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Integrin-independent repression of cadherin transcription by talin during axis formation in Drosophila

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Abstract :

The *Drosophila* anterior-posterior axis becomes polarised early during oogenesis by the posterior localisation of the oocyte within the egg chamber. The invariant position of the oocyte is thought to be driven by an up-regulation of the adhesion molecule DE-Cadherin in the oocyte and the posterior somatic follicle cells, providing the first *in vivo* example of cell sorting specified by quantitative differences in cell-cell adhesion[1,2]. However, it has remained unclear how DE-Cadherin levels are regulated. Here, we show that talin, known for linking integrins to the actin cytoskeleton, has the unexpected function of specifically inhibiting DE-Cadherin transcription. Follicle cells mutant for talin displayed a strikingly high level of DE-Cadherin, due to elevated transcription of DE-Cadherin in the mutant cells. We demonstrate that this deregulation of DE-Cadherin is sufficient to attract the oocyte to lateral and anterior positions. Surprisingly, we found that this function of talin is independent of integrins. These results uncover a novel role for talin in regulating cadherin-mediated cell adhesion.

Adhesion between cells is a defining process for the development of multicellular organisms. Two main mechanisms have been found to regulate cell adhesion. The first one allows adhesion between cells of the same type to form layers or epithelia. This form of adhesion is often mediated by members of the cadherin family, which form direct homophilic bonds [3]. The second one concerns a higher level of organisation, the adhesion between different layers of cells to form complex tissues. This is mostly mediated by members of the integrin pathway, which link indirectly two layers of cells through their binding to the extracellular matrix[4,5]. While the mechanisms of cadherin and integrin adhesion has been extensively studied, relatively little is known about how these two pathways may regulate each other. This may be an important step in the acquisition of invasive behaviour of cancer cells, for example colon cancer cells downregulate E-Cadherin upon activation of integrin-mediated migration [6].

The regulation of cell adhesion is also crucial for dynamic morphogenetic events. For example differential adhesion is used to sort out different cell populations [7]. Two possible mechanisms have been proposed for this; one relying on the different cells having different types of adhesion molecule [8], and the other relying on the cells having different amounts of the same adhesion molecule [9]. The best characterised example of the latter mechanism *in vivo* is the positioning of the oocyte at the posterior of the *Drosophila* egg chamber, which depends on higher levels of DE-cadherin in the oocyte and the posterior follicle cells[1,2]. The correct position of the oocyte is then required for all subsequent signaling events (reviewed in [10]). However, it was unknown how DE-Cadherin levels are regulated.

In this study, we used a genetic approach to examine the role of integrins and integrin-associated proteins in these early steps of *Drosophila* oogenesis. We found that mutations affecting *rhea*, which encodes the single *Drosophila* homologue of talin[11], lead to a dramatic mislocalisation of the oocyte (Fig 1B). Talin is a cytoskeletal linker protein that may directly link integrins to the actin cytoskeleton. It is a large protein of more than 2500 amino acids,

containing a globular N-terminal region of 50 Kda (head domain) composed of a FERM (band 4.1, ezrin, radixin, moesin and merlin) domain, which can bind to the cytoplasmic domain of the integrin β subunit[12]. The rod domain (220kda) contains low affinity integrin binding sites and binding sites for actin and vinculin.

Talin is not required in the germline to position the oocyte as germline clones mutant for the null allele *rhea*^{79} did not show any phenotype and completed oogenesis normally (n=126) (Fig 1A). However, small follicle cell clones were sufficient to induce a mislocalisation of the oocyte with a high penetrance (Fig 1B and 1C). Mutant follicle cell clones not situated at the posterior of the egg chamber induced a mislocalisation of the oocyte in 75% (n=82) of egg chambers scored for the null allele *rhea*^{79} and in 50% (n=35) for *rhea*^{2} , an allele that encodes a protein truncated after amino acid 1279 of 2836. In contrast, mutant follicle cell clones at the posterior did not induce a mispositioning of the oocyte (n=80). To analyse this bias further, we checked the correlation between the position of the mutant clones and the oocyte within the egg chamber. Strikingly, we found that the oocyte adheres with high fidelity to the talin mutant cells (Fig 1B, 1C). We quantified this phenotype and found that 96% (n=61), and 95% (n=18) of the mislocalised oocytes contact *rhea*^{79} and *rhea*^{2} mutant follicle cells respectively (Fig 1F).

Large clones of talin mutant follicle cells induced a second phenotype, the formation of compound egg chambers containing several cysts encapsulated within one egg chamber. However, even in these conditions, the oocytes preferentially contact follicle cells lacking talin (Fig 1D). We further showed that both phenotypes could be rescued by restoring talin expression from a transgene construct (Fig 1E, F).

The mislocalisation of the oocyte could be due to an indirect effect of talin mutations on the polarity and/or identity of the mutant cells. Using specific markers (Fig 2A, B, C and data not shown), we found that the absence of talin did not affect the apico-basal polarity neither

the identity of the mutant follicle cells. These results suggest a direct role for talin in positioning of the oocyte.

The primary role envisioned for talin is to directly link integrins to the actin cytoskeleton, by simultaneously binding to the integrin β subunit cytoplasmic domain and actin filaments [13,14]. The knock-out of talin in *C.elegans* and *Drosophila* induces a disorganisation of the actin cytoskeleton, and very similar phenotypes to those seen in the absence of integrins [11,15]. Therefore, we expected that the role for talin in oocyte positioning would be mediated through integrins. To examine the phenotype induced by removing all integrin function from the follicle cells, we induced homozygous clones for mutations in the gene encoding the βPS subunit, *myospheroid*, in flies homozygous for a null mutant in the gene encoding the βν subunit[16]. We found that a total loss of integrins either in the germline or in the follicle cells, did not induce a mislocalisation of the oocyte (n=88) (Fig 3A, 3B). Thus, integrins are not required for the localisation of the oocyte, and talin is functioning in this process independently of integrins.

As a further test of the role of integrins in talin function in the follicle cells, we examined whether talin distribution is altered by loss of integrins. Talin was found localised ubiquitously at the membrane of wild type follicle cells and this cortical location was not altered in cells lacking both integrin β subunits (n=20) (Fig 3C). Thus, the general cortical localisation of talin in the follicular epithelium is not directed by integrins. We then checked if the loss of talin affected the organisation of the actin cytoskeleton during early oogenesis. We visualised actin filaments using Rhodamin-phalloidin and their distribution in wild type and talin mutant cells was indistinguishable (Fig 3D). Thus, the early function of talin in the follicle cell epithelia appears to be distinct from its more general cytoskeletal linker function.

Later in oogenesis, we were able to identify a more classical function for talin with integrins to organise the actin cytoskeleton. We stained late egg chamber carrying mutant

clones for talin and found that, like in clones lacking βPS [17], loss of talin perturbed the arrangement of the basal actin fibers (Fig 3E) and consequently caused the formation of round eggs.

In egg chambers containing clones of cells lacking DE-cadherin, the oocyte attached to the remaining wild type follicle cells [1,2]. This is the opposite of what occured in the absence of talin, suggesting that talin might negatively regulate cadherin function. We therefore analysed the expression and localisation of DE-cadherin in follicle cells mutant for talin. We found that follicle cells lacking talin contained strikingly high levels of DE-cadherin at the time when the oocyte normally would reach the posterior pole (Fig 4A). This upregulation was also retained until later stages (Fig 4B). In contrast, cells mutant for the two integrin β subunits showed normal level of DE-Cadherin (Fig 4C). Furthermore, cells lacking DE-Cadherin showed a normal distribution of talin (Fig 4D), indicating that there is not a feedback loop between talin and DE-Cadherin. The increase in DE-cadherin levels in the follicle cells lacking talin could thus explain why the oocyte sticks preferentially to talin mutant cells.

To test this hypothesis, we examined if a direct overexpression of DE-cadherin in a group of follicle cells could induce oocyte mispositioning. We used the flip-out technique[18] to induce group of cells overexpressing DE-cadherin. Using precise heat shock conditions (see materials and methods), we were able to induce groups of follicle cells that caused oocyte delocalisation. Furthermore, the mislocalised oocyte was always in contact with a cell or group of cells that overexpressed DE-cadherin (Fig 4E", arrow). Using the same approach, we found that overexpression of talin in follicle cell clones did not induce any changes in oocyte localisation nor did it cause a detectable decrease in the amount of DE-cadherin (data not shown). Thus, follicle cells mutant for talin express a higher level of DE-cadherin, which

can explain why follicle cells lacking talin attract the oocyte, since overexpresion of DE-cadherin in follicle cells is sufficient to attract the oocyte.

Finally, to test if the overexpression of DE-cadherin in talin mutant clones is the primary cause of oocyte mislocalisation, we removed DE-cadherin from the talin mutant follicle cells. The double mutant clones for null alleles of *rhea* and *shotgun* were identified by the loss of GFP markers on both chromosome arms (Fig 4F) and verified by the absence of talin and DE-cadherin detected with antibodies (data not shown). We found that the double mutant follicle cells did not cause oocyte mislocasition nor did they preferentially contact the oocyte (n=17) (Fig 4F). This contrasts with 75% oocyte mislocalisation induced by follicle cells lacking talin alone. Thus, removing DE-Cadherin prevents the mislocalisation of the oocyte induced by the loss of Talin (Fig4F). We conclude that Talin affects oocyte position primarily by causing overexpression of DE-Cadherin.

In a wild type germarium, the follicle cells contacting the oocyte express higher amounts of DE-Cadherin, however, it is not known if this regulation occurs at the protein and/or mRNA level. We found that these follicle cells express higher amounts of DE-Cadherin mRNA (Fig 5A). This indicates that at least part of the regulation occurs at the mRNA level. Then, to test for a post-transcriptional regulation, we examined the expression and localisation of a GFP-tagged DE-cadherin, driven by a ubiquitous promoters (*tubulin* or *ubiquitin*), which are presumably not sensitive to the endogenous transcriptional regulation of DE-Cadherin. For both transgenes, we found that DE-Cadherin-GFP was distributed as a gradient with the highest levels at the posterior of the egg chamber (Fig 5B). Thus, a second layer of gene regulation at the protein level was revealed.

We then asked at which step in the synthesis of DE-cadherin (*shg*) does the talin regulation occur. We analysed the level of DE-cadherin *(shg)* mRNA in follicle cells lacking

talin, identifying mutant clones by the lack of GFP (Fig 5C) and *shg* transcript levels by in situ hybridisation (Fig 5C'). We found that mutant cells expressed a much higher level of *shg* mRNA than the surrounding wild type cells. Elevated levels of DE-cadherin mRNA were seen early during oocyte positioning and were maintained until later stages. We thus conclude that talin regulates DE-cadherin expression by modulating the level of DE-cadherin transcript. However, these results do not indicate whether talin affects DE-Cadherin transcription in the nucleus or the stabilisation of its mRNA in the cytoplasm. To distinguish between these possibilities, we used an enhancer-trap line inserted in the DE-cadherin gene, which reproduces the endogenous mRNA distribution (*shotgun* $^{P34\text{-}1}$, data not shown and [19]). The *LacZ* reporter gene is thus under the control of the endogenous *shotgun* promoter and the *lacZ* mRNA does not share any sequence with the *shotgun* mRNA. We found that cells mutant for talin show a clear upregulation of the LacZ expression (Fig 5D and E). This result demonstrates that talin regulates DE-Cadherin at the transcriptional level.

Finally, we checked if talin could also regulate the levels of the DE-cadherin protein independent of mRNA levels. In order to test this, we examined the expression and localisation of a GFP-tagged DE-cadherin, driven by a ubiquitous promoter, that presumably lacks the ability to be transcriptionally regulated by talin. In follicle cell clones mutant for *rhea*^{79}, identified by the lack of talin (Fig 5F'), the expression and distribution of the fusion protein was not affected (Fig 5F"), even though the oocyte was misplaced. Therefore, talin cannot regulate DE-cadherin protein levels when it is expressed from another promoter.

Our analyses contribute three main findings: (1) talin has at least one essential function that does not involve integrins; (2) talin is part of a novel pathway that regulates cadherin transcription; (3) overexpression of DE-cadherin, either directly or by eliminating talin function, is sufficient to induce a delocalisation of the oocyte.

Perhaps the key question to arise from this work is how talin is being used in a pathway that regulates transcription? There are other examples of cytoskeletal linker proteins that are involved in adhesion also playing a role in transcription (reviewed in [20]). A particularly well characterised example is β-catenin, which not only contributes to the links between DE-cadherin and the actin cytoskeleton, but can also associate with LEF/TCF transcription factors and directly translocate to the nucleus to regulate the transcription of several genes implicated in cancer [21]. Talin does not contain any domains shared with DNA binding proteins or transcriptional regulators. Furthermore, with the antibody we have used, which recognises the carboxy terminus, we have not seen any evidence of nuclear talin. Talin in mammalian cells has been found to be cleaved by calpain into a 50 kD head domain and 200 kD tail domain [22], so we have not excluded the possibility that the head domain enters the nucleus. However, at present it seems more likely that talin acts in the cytoplasm to regulate the activity of a transcriptional factor rather than controlling gene expression on its own. The Traffic Jam protein is a large Maf factor and would be an attractive candidate for talin regulation, as it is specifically required in the somatic cells to inhibit the expression of DE-Cadherin during oogenesis [23]. However, follicle cells mutant for *tj* overexpress not only DE-Cadherin but also two other adhesion molecules, Fas3 and Neurotactin. Thus, talin would have to regulate part of Tj activity, as we found that Fas3 is not upregulated in cells lacking talin.

The existence of a gradient of adhesiveness has been proposed to be sufficient to localise the oocyte[1]. Here, we validated this model and further showed that the establishment of the DE-Cadherin gradient involves regulation at both transcriptional and post-transcriptional levels. We propose that the first level might depend on talin, while the second does not. The post-transcriptional level of regulation seems sufficient to position the oocyte, as a ubiquitously expressed DE-cadherin-GFP protein reproduces the endogenous gradient and is

able to rescue a null allele of DE-cadherin (*shotgun*^{R69})[24,25]. This post-transcriptional regulation remains to be characterised. It is thus not possible to simply remove it to test if the transcriptional regulation is also sufficient to localise the oocyte. However, two lines of evidence emphasize the importance of the transcriptional regulation: firstly, it is likely that a transcriptional gradient would contribute to the formation of a gradient of the corresponding protein; and secondly, cells mutant for talin overexpress DE-cadherin mRNA, which translates into a sufficiently high level of protein to override the post-transcriptional regulation, as the oocyte becomes mislocalised in contact with the mutant cells. Both levels of regulation are thus required for the correct positioning of the oocyte.

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Materials and methods :

Fly stocks

The following mutants were used: *rhea*^{79} [11], *rhea*^{2} [26], *rhea*^{17} [11], *shg*^{JH} (Z221, Tubingen), *shg*$^{P34\text{-}1}$ [19], *shg*^{R69} [1], *mys*^{XG43} [27], βv^{1}, βv^{2} [16]. Rescue experiments were performed by heat-shocking flies of the following genotype: hs-Flp; ubi-talin; FRT2A-*rhea*^{79}/FRT2AGFPnls. To analyse the distribution of a ubiquitously expressed cadherin-GFP fusion protein, we used two

transgenes: *tubulin*-Cadherin-GFP (kind gift of Anne Pacquelet and Pernille Rorth) and *ubiquitin*-Cadherin-GFP[24]. To analyse the distribution of Cadherin-GFP fusion protein in *rhea*^{79} mutant clones, the following flies were heat-shocked: y,w,hs-Flp; tub-cadh-GFP, FRT2A-*rhea*^{79}/tub-cadh-GFP, FRT2A.

Generation of mutant clones

Mutant clones were generated by the FLP/FRT technique [28], using either the FRTG13GFPnls chromosome or the FRT2AGFPnls chromosome [29]. Clones were induced by heat-shocking third instar larvae for 2 hours on 3 consecutive days. Adult flies were dissected 2 or 3 days after eclosion. We generate double clones for *shotgun* and *rhea* by heat-shocking flies of the following genotype: y,w,hs-Flp; FRTG13-*shg*^{R69} /FRTG13GFPnls; FRT2A-*rhea*^{79}/FRT2AGFPnls.

Overexpression

Somatic overexpression of *shg* was performed by generating Flip-out/Gal4 clones in females y,w,hs-flp/+; act>CD2>Gal4 [30], UAS-GFP/ +; UAS-DE-cadherin [5,9] [31]. Adult flies were heat shock 2 hours at 37° and dissected 24H after.

Immuno-staining and in situ hybridisation

Antibody stainings were performed according to standard procedures [32]. Antibodies were used at the following concentration: mouse anti-Orb (orb4H8 and orb6H4 DHSB) 1/250, rat anti-DE-cadherin (D-CAD2) 1/20, mouse anti-Talin (talin C19) 1/20, mouse anti-integrin βPS (CF.6G11 DSHB) 1/20, mouse anti-Fasciclin III (7G10 DSHB) 1/10, rabbit anti-Bazooka [33]; 1/500, mouse anti-GFP (Roche) 1/200, rabbit anti-βgal (ICN Pharmaceuticals) 1/1000. DNA was stained with Hoescht (1/1000) and F-actin was labelled with rhodamine phalloidin (Molecular probes) 1/100. Secondary antibodies conjugated with Cy3 (Jackson laboratories) were used at 1/200. To combine antibody staining and in situ hybridisation, we used a standard procedure for antibody staining except that we used DEPC water and added 1ul

RNA guard (Pharmacia) with the first and second antibody. Then, in situ hybridisation was done according to standard protocols (hybridation temperature 55°) using dioxygenin-labelled cDNA of *shotgun* (primer sequences used to synthetise the probe CADH5 (5'-TCAAGTGC GAGGAATCGTGC-3') and CADH3T7 (5'-GAATTGTAATAC GACTCACTA TAGGG TGATGTGCTGATGGCGGATG-3'). In situ staining was stained using either an NBT/BCIP kit or the TSA-Fluorescein system (NEN). Samples were examined either with a Leica DMR microscope or by confocal microscopy using a Leica SP2 AOBS microscope.

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Figure Legends :

Figure 1 **Talin is required in the follicle cells for oocyte localisation**

In all the figures posterior is to the right.

A Germline clones mutant for talin marked by the absence of GFP. The oocyte (star), identified by the accumulation of the protein orb (red), is localised at the posterior of the egg chamber.

B-C Follicle cells clones mutant for talin, identified by the absence of GFP (one example is shown with a dotted line), are sufficient to induce a mispositioning of the oocyte (yellow arrow) identified by orb (B') or DNA staining (C'). White horizontal bars indicate junction points between egg chambers and their stalks, which normally abut the oocyte. The mislocalised oocyte adheres to follicle cells lacking talin (B" and C").

D Large clones of follicle cells mutant for talin (D) lead to formation of compound egg chambers with several oocytes (yellow arrows) (D') which all adhere to mutant follicle cells (D").

E A transgene encoding talin driven by the ubiquitin promoter rescues the mislocalisation of the oocyte induced by follicle cells clones mutant for talin. The oocyte still lies at the posterior despite the presence of a large clone at the anterior (dotted line).

F Penetrance of the phenotype of oocyte mislocalisation (green) for $rhea^{79}$, $rhea^{2}$, (*ubi-tal*; $rhea^{79}$). We only considered follicle cells that are not at the posterior of the egg chamber. Percentage of mislocalised oocytes (red) in contact to mutant follicle cell clones for $rhea^{79}$ and $rhea^{2}$.

Figure 2 **Follicles cells lacking talin conserved their polarity and identity**

We checked the distribution of apical, basal and lateral markers of epithelial polarity and found that apical markers such as Bazooka (Baz) and atypical Protein kinase C (aPKC) (data

not shown), lateral marker such as α-spectrin (data not shown) and baso-lateral marker such as integrin βPS subunit localised normally in follicle cells lacking talin in contact with the germline.

A-A" Bazooka apical localisation (red) is not affected in follicle cells mutant for talin identified by the lack of GFP.

B-B" Integrin βPS (red) still localises to the basal cortex of talin mutant follicle cells.

C-C" In this egg chamber, the oocyte (yellow arrow) is displaced on one side. In addition, the mispositioned oocyte sticks to mutant follicle cells that do not overexpress the Fas III protein like the wild type polar cells (white arrow). We found the same result for another marker of the polar cells PZ80 (data not shown). Talin mutant cells thus do not express polar cells markers.

Figure 3 **Integrins are not required for oocyte localisation**

A Germline clones for integrins marked by the absence of GFP. The oocyte (star), identified by the accumlation of the protein orb (red), is localised at the posterior of the egg chamber.

B Large and small follicle cells clones mutants for the two β-subunits do not induce a defect in oocyte localisation.

C-C" The distribution of talin (red) is not affected in follicle cells mutants for the two β-subunits.

D-D" Clones in a stage 6 egg chamber labelled with rhodamine-phalloidin. Talin mutant cells show no defect in F-actin organisation.

E-E" Clone in a stage 12 egg chamber stained with rhodamin phalloidin. The optical section is focused on the basal surface, where bundles of actin are perpendicular to the A-P axis. Follicle cell clones mutant for talin identified by the lack of GFP affect basal actin filament organisation (E',E").

Figure 4 **Follicle cells lacking talin overexpress DE-cadherin**

A-A" Early in oogenesis**,** follicle cells lacking talin express high levels of DE-cadherin (red) compared to wild type adjacent follicle cells.

B-B" Follicle cells lacking talin overexpress DE-cadherin (red) in a stage 6 egg chamber.

C-C" DE-cadherin level (red) is not affected in follicle cells mutants for the two integrin β-subunits.

D-D" The distribution of talin (red) is not affected in follicle cells lacking DE-cadherin, compared to wild type follicle cells (D").

E-E" Using the Flp-out technique, cells overexpressing DE-cadherin are positively identified by the expression of GFP (D'). The oocyte (yellow arrow) is mislocalised on the lateral side, instead of the posterior. Moreover, the oocyte (yellow arrow) identified by orb (D") adheres to cells overexpressing DE-cadherin (white arrow) (D").

F-F" Follicle cells double mutant for a null allele of *shotgun* and *rhea*. The double mutant cells are identified by the complete lack of GFP (dotted line). Despite the presence of a lateral clone, the oocyte remains at the posterior (Orb in red).

Figure 5 **Talin regulates DE-cadherin transcription**

A DE-Cadherin mRNA expression in a wild type germarium. Posterior follicles cells contacting the oocyte express higher levels of DE-cadherin mRNA (arrows).

B Expression of a cadherin-GFP protein fusion driven by the ubiquitin promoter in a wild type germarium. DE-cadherin-GFP is distributed as a gradient with the highest levels at the posterior of the egg chamber (arrows).

C-C' Follicle cells clones for talin are identified by the lack of GFP with an antibody (C). The level of DE-cadherin transcript, detected by an RNA probe using a histochemical method (C'), is overexpressed exactly in the cells which lack talin in an early egg chamber.

D-D'' Follicle cells mutant for talin in an early egg chamber show a clear upregulation of the lacZ expression, identified by an antibody against the β−galactosidase(red).

E-E'' Follicle cells mutant for talin in a late egg chamber show a clear upregulation of the lacZ expression identified by an antibody against the β−galactosidase (red).

F-F''' Follicle cells lacking talin, identified by an antibody against talin (red), and wild type cells express the same level of cadherin-GFP protein fusion when expressed under the tubulin promoter.

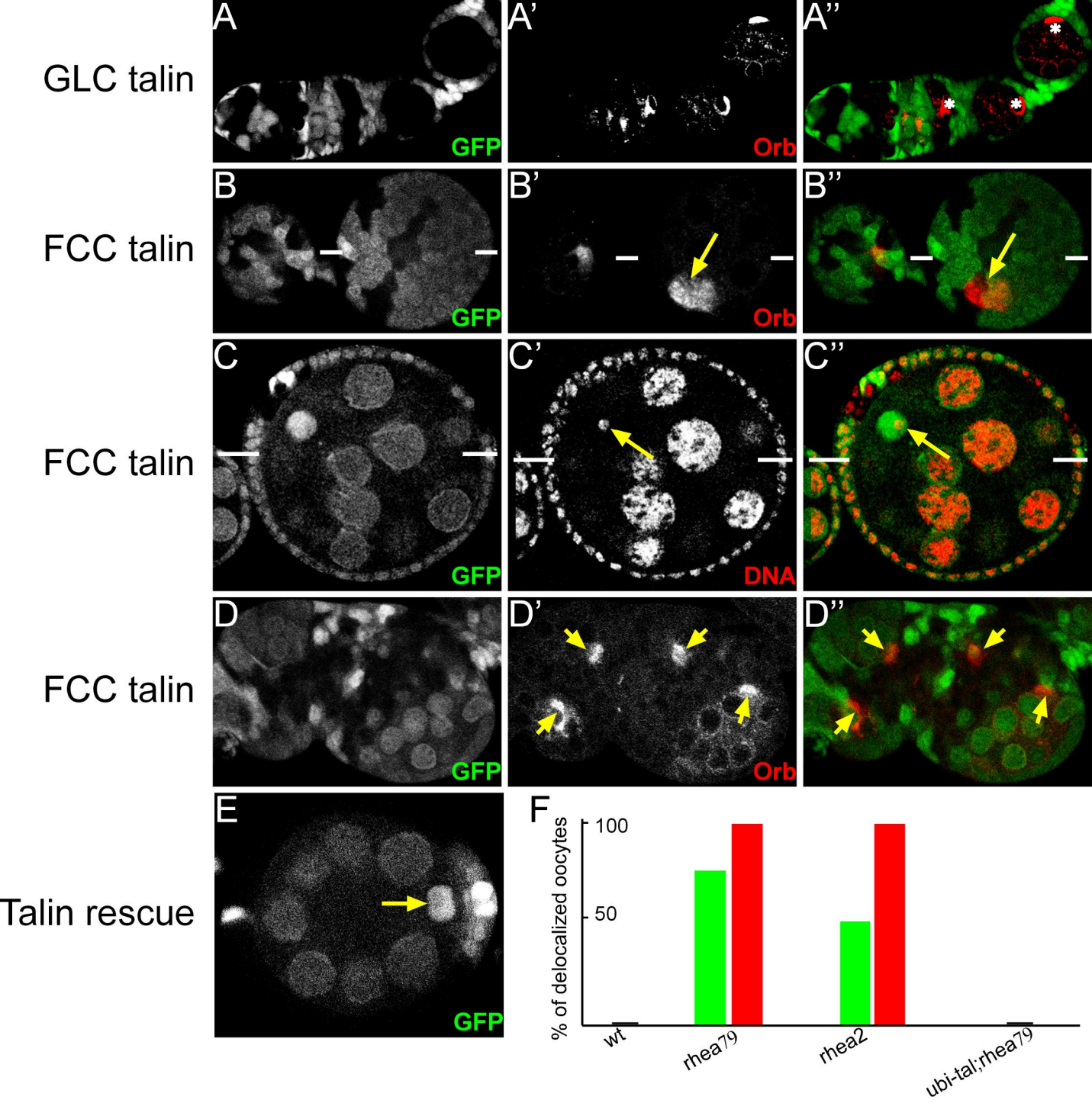

Figure 1

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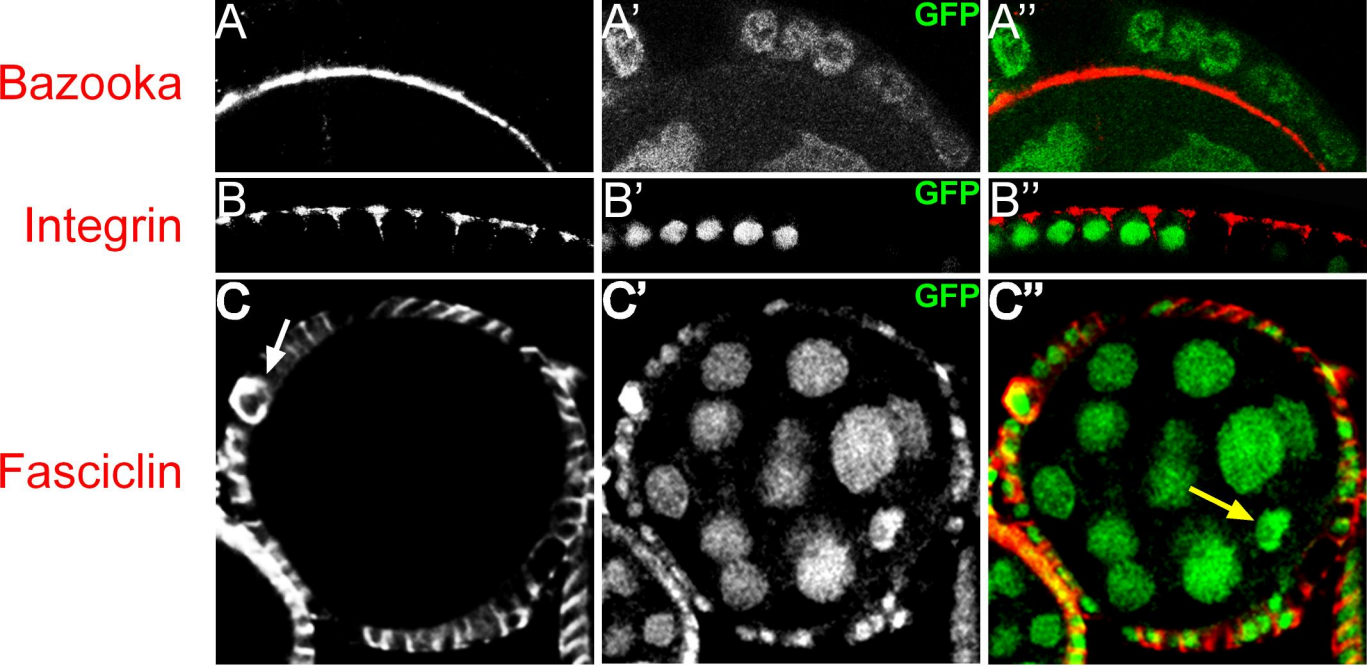

Figure 2
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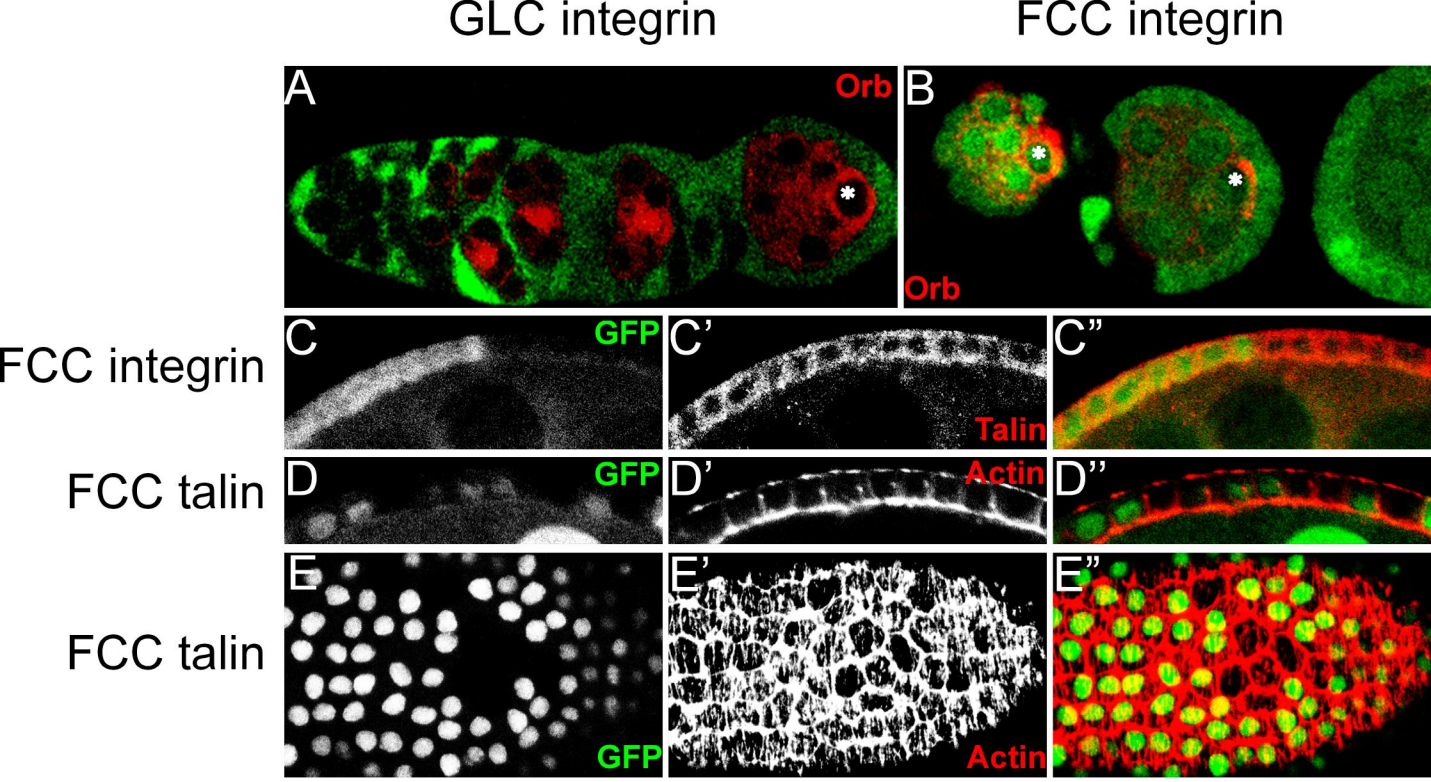

Figure 3
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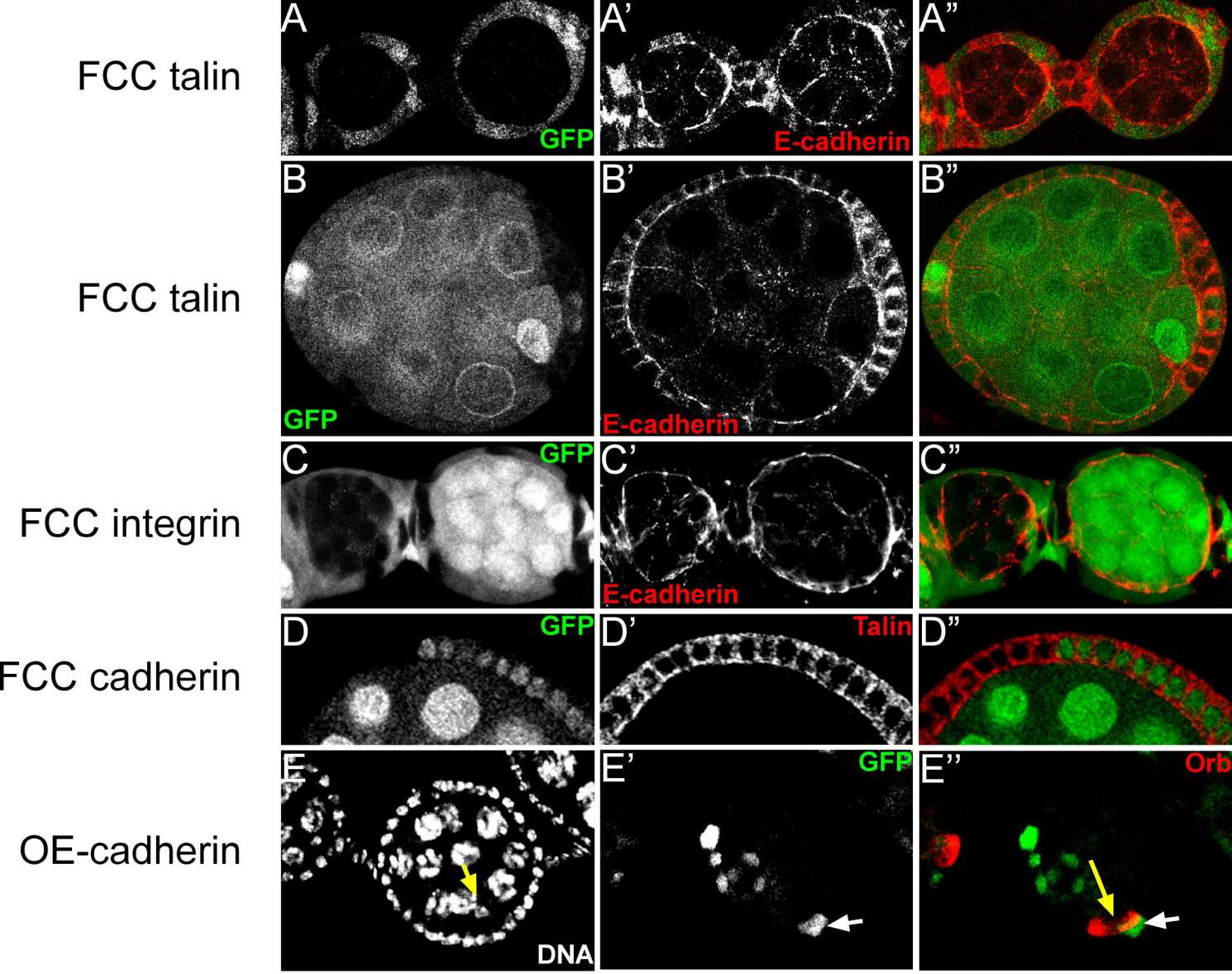

Figure 4
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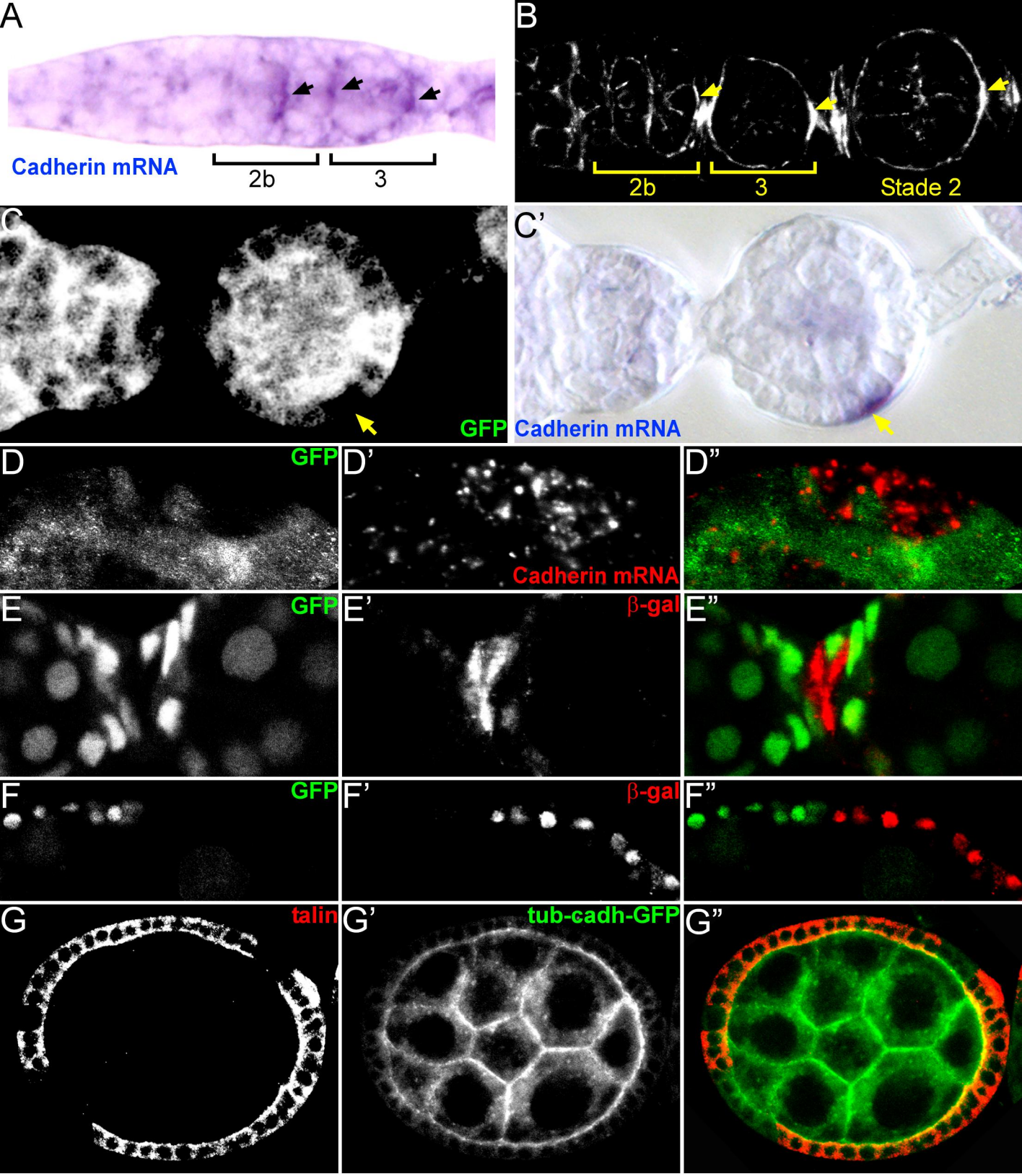

Figure 5
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