cyt} did not produce any visible phenotypes within the terminal domains (Fig. 16B). Although we cannot rule out the possibility that FAK56:GFP or Tor^{D}/β_{cyt} overexpression do not block integrin-dependent adhesion in the follicular epithelium, given the effect that these transgenes have on other cell-matrix interactions, our results support a model in which the correct orientation of the plane of division of follicle cells depends on the signalling capacity of active integrins. To test further this hypothesis, we examined if the stratification phenotype caused by the lack of myspheroid could be rescued solely by activation of integrin signalling. To this end, we ectopically expressed Tor^{D}/β_{cyt} in *myspheroid* mutant cells and scored the ability of Tor^{D}/β_{cyt} overexpression to rescue the stratification phenotype. We found that expression of Tor^{D}/β_{cyt} could substantially rescue the stratified epithelia of *myspheroid* mutant clones, as only 16% (n=18) of mosaic egg chambers displayed a mild multilayer phenotype (Fig. 16C, D). Thus, considering that Tor^{D}/β_{cyt} cannot bind to the extracellular matrix, our results demonstrate that integrin-mediated signalling is sufficient to prevent the stratification of the follicular epithelium, most probably by ensuring that follicle cells divide within the epithelial plane.

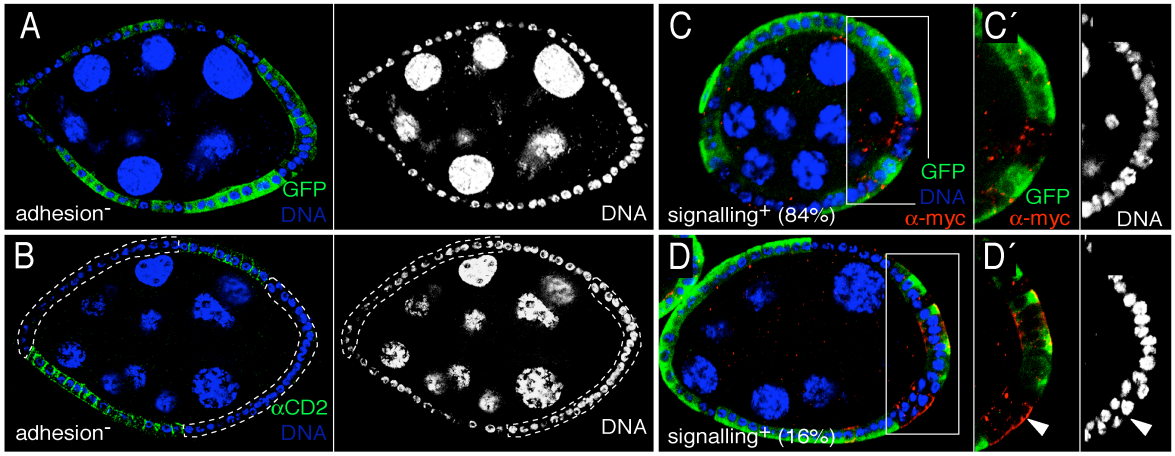

Figure 16. Integrin adhesion is not required to maintain the integrity of the follicular epithelium

(A) Mosaic egg chamber labelled with anti-GFP to mark cells that overexpress Fak56-GFP (green) and TO-PRO-3 (blue). (B) Mosaic egg chamber harbouring two clones of cells ectopically expressing $Tor^{D}/\square_{cyt}$ at both termini. The cells without CD2 signal (green; dashed lines) are $Tor^{D}/\square_{cyt}$ positive; TO-PRO-3 in blue. The number of clones at the termini analyzed in these experiments is 44 for the overexpression of Fak56-GFP and 18 for that of $Tor^{D}/\square_{cyt}$. As an internal control, we detected wing blisters in both experimental females, indicating that the transgenes were able to block integrin-mediated adhesion in the wing epithelium. (C, D) Mosaic egg chambers stained with anti-Myc to detect $Tor^{D}/\square_{cyt}$ (red), anti-GFP (green) and TO-PRO3 (blue) to show that the overexpression of $Tor^{D}/\square_{cyt}$ can rescue the stratification of *mys*$^{-}$ mosaic epithelia. (C) Egg chamber containing a clone of *mys*$^{-}$ cells that express $Tor^{D}/\square_{cyt}$. This egg chamber has not developed extra layers. (D) Egg chamber containing a clone of *mys*$^{-}$ cells that express $Tor^{D}/\square_{cyt}$. In this case, the mutant cells give rise to a mild multilayer phenotype.

III.1.viii. The class VI unconventional myosin Jaguar is required to orientate the mitotic spindle of follicle cells

In an attempt to unveil the molecular mechanisms by which integrin signalling regulates spindle orientation, we searched for proteins that could link integrin activation with mitotic spindle positioning. Considering the role that the actomyosin cytoskeleton plays in spindle orientation in other cell types (see Discussion) [229, 230], we analysed the role of several genes known to be implicated in the remodelling of the actin cytoskeleton in response to integrin signalling [231-234]. These include *rok* (*Drosophila* Rho kinase), a kinase utilised downstream of activated Rho GTPase [235], the three Rac GTPases known to be present in the *Drosophila* genome *rac1*, *rac2* and *Mtl* [236] and the regulatory light chain of the *Drosophila* non-conventional myosin II *spaghetti squash* (*sqh*) [237]. In addition, we studied the unconventional myosin *crinkled* (myosin VIIA), which contains two FERM domain repeats, a domain also found in Talin that mediates Talin-integrin direct binding [198, 238, 239]. Mosaic epithelia containing clusters of follicle cells mutant for *rok*, *sqh* or *ck* and for the three rac genes *rac1*, *rac2* and *Mtl*, did not give rise to phenotypes that may implicate these genes in follicle cell spindle orientation (Figs. 17, 18 and data not shown). These observations thus suggest that integrin-mediated positioning of the mitotic spindle does not require myosin II or VIIA, nor the actin cytoskeleton regulators rok, rac1, rac2 and Mtl.

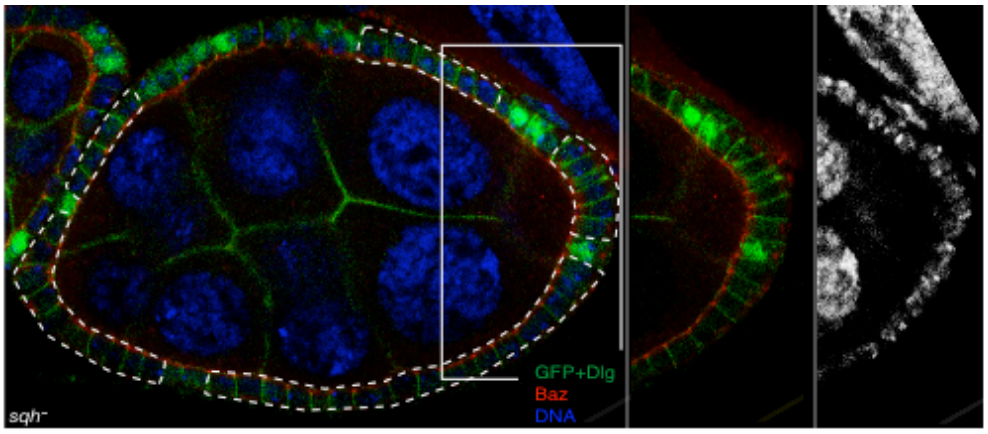

Figure 17. The myosin II regulatory light chain Sqh is not required to maintain the apical-basal polarity of follicle cells nor the follicular epithelium monolayer.

Egg chamber containing *sqh* mutant follicle cell clones stained with anti-Baz (red), anti-GFP and anti-Dlg (green) and TO-PRO-3 (blue). Dashed lines demarcate mutant cells.

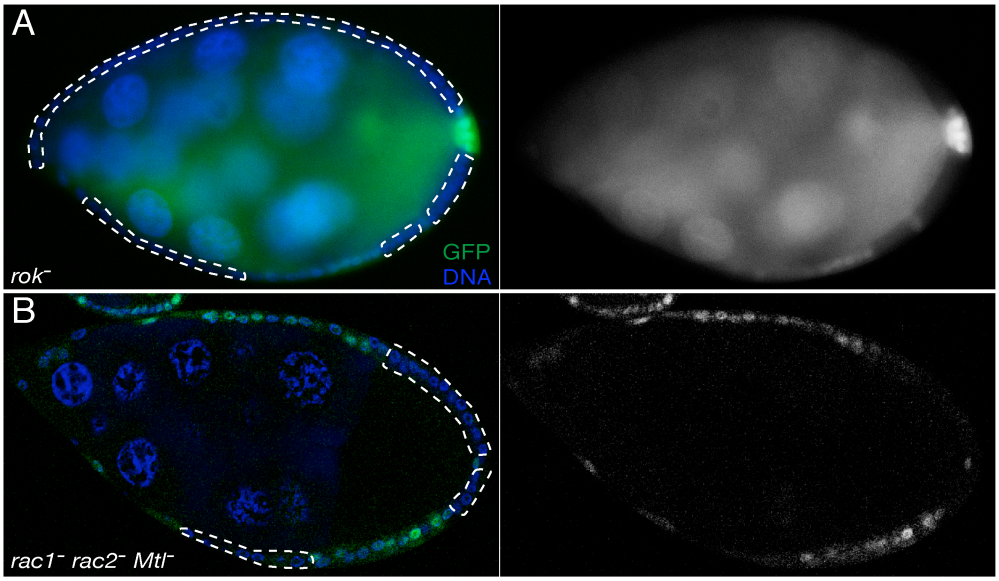

Figure 18. *Drosophila* Rho kinase and Rac GTPases are not required to maintain the follicular epithelium monolayer..

Egg chambers containing follicle cell clones mutant for *rok* (A) and *rac1, rac2* and *Mtl* (B) stained with anti-GFP (green) and TO-PRO-3 (blue). Dashed lines demarcate mutant cells.

Next, we examined the class VI myosin *jaguar (jar)*, known to be required for the correct orientation of the mitotic spindle in *Drosophila* neuroblasts [230]. We found that loss of unconventional myosin VI Jar in follicle cells shares a number of phenotypes with *myspheroid* mutant cells. First, *jaguar* mutant clones at the termini induce the growth of extra layers in 68% of the cases (n=22; Fig. 19A). Second, the apical-basal polarity of *jar* mutant cells is not affected, at least as shown by Baz and Dlg localisation (Fig. 19B, C). Third, the orientation of the mitotic spindle in *jaguar* mutant cells can be found perpendicular to the germline in 25% of the cases (n=16). As in the case of *myspheroid* mutant cells, this percentage is likely to be an underestimation of the number of randomised spindles, as we only scored strictly perpendicular ones (Fig. 19E). There are however two clear differences with *myspheroid* mutant cells. First, *jar* mutant cells can occasionally induce the arrangement of small patches of mutant main body follicle cells in a second layer. Although this phenotype is never found in *myspheroid* mosaic follicles and it suggests an integrin-independent activity of Jar — at least in this cell type —, the penetrance of this phenotype is low (27%, n=15; Fig. 19A). Second, we could not detect a non-autonomous effect of *jar* loss-of-function. In fact, all dividing wild-type cells next to mutant ones had correctly aligned spindles (n=11; Fig. 19F). Our results strongly indicate that *jar* is required for the proper alignment of the mitotic spindle in follicle cells without affecting their polarity. Interestingly, the phenotypic similarities between *myspheroid* and *jar* mutant cells raise the possibility that both genes act in the same pathway to position correctly of the mitotic spindle. In this scenario, the fact that *jar* acts cell autonomously places Jar downstream of integrin activity.

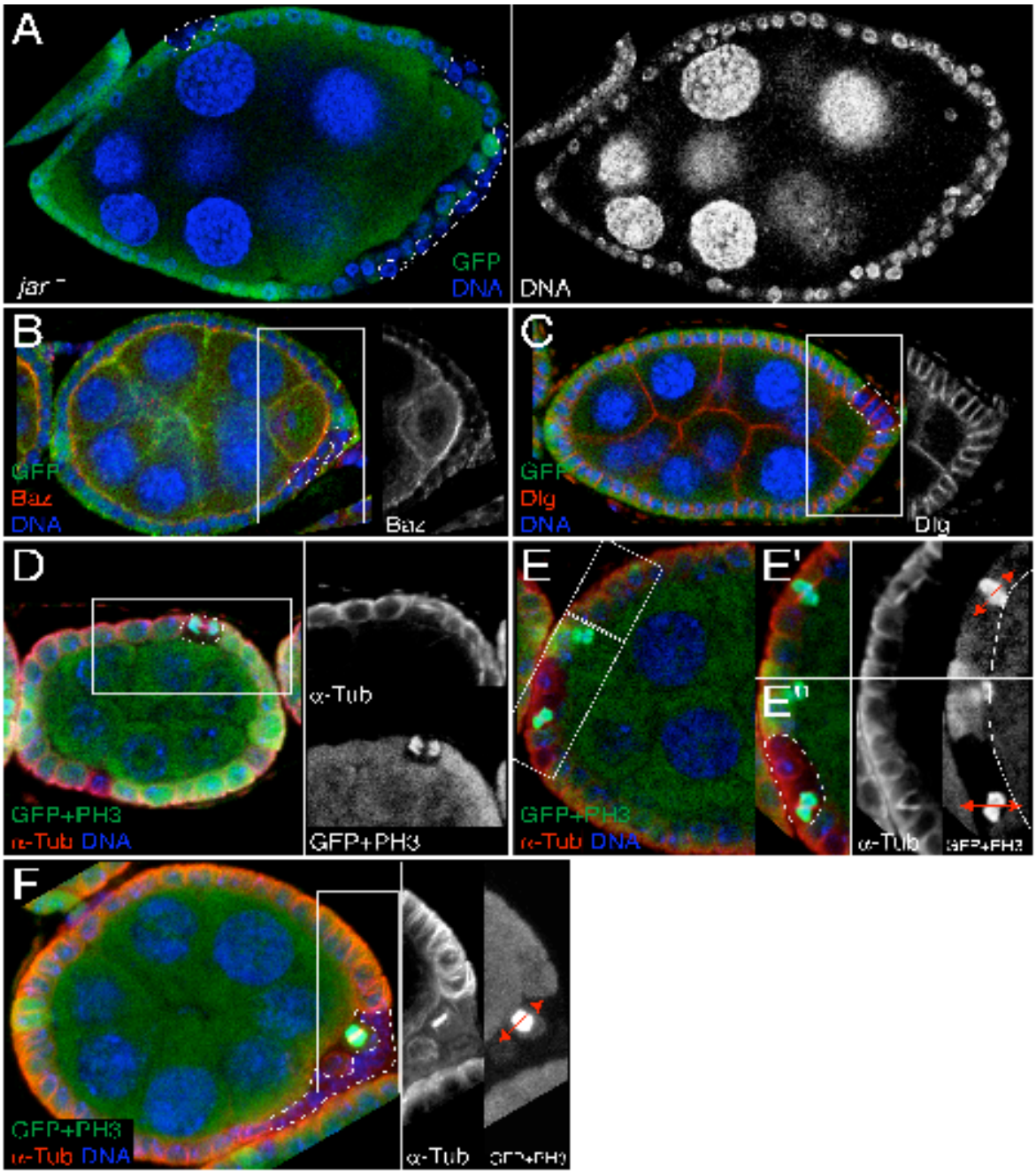

Figure 19. Unconventional myosin VI *jaguar* is required autonomously for the correct orientation of the mitotic spindle of epithelial follicle cells

(A) Mosaic egg chamber stained with GFP (green) and TO-PRO-3 (blue) to show that the absence of *jar* function can give rise to multilayered follicular epithelia in both main body and terminal follicle cells. (B, C) Egg chambers carrying *jar* clones labelled with anti-GFP (green), TO-PRO-3 (blue) and with

anti-Bazooka (B) or anti-Discs large (C) in red. Jar is not required for the correct localisation of Baz and Dlg proteins. The clone shown in (C) has developed ectopic layers, but these are visible in a different focal plane. (D-F) Mosaic follicles labelled with anti-□-tubulin (red), anti-GFP and anti-PH3 (green) and TO-PRO-3 (blue) to show the chromatin and the orientation of the spindle in mitotic cells. The green channel is used both to distinguish mutant cells and to label cells in mitosis. (D) Mutant cell located in the main body domain showing a mitotic spindle correctly oriented parallel to the germ line. (E) The mitotic spindle of *jar*$^{-}$ cells in the terminal domains can adopt a nearly perpendicular orientation with respect to the germ line. (E') Magnification of the box in E to show the abnormal orientation of the mutant spindle. (E'') An internal, wild-type control (not visible in the plane of focus imaged in (E)) is shown for comparison. (F) This aberrant orientation of the spindle is cell autonomous, as terminal wild-type cells in contact with *jar* mutant cells always align their spindle parallel to the germ line. Double-arrows indicate the orientation of the mitotic spindles. Dashed lines delineate mutant cells.

III.2. DISCUSSION

III.2.i. Integrins are required to orientate the mitotic spindle

Using a null allele of the *mys* gene we have identified a new role for integrins in the maintenance of epithelia. We show that lack of integrin function induces the stratification of the follicular epithelium, a phenotype due neither to excessive cell proliferation nor to defects in the apical-basal polarity but to improper positioning of the mitotic spindle. In addition, and since the development of ectopic layers could be rescued by the expression of a chimaeric integrin that is unable to adhere to the extracellular matrix but capable of triggering intracellular signalling, our results demonstrate that integrin-mediated signalling is required for the proper orientation of the mitotic spindle in epithelial follicle cells. Although it has been recently shown that mouse keratinocytes require b1 integrin for proper spindle orientation [211], this integrin is also necessary for the establishment of apical-basal polarity in this cell type, in contrast with the role of *mys* in follicle cells. Our results thus provide a link between integrin activity and cell division that may explain the aberrant behaviour of certain epithelia when integrin function is impaired, such as the mammary glandular epithelium [240, 241]. Interestingly, it has been reported recently that integrin b1 tail regulates several aspects of mitosis in cells in culture, such as centrosome function, the organisation of the mitotic spindle and cytokinesis [187]. While our results do not involve βPS integrin in cell proliferation, the above findings reinforce the role of integrins during mitosis.

The mechanism by which integrins influence the orientation of the mitotic apparatus is still unknown, but several lines of evidence point to an interaction between the actomyosin cytoskeleton and integrin activity

in this process. First, this cytoskeleton is one of the main targets of integrin signalling and a variety of molecules that transmit signals from activated integrins to the actin cytoskeleton have been identified, such as Talin [242]. Moreover, non-muscle myosin-II has been reported to interact with integrins to affect nerve growth cone motility and to regulate epithelial cell scattering [234, 243]. In addition, unconventional myosin-X has been shown to provide a motor-based link between integrins and the actin cytoskeleton in vertebrate cells in culture [244]. Finally, integrins are required in epithelial follicle cells to organize actin filaments [30]. Second, the importance of the actomyosin cytoskeleton in the orientation of cell division came from the demonstration that myosin-II is required to separate and position the centrosomes during mitotic spindle assembly and alignment [229] and by the finding that the class VI unconventional myosin Jaguar is required to position the cell division axis in *Drosophila* neuroblasts [230]. Our observation that Jaguar is necessary for the correct orientation of the mitotic spindle in follicle cells, but not for their apical-basal polarisation, strongly supports this view. Hence, we favour a model where integrin adhesion to the extracellular matrix elicits a signal cascade in the follicle cells that implicates Jaguar and that organizes the actin cytoskeleton. As a consequence, cells that enter mitosis separate their duplicated centrosomes so that the spindle is aligned parallel to the germline surface, a final orientation that is aided by the anchoring of interphase centrosomes to one of the apical corners of follicle cells. Interestingly, Jar interacts with CLIP-190, a microtubule-binding protein that associates strongly to microtubule plus-ends in interphase, thus providing a link between Jar and the microtubule cytoskeleton [245, 246].

Planar cell polarity (PCP) genes have been involved in mitotic spindle orientation in the *Drosophila* wing disc, in zebra fish gastrulation and in

Arabidopsis shoot apex growth [247-250]. In order to test if PCP genes were co-operating with integrins in spindle orientation in follicle cells, we checked the consequences of removing the function of PCP receptors *frizzled* and *frizzled 2* simultaneously, or that of the core PCP gene *dishevelled* [251]. We found that disrupting the PCP pathway does not produce similar phenotypes to *mys* mutants (data not shown), suggesting that PCP genes are not required for the correct orientation of the mitotic apparatus in the follicular epithelium. This conclusion is supported by the fact that mutations in the receptor tyrosine phosphatase *Dlar*, which is required to polarize the actin cytoskeleton of follicle cells and to organize epithelial planar polarity, do not induce stratification of the follicular epithelium [30, 43].

III.2.ii. A non-autonomous effect of integrin signalling

The fact that the ectopic layers developed in mosaic egg chambers are composed of both mutant and wild-type cells suggests that the abrogation of integrin function affects both classes of cells. This non-autonomous effect of the lack of integrins has previously been observed in the organization of the actin fibers that form at the basal side of the follicle cells [30], as removal of *mys* function caused a disorganization of the actin fibers of both mutant and surrounding wild-type cells. How the impairment of integrin signalling in one cell could affect its wild-type neighbour is unknown, but our results indicate that this is a short-range effect. Considering the role of integrins in ECM organization [226], it is possible that the local non-autonomous effect is due to a failure to assemble correctly the ECM around mutant cells. As a consequence, integrins in the adjoining cell cannot adhere properly, resulting in defective integrin-mediated signalling and spindle alignment. A second possibility would involve a memory mechanism for the positioning of the

mitotic spindle, which would require that a cell 'remembers' how the plane of division was fixed in the previous divisions. In this case, our results implicate integrin signalling in the transmission of the 'spindle memory' from a progenitor cell to its daughters.

IV. THE ROLE OF MISSHAPEN DURING BORDER CELL MIGRATION

IV.1. RESULTS

IV.1.i. *misshapen* is expressed in border cells prior to and during their migration

In order to investigate possible requirements for *misshapen* in border cell migration, we first examined its expression pattern. When characterized as a gene involved in the control of cell shape in *Drosophila* photoreceptor cells, *misshapen* was also vaguely reported as being expressed in border and follicle cells during oogenesis (data not shown in [109]). Therefore, we first decided to analyze in more detail the pattern of *misshapen* expression during oogenesis. To this end, we made use of three enhancer trap lines as reporters of *misshapen* expression, *misshapen*^{6286} that had been reported to recapitulate the *misshapen in situ* pattern [252] [109], *misshapen*^{10162} and *misshapen*^{138716}(ref). All lines show an identical and very dynamic expression pattern in the ovary. In the germarium, *misshapen* is expressed in the inner germarial sheath cells, and more specifically in the escort cells (Fig. 20B,B'). As the egg chambers bud off the germarium, from S3 onwards, *misshapen* is expressed at low levels in all follicle cells and at high levels in the anterior and posterior polar cells (Fig. 20B-E). At S8 and early S9, high levels of *misshapen* are not only detected in the polar cells but also in a group of follicle cells adjacent to the anterior polar cells, the border cells (Fig. 20D,E). This elevated expression in the border cells persists during their migration (Fig. 20E). In addition, the low expression detected in most if not all follicle cells also continued at stage 9 and later.

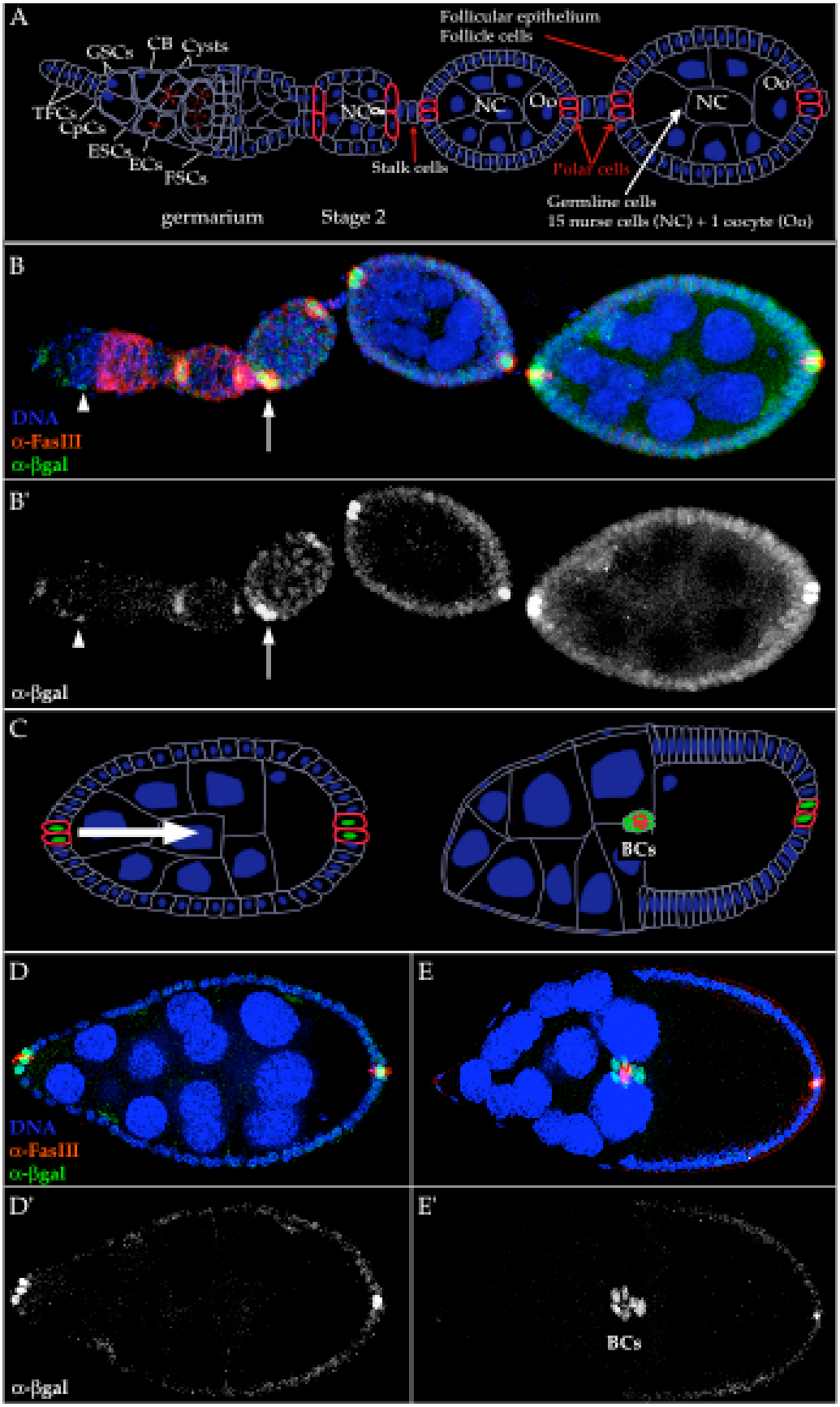
A
GSCs
CB
Cysts
TFCs
CpCs
ESCs
ECs
FSCs
germarium
Stage 2
NC
Oo
Stalk cells
Follicular epithelium
Follicle cells
Polar cells
Germline cells
15 nurse cells (NC) + 1 oocyte (Oo)
B
DNA
α-FasIII
α-βgal
B'
α-βgal
C
BCs
D
DNA
α-FasIII
α-βgal
E
D'
α-βgal
E'
BCs

Figure 20. Misshapen expression pattern during *Drosophila* oogenesis.

(A) Schematic representation of an ovariole showing the germarium and the developing egg chambers until S6. GSCs – Germline Stem Cells, CB – Cystoblast, TFCs – Terminal Filament Cells, CpCs – Cap Cells, ESCs – Escort Stem Cells, ECs – Escort Cells, FSCs – Follicle Stem Cells. Polar cells are shown in red. (B-E) Ovaries from the enhancer trap line *misshapen*^{10162} stained to visualise DNA (blue), □-FasIII (red) and □-□gal (green). In the germarium, *misshapen* is expressed in the Escort Cells (arrowhead in B and B'). From S3 onwards *misshapen* expression is detected in all follicle cells at low levels and at high levels in both anterior and posterior polar cells (arrows in B, B'). (D, E) Schemes of S8 and S10 egg chambers, respectively. The arrow in C indicates the direction of migration of the border cell cluster. *misshapen* expression is also detected at high levels in border cells before migration starts (C, C'). These high levels are maintained during their migration towards the anterior membrane of the oocyte by S10 (E, E'). BCs stands for border cells.

The high expression levels of *misshapen* found in border cells prior to and during their migration, together with its role in cell shape control [109], indicated a possible role for this gene in border cell migration.

IV.1.ii. *misshapen* is required for border cell migration

To examine the role of *misshapen* in border cell migration, we analysed mosaic egg chambers containing clones of the null alleles *msn*^{102} or *msn*^{172} [109, 217]. To visualize the border cell cluster we used a DNA dye (TOPO-3) and the anti-FasIII antibody which labels the polar cells [51]. By stage 10, wild-type border cells have reached the oocyte. At this stage, main body follicle cells have also reached the oocyte, which provides a good landmark to age the egg chambers. However, in 100%

of mosaic S10 egg chambers where all cells within the cluster are mutant for *misshapen* migration was completely blocked and border cells were found at the anterior pole of the follicles (n = 58) (Fig. 21A, E). In 88.9% of S10 egg chambers with mosaic border cell clusters containing both wild type and mutant cells, migration was also blocked (n = 36) (Fig. 21B, E). This failure in migration was independent of the proportion of wild type versus mutant cells since even border cell clusters harbouring only one or two mutant cells were unable to migrate. In the remaining 11.1% of S10 egg chambers, the mosaic border cells clusters showed a delayed migration phenotype, as they reached only half way to the oocyte (Fig. 21E). Interestingly, in mosaic border cell clusters that were able to migrate, wild type cells were always found at the front of the cluster, while mutant cells were dragging behind (Fig. 21B). This phenotype has been observed in mutants for other genes involved in border cell migration [63, 70, 253].

The function of Misshapen is not restricted only to border cell migration as *misshapen* mutant main body follicle cells also show a delayed posterior migration phenotype (Fig. 21C). This phenotype was observed only when mutant clones were affecting the most anterior main body follicle cells. These results indicate specific requirements for *misshapen* in the migration of the anterior follicle cells.

The system that we used to generate follicle cell clones also gives rise to sporadic germline clones, thus allowing the analysis of the role of *mishappen* in the germ line. We found that 63.6% of S10 egg chambers (n=11) harbouring mutant germline for *misshapen* and a wild type border cell cluster migration was also blocked (Fig. 21D,E). In the remaining 36.4%, migration was delayed. This phenotype has been quantified in the table in Fig. 21E where 0% of migration refers to the clusters found at the anterior pole, <50% and >50% of migration indicate clusters that are found before and after half way to the oocyte

(50% migration), respectively, and 100% migration indicate clusters with no defects in migration.

Altogether, these results show that *misshapen* is absolutely required for the migration border cell and a subpopulation of anterior follicle cells.

Next, we decided to address what are the mechanisms by which *misshapen* is controlling border cell migration. For that purpose, we decided to analyse the phenotype of *misshapen* mutant border cells at a cellular level, looking at the distribution of cell adhesion molecules and the actin cytoskeleton.

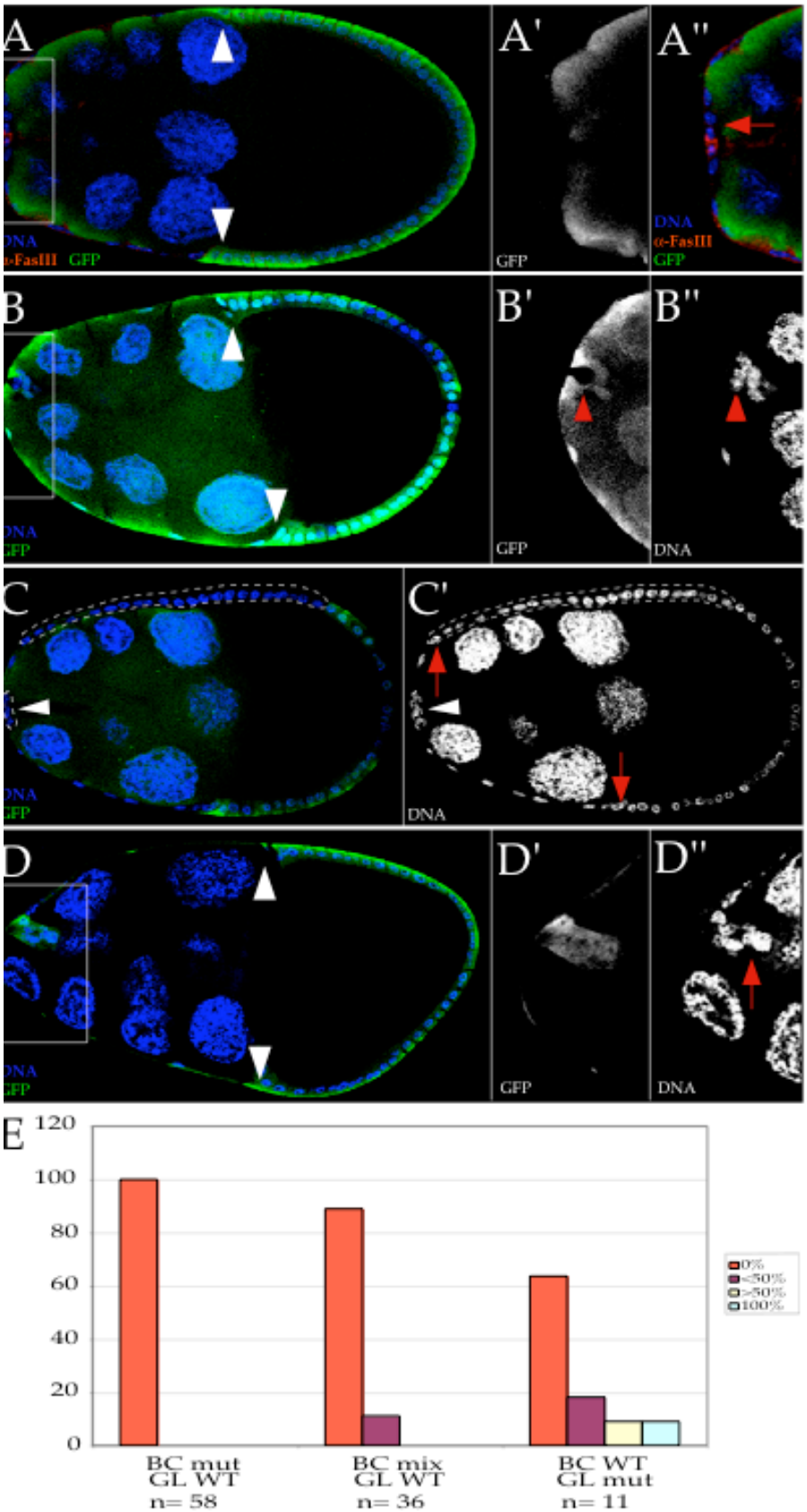
A
A'
A''
DNA
α-FasIII
GFP
B
B'
B''
C
C'
D
D'
D''
E
120
100
80
60
40
20
0
0%
<50%
>50%
100%
BC mut
GL WT
n= 58
BC mix
GL WT
n= 36
BC WT
GL mut
n= 11

Figure 21. Loss of Misshapen function in border cells results in a block of migration.

S9 (A, C) and S10 (B, D) egg chambers harbouring *misshapen* mutant follicle cells (A-C) and germline (D) clones stained against DNA (blue) (A-D) and anti-FasIII (red in A). Mutant cells are marked by the absence of GFP in all figures. (A) At S9 *misshapen* mutant border cells fail to migrate and stay at the anterior pole of the egg chamber (red arrow in A´) when they should be at the same distance from the oocyte as their wild-type counterpart main body follicle cells (arrowheads in A). (A´, A´´) Magnifications of the white box in A. (B) Wild type border cells are found at the front of the cluster and have initiated migration while mutant border cells (red arrowhead in B´ and B´´) are dragged behind. (B´,B´´) Magnifications of the white box in B. (C, C´) *misshapen* mutant main body follicle cells (dashed line in C) also display a defective migration phenotype (red arrows in C´). The white arrowheads in C and C´ point to *misshapen* mutant border cells that have not migrated. (D) Border cell migration is also affected (red arrow in D´´) when the germline is mutant for *misshapen* (D´). (E) Table correlating the percentage of migration with the cells mutant for *misshapen* (see text). BC = Border Cells, GL = germline, mut= mutant and n= number of egg chambers analysed.

IV.1.iii. *misshapen* mutant border cells display a wild-type polarity

Prior to migration, border cells are part of the follicular epithelium and like the rest of follicle cells they exhibit an epithelial apical-basal polarity [63] (for review see [60]. This polarity is maintained by transmembrane proteins that operate at the apical, lateral or basal surface of the follicle cells [37]. For border cells to migrate, changes in the localization of some of these polarity proteins are necessary. For instance, a switch in the polarized expression of Fasciclin II (FasII), Discs-large (Dlg) and Lethal-giant-larvae (Lgl) in polar cells from a

circumferential localization to the leading half controls delamination and migration of the border cell cluster [36]. At the time of border cell differentiation, Fasciclin II expression is lost from all the anterior follicle cells except from the polar cells [36]. Fasciclin II in turns regulates the localization of the tumour supressor genes *discs-large* and *lethal-giant-larvae* in polar cells [34, 65] to the apical membrane of the cell, facing the germline [36]. Complete loss of any these three proteins impairs the polarity of anterior polar cells, resulting in a delayed delamination but without affecting the rate of migration. Since *misshapen* mutant border cells do not even initiate migration this could reflect a problem in the polarisation of the polar cells. To address whether polar cell polarity was affected, we analized Discs-large localization in *misshapen* mutant border cells. We found that, like in the wild-type situation, Discs-large localizes at the apical membrane of the polar cells, facing the germline, in border cell clusters lacking *misshapen* function (Fig. 22A, A').

In addition to FasII, Dlg and Lgl, *D*E-Cadherin, a homophilic cell-cell adhesion molecule, is also involved in border cell migration. Prior to migration, *D*E-cadherin levels are upregulated in border cells and at the junctions between the border cell and the nurse cells. During migration, this high expression is maintained in the polar cells and in the junctions between border cells, but it decays at the border cell-nurse cell junctions [63]. Elimination of *D*E-Cadherin function does not affect border cell cluster formation but the cluster fails to invade the germline cells. Furthermore, *D*E-Cadherin is required in both, border cells and nurse cells, for border cell migration [63]. Thus, *D*E-Cadherin seems to provide the traction necessary for border cells to migrate between the nurse cells to reach the oocyte. Because this phenotype resembles that of lack of *misshapen* activity, we decided to analyse whether a defect in *D*E-Cadherin localization was responsible for the failed migration of

misshapen mutant border cells. However, we found no detectable defects in *D*E-Cadherin localization in these mutant cells (Fig. 22B, B').

Taken altogether, these results show that the role of *misshapen* in border cell migration is not at the level of the acquisition of a motile polarity.

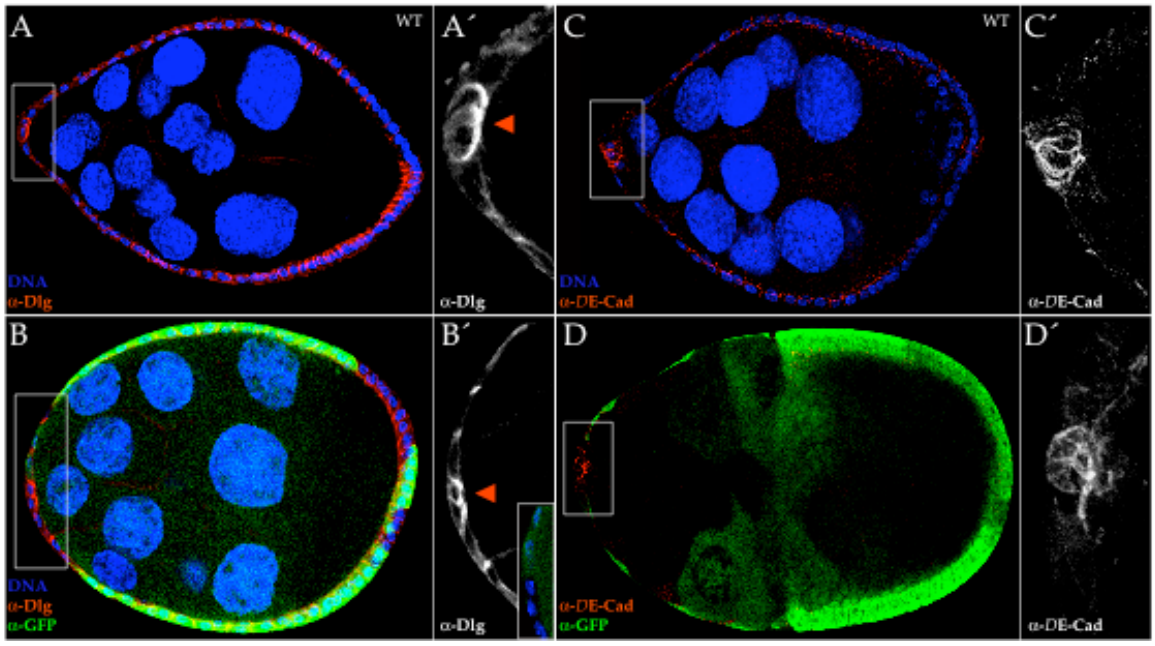

Figure 22. *misshapen* border cells display a wild-type polarity.
(A, C) Wild-type S8 egg chambers stained against DNA (blue) and anti-Discs-large (Dlg) (red in A) or anti-DE-Cadherin (red in C). (A´, C´) Magnifications of the white boxes in A and B respectively. (B, D) S8 (B) and S9 (D) egg chambers harbouring *misshapen* mutant border cells. (B´, D´) Magnifications of the white box in B and D respectively. (A) Wild-type border cells localise Dlg to the apical membrane facing the germline. (B) In *misshapen* mutant cells Dlg is properly localised as do wild-type border cells (A) (arrowheads in A´ and B´). (C) *D*E-Cadherin is enriched in the area of contact between outer border cells, between outer border cells and the central polar, and between outer border cells and nurse cells (C'). (D) *misshapen* mutant border cells display a wild-type DE-Cadherin localization (D´).

IV.1.iv. *misshapen* is required for the actin cytoskeleton remodelling in border cells

In order to migrate, border cells also need to reorganize the actin-cytoskeleton so that actin-based protrusions are extended in between the nurse cells in the direction of movement [254] [63, 66]. Interfering with the ability of cells to regulate the formation of these protrusions inhibits cell migration [255] [66]. Interestingly, *misshapen* has been shown to regulate actin dynamics during the process of dorsal closure, growth cone motility in the eye and hair development in the wing [93, 103, 109, 114, 115, 215, 256, 257]. Moreover, Misshapen has been shown to bind and phosphorilate Bifocal, a cytoskeletal regulator that binds F-actin [117]. These data suggested that *misshapen's* role in border cells could be related to the regulation of the actin cytoskeleton. Thus, we analysed F-actin distribution in border cells mutant for *misshapen* and found that they exhibited a higher accumulation of F-actin and an increased number of actin bundles (Fig.23C, D).

We have also looked at the basal array of actin bundles in the main body follicle cells. In wild-type follicle cells actin bundles are always found perpendicular to the anterior-posterior axis of the egg chamber [42], however actin bundles in *misshapen* mutant follicle cells are missoriented (Fig. 23E).

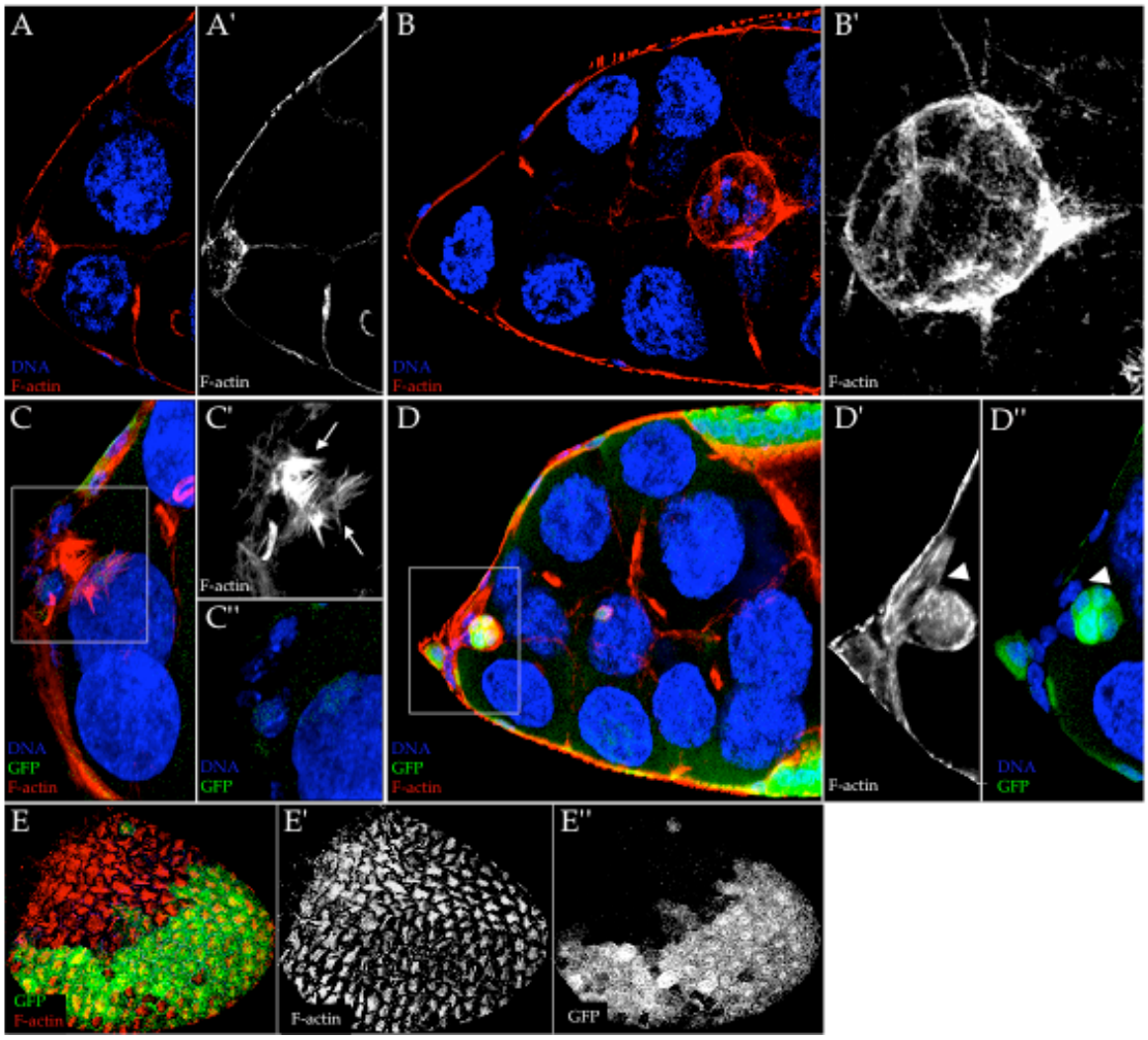

Figure 23. *misshapen* is required for proper actin dynamics in the border cells.

(A-D) S9 egg chambers and (E) S10 egg chamber stained against DNA (blue) and F-actin (red in A-D and white in A´-D´). (A, B) F-actin is localised at the front of the migrating cluster before and during migration (red arrowheads in A and B respectively). (C) *misshapen* mutant border cells display a higher concentration of F-actin and an increased the number of actin bundles (arrow in C´). (C´, C´´) Magnifications of the white box in C. (D) *misshapen* mutant mosaic cluster that has failed to migrate. (D´, D´´) Magnifications of the white box in D. The white arrowhead in D´ and D´´ points to a *misshapen* mutant border cell displaying an abnormal F-actin organization compared to

the wild-type border cells. (E) *misshapen* mutant follicle cell clones showing a missoriented basal array of F-actin compared with that of their wild-type neighbours.

IV.1.v. Misshapen and the JAK-STAT pathway, independent pathways regulating border cell migration

The JAK/STAT pathway has been reported to modulate border cell migration by regulating both the recruitment of the border cell cluster and the proper migration of the cluster [56]. Activation of the JAK/STAT cascade is necessary and sufficient to transform epithelial follicle cells into mesenquimal migratory cells [56, 57]. Unpaired (UPD), a ligand for the JAK/STAT pathway in flies, is secreted from polar cells activating the JAK/STAT pathway on adjacent border cells to direct their proper differentiation and migration [56, 57]. Interestingly, the expression pattern of *unpaired* resembles that of *misshapen* in the polar cells, being expressed in anterior and posterior polar cells from early stages of oogenesis until late S10 [56, 57]. Furthermore, border cells mutant for Stat92E do not initiate migration in late stage 9 or stage 10 egg chambers, as we have observed for *misshapen* mutant cells. Since Stat92E is a transcription factor, we decided to analyse whether JAK/STAT activity could be regulating *misshapen* expression.

The loss of *hop*, the JAK tyrosine kinase, or the expression of a dominant negative form of *domeless*, the receptor for Upd, in border cell clusters resulted in failure in migration, as previously reported [56, 57]. However, the expression of *misshapen*, as visualised by the enhancer trap misshapen was not affected (Fig. 24A and data not shown). Next, we analyzed whether *misshapen* could be an upstream factor regulating the activity of the JAK/STAT pathway. To test this, we analyzed STAT protein levels in *misshapen* mutant clones. STAT protein is normally

expressed in border cells as they migrate [258]. We found that STAT was clearly detected in migration defective *misshapen* mutant border cell clusters (Fig. 24B). All these results indicate that *misshapen* is acting neither upstream nor downstream of the JAK/STAT pathway to regulate border cell migration.

In order to gain insight into the mechanisms by which *misshapen* regulates border cell migration, we examined the expression of proteins that are highly expressed in border cells and that are required for their migration. *slow border cells* (*slbo*), the *Drosophila* homolog of the mammalian C/EBP transcription factor, was the first gene identified to play a role in border cell migration. Slbo is detected in border cells just prior to and during their migration [59]. Slbo is required in the border cells to become migratory, since it directs the expression of genes controlling border cell migration such as *D*E-Cadherin [63, 64, 259-262]. When we analyzed Slbo protein levels in *misshapen* mutant clones, we could clearly detect Slbo protein in *misshapen* mutant border cells (Fig.24C). To discard the possibility of subtle effects on Slbo protein levels, we analysed border cell clusters composed of a mixture of wild type cells and cells homozygous mutant for *misshapen*, thus allowing a direct comparison of proteins levels. We found that Slbo protein was expressed similarly in all cells (Fig. 24D).

This result indicates that the failure of *misshapen* mutant border cells to migrate is not due to a lack of border cell identity.

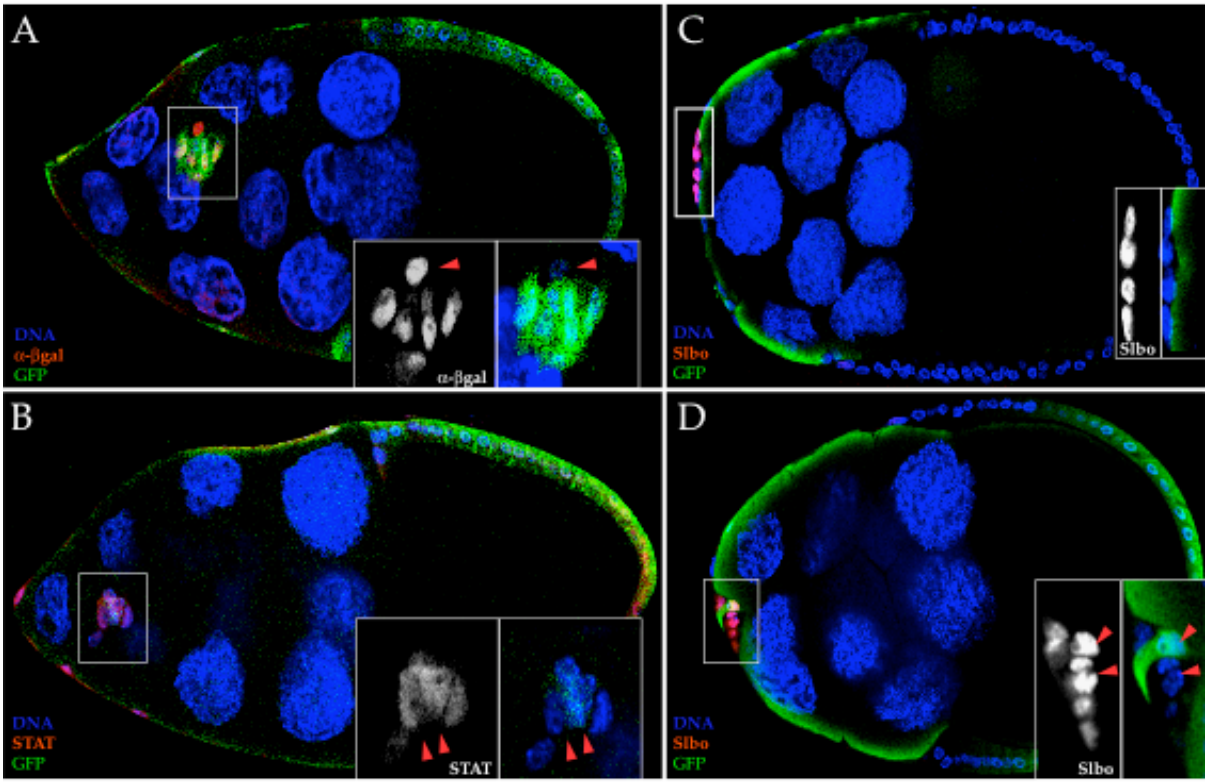

Figure 24. Regulation of *misshapen* and *stat92E* expression in border cells is independent of each other. *misshapen* does not regulate *slbo* expression in border cells.

S9 egg chambers harbouring *hop* (A) and *misshapen* (B-D) mutant follicle cell clones. (A) Expression of a *misshapen* enhancer trap (red) is not affected in *hop* mutant border cells (arrowheads). (B) The protein STAT (red) can be detected in *misshapen* mutant border cells as it is in wild-type border cells (arrowheads). (C, D) Slbo (red) protein is clearly detected in *misshapen* mutant border cells and its expression levels are similar to that of a wild-type border cell (arrowheads in D).

IV.1.vi. Different requirements for *misshapen* and the *D*JNK pathway during border cell migration

Misshapen is a member of the Ste20-related kinases which have been found to activate Mitogen Activated Protein Kinases (MAPK) cascades in different organisms (reviewed in [263]. In *Drosophila* it has been shown to act upstream of the *Drosophila* Jun N-Terminal Kinase (*D*JNK) MAPK module to regulate dorsal closure in the embryo [112] and in planar polarity [215]. However, during photoreceptor axon targeting in the *Drosophila* eye the downstream pathways regulated by Misshapen seem to be more diverse and not only limited to *D*JNK activation. Thus, we decided to determine whether Misshapen regulates border cell migration through the on activation of *D*JNK.

To determine whether components of *D*JNK signalling act downstream of Misshapen in border cell migration, we generated mutant clones in different components of the pathway, such as the *D*JNKK *hemipterous* (*hep*) [79] and the target of the pathway, the transcription factor *D*jun [87]. We used a strong hypomorphic mutation hep^{r75} [79], the loss of function allele *Djun1* [86], and the UAS-FLP system [46]. Removing either *hemipterous* or *Djun* function from border cells caused a delay in their migration. At stage 10, when 100% of control (GFP) clones have reached the oocyte, only 46.5% of *hemipterous* and 33% of *Djun* have done so (Fig. 25A, D and data not shown). This is in contrast with previous results showing that border cells mutant for *hemipterous* migrate normally [264]. In addition, we also found that, as it is the case for *misshapen*, *hemipterous* was required in the germline for border cell migration (Fig. 25B). In this case, 14.3% of S10 egg chambers harbouring *hemipterous* germline clones and wild type border cell clusters migration was blocked and in 57.2% it was delayed (Fig. 25B, E). These results show that the *D*JNK is required for border cell

migration, although it is not as essential as it is *misshapen*. To test further our results we decided to inhibit *D*JNK pathway activity specifically in the border cells by expressing a negative regulator of the pathway, the MAPK phosphatase Puckered (Puc) [88]. Puckered activity controls that of the *D*JNK pathway by inhibiting Hemipterous, hence regulating *D*Jun levels [79, 89, 265]. In addition, *puckered* is itself a downstream target of the *D*JNK pathway, thus, *puckered* expression is a reporter of the activity of the pathway. Interestingly, *puckered* has been shown to be expressed during oogenesis in the border cells as they migrate. It is also expressed in other epithelial follicle cells, such as centripetal, stretched and posterior follicle cells, as shown by the expression pattern of enhancer trap lines in the *puckered* locus [89, 264] (and our own unpublished observations). Overexpression of Puckered in border cells using the slboGal4 causes a delay in border cell migration in 55.9% of S10a experimental egg chambers (n=68) (Fig. 25C). This effect resembles very much the one observed when removing *hemipterous* or *Djun* function. Quantification of these phenotypes is shown in the tables of Fig.25 (Fig. 25E, F) where we have applied the criteria used before when analysing *misshapen* clones. Interestingly, we also found that, as in the case of *misshapen* mutant border cells, there was a higher and not polarised accumulation of F-actin in these mutants (Fig. 25D and data not shown). All together, our results show that the *D*JNK pathway is required for border cell migration. However, *D*JNK requirements do not seem to be as strong as *misshapen*, suggesting that the *D*JNK module components might be redundant in this process.

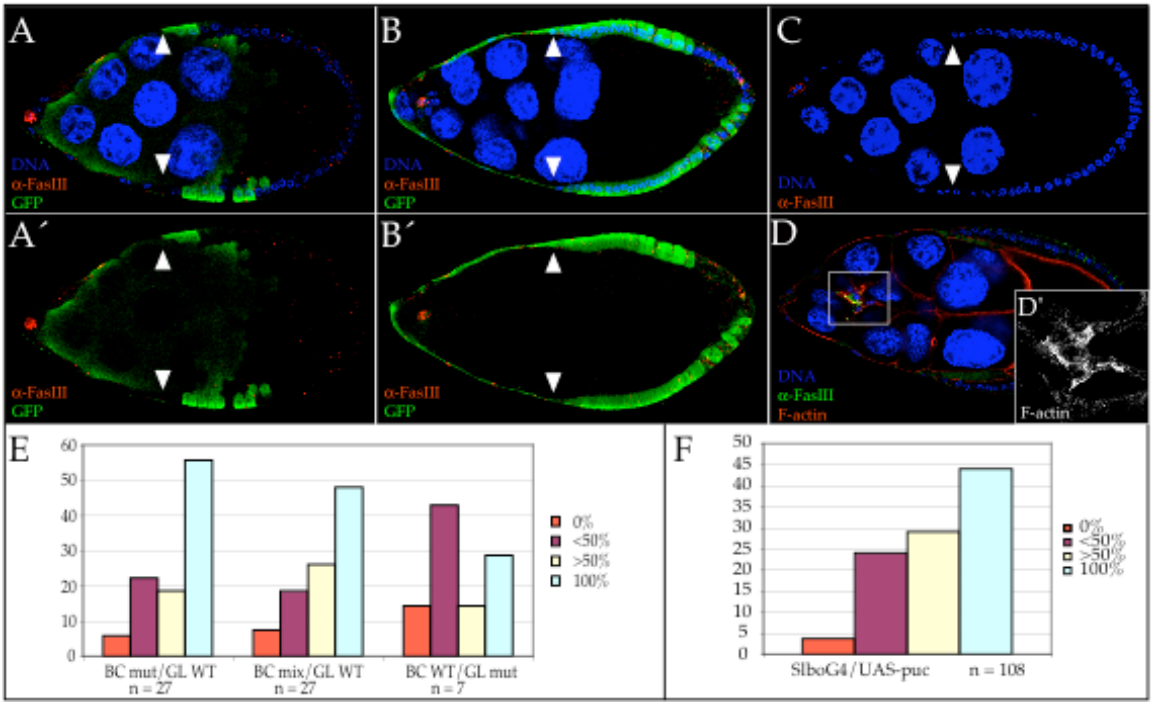

Figure 25. The DJNK pathway is not the only downstream target of *misshapen* in border cells

(A-C) S9 egg chambers stained against DNA (blue) and anti-FasIII (red). (A, A´) *hemipterous* mutant border cell cluster fails to properly migrate (arrowheads indicate the position of the main body follicle cells). (B, B´) Migration of the border cell cluster is also affected when germline cells are mutant for *hemipterous*. (C) Overexpression of Puckered in the border cells causes a delay in the migration of the cluster. (D) Border cells overexpressing Puckered showing a higher acummulation of F-actin (red). (D´) Magnification of the white box in D. (E, F) Tables correlating the percentage of migration with the cells mutant for *hemipterous* (E) and with border cells overexpressing Puckered (F). (BC = Border Cells, GL = germline, mut= mutant and n= number of egg chambers analysed).

IV.1.vii. Other pathways downstream of Misshapen

The finding that the loss of activity of the *D*JNK pathway results in a weaker border cell migration phenotype when compare to *misshapen* mutants suggests the existence of other downstream targets of the Misshapen kinase. In this regard, it is interesting to note that there is genetic evidence that suggets the existence of redundancy among JNK and p38, two kinases known to act downstream of Misshapen in the generation of cell polarity [215]. Although each of these kinases has acquired specific functions during other embryonic processes [266], it tis possible that JNK and p38 act redundantly to mediate border cell migration. The analysis of *D*p38 mutant border cells is not possible since there is not a mutant available affecting only this kinase. We thus decided to analyse the phenotypic consequences for border cell migration of removing both kinases, *D*JNKK/hep and *D*p38K/lic.

We found that border cells double mutant for *hep* and *lic* show a delay in their migration, but not a complete block, as is the case for *misshapen* mutant border cells (Fig. 26A). This result suggests that other genes downstream of Misshapen (in addition to *D*JNK) regulate border cell migration.

To address this issue further we looked for potential involvement of other genes that have been shown to act downstream of Misshapen in other biological processes. This is the case for Dreadlocks (Dock), the *Drosophila* homolog of the human proto-oncogen Nck. Dock is an adapter protein that possibly interacts with Misshapen through its SH3 domain, as it is the case for the mammalian homologs NIK (Nck Interacting Kinase) and Nck [116]. In fact, Misshapen and Dock have been shown to interact during photoreceptor axon guidance, although not in planar polarity [113, 215]. To determine whether Dock was a partner for Misshapen during border cell migration, we first analysed

Dock's requirement in this process. We found that mutant border cells for a null allele of *dock* failed to migrate properly in 75% of S9-S10 egg chambers analysed (Fig. 26B). We also observed defects in the acummulation of the F-actin cytoskeleton (Fig. 26B). Thus, Dock is required for the correct migration of border cells. This makes it an obvious candidate to be cooperating with Hemipterous and *D*Jun in this process. We then asked whether removing both Hemipterous and Dock would show a phenotype like Misshapen. We found that the *hep*;*dock* double mutants display a phenotype that resembles that of the single mutants (Fig. 26C). These results suggest that either Dock functions on a parallel pathway required for border cell migration or Misshapen must act through Hemipterous, Dock and additional proteins.

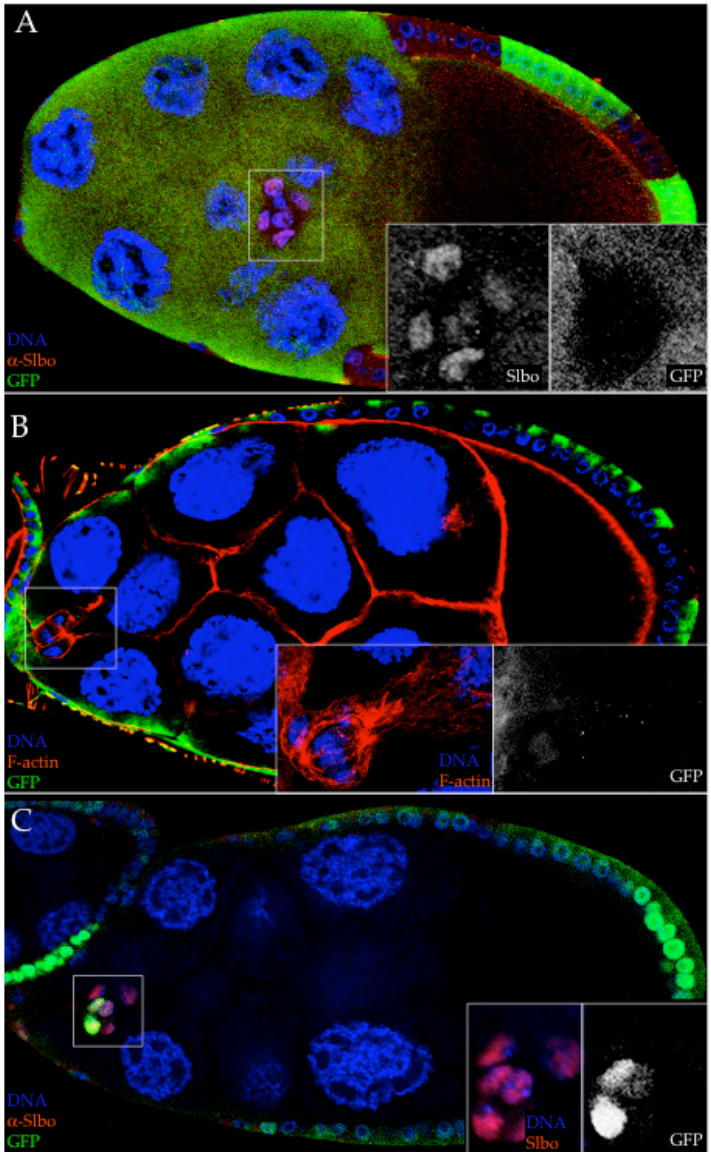

Figure 26. *hep*, *lic* and *dock* are not the only downstream targets of Misshapen.

S10 (A) and S9 (B, C) egg chambers stained against DNA (blue), anti-Slbo (red in A, C) and F-actin (red in B). (A) *hep lic* mutant border cells are still able to migrate. (B) *dock* mutant border cells showing a delayed migration and a defective actin accumulation (white boxes in B). (C) *hep;dock* mutant border cells clones marked by the absence of both nuclear and cytoplasmic GFP. Lack of both *hep* and *dock* does not resemble lack of *misshapen* phenotype in border cells, since a cluster containing most of the cells mutant for *hep* and *dock* is still able to migrate (white box in C).

IV.2. DISCUSSION

IV.2.i. Misshapen regulates the actin-cytoskeleton dynamics to control border cell migration

misshapen is expressed in polar cells from S2 onwards and in border cells just prior to, and during, their migration. As expected from its expression pattern, our results demonstrate that *misshapen* is involved in border cell migration. In fact, using two different null alleles we have shown that lack of *misshapen* in border cells completely abolish their migration indicating that *misshapen* is essential for the migration of the border cell cluster.

The inability of *misshapen* mutant border cells to migrate is not due to defects in the polarization of the cluster. We did not find any defect in the polarity of *misshapen* mutant border cells. Changes necessary for a proper acquisition of a "motile" polarity and for the ulterior migration appear to occur correctly as indicated by the normal localization of Discs-large and *D*E-Cadherin in *misshapen* mutant border cells. Instead, our data point to *misshapen* being involved in the actin cytoskeleton remodelling required for border cell migration. Wild-type border cells reorganize their actin cytoskeleton and extend F-actin rich protrusions in the direction of migration. However, border cells lacking *misshapen* function display a higher concentration of F-actin and are not able to accumulate actin to the front of the cell. These higher levels of F-actin in mutant border cells were not a secondary consequence of the block in migration, since wild-type border cells belonging to a mosaic cluster and therefore with a defective migration, do not show any defect in F-actin accumulation and distribution (Fig. 23D). Moreover, we also detected defects in the organization of the basal array of actin bundles in *misshapen* mutant main body follicle cells, indicating that Misshapen

is required for the proper dynamics of the actin cytoskeleton in the follicle cells.

These data are in concordance with previous reports involving *misshapen* in cytoskeleton rearrangements in the photoreceptor cells and in the wing [109, 114, 115, 215].

IV.2.ii. Misshapen, a new independent pathway regulating border cell migration

Four different signalling pathways have been reported to regulate border cell migration: the JAK/STAT pathway, which regulates both recruitment to the border cell cluster and cell migration; the Taiman pathway, a global steroid-hormone signalling pathway that defines the timing of migration; and the EGFR and PVR pathways, which regulate the direction of migration (reviewed in [60], see section on *Genes involved in border cell migration*). In addition to these signalling pathways, the Slbo transcription factor is required to allow border cell migration. Although there is some controversy as to whether Slbo acts downstream of the JAK/STAT pathway or not [56] [57], recent data in our laboratory support the work of Beccari et al and demonstrate that Slbo is not a target of the JAK/STAT pathway.

The data presented in this thesis, together with recent results obtained in our laboratory, have revealed Misshapen as a key component of a new pathway controlling border cell migration, independent of slbo and th activity of the JAK/STAT signalling cascade.

Despite the similar phenotypes observed in mutant for *misshapen* or the JAK-STAT pathway, our results show that *misshapen* does not act neither upstream nor downstream of the JAK-STAT pathway to control border cell migration. We have shown that STAT levels are not affected

in *misshapen* mutant border cells and that STAT is not required to regulate *misshapen* expression.

In addition, our finding that *misshapen* expression is not affected in *slbo* mutant border cells supports the idea that neither slbo nor the JAK/STAT pathway regulate *misshapen* expression. Furthermore, our results also show that the observed defects of *misshapen* border cell mutant clones on migration were not due to effects on Slbo expression.

The Taiman pathway, as is the case for *misshapen*, functions independently of *slbo* to regulate border cell migration. *taiman* mutant border cells fail to migrate and show an abnormal accumulation of *D*E-Cadherin and *D*FAK [73]. The fact that mutations in *misshapen* do not affect either expression or localization of *D*E-Cadherin suggests that *misshapen* acts independently of the Taiman pathway to regulate migration of the border cell cluster. However, this hypothesis needs to be tested.

Both the EGFR and the PVR pathways are mainly involved in the guidance of the border cell cluster towards the oocyte. Our results involve misshapen in a previous step of border cell migration. However, a putative interaction of misshapen with the EGFR and PVR pathways should be tested. One possibility is analyze the levels phospho-tyrosine, as this has been shown to be a reasonable readout of endogenous RTK activation [267]

IV.2.iii. The *D*JNK pathway is not the only downstream target of *misshapen* in border cell migration

The Misshapen kinase has been shown to exert its role during dorsal closure, a migration process taking place during embryogenesis, by regulating the activity of the *D*JNK pathway. During oogenesis, the loss of *D*JNK activity in border cells results in a delay in their migration.

However, this defective migration phenotype is not as severe as that observed in *misshapen* mutants. Furthermore, *misshapen* mutants also display a defective migration phenotype in the main body follicle cells. This additional phenotype is not observed in *hemipterous* mutant main body follicle cells, suggesting that *misshapen* is interacting with other pathway/s to control follicle cell rearrangements. The finding that *hep lic* double mutant border cells do not resemble the phenotype of *misshapen* mutants indicate either that both kinases are not acting redundantly in border cell migration or that *lic* has no role in this process.

We also have found a role for Dock, an adaptor molecule known to interact with Misshapen during photoreceptor axon guidance, during border cell migration. However, and like *hep* mutants, *dock* mutant border cells do not display a complete block of migration. In addition, hep;dock mutant border cells do not present a stronger phenotype than that of the single mutants. Altogether, these results suggest that either Dock affects border cell migration independently of Misshapen or that Misshapen must act through Hep, Dock and additional proteins.

The kinase activity reported for Misshapen [109] suggests that Misshapen-dependent phosphorilation regulates additional signalling pathways in border cell migration. However, it is necessary to test whether the kinase function of Misshapen is involved in this process.

IV.2.iv. *misshapen* is required in the germ line for the migration of the border cell cluster

Although *misshapen* expression has solely been described in the somatic follicle cells, *misshapen* has also been reported to have a role in the germ line since *misshapen* mutant germline clones fail to develop into embryos [109]. Our results also denote a role for *misshapen* in the

germline, since removal of *misshapen* function only in the germline causes a failure in the migration of the border cell cluster. What it is the role of *misshapen* in the nurse cells we do not know, but a similar result has been also reported for *D*E-Cadherin, which is required in both the border cells and the germline for a proper migration of the border cell cluster [63]. Since the nurse cells accommodate to allow the migration of the border cell cluster in between them, one could hypothesize that *misshapen* also has a role in the reorganization of the actin cytoskeleton and changes in cell shape of the nurse cells.

V. CONCLUSIONS

1.- Integrins are expressed in the basal, lateral and apical domains of the follicle cells during *Drosophila* oogenesis. They are also found in the germline until S3.

2.- Integrins are required to maintain the simple structure of the follicular epithelium. Egg chambers lacking integrin function develop between one and four extra layers at both terminal domains.

3.- The αPS1βPS heterodimer is the main responsible for the maintenance of the follicular monolayer. The αPS2βPS does not play a significant role in this process.

3.- Loss of integrin function does not affect proliferation nor apical-basal polarity of the follicle cells. Integrins – presumably via interactions with the basement membrane – play a reinforcing role in follicle cells apical-basal polarisation.

4.- Integrin-mediated signalling is required to orientate the mitotic spindle of follicle cells.

5.- Integrin-mediated positioning of the mitotic spindle does not require myosin II or VIIA, nor the actin cytoskeleton regulators rok, rac1, rac2 and Mtl. Unconventional myosin VI jaguar is required autonomously for the correct orientation of the mitotic spindle of epithelial follicle cells.

6.- *misshapen* is expressed in the inner germarial cells within the germarium and at low levels in follicle cells and at high levels at anterior

and posterior polar cells from S3 onwards. High *misshapen* expression levels are also found in border cells prior to and during their migration.

7.- Misshapen is required for border cell migration in both the border cells and the germline. Lack of Misshapen activity also affects migration of the main body follicle cells.

8.- Polarity is not affected in *misshapen* mutant border cells. Instead, Misshapen is required for the actin cytoskeleton remodelling in the border cells.

9.- Misshapen acts independently of the JAK-STAT pathway to control border cell migration. Slbo expression is not affected in *misshapen* mutants.

10.- The *D*JNK pathway is not the only pathway downstream of Misshapen. Misshapen may act through Hemipterous, Dock and additional proteins to control border cell migration.

VI. EXPERIMENTAL PROCEDURES

VI.1. Staining procedures and microscopy

Unless otherwise noted, newly hatched *Drosophila* females were yeasted for 2 days before dissection. Stainings were performed at room temperature. Ovaries were fixed in PBT (PBS + 0.1% Tween 20) with 4% PFA (Paraformaldehyde) for 20 minutes and then blocked for 1 hour in PBT 10 (0.1% Tween, 10% BSA Bovine Serum Albumine). After blocking, ovaries were incubated overnight in PBT1 (0.1% Tween, 1% BSA) containing the primary antibody. After washes with PBT 1 for 1-2 hours, egg chambers were incubated with the secondary antibody in PBT 0.1 (0.1% Tween, 0.1% BSA) for 2-4 hours. When used, the DNA dye and rhodamine-phalloidin were added in PBT after the secondary antibody, for 10 minutes and 30 minutes respectively. After 3 washes in PBT of 10 minutes each, ovaries were mounted in Vectashield (Vector).

Primary antibodies were used at the following concentrations: rabbit anti-GFP (Molecular Probes™) 1/10000, mouse anti-GFP (Molecular Probes™) 1/100, mouse anti-βPS (Developmental Studies Hybridoma Bank, University of Iowa, U. S. A. (DSHB)) 1/10, rabbit anti-Bazooka [268] 1/500, mouse anti-Discs Large (DSHB) 1/100, rabbit anti-β_HSpectrin [32] 1/500, rabbit anti-*D*Patj [27] 1/250, rabbit anti-aPKC (PKCζ C20; Santa Cruz Biotechn., Inc.) 1/10000, rat anti-*D*E-Cadherin [223] 1/10 (a 1:1 mixture of both *D*CAD antibodies 1/20 each), mouse anti-Armadillo (DSHB) 1/50, rabbit anti-α-Spectrin [269] 1/250, rat anti-α-Tubulin [270] 1/1000, mouse anti-α-Tubulin (clone DM1A, Sigma) 1/500, rabbit anti-Phospho-histone H3 (PH3; Upstate) 1/250, rabbit anti-Laminin A [44] (1/250), rabbit anti-bGalactosidase (Cappel™) 1/10000, mouse anti-Myc 1/100 (Oncogen Science), mouse

anti-CD2 [271] (1/1000), mouse anti-FasIII [272] 1/20, rat Anti-Slbo [267] 1/3000, rabbit anti-STAT [273] 1/1000. Secondary antibodies FITC (Molecular Probes™), and Cy3 and Cy5 (Jackson ImmunoResearch Laboratories, Inc.) were used at 1/200. F-actin was stained with Rhodamine-phalloidin (Molecular Probes). The DNA dye TO-PRO-3 (Molecular Probes™) was used at 1/1000. Images were captured with a Leica TCS-SP2 confocal microscope and processed with Adobe Photoshop.

VI.2. *Drosophila* genetics

Generation of somatic and germline clones

To generate somatic and germline clones we utilized the FRT/FLP technique [217]. The following mutant alleles and chromosomes were used:

mys^{11} FRT101 (also known as *mys*^{XG43})[216]
mys^{10} FRT101 (also known as *mys*^{XB87})[216]
rhea^{79} FRT2A [198]
mew^{M6} FRT18A [186]
if^{K13} FRT18A [186]
dsh^{V26} FRT101 [274]
dsh^{75} FRT101 [274]
fz^{P21} *fz 2*^{C1} FRT2A [275]
Rac1^{J11} *Rac2*^{Delta} *Mtl*^{Delta} [236]
rok^{2} [235]
jar^{322} [230]
sqh^{1} is a hypomorphic allele [237]. As an internal control we observed that *sqh*^{1} mutant border cells failed to migrate (not shown) [66].
msn^{102} FRT80 [109]

msn^{172} FRT80 [109]

hep^{75} FRT101[79]

jun1 FRTG13 [85]

dock^{P1} FRT40 [116]

hep lic FRT101 [266]

hep^{75} FRT101; dock^{D333} FRT40

hop^{C111} FRT101 – null allele [276]

nlsGFP FRT101; ubGFP FRT40; T155-Gal4 UAS-flipase

ubi-GFP FRT101; *e22c*-Gal4 UAS-flipase

ovoD1 FRT101/ X^X; hs-Flipase38

w P{arm-lacZ} FRT18A; hs-Flipase38

hs-Flipase; hs-GFP FRT2A

e22c-Gal4 UAS-Flipase; ubi-GFP FRT80

minute hs-myc FRT40; T155-Gal4 UAS-Flipase

ubiGFP FRT40 FRT40; T155-Gal4 UAS-Flipase

ubi-GFP FRTG13; T155-Gal4 UAS-Flipase

e22c-Gal4 UAS-Flipase; P{*tubulin* P-GAL80^{ts}}2 (RL Davis 2003)

The *e22c*-Gal4 UAS-Flipase and T155-Gal4 UAS-Flipase [46] drivers are expressed in the follicle stem cells in the germarium. With a low frequency, these lines also induce recombination in the germline.

hep mutant germline clones were generated by heat-shocking third instard larva for 1 hour at 37^{0}C, 1 hour at 25^{0}C and 1 hour at 37^{0}C. Females were dissected two days after hatching. *fz*^{P21} *fz 2*^{C1} or *rhea*^{79} mutant clones were induced by heat-shocking third instard larvae for 1 hour at 37^{0}C, 1 hour at 25^{0}C and 1 hour at 37^{0}C or alternatively newly hatched females were heat-shocked following the same treatment during two consecutive days and dissected 2 days after heat-shock.

Mutant clones were marked by the absence of GFP or myc. Females harbouring the hs-GFP or hs-myc transgenes were heat-shocked for 1

hour at 37^{0}C to induce GFP or myc expression respectively and then kept at 25^{0}C for 1 hour prior dissection.

In order to generate clones of cells expressing Tor^D/$\square_{cyt}$ [177] or Fak56:GFP [205] in the follicular epithelium we made use of the 'flip-out' technique [277]. *y w* hs-Flipase 122; Act <hs-CD2> Gal4 females were crossed to UAS-Tor^D/$\square_{cyt}$. *yw* hs-Flipase 122; Act <y^+> Gal4 UAS-lacZ females were crossed to UAS-Fak56:GFP males. In both cases IIIrd instar larvae were heat-shocked at 37 ^{0}C for 30' minutes. In order to obtain follicular epithelia overexpressing Tor^D/$\square_{cyt}$ and containing *myspheroid* mutant cells, females of the following genotype *mys^{11}* FRT-101/*Ubiquitin*-GFP FRT-101; *e22c*-Gal4/UAS-Tor^D/$\square_{cyt}$; P{*tubulin* P-GAL80^{ts}}2/+ were grown at 18^{o}C until eclosion. Adult females were kept at 31^{0}C for 3-5 days prior to ovary dissection.

VI.3. Gain-of-function experiments

For the gain-of-function experiments we made use of the Gal4/UAS (Upstream Activating Sequences) system [278]. The following lines were used:

UAS-dome $\square^{cyt}$ [279] – dominant negative form of the JAK-STAT pathway receptor Domeless

UAS-*puc* [88]

slboGal4 [280]. This driver is expressed specifically in the border cells.

To address Misshapen expression pattern we used the following *misshapen* alleles:

misshapen^{6286} [252]

misshapen^{138716} has a P {lacW} insertion in the 5' UTR of the *misshapen* gene (Lopez-Schier, 2001.8.25 personal communication to FlyBase).

misshapen^{10162} - bloomintong

VII. RESUMEN EN ESPAÑOL

VII.1. INTRODUCCIÓN

Durante el desarrollo de un organismo se requieren complejas reorganizaciones de los tejidos para finalmente obtener la estructura tridimensional de un organismo adulto. Estas reorganizaciones se consiguen gracias a varios procesos celulares tales como la pérdida de la polaridad epitelial durante la transición epitelio-mesénquima, la constricción apical de células epiteliales, la intercalación celular, la migración celular y la división polarizada. Las células epiteliales pueden sufrir varios procesos morfogenéticos que contribuyen a esculpir los órganos y las distintas partes del cuerpo durante el desarrollo. Aunque está claro que la morfogénesis epitelial se lleva a cabo en su gran mayoría por reorganizaciones epiteliales y cambios en adhesión celular, todavía hoy es un objetivo importante entender como estos procesos se coordinan para construir estructuras biológicas complejas a partir de simples capas celulares.

A pesar de las diferencias observadas entre especies respecto a la organización de la epidermis, hay evidencias que sugieren que la formación de la epidermis y su diferenciación pueden compartir un gran número de homologías entre *Drosophila* y vertebrados. En esta tesis se va a usar el proceso de la oogénesis de *Drosophila* como sistema modelo para identificar genes que se requieran para el control de la morfogénesis de células epiteliales. Durante la oogénesis de *Drosophila*, el epitelio folicular de los huevos en desarrollo exhibe un diverso rango de reorganizaciones epiteliales en un tejido genéticamente accesible, haciéndolo por tanto un excelente sistema modelo para el estudio de la morfogénesis epitelial.

VII.2. Oogénesis en *Drosophila* (Fig. 1)

La hembra de *Drosophila* produce cientos de gametos a lo largo de toda su vida en un proceso conocido como oogénesis. Este proceso tiene lugar en los dos ovarios, que ocupan la mayor parte del abdomen de una hembra adulta de *Drosophila*. Cada ovario está compuesto de unas 15 ovariolas en las que se forman los gametos femeninos. A su vez, cada ovariola contiene una línea de huevos en desarrollo o folículos en diferentes estadios de desarrollo (estadios 1-14) [10, 11]. Las ovariolas se componen de dos regiones: el germario y el vitelario. El germario, donde se ensamblan los folículos, se divide en 4 regiones (1, 2a, 2b y 3). En la región 1 se encuentran las células precursoras de los gametos, las Células Troncales de la Línea Germinal (GSCs – Germline Stem Cells). La oogénesis empieza cuando una GSC se divide asimétricamente. Una de las células hijas renueva la GSC y la otra célula hija entra en diferenciación y se convierte en un cistoblasto. El cistoblasto sufre 4 rondas de mitosis con citoquinesis incompleta dando lugar a un cisto de 16 células conectadas por puentes o canales intercelulares. En la región 2a del germario, una de las células de la línea germinal con 4 canales se selecciona como oocito y se sitúa en la parte posterior del grupo de 15 cistocitos restantes, que se convierten en las células nutricias poliploides [13-15]. En el límite entre la región 2a/2b del germario también encontramos otro tipo de Células Troncales, en este caso de origen somático (SSCs – Somatic Stem Cells) [16]. Estas SSCs dan lugar a 3 tipos de células somáticas: i) las células que van a formar el epitelio folicular, ii) dos parejas de células polares a ambos lados del folículo y iii) las células del tallo intefolicular, formado por 6-8 células del tallo que conectan dos folículos consecutivos.

En la región 2b, aproximadamente 30 células foliculares encapsulan al cisto de 16 células [13]. Más tarde, en la región 3 (también conocida

como estadio 1), ambos, el cisto de la línea germinal y las células foliculares que lo rodean adoptan una forma redondeada y forman un folículo completo o huevo en desarrollo. Los huevos en desarrollo salen del germario y entran en el vitelario como folículos de estadio 2. A medida que el folículo avanza hacia la parte posterior de la ovariola, éste madura y aumenta de tamaño drásticamente produciendo, y finalmente, un folículo maduro de estadio 14.

Hay un patrón interesante y muy dinámico de proliferación celular y diferenciación durante oogénesis. En el caso de las células somáticas, mientras que las células polares y las células del tallo paran de dividirse y están totalmente diferenciadas cuando el folículo sale del germario, las células foliculares continúan proliferando hasta el final del estadio 6. Sufren 5-6 rondas de división alcanzando un número de aproximadamente 650 células y forman un epitelio simple monoestratificado [16-19]. Estas células foliculares presentan una polaridad apical-basal muy acusada, con marcadores que se localizan específicamente en el dominio apical en contacto con las células de la línea germinal y en los dominios lateral y basal en contacto con una matriz extracelular, como por ejemplo Bazooka, *D*E-Cadherina, *D*Patj, Discs-large, etc… En las células en división, la orientación del huso mitótico determina la posición de las dos células hijas después de la división. Las células foliculares fijan su huso paralelo a la superficie en contacto con la línea germinal de forma que ambas células hijas permanecen dentro de la monocapa y en contacto con las células de la línea germinal.

VII.3. Reorganizaciones del epitelio folicular (Fig. 3)

El epitelio folicular es un excelente sistema modelo donde estudiar procesos requeridos para la morfogénesis tisular. Inicialmente, las células del epitelio folicular adquieren una forma cuboidal. A medida que los folículos maduran, éstos sufren varios cambios morfológicos que incluyen cambios en la forma y motilidad celular. Estas reorganizaciones son una consecuencia de varios eventos de migración celular que empiezan en el estadio 9:

i) Al principio de este estadio, las células foliculares cuboidales cambian de forma y empiezan a migrar posteriormente, de forma que aproximadamente el 95% de las células foliculares forman un epitelio columnar sobre el oocito. El restante 5% se estira para formar un epitelio escamoso sobre las células nutricias.

ii) Simultáneamente, un grupo de 6 a 8 células foliculares, conocidas como células del borde (Border cells), se delaminan del polo anterior del epitelio folicular tras una transición epitelio-mesénquima,. Las células del borde migran posteriormente siguiendo la ruta más directa entre las células nutricias hasta que llegan a la membrana anterior del oocito en el estadio 10. Después migran dorsalmente siguiendo el borde que forman las células nutricias y el oocito.

iii) En el estadio 10a se produce la migración de las células centrípetas, un grupo de células que migran entre las células nutricias y el oocito hasta cubrir la parte anterior del mismo.

VII.4. Migración de las células del borde (Fig. 4)

Las células del borde ejecutan una migración estereotipada desde el polo anterior del huevo en desarrollo hasta la esquina anterior-dorsal del oocito donde son necesarias para la formación de un micropilo funcional [59, 60].

La migración de las células del borde está altamente regulada, tanto espacial como temporalmente. Las células del borde migran unas 150 micras en aproximadamente 6 horas siguiendo la ruta más directa desde el polo anterior del huevo en desarrollo hacia la membrana del oocito. Por tanto, la migración de las células del borde representa un sistema modelo excelente en el que estudiar los mecanismos implicados en la regulación de la migración celular.

Las células del borde son un grupo de unas 6 células exteriores y dos células que ocupan una posición central y que se denominan células polares anteriore. En el estadio 8, 4-8 células foliculares adyacentes a las células polares anteriores son reclutadas para formar el grupo migratorio de las células del borde [62]. Antes de la migración, las células del borde forman parte del epitelio folicular y se encuentran adheridas a las células foliculares vecinas. Por tanto, la formación de este conjunto de células con características mótiles implica varios pasos como por ejemplo la pérdida de la polaridad epitelial, la ruptura del contacto con las células vecinas y la posterior delaminación del epitelio. Este proceso se conoce como transición epitelio-mesénquima.

Varias moléculas han sido implicadas en la migración de las células del borde. Por ejemplo, y al igual que las células polares, las células del borde anteriores también expresan mayores niveles de *D*E-Caderina durante la migración [63]. La *D*E-Caderina se requiere tanto en las células del borde como en las células nutricias para la migración de las células del borde.

Fasciclin II es otra molécula de adhesión que se posee un papel en la migración de las células del borde. En las células polares anteriores, Fasciclin II regula la localización de las proteínas supresoras de tumores Discs-large y Letal-giant-larvae a la membrana apical de las células del borde, de cara a las células nutricias [34, 65]. Como la acumulación de estas proteínas en la superficie apical es necesaria para adquirir una polaridad "mótil", Fasciclin II controla temporalmente la motilidad del grupo de células del borde [36].

Tras la transición epitelio-mesénquimal, las células del borde se delaminan del epitelio folicular. Ello implica romper el contacto con las células vecinas, extender protrusiones basadas en actina filamentosa y finos filopodios entre las células nutricias y adquirir una morfología irregular tipo fibroblasto [66]. Después de la segregación del epitelio folicular, las células del borde pierden la polarización de la DE-caderina pero retienen algunas características epiteliales. Por el contrario, las células polares retienen aspectos de polaridad epitelial. Las células polares están situadas en el centro del grupo y no migran activamente sino que son llevadas por las células del borde exteriores [67].

La transición de células epiteliales no mótiles a células mesenquimales mótiles son una consecuencia de cambios en la expresión génica, adhesión celular y organización del citoesqueleto. Hasta ahora, 4 rutas principales diferentes se han implicado en la determinación y migración de las células del borde.

1) La ruta **Janus Kinase - Signal Transducer and Activator of Transcription** (JAK/STAT) está involucrada en el reclutamiento de las células del borde exteriores por las células polares anteriores y en la transición de células estacionarias a mótiles [56]. La ruta JAK/STAT promueve la expresión de Slbo (Slow Border Cells), el homólogo en *Drosophila* del factor de transcripción C/EBP [57, 59]. Slbo se expresa en las células del borde antes y durante su migración [70]. Slbo

controla la expresión de la mayoría de los genes requeridos para la migración de las células del borde. Una de las dianas de Slbo es la *D*E-caderina y, de hecho, en mutantes para slbo el aumento de expresión de esta caderina no ocurre [63].

2) La conversión de las células del borde de un grupo de células epiteliales estáticas a células invasivas y la definición del momento de migración requiere la integración de la actividad de Slbo y de la **ruta de Taiman**. Taiman es un coactivador de la hormona esteroidea ecdisona y funciona independientemente de Slbo [73]. Mientras que Slbo se requiere para la expresión de la *D*E-caderina, taiman es necesario para localizar adecuadamente *D*E-cadherina a través de la estimulación de la renovación de los complejos de adhesión [73].

3 y 4) Dos rutas RTK (Receptor Tyrosin Kinases), las rutas **EGFR (Epidermal Growth Factor Receptor) y PVR (Platelet derived growth factor and Vascular growth factor Receptor)**, cooperan de forma redundante para guiar a las células del borde hasta su destino final. La migración de las células del borde se lleva a cabo en dos pasos: el primero consiste en una migración dirigida posteriormente en la cual EGFR y PVR actúan de manera redundante, el segundo implica una migración dirigida dorsalmente que depende de la ruta EGFR [74, 75].

VII.5. Moléculas que controlan la morfogénesis epitelial en *Drosophila*

VII.5.i. La ruta *D*JNK (*Drosophila* c-Jun N-terminal Kinase) y misshapen en *Drosophila* (Fig. 5).

La ruta *D*JNK es un módulo MAPK (Mitogen-Activated Protein Kinase). En *Drosophila*, la ruta JNK consiste en una cascada de 3 MAPKs: slipper, una *D*JNKKK [78], hemipterous, una *D*JNKK [79] y basket, una *D*JNK [80, 81]. La actividad de la ruta *D*JNK tiene como consecuencia la fosforilación de *D*Jun, un factor de transcripción que promueve la transcripción de distintos genes diana. Uno de estos genes es puckered (puc), una MAP kinasa fosfatasa del tipo Cl-100 [88, 89]. Puckered desfosforila a Hemipterous y por tanto inhibe la activación de *D*Jun, por lo que la ruta presenta un bucle de retroalimentación negativa que supone un mecanismo de control interno permitiendo una regulación de la intensidad de respuesta una vez que la cascada de señalización está activada.

El intensivo estudio de las implicaciones de la ruta *D*JNK en el proceso de migración epitelial conocido como cierre dorsal, en el cual tienen lugar una serie de cambios dinámicos en forma celular, motilidad y adhesión, ha resaltado a esta cascada de señalización como un regulador de la morfogénesis y la motilidad celular. Durante el cierre dorsal, la ruta *D*JNK se requiere para la correcta maduración de los centros de nucleación de actina y su posterior dinámica [78, 79, 93]. Esta ruta también se ha involucrado en la regulación de la reorganización de la actina en el proceso de cierre torácico, otro proceso de migración epitelial [107].

Drosophila Misshapen es una MAPKKKK de la familia de las kinasas Ste20 homóloga a la Ste20 kinase Nck Interacting Kinase (NIK) de mamíferos [108]. Se ha demostrado tanto bioquímicamente como

genéticamente que Misshapen activa a la ruta *D*JNK en el proceso de cierre dorsal [110, 112, 113]. Al igual que la ruta *D*JNK, las funciones de Misshapen están ligadas a las reorganizaciones del citoesqueleto que conducen a cambios de forma celular y motilidad celular. Sin embargo, las funciones de Misshapen no se llevan a cabo únicamente por la activación de la ruta *D*JNK. Por ejemplo, durante el desarrollo del ojo se ha visto que Misshapen interacciona con Dreadlocks, una proteína adaptadora SH2/SH3, donde ambos se requieren para que los axones de los fotorreceptores lleguen correctamente a su destino [110, 113, 114, 116].

VII.6. Integrinas

Las integrinas pertenecen a una familia ampliamente expresada de receptores de la superficie celular. Las integrinas son receptores transmembrana que median la adhesión entre la célula y la matriz extracelular. Son receptores heterodiméricos compuestos de una subunidad □ y una □ [118]. Las integrinas regulan varios procesos fundamentales durante la morfogénesis epitelial orquestando el reclutamiento tanto de moléculas del citoesqueleto como de moléculas de señalización que regulan la adhesión de la células a la matriz extracelular, la migración celular y la forma celular [120, 121]. También están involucradas en la regulación de la proliferación y la supervivencia celular, y dirigen la diferenciación de tejidos y órganos [120, 121].

Las integrinas están extremadamente conservadas a lo largo de la evolución [122, 123]. En *Drosophila* hay dos subunidades □: □PS y □v y 5 subunidades □: □PS1-□PS5. Se cree que todas las subunidades □ forman heterodimeros con las subunidad □PS (codificada por el gen myospheroid). Esto se ha comprobado en el caso de las subunidades □PS1, □PS2 y □PS3 [127-131]. La subunidad □□ se expresa

mayoritariamente en las células endodermales del intestino del embrión de *Drosophila* [133]. □□ no es esencial para la viabilidad o la fertilidad y se requiere sólo en el intestino donde puede sustituir parcialmente la función de la subunidad □PS, pero no se han detectado otras funciones para esta subunidad durante embriogénesis, desarrollo del disco imaginal de ala u oogénesis [132].

VII.7. OBJETIVOS

Este proyecto pretende estudiar a nivel genético y molecular, la migración de las células del borde y el mantenimiento de la epitelio folicular monoestratificado durante la oogénesis de *Drosophila*.

VII.7.i. Papel de las integrinas durante la morfogénesis del epitelio follicular

Para analizar el papel de las integrinas en el epitelio folicular se pretende investigar los siguientes puntos:

a.- El patrón de expresión de las integrinas en el epitelio folicular.

b.- El análisis de las consecuencias fenotípicas de la eliminación de la función de las integrinas en las células foliculares.

c.- El papel de las integrinas en el establecimiento y/o el mantenimiento de la polaridad apico-basal de las células foliculares.

d.- El papel de las integrinas en el control de la proliferación de las células foliculares.

e.- Averiguar cuál de las dos funciones de las integrinas, adhesión y señalización, es responsable de la función de las integrinas durante oogénesis.

f.- Investigar los mecanismos moleculares por los cuáles las integrinas ejercen su función en las células foliculares.

VII.7.ii. Papel de Misshapen en la migración de las células del borde

Para averiguar el papel de Misshapen durante la migración de las células del borde he analizado:

a.- El patrón de expresión de Misshapen durante la migración de las células del borde.

b.- El fenotipo de la pérdida de función de Misshapen en el proceso de la migración de las células del borde.

c.- Las consecuencias a nivel celular de eliminar la función de misshapen en las células del borde.

c.- Las interacciones de Misshapen con otras rutas involucradas en la migración de las células del borde.

VII.8. PAPEL DE LAS INTEGRINAS EN LA MORFOGÉNESIS EL EPITELIO FOLICULAR

VII.8.i. RESULTADOS Y DISCUSIÓN

En primer lugar analizamos el patrón de la integrinas durante la oogénesis de *Drosophila*. Puesto que la subunidad PS es la única presente en el ovario, el patrón de expresión de esta subunidad debería reflejar la distribución de las integrinas en el ovario adulto. Usando un anticuerpo específico contra la subunidad PS hemos visto que se expresa en la células somáticas del germario, en el epitelio folicular y en el tallo interfolicular que conectan folículos adyacentes. Además, PS se encuentra en la línea germinal hasta los estadios 3-4. Hemos observado también que las integrinas se localizan a lo largo de los dominios laterales y apicales de las células foliculares, así como en la parte basal.

Para estudiar las consecuancias fenotípicas de la eliminación de la función de las integrinas en el epitelio folicular utilizamos dos alelos

nulos del gen myspheroid para generar clones de células mutantes [216, 217]. Nuestros resultados muestran que los epitelios mosaico que contienen células foliculares mutantes para las integrinas muy a menudo pierden su estructura monocapa y crecen entre una y cuatro extra capas. Estas capas ectópicas están compuestas tanto de células mutantes como de células silvestres, sugiriendo un efecto no autónomo de la falta de función de las integrinas. Además, la eliminación de un componente central del complejo de las integrinas, Talin, da lugar a un fenotipo similar, indicando que el fenotipo observado se debe a la ausencia de la función de las integrinas. También hemos determinado que la principal integrina responsable del mantenimiento de la monocapa es el heterodímero □PS1□PS y que □PS1□PS no juega un papel significante en este proceso.

Tras una análisis más exhaustivo de este fenotipo, hemos determinado que el fenotipo de estratificación derivado de la falta de función de las integrinas no se debe ni a un exceso detectable de proliferación ni a defectos en la polaridad apico-basal de las células mutantes. Por el contrario, nuestros resultados indican que las integrinas se requieren para el correcto alineamiento del huso mitótico de las células foliculares paralelo a la superficie de la línea germinal. Puesto que el desarrollo de capas ectópicas se puede rescatar con la expresión de una integrina quimérica incapaz de unirse a la matriz extracelular pero capaz de inducir señalización intracelular, es la señalización mediada por integrinas - y no la adhesión mediada por integrinas - la responsable del mantenimiento de la monocapa del epitelio folicular. Considerando que lo más probable es que el desarrollo de capas extras dependa de la aparición de husos mitóticos desorientados, nuestros resultados demuestras que la señalización mediada por integrinas se requiere para la correcta orientación del huso mitótico en las células del epitelio folicular.

Los mecanismos por lo cuáles las integrinas influencian la orientación del aparato mitótico aún no se conoce, pero varias líneas de evidencia apuntan a una interacción entre el citoesqueleto de actomiosina y las integrinas en este proceso. Primero, este citoesqueleto es una de las dianas de la señalización de integrinas y se han identificado varias moléculas, tales como Talin [242], que transmiten señales desde las integrinas activadas al citoesqueleto de actina.

El hecho de que las capas ectópicas desarrolladas en huevos en desarrollo con mosaicos estén constituidas tanto por células mutantes como por células silvestres sugiere que la falta de función de las integrinas tiene efectos en las células mutantes y también en sus vecinas silvestres. Este efecto no autónomo de la falta de integrinas se ha observado también en la organización de las fibras de actina que forman el lado basal de las células foliculares. La eliminación de la función de las integrinas causa una desorganización de las fibras en las células mutantes y en las células silvestres que las rodean [30]. Cómo la alteración de la señalización de integrinas en una célula determinada puede afectar a sus vecinas silvestres se desconoce, pero nuestros resultados indican que es un efecto limitado espacialmente. Considerando que las integrinas se han implicado en la organización de la matiz extracelular [226], una explicación posible para esta no autonomía local es que la matriz extracelular que rodea a las células mutantes no esté ensamblada correctamente. Como consecuencia, las integrinas de la células adyacente no pueden adherirse propiamente y señalizar intracelularmente para posicionar el eje de división. Una segunda posibilidad involucraría alguna clase de mecanismo de "memoria" para el alineamiento del huso mitótico, que podría requerir que una célula "recuerde" como se había fijado el plano de división en previas divisiones. En este caso, nuestros resultados implican la

señalización por integrinas en la transmisión de una "memoria del huso" de una célula progenitora a su hijas.

VII.9. PAPEL DE MISSHAPEN EN LA MIGRACIÓN DE LAS CÉLULAS DEL BORDE DURANTE LA OOGÉNESIS DE *DROSOPHILA*

VII.9.i. RESULTADOS Y DISCUSIÓN

Para investigar los posibles requerimientos de *misshapen* en la migración de las células del borde, primero examinamos su patrón de expresión. Usando tres líneas diferentes en las que un gen reportero estaba insertado bajo el control de misshapen, comprobamos que *misshapen* presenta un patrón de expresión dinámico durante oogénesis. En el germario, *misshapen* se expresa en las células de la capa interna del germario. A partir del estadio 3, *misshapen* se expresa a bajos niveles en todas las células foliculares y a elevados niveles en las células polares anteriores y posteriores. En los estadios 8 y 9, elevados niveles de misshapen se expresan también en las células del borde y esta elevada expresión continúa durante la migración de estas células.

Los elevados niveles de *misshapen* encontrados en las células del borde antes y durante su migración, junto con su papel en el control de la forma celular, indican un posible papel para este gen en la migración de las células del borde.

De hecho, usando dos alelos nulos hemos demostrado que la falta de función de misshapen en las células del borde bloquea totalmente su migración indicando que misshapen es esencial para la migración de este grupo de células.

La incapacidad de las células del borde mutantes para migrar no se debe a defectos en la polarización del grupo de células del borde, ya que los cambios necesarios para la adquisición de una polaridad "motil" y

para la posterior migración parecen ocurrir correctamente como demuestran la localización de los marcadores de polaridad Discs-large y *D*E-Caderina en las células del borde mutantes para misshapen. Al contrario, nuestros resultados apuntan a que misshapen está involucrado en la remodelación del citoesqueleto de actina en la migración de las células del borde. Las células silvestres reorganizan su citoesqueleto de actina y extienden protrusiones ricas en actina filamentosa en la dirección de la migración. Sin embargo, las células del borde que no tienen la función de misshapen poseen una mayor concentración de actina filamentosa y no son capaces de reorganizar la actina al frente de la célula. Estos elevados niveles de actina filamentosa en la células del borde mutantes no son una consecuencia secundaria del bloqueo en la migración de estas células, puesto que las células silvestres que forman parte de un mosaico de células del borde que no ha migrado, no presentan ningún defecto en la acumulación y localización de la actina filamentosa. Además, hemos detectado defectos en la organización de los haces de actina basales en las células foliculares del dominio principal, indicando que efectivamente misshapen se requiere para una dinámica correcta del citoesqueleto de actina en las células foliculares.

Estos datos están en concordancia con informes previos que involucran a *misshapen* en la reorganización del citoesqueletoo en los fotorreceptores y y en células del ala de *Drosophila* [109, 114, 115, 215].

A pesar de los fenotipos similares observados en mutantes para misshapen y para los componentes de la ruta JAK/STAT en la migración de las células del borde, nuestros resultados muestran que misshapen no actúa ni encima ni debajo de la ruta JAK/STAT para controlar la migración de las células del borde. Los niveles de STAT, el factor de transcripción de la ruta JAK/STAT no están afectados en mutantes para

misshapen y STAT no se requiere para regular la expresión de *misshapen*. Nuestros resultados también muestran que los defectos observados en la migración de las células del borde mutantes para *misshapen* no se deben a defectos en la expresión de Slbo, un marcador de identidad de las células del borde.

Se ha visto que misshapen ejerce su función durante el proceso de cierre dorsal a través de la regulación de la ruta *D*JNK. Nuestros resultados también implican a esta ruta en la migración de las células del borde. La falta de actividad de *D*JNK tanto en las células del borde como en las células de la línea germinal tiene como resultado un fallo en la migración de las células del borde. Sin embargo, este defecto en la migración no es tan severo como el observado para los mutantes de misshapen, puesto que las células mutantes para los distintos componentes de la ruta DJNK presentan un retraso en la migración pero ésta no se encuentra totalmente bloqueada. Además, los mutantes para misshapen también presentan un fenotipo de migración defectiva en las células foliculares del dominio principal. Este fenotipo adicional no se observa en mutantes para la ruta *D*JNK, indicando que misshapen está actuando con otra/s ruta/s para controlar la migración de las células del borde. Hay evidencias genéticas que sugieren la existencia de redundancia entre las kinasas JNK/p38 debajo de *misshapen* en la generación de polaridad [215]. Sin embargo, el hecho de que mutantes dobles *D*JNKK/*hemipterous D*p38/*lic* no presentan el mismo fenotipo que los mutantes para *misshapen* indican que ambas kinasas no actúan redundamente en la migración de las células del borde.

También hemos encontrado un papel para Dock, una molécula adaptadora que interacciona con Misshapen durante la guía de axones de los fotorreceptores, durante la migración de las células del borde. Sin embargo, al igual que mutantes para *hemipterous*, las células del borde mutantes para *dock* no presentan una migración totalmente

bloqueada. Además, mutantes *hep;dock* no presentan un fenotipo más fuerte que el de los mutantes simples. Estos resultados sugieren que, o bien Dock afecta a la migración de forma independiente a Misshapen, o que Misshapen actúa a través de Hemipterous, Dock y otras proteínas adicionales.

Aunque la expresión de *misshapen* se ha descrito únicamente en las células foliculares somáticas, se ha descrito también un papel para misshapen en las células de la línea germinal, puesto que clones de la línea germinal mutantes para misshapen no se desarrollan correctamente [109]. Nuestros resultados también denotan un papel para *misshapen* en las células de la línea germinal, ya que la eliminación de la función de misshapen en estas células también produce un bloqueo de la migración de las células del borde. Cuál es el papel de *misshapen* en las células de la línea germinal aún no se sabe, pero resultados similares se han descrito para la *D*E-Caderina - se requiere en los dos tipos celulares para una correcta migración de las células del borde [63]. Puesto que las células de la línea germinal tienen que acomodarse para permitir la migración de las células del borde entre ellas, pudiera ser que misshapen estuviese también involucrado en al reorganización del citoesqueleto de actina y en los cambios en la forma celular de las células nutricias.

VIII. CONCLUSIONES

1.- Las integrinas se expresan en los dominios basales, laterales y apicales de las células foliculares durante la oogénesis en *Drosophila*. También se encuentran en la línea germinal hasta el S3.

2.- La integrinas □PS1□PS se requiere para mantener la estructura simple del epitelio folicular. Huevos en desarrollo en los que grupos de

células foliculares pierden la función de las integrinas desarrollan epitelios estratificados en ambos dominios terminales.

3.- La falta de función de las integrinas no afecta a la proliferación ni a la polaridad apico-basal de las células foliculares. Las integrinas – presumiblemente via interacciones con la membrana basal – juegan un papel de refuerzo en la polarización apico-basal de las células foliculares.

4.- La señalización mediada por integrinas es suficiente para orientar el huso mitótico de las células foliculares.

5.- El posicionamiento del huso mitótico mediado por integrinas no requiere la Miosina II ni la VIIA, ni a los reguladores del citoesqueleto de actina, Rok, Rac1, Rac2 y Mtl. La Miosina no convencional VI, Jaguar, se requiere de forma autónoma para la correcta orientación del huso mitótico de las células foliculares.

6.- *misshapen* se expresa en la células internas del germario, en bajos niveles en las células foliculares y a elevados niveles en las células polares anteriores y posteriores desde el S3 en adelante. Elevados niveles de expresión de *misshapen* se encuentran también en las células del borde antes y durante su migración.

7.- Misshapen se requiere para la migración de las células del borde tanto en las células del borde como en la línea germinal. La pérdida de la actividad de Misshapen también afecta a la migración de las células foliculares del dominio principal.

8.- La polaridad no está afectada en células del borde mutantes para *misshapen*. Sino que, Misshapen se requiere para la remodelación del citoesqueleto de actina en las células del borde.

9.- Misshapen actúa independientemente de la ruta JAK-STAT para controlar la migración de las células del borde. La expresión de *slbo* no está afectada en mutantes para *misshapen*.

10.- La ruta DJNK no es la única que actúa por debajo de Misshapen. Misshapen puede que actue a través de Hemipterous, Dock y proteínas adicionales para controlar la migración de las células del borde.

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