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

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The *Drosophila melanogaster* anterior–posterior axis becomes polarized early during oogenesis by the posterior localization of the oocyte within the egg chamber. The invariant position of the oocyte is thought to be driven by an upregulation of the adhesion molecule DE-cadherin in the oocyte and the posterior somatic follicle cells, providing the first *in vivo* example of cell sorting that is 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 its role in linking integrins to the actin cytoskeleton, has the unexpected function of specifically inhibiting DE-cadherin transcription. Follicle cells that are mutant for talin show a strikingly high level of DE-cadherin, due to elevated transcription of DE-cadherin. We demonstrate that this deregulation of DE-cadherin is sufficient to attract the oocyte to lateral and anterior positions. Surprisingly, this function of talin is independent of integrins. These results uncover a new 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 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 concerns a higher level of organization: the adhesion between different layers of cells to form complex tissues. This is mostly mediated by members of the integrin pathway, which indirectly link two layers of cells through their binding to the extracellular matrix[4,5]. Whereas the mechanisms of cadherin and integrin adhesion have been extensively studied, relatively little is known about how these two pathways might regulate each other. This may be an important step towards understanding the invasive behaviour of cancer cells; for example, colon cancer cells downregulate E-cadherin upon activation of integrin-mediated migration[6].

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 relies on the different cells having different types of adhesion molecule[8], and the other relies on the cells having different amounts of the same adhesion molecule[9]. The best characterized example of the latter mechanism *in vivo* is the positioning of the oocyte at the posterior of the *D. melanogaster* 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 signalling events (reviewed in ref. 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 *D. melanogaster* oogenesis. We found that mutations that affect *rhea*, which encodes the single *D. melanogaster* homologue of talin[11], lead to a marked mislocalization of the oocyte (Fig. 1B). Talin is a cytoskeletal linker protein that may connect integrins directly to the actin cytoskeleton. It is a large protein of more than 2,500 amino acids, containing a globular amino-terminal region (head domain) with a relative molecular mass of 50,000 (M_r 50K), which is composed of a FERM (band 4.1, ezrin, radixin, moesin and merlin) domain that can bind to the cytoplasmic domain of the integrin β subunit[12]. The rod domain (M_r 220K) contains low-affinity integrin-binding sites and binding sites for actin and vinculin.

Talin is not required in the germline to position the oocyte, because germline clones that were mutant for the null allele *rhea*^{79} did not show any abnormal phenotypes and completed oogenesis normally (Fig. 1A; $n = 126$). However, small follicle cell clones were sufficient to induce a mislocalization of the oocyte with a high penetrance (Fig. 1B, C). Mutant follicle cell clones that were not situated at the posterior of the egg chamber induced a mislocalization of the oocyte in 75% ($n = 82$) of egg chambers that were scored for the null allele *rhea*^{79} and in 50% ($n = 35$) for *rhea*^{2}, an allele that encodes a protein that is 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. Notably, we found that the oocyte adheres with high fidelity to the talin-mutant cells (Fig. 1B, C): 96% ($n = 61$) and 95% ($n = 18$) of the mislocalized oocytes contact *rhea*^{79} and *rhea*^{2} mutant follicle cells, respectively (Fig. 1F).

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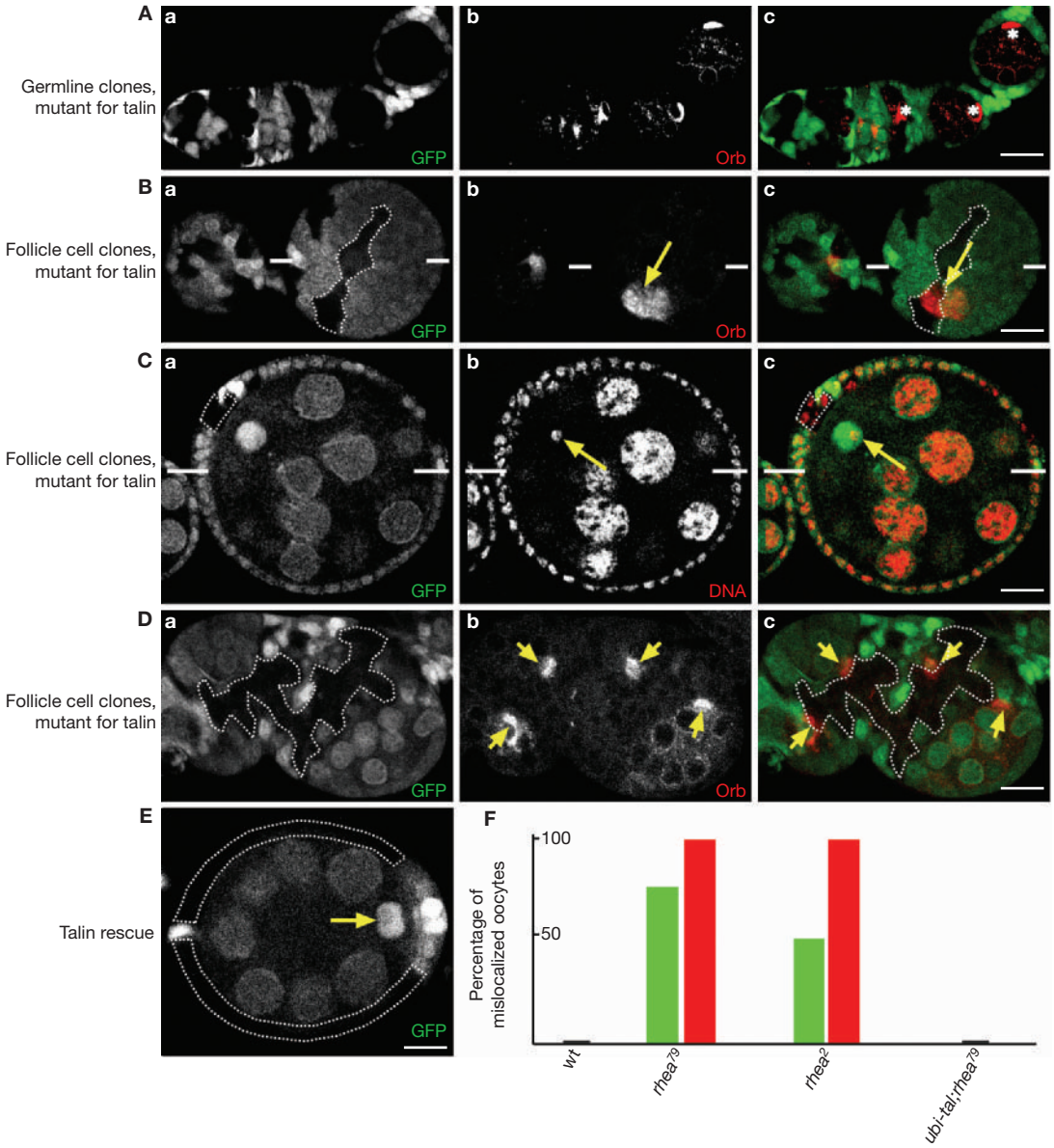

Figure 1 Talin is required in the follicle cells for oocyte localization. In all the figures, posterior is to the right. (**A**) Germline clones that are mutant for talin are marked by the absence of GFP. The oocyte (asterisk), identified by the accumulation of the protein orb (red), is localized at the posterior of the egg chamber. (**B**, **C**) Follicle cell clones that are mutant for talin, identified by the absence of GFP (one example is shown with a dotted line), are sufficient to induce mispositioning of the oocyte (yellow arrow), identified by orb (**B, b**) or DNA staining (**C, b**). White horizontal bars indicate junction points between egg chambers and their stalks, which normally abut the oocyte. The mislocalized oocyte adheres to follicle cells that lack talin (**B, c** and **C, c**). (**D**) Large clones of follicle cells that are mutant for talin (**D, a**) lead to formation of compound egg chambers with several oocytes (**D, b**; yellow arrows), which all adhere to mutant follicle cells (**D, c**). (**E**) A transgene that encodes talin and is driven by the *ubiquitin* promoter rescues the mislocalization of the oocyte, induced by follicle cell clones that are mutant for talin. The oocyte still lies at the posterior (yellow arrow) despite the presence of a large clone at the anterior (dotted line). (**F**) Penetrance of the phenotype of oocyte mislocalization (green) for *rhea*^{79}, *rhea*^{2} and *ubi-tal*; *rhea*^{79}. We only considered follicle cells that were not at the posterior of the egg chamber. The percentage of mislocalized oocytes in contact with mutant follicle cell clones for *rhea*^{79} and *rhea*^{2} is shown in red. Scale bars, 10 μm (**A**, **B**) and 20 μm (**C**–**E**).

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

The mislocalization 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. 2 and data not shown), we found that the absence of talin did not affect the apico-basal polarity nor the identity of the mutant follicle cells. These results suggest a direct role for talin in positioning of the oocyte.

Talin has been shown to directly link integrins to the actin cytoskeleton by simultaneously binding to the integrin β subunit cytoplasmic domain and to actin filaments[13,14]. Moreover, the knockout of talin in *Caenorhabditis elegans* and *D. melanogaster* induces a disorganization 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

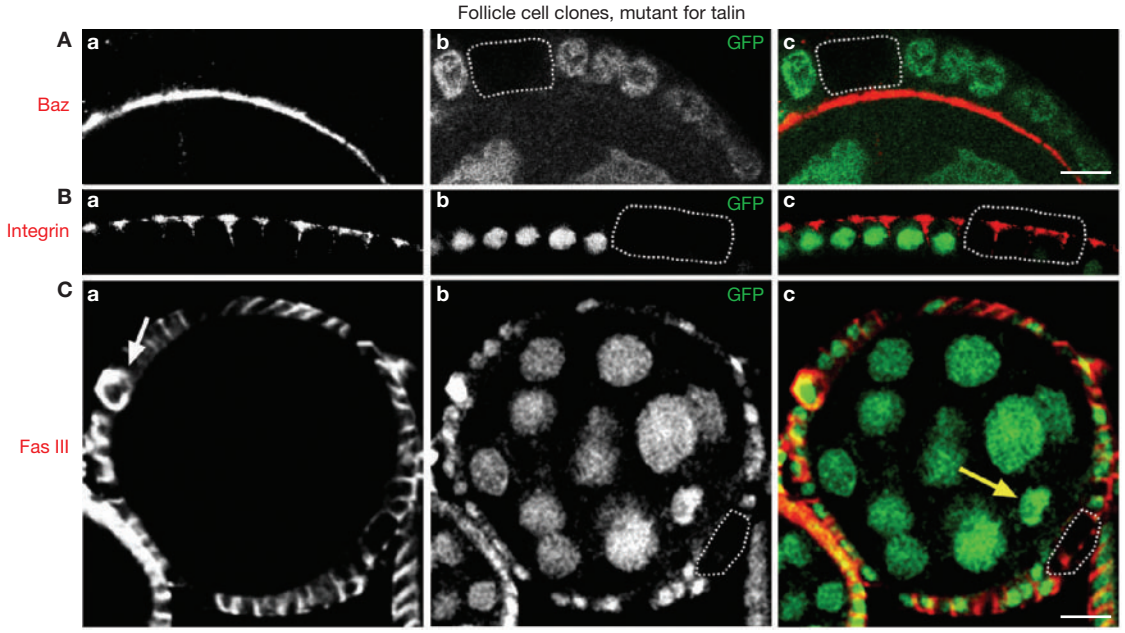

Figure 2 Follicle cells that lacked talin conserved their polarity and identity. We checked the distribution of apical, basal and lateral markers of epithelial polarity and found that apical markers such as Bazooka (Baz) and atypical protein kinase C (aPKC) (data not shown), lateral markers such as α-spectrin (data not shown), and basolateral markers such as the integrin βPS subunit localized normally in follicle cells that lacked talin, in contact with the germline. (**A**) Bazooka apical localization (red) is not affected in follicle cells that are mutant for talin, identified by the lack of GFP (dotted line). (**B**) Integrin βPS (red) still localizes to the basal cortex of talin-mutant follicle cells. (**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, unlike wild-type polar cells, do not overexpress the protein Fas III (white arrow). We found the same result for another polar cell marker, PZ80 (data not shown). Talin-mutant cells thus do not express polar cell markers. Scale bars, 10 μm (**A**) and 20 μm (**C**).

talin in oocyte positioning would be mediated through integrins. To examine the phenotype that is induced by removing all integrin function from the follicle cells, we induced homozygous clones for mutations in the gene that encodes the βPS subunit, *myospheroid*, in flies that were 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 mislocalization of the oocyte (Fig. 3A, B; $n = 88$). Thus, integrins are not required for the localization of the oocyte, and talin functions in this process independently of integrins.

As a further test for whether integrins might affect talin function in the follicle cells, we examined whether talin distribution is altered by loss of integrins. Talin was found localized ubiquitously at the membrane of wild-type follicle cells and this cortical location was not altered in cells that lacked both integrin β subunits (Fig. 3C; $n = 20$). Thus, the general cortical localization of talin in the follicular epithelium is not directed by integrins. We then used rhodamine-phalloidin to test whether loss of talin affects the organization of the F-actin cytoskeleton during early oogenesis, but saw that the distribution in wild-type and talin-mutant cells was indistinguishable (Fig. 3D). Thus, the early function of talin in the follicle cell epithelia seems to be distinct from its more general cytoskeletal linker function.

Later in oogenesis, we were able to identify a more classic function for talin, acting with integrins to organize the actin cytoskeleton. We stained late egg chambers that were carrying mutant clones for talin and found that, as in clones that lack βPS[17], the arrangement of the basal actin fibres was perturbed (Fig. 3E) and consequently caused the formation of round eggs.

In egg chambers containing clones of cells that lack DE-cadherin, the oocyte attaches to the remaining wild-type follicle cells[1,2]. This is the opposite of what occurred in the absence of talin, suggesting that talin might negatively regulate cadherin function. We therefore analysed the expression and localization of DE-cadherin in follicle cells that were mutant for talin. We found that follicle cells that lacked talin contained markedly 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 that were mutant for the two integrin β subunits showed normal levels of DE-cadherin (Fig. 4C). Furthermore, cells that lacked DE-cadherin showed a normal distribution of talin (Fig. 4D), indicating that there is no feedback loop between talin and DE-cadherin. The increase in DE-cadherin levels in the follicle cells that lacked talin could thus explain why the oocyte sticks preferentially to talin-mutant cells.

To test this hypothesis, we examined whether 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 groups of cells that overexpress DE-cadherin. Using precise heat-shock conditions (see Methods) we were able to induce groups of follicle cells that caused oocyte delocalization. Furthermore, the mislocalized oocyte was always in contact with a cell or group of cells that overexpressed DE-cadherin (Fig. 4E, c; arrow). Using the same approach, we found that overexpression of talin in follicle cell clones did not induce any changes in oocyte localization nor did it cause a detectable decrease in the amount of DE-cadherin (data not shown). Thus, follicle cells that are mutant for talin express a higher level of DE-cadherin, which can explain why follicle cells that lack talin attract the oocyte ectopically, because overexpression of DE-cadherin in follicle cells is sufficient to attract the oocyte.

Finally, to test whether the overexpression of DE-cadherin in talin-mutant clones is the primary cause of oocyte mislocalization, we removed DE-cadherin from the talin-mutant follicle cells. The double-mutant clones for null alleles of *rhea* and *shotgun* (*shg*) were identified by the loss of GFP markers on both chromosome arms (Fig. 4F) and verified

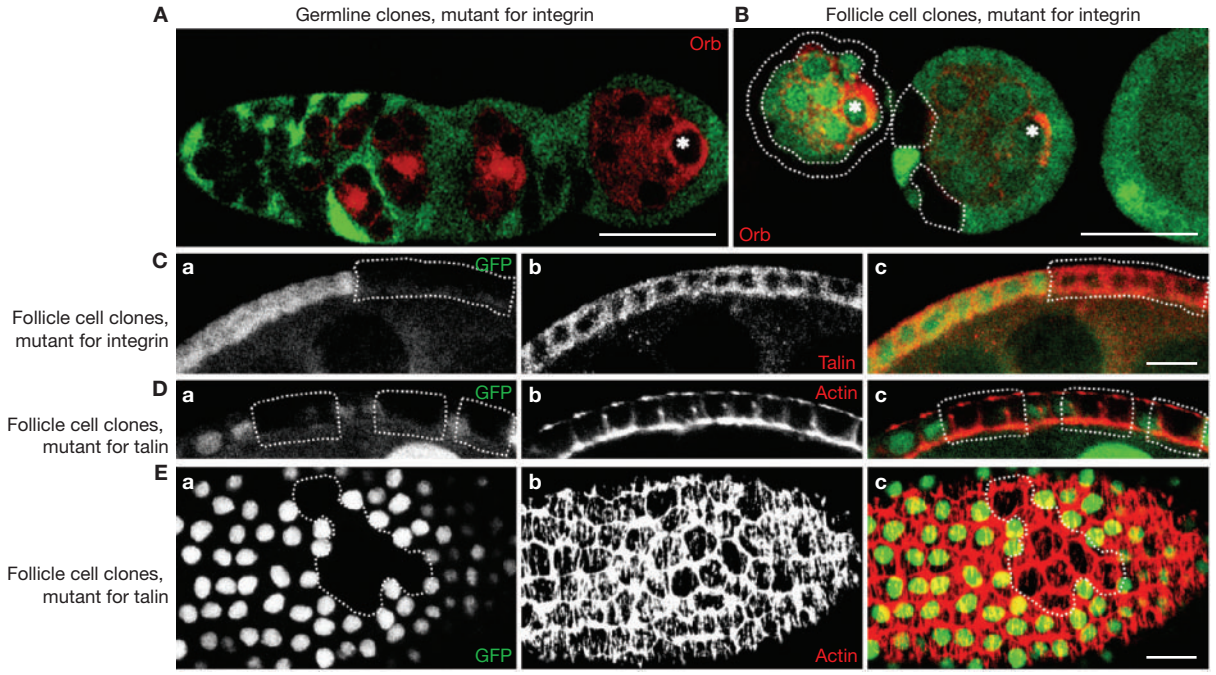

Figure 3 Integrins are not required for oocyte localization. (**A**) Germline clones for integrins, marked by the absence of GFP. The oocyte (asterisk), identified by the accumulation of the protein orb (red), is localized at the posterior of the egg chamber. (**B**) Large and small follicle cell clones (outlined by dotted line) that are mutant for the two β subunits do not induce a defect in oocyte localization. (**C**) The distribution of talin (red) is not affected in follicle cells that are mutant for the two β subunits. (**D**) Clones in a stage 6 egg chamber labelled with rhodamine-phalloidin. Talin-mutant cells show no defect in F-actin organization. (**E**) Clone in a stage 12 egg chamber stained with rhodamine-phalloidin. The optical section is focused on the basal surface, where bundles of actin are perpendicular to the anterior–posterior axis. Follicle cell clones that are mutant for talin (identified by the lack of GFP) affect basal actin filament organization (**E, b, c**). Scale bars, 10 μm (**A**–**C**) and 20 μm (**E**).

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 mislocalization nor did they preferentially contact the oocyte (Fig. 4F; $n = 17$). This contrasts with the 75% oocyte mislocalization that was induced by follicle cells that lacked talin alone. Thus, removing DE-cadherin prevents the mislocalization of the oocyte that is induced by the loss of talin (Fig. 4F). We conclude that talin affects oocyte position primarily by causing overexpression of DE-cadherin.

In a wild-type germarium, the follicle cells that contact the oocyte express higher amounts of DE-cadherin; however, it is not known whether this regulation occurs at the protein and/or the mRNA level. We found that these follicle cells express higher amounts of *DE-cadherin* (*shg*) mRNA (Fig. 5A). This indicates that at least part of the regulation occurs at the mRNA level. Next, to test for post-transcriptional regulation, we examined the expression and localization of a green fluorescent protein (GFP)-tagged DE-cadherin, driven by 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 was revealed and DE-cadherin is also regulated at the protein level.

We then asked at which step in the synthesis of DE-cadherin the talin regulation occurs. We analysed the level of *DE-cadherin* mRNA in follicle cells that lacked talin, identifying mutant clones by the lack of GFP (Fig. 5C, a) and *DE-cadherin* transcript levels by *in situ* hybridization (Fig. 5C, b). We found that mutant cells expressed a much higher level of *DE-cadherin* 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 stabilization 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 ref. 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 that are mutant for talin show a clear upregulation of lacZ expression (Fig. 5D, E). This result demonstrates that talin regulates DE-cadherin at the transcriptional level.

Finally, we tested whether talin could also regulate the levels of the DE-cadherin protein independently of mRNA levels. To test this we examined the expression and localization 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 that were mutant for *rhea*^{79} — identified by their lack of talin (Fig. 5F, a) — the expression and distribution of the fusion protein was not affected (Fig. 5F, b) 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: first, talin has at least one essential function that does not involve integrins; second, talin is part of a novel pathway that regulates cadherin transcription; and third, overexpression of DE-cadherin, either directly or by eliminating talin function, is sufficient to induce delocalization of the oocyte.

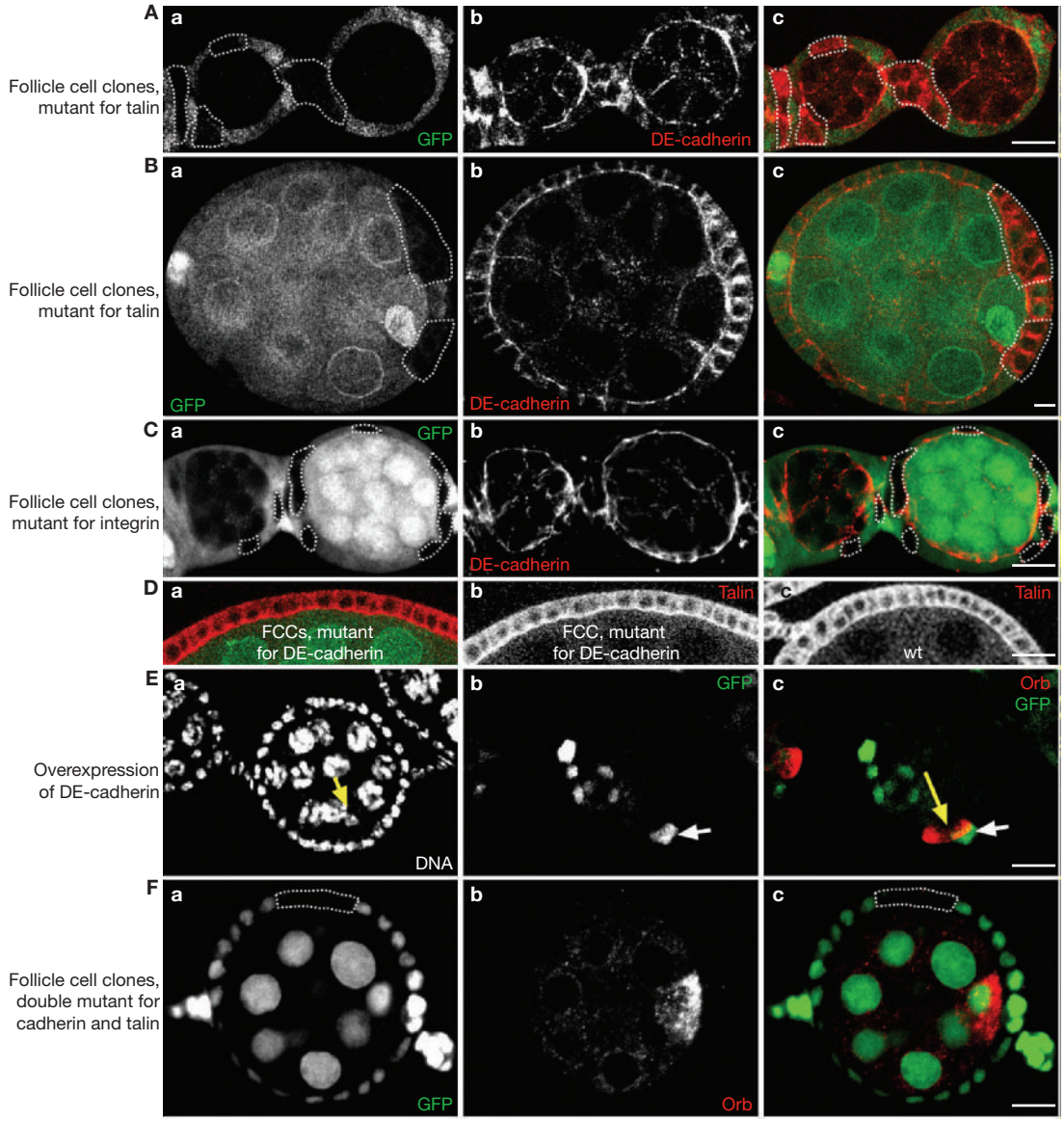

Figure 4 Follicle cells that lack talin overexpress DE-cadherin. (**A**) Early in oogenesis, follicle cells that lack talin express high levels of DE-cadherin (red; dotted line) compared with wild-type adjacent follicle cells. (**B**) Follicle cells that lack talin overexpress DE-cadherin (red; dotted line) in a stage 6 egg chamber. (**C**) The DE-cadherin level (red) is not affected in follicle cells that are mutant for the two integrin β subunits. (**D**) The distribution of talin (red) is not affected in follicle cell clones (FCCs) that lack DE-cadherin, compared with wild-type (wt) follicle cells (**D, c**). (**E**) Using the flip-out technique, cells that overexpress DE-cadherin are positively identified by the expression of GFP (**E, b**). The oocyte (yellow arrow) is mislocalized on the lateral side, instead of the posterior. Moreover, the oocyte (yellow arrow) identified by orb (**E, c**) adheres to cells that overexpress DE-cadherin (white arrow) (**E, c**). (**F**) Follicle cells that are 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; red). Scale bars, 10 μm (**A**, **C**, **D**) and 20 μm (**B**, **E**, **F**).

Perhaps the key question to arise from this work is how talin is used in a pathway that regulates transcription. There are other examples of cytoskeletal linker proteins, involved in adhesion, which also have a role in transcription (reviewed in ref. 20). A particularly well characterized 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 that are implicated in cancer[21]. Talin does not contain any domains that are shared with DNA-binding proteins or transcriptional regulators. Furthermore, with the antibody we have used, which recognizes the carboxyl terminus, we have not seen any evidence of nuclear talin. Talin in mammalian cells has been found to be cleaved by calpain into an N-terminal head domain (M_r 50K) and a C-terminal tail domain (M_r 200K)[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 by controlling gene expression on its own. The Traffic Jam protein is a large Maf factor and would be an attractive candidate for talin regulation, because it is specifically required in the somatic cells to inhibit the expression of DE-cadherin during oogenesis[23]. However, follicle cells that are mutant for *traffic jam* (*tj*) overexpress not only DE-cadherin but also two other adhesion molecules, Fas3 and Neurotactin. Thus, talin would have to regulate only part of Tj activity, because we found that Fas3 is not upregulated in cells that lack talin.

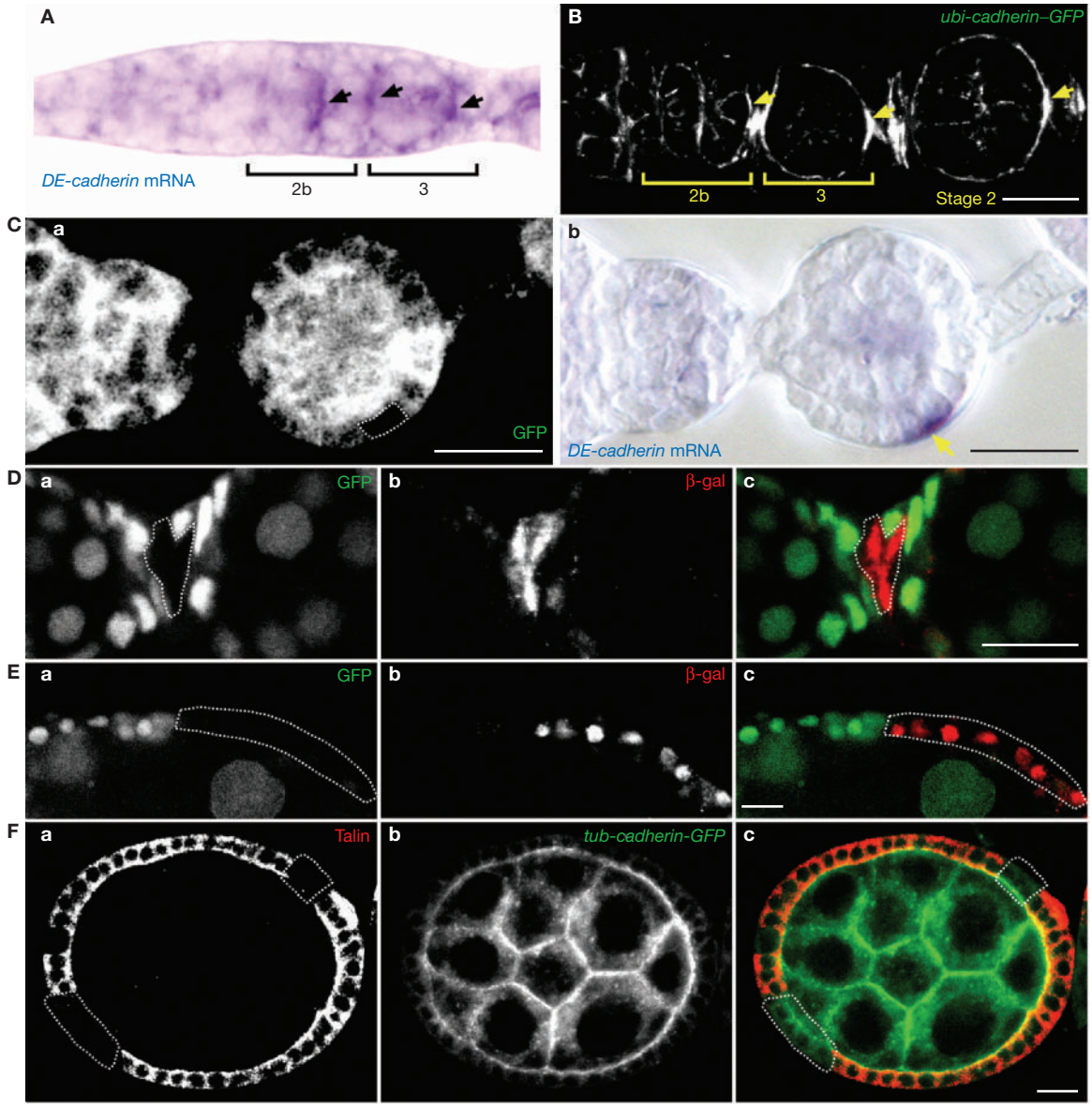

Figure 5 Talin regulates DE-cadherin transcription. (**A**) *DE-cadherin* mRNA expression in a wild-type germarium. Posterior follicle cells that contact the oocyte express higher levels of *DE-cadherin* mRNA (arrows). Regions 2b and 3 of the germarium are indicated. (**B**) Expression of a cadherin–GFP fusion protein driven by the *ubiquitin* (*ubi*) 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**) Follicle cell clones that express talin are identified by a lack of GFP with an antibody (**C, a**). The level of DE-cadherin transcript, detected by an RNA probe using a histochemical method (**C, b**), is overexpressed exactly in the cells that lack talin in an early egg chamber. (**D**) Follicle cells that are mutant for talin in an early egg chamber show a clear upregulation of lacZ expression, identified by an antibody against β-galactosidase (β-gal; red). (**E**) Follicle cells that are mutant for talin in a late egg chamber show a clear upregulation of lacZ expression, identified by an antibody against β-gal (red). (**F**) Follicle cells that lack talin, identified by an antibody against talin (red), and wild-type cells both express the same level of cadherin–GFP fusion protein when expressed under the *tubulin* promoter. Scale bars, 10 μm (**B**–**E**) and 20 μm (**F**).

The existence of a gradient of adhesiveness has been proposed to be sufficient to localize 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 transcriptional level might depend on talin, whereas the post-transcriptional level does not. The post-transcriptional level of regulation seems sufficient to position the oocyte, because 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 characterized. It is thus not possible to simply remove it to test whether the transcriptional regulation is also sufficient to localize the oocyte. However, two lines of evidence emphasize the importance of the transcriptional regulation: first, it is likely that a transcriptional gradient would contribute to the formation of a gradient of the corresponding protein; and second, cells that are mutant for talin overexpress *DE-cadherin* mRNA, which translates into a sufficiently high level of protein to override the post-transcriptional regulation, because the oocyte becomes mislocalized in contact with the mutant cells. Both levels of regulation are thus required for the correct positioning of the oocyte. □

METHODS

Fly stocks. The following mutants were used: *rhea*^{79} (ref. 11), *rhea*^{2} (ref. 26), *rhea*^{17} (ref. 11), *shg*^{IH} (Z221, Tubingen), *shg*$^{P34\text{-}1}$ (ref. 19), *shg*^{R69} (ref. 1), *mys*^{XG43} (ref. 27), βv^{1} and βv^{2} (ref. 16). Rescue experiments were performed by heat-shocking flies with 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* (a gift from A. Pacquelet and P. 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 h on three consecutive days. Adult flies were dissected 2 or 3 days after eclosion. We generated 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 (ref. 30), UAS–GFP/+; UAS-DE-cadherin[5,9,31]. Adult flies were heat-shocked for 2 h at 37 °C, and dissected after a further 24 h.

Immunostaining and *in situ* hybridization. Antibody stainings were performed according to standard procedures[32]. Antibodies were used at the following concentrations: 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, 1:200 (Roche, Paris, France); rabbit anti-βgal, 1:1,000 (Cappel, ICN, Aurora, OH). DNA was stained with Hoescht (1:1,000) and F-actin was labelled with rhodamine-phalloidin, 1:100 (Molecular probes, Eugene, OR). Secondary antibodies conjugated with Cy3 (Jackson Immuno Research Europe, Soham, England) were used at 1:200. To combine antibody staining and *in situ* hybridization, we used a standard procedure for antibody staining except that we used DEPC water and added 1 µl RNA guard (Pharmacia, Paris, France) with the first and second antibody. Then, *in situ* hybridization was performed according to standard protocols (hybridization temperature, 55 °C) using dioxygenin-labelled cDNA of *shotgun*. Primer sequences used to synthesize the probes CADH5 and CADH3T7 were: 5′-TCAAGTGC GAGGAATCGTGC-3′ and 5′-GAATTGTAATAC GACTCACTA TAGGG TGATGTGCTGATGGCGGATG-3′, respectively. *In situ* staining was performed using either an NBT/BCIP kit or the TSA-Fluorescein system (NEN, Boston, MA). Samples were examined either with a Leica Microsystems AG (Wetzlar, Germany) DMR microscope or by confocal microscopy using a Leica SP2 AOBS microscope.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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