Lateral adhesion drives reintegration of misplaced cells into epithelial monolayers

Dan T. Bergstrahl[^1], Holly E. Lovegrove[^1] and Daniel St Johnston[^2]

Cells in simple epithelia orient their mitotic spindles in the plane of the epithelium so that both daughter cells are born within the epithelial sheet. This is assumed to be important to maintain epithelial integrity and prevent hyperplasia, because misaligned divisions give rise to cells outside the epithelium[^1,2]. Here we test this assumption in three types of *Drosophila* epithelium: the cuboidal follicle epithelium, the columnar early embryonic ectoderm, and the pseudostratified neuroepithelium. Ectopic expression of Insuteable in these tissues reorients mitotic spindles, resulting in one daughter cell being born outside the epithelial layer. Live imaging reveals that these misplaced cells reintegrate into the tissue. Reducing the levels of the lateral homophilic adhesion molecules Neuroglian or Fasciclin 2 disrupts reintegration, giving rise to extra-epithelial cells, whereas disruption of adherens junctions has no effect. Thus, the reinsertion of misplaced cells seems to be driven by lateral adhesion, which pulls cells born outside the epithelial layer back into it. Our findings reveal a robust mechanism that protects epithelia against the consequences of misoriented divisions.

Previous work demonstrated that metaphase spindles in the cuboidal follicle epithelium are oriented between 0° and 35° relative to the plane of the layer, roughly perpendicular to the apical–basal axis of the cell[^3]. Metaphase spindle orientation in this tissue relies on the canonical factors Mud and Pins, and mutants in either gene randomize spindle orientation[^4]. Unexpectedly, we found that the organization of the epithelium is maintained in *mud* and *pins* mutants (Fig. 1a and Supplementary Fig. 1A). This is not due to post-metaphase correction of division angles, as vertically oriented spindles persist into telophase in *mud* mutants (Fig. 1b).

To disrupt spindle orientation more severely, we ectopically expressed Insuteable in follicle cells. In neuroblasts, this protein recruits Pins and Mud to the apical cortex of neuroblasts so that mitotic spindles are oriented along the apical–basal axis[^5-9]. It has a similar effect on spindle orientation when ectopically expressed in follicle cells (Fig. 1c). Rather than randomizing spindle orientation as in *pins* and *mud* mutants, Insuteable orients almost all spindles perpendicular to the epithelial plane (Fig. 1d). Divisions are thus horizontal and produce an apical and a basal daughter (Supplementary Fig. 1B). Like spindle randomization, this has no effect on tissue organization (Fig. 1c,e and Supplementary Fig. 1C). In the neuroblast, spindle orientation controls cell fate by ensuring the asymmetric segregation of fate determinants to one daughter cell. Insuteable expression in the follicle epithelium does not confer neural cell fate, because it does not cause expression of the transcription factor Deadpan (Supplementary Fig. 1D). We also observed that female flies expressing UAS-Insuteable under the control of the strong follicle cell driver Traffic Jam-Gal4 are fertile (not shown), indicating that reorienting most divisions in the follicular epithelium does not disrupt egg chamber development.

In the imaginal wing disc, misoriented cell division is associated with basal cell extrusion and apoptosis[^10,11]. We therefore considered the possibility that the apically misplaced cells produced by horizontal divisions in the follicle cell layer are also eliminated by programmed cell death. However, misplaced cells show neither cleaved caspase-3 immunoreactivity nor pyknosis (Supplementary Fig. 1E). Furthermore, expression of the apoptotic inhibitor p35 has no effect on follicular epithelia expressing Insuteable (*n* = 17 ovarioles from 6 dissected flies) or containing *pins*[^62] mutant clones (*n* = 12 clones larger than 5 cells; Supplementary Fig. 1F,G). Live imaging reveals that rather than dying, misplaced daughter cells simply reintegrate back into the epithelial monolayer (Fig. 1f and Supplementary Video 1).

Our findings prompted a closer examination of mitosis in wild-type follicle cells. These cells divide only during the early stages of egg chamber maturation, switching from mitosis to endocycling at stage 6. Live imaging reveals that the monolayer has an uneven, ‘bubbly’ appearance in early stages (Supplementary Fig. 2A). This is because mitotic cells round up, exhibiting a concomitant increase in cortical phospho-myosin (Supplementary Fig. 2B), and often move apically,

[^1]: The Gurdon Institute and the Department of Genetics, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK.
[^2]: Correspondence should be addressed to D.St.J. (e-mail: d.stjohnston@gurdon.cam.ac.uk)
[^3]: These authors contributed equally to this work.

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Figure 1 Spindle misorientation does not disrupt the organization of the follicle epithelium. (a) The organization of the follicle cells into an epithelial monolayer is normal in mud³/mud⁴ transheterozygotes. Disorganization was not observed in any of 42 ovarioles imaged. (b) Incorrectly oriented divisions are observed in mud mutant egg chambers. Five such complete reorientations were observed. These are exceptional cases, given that mud mutation randomizes, rather that perpendicularizes, divisions. Left panel, the XY plane. Right panel, the ZY plane. The arrows point to the dividing cell. (c) Ectopically expressed Inscuteable localizes apically and causes spindle reorientation in follicle cells. This image is one of the twenty divisions quantified in d. A dividing cell is shown within the rectangle. (d) A cumulative data plot of spindle angles in wild-type (n=29 spindles assessed from 29 different egg chambers) and UAS–Inscuteable-expressing (n=20 spindles assessed from 20 different egg chambers) follicle cells shows the extent of spindle misorientation caused by Inscuteable expression. P=1.047 x 10⁻¹⁰ as determined by the Kolmogorov–Smirnov test. (e) Inscuteable expression does not disrupt the organization of the follicular epithelium. This image is representative of 103 ovarioles imaged. (f) The apical product of a misoriented division reintegrates into the monolayer. Clonal expression of Actin-Gal4 was used to drive both UAS–Inscuteable and UAS–GFP, which illuminates the relevant cell (arrow) and its neighbours to the left. Tubulin–RFP marks the misoriented spindle at time 0°. As in subsequent time lapses, the 0° time point represents abscission. This is one of seven complete reintegrations imaged. Scale bars in this figure represent 10 μM.

pulling away from the basement membrane (Fig. 2a,b). Daughter cells are frequently born detached from the basement membrane (Fig. 2c and Supplementary Fig. 2C). These cells then reinsert into the monolayer (Fig. 2d and Supplementary Video 2). These results are consistent with the earlier observation that metaphase spindle angles, which determine the angle of division, are not strictly parallel
Reintegration is a feature of wild-type cell division in the follicle epithelium. During division, cells usually move apically relative to the epithelial layer, often seeming to detach from the basement membrane (Viking-GFP). One prometaphase cell is shown in a and two recently born sister cells in b. These fixed-tissue images are representative of the live divisions quantified in c. c Cells are frequently born without an obvious connection to the basement membrane. Divisions were imaged live to a depth of at least 10 μm to confirm the position of each cell in the tissue. n = number of cell divisions. d Reintegration occurs in wild-type tissue. Egg chambers were imaged live using Jupiter-Cherry to mark the spindle and Basigin–YFP to mark the cell outline. Images taken from Supplementary Video 2. Time points in this and all subsequent time courses are numbered relative to abscission (at zero). This video is one of the sixty divisions quantified in c. The arrow points to the dividing cell. Scale bars in this figure represent 10 μM.

Reintegration of newly born epithelial cells has previously been observed in two specific developmental contexts. In mammalian ureteric buds, cells move apically into the lumen to divide and one daughter cell then re-enters into the epithelium at a distant site. This may contribute to branching. Second, neuroepithelial cells of the zebrafish neural keel normally orient their spindles vertically, and the apical daughter then intercalates into the opposite side of the neural tube in a process that depends on planar cell polarity signalling. In both of these cases, reintegration occurs at a distant site. In contrast, reintegration in the follicle epithelium is always local, and therefore acts to maintain, rather than to alter, epithelial architecture.

As local reintegration can be detected only by live imaging, it is possible that it is a general feature of epithelial tissues that has been largely overlooked. To test this possibility, we examined two other types of Drosophila epithelium: the columnar epithelium of the early embryonic ectoderm and the neuroepithelium of the developing optic lobe. It has previously been shown that ectopic expression of Inscuteable reorients spindles in these tissues without affecting tissue integrity. The neuroepithelium is pseudostratified and undergoes interkinetic nuclear migration before division. Expression of Inscuteable in this tissue efficiently reorients divisions, producing one daughter cell that protrudes apically from the layer, as in the follicular epithelium (Fig. 3c). Live imaging reveals that these apical cells then reintegrate into the epithelium over the next 30 min (Fig. 3c and Supplementary Fig. 3A and Supplementary Video 3). Inscuteable expression also causes misoriented divisions in the columnar cells of the early embryonic ectoderm, resulting in misplaced daughter cells that lie below, rather than above, the monolayer (Supplementary Fig. 3B,C). Three-dimensional tracking over time shows that these basally misplaced daughter cells can move apically to reintegrate (Fig. 3d and Supplementary Fig. 3D).

Reintegration seems to be an active process, because cells undergo a series of shape changes as they reinsert into the monolayer (Fig. 4a and Supplementary Video 4). One possibility is that this is a cell migration process driven by actomyosin constriction at the rear (the apical surface), which squeezes the basal side of the cell back into the epithelium. However, we did not observe an obvious enrichment of the Myosin Regulatory Light Chain (Spaghetti Squash) or Heavy Chain (Zipper) at the apical surface of reintegrating cells (Fig. 4b and Supplementary Fig. 4A and Supplementary Video 5). Myosin is most obviously enriched at the adherens junctions. This correlates with a planar constriction of the reintegrating cell at this level, which would be predicted to hinder rather than help reintegration. Furthermore, reintegrating cells often show a large, transient expansion of their apical free surface, which suggests that the apical membrane is pushed out to accommodate the compression of the basal side of the cell as it squeezes between its neighbours (Supplementary Fig. 4B). This...
Figure 3 Reintegration occurs in multiple epithelia. (a) Inscuteable expression causes spindle misorientation in the optic lobe neuroepithelium. The two daughter cells are tracked in c. This image is representative of the Inscuteable-induced spindle misorientation in this tissue quantified in ref. 14. The dividing cell is shown in the red rectangle. (b) Expression of Inscuteable reorients mitotic spindles in the early embryonic ectoderm. Spindles are perpendicular to the plane of the epithelium. These spindles are among the 34 quantified in Supplementary Fig. 3B. Left panel, the XY plane. Right panel, the YZ plane indicated by the dashed line. (c) Both products of a misoriented division are maintained in the neuroepithelial layer. Pseudo-colouring indicates the two division products. By the 12-min time point, the basal daughter has reattached to the apical surface. After this point it moves orthogonally to the plane of focus and is no longer observed. This is one of two complete reintegrations imaged in this tissue. The dividing cell is shown in the red rectangle. (d) Reintegration of two basally misplaced cells following misoriented divisions in the embryonic ectoderm. Arrows point to correctly positioned daughter cells. One basally mispositioned daughter is marked by a white asterisk. The other is marked by a yellow asterisk. These reintegrations, representing two of the three tracked, were exceptional; other misplaced daughter cells could not be tracked owing to the rapid morphogenetic movements of the tissue and the limited depth resolution. Scale bars in this figure represent 10 μM.
Figure 4 Reintegration occurs despite weakened adherens junctions. (a) Reintegration is characterized by an apical protrusion as the basal side of the cell reintegrates. Only one of two division products (marked by an asterisk) is seen in this plane of focus. This video is representative of the 60 divisions quantified in Fig. 2c. (b) Localized cortical enrichment of Sqh is not observed during reintegration. Arrows point to Sqh enrichment at the contractile ring. The reintegrating cell is marked with an asterisk. This represents one of 20 divisions imaged using these markers. (c) The apical product of a misoriented division remains attached to the epithelial layer via adherens junctions. This is one of three similar images collected. The dividing cell is shown in the rectangle. (d) Following an Insuteable-induced perpendicular division, the basal daughter cell establishes a transient adherens junction (marked by an arrow) along the length of its lateral cortex with the neighbouring cell. Adherens junctions were marked with Armadillo–GFP. Two Z-planes spaced 1μm apart were merged. This is one of two complete divisions imaged with these markers. (e) Overexpression of Insuteable (marked by co-expression of UAS–GFP) in armadillo3 mutant clones (marked by loss of RFP) does not promote multilayering. This image is one of the 15 clones of >5 cells, none of which showed multilayering. Scale bars in this figure represent 10μM.

In addition to their apicolateral adherens junctions, follicle cells adhere laterally through functionally redundant homophilic adhesion molecules, such as the IgCAM Neuroglian167 (Nrg167) and the N-Cam-like protein Fasciclin II (Fas2; refs 19,20). Both Nrg167 and Fas2 are highly expressed along the length of follicle cell lateral membranes during the first half of oogenesis, when follicle

epithelium18. We were unable to detect misplaced cells or multilayering in armadillo3 clones expressing Insuteable (n = 15 mutant clones of at least 6 cells) and observed no cell death (Fig. 4e). Reintegration of an armadillo3 mutant cell expressing Insuteable was also observed directly (Supplementary Fig. 4D). These results argue against a major role for adherens junctions in this process.
cells are dividing, but their expression is downregulated in post-mitotic stages\(^2\) (Fig. 5a,b). This pattern of expression suggests that these proteins are important during division. They are also expressed along lateral membranes in the embryonic epithelium and neuroepithelium (Supplementary Fig. 5A,B). Furthermore, Nrg is localized along the cortex throughout the course of reintegation (Supplementary Video 7).

In agreement with earlier work, short hairpin RNA (shRNA)-mediated depletion of Nrg\(^{16}\) causes the appearance of occasional follicle cells lying apical to the epithelial monolayer, which is otherwise unperturbed (Fig. 5c; ref. 22). Apical cells are also observed in mutant clones of Fas2\(^{20,33}\), a P-element allele that behaves as a protein null\(^2\) (Fig. 5d and Supplementary Fig. 5C). Similar phenotypes have been previously attributed to the loss of apical–basal polarity, but the Nrg shRNA and Fas2 mutant cells within the monolayer seem to have normal polarity, as shown by the wild-type distributions of aPKC, Par-6, Bazooka, DE-cadherin, Arm and Dlg (Supplementary Fig. 5DI; ref. 24). We therefore reasoned that the apically extruded cells represent failed reintegrations. To test this possibility, we increased the number of cells born above the layer by overexpressing InsCuteable in Nrg knockdown or Fas2 mutant cells. InsCuteable expression increased the mean number of apically positioned cells more than twofold when combined with Nrg shRNA and more than tenfold in Fas2\(^{20,33}\) mutant egg chambers (Fig. 5e,f,h). Live imaging confirmed that cells born
apically remain above the epithelium and never reintegrate (Fig. 5g and Supplementary Video 8 and Supplementary Fig. 5i). Cumulatively, these results show that normal levels of lateral adhesion are required for reintegration.

On the basis of these results, we propose that tissue surface tension drives reintegration by acting to maximize cell–cell adhesion25 (Fig. 5i). As this process is driven by lateral adhesion, it should be able to pull cells back into the monolayer from either side of the epithelium, and this may explain how misplaced cells in the embryonic ectoderm reintegrate from the basal side, whereas follicle and optic lobe cells reintegrate from the apical side. Although these three epithelia reintegrate misplaced cells, this does not seem to be the case in the wing disc epithelium11. This difference may arise because lateral adhesion molecules such as Neuroglian are concentrated in apical septate junctions in the wing disc, rather than along the entire lateral membrane as seen in most other mitotic epithelia16. These lateral adhesion proteins will therefore segregate into only the apical daughter of a horizontal division in the wing disc, thereby preventing the basal daughter from integrating by maximizing lateral adhesion.

Contrary to expectation, spindle misorientation does not disrupt the organization of typical cuboidal, columnar or pseud stratified epithelia in Drosophila. Instead, misplaced cells reintegrate, providing a robust mechanism to protect epithelial monolayers from the consequences of misoriented divisions. Indeed, this mechanism may act more generally to safeguard epithelia against any processes that might disrupt their organization. It will therefore be interesting to investigate whether reintegration also occurs in vertebrate epithelia, where the main lateral adhesion molecule is E-cadherin, and whether a role in reintegration contributes to E-cadherin’s function as a tumour suppressor.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper

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AUTHOR CONTRIBUTIONS

D.T.B. and H.E.L. performed the experiments and data analysis. D.T.B., H.E.L. and D.S.I. planned the experiments. D.T.B. and D.S.I. conceived the project and wrote the manuscript.

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1. Pease, J. C. & Tirnauer, J. S. Mitotic spindle misorientation in cancer—out of alignment and into the fire. J. Cell Sci. 124, 1007–1016 (2011).
2. McCAffrey, L. M. & Macara, I. G. Epithelial organization, cell polarity and tumorigenesis. Trends Cell Biol. 21, 727–735 (2011).
3. Fernández-Miñán, A., Martín-Bermudo, M. D. & González-Reyes, A. Integrin signaling regulates spindle orientation in Drosophila to preserve the follicular epithelium monolayer. Curr. Biol. 23, 683–688 (2007).
4. Bergstrahl, D. T., Lovegrove, H. E. & St Johnston, D. Discs large links spindle orientation to apical-basal polarity in Drosophila epithelia. Curr. Biol. 23, 1707–1712 (2013).
5. Kraut, R., Chia, W., Jan, L. Y., Jan, Y. N. & Knoblich, J. A. Role of inscutable in orienting asymmetric cell divisions in Drosophila. Nature 383, 50–55 (1996).
6. Yu, F., Morin, X., Cai, Y., Yang, X. & Chia, W. Analysis of partner of inscutable, a novel player of Drosophila asymmetric divisions, reveals two distinct steps in inscutable apical localization. Cell 100, 399–409 (2000).
7. Siller, K. H., Cabernard, C. & Doe, C. Q. The NuMA-related Mud protein binds Pins and regulates spindle orientation in Drosophila neuroblasts. Nat. Cell Biol. 8, 594–600 (2006).
8. Bowman, S. K., Neumüller, R. A., Novatchkova, M., Du, Q. & Knoblich, J. A. The Drosophila NuMA homolog Mud regulates spindle orientation in asymmetric cell division. Dev. Cell 10, 731–742 (2006).
9. Izumi, Y., Ohta, N., Hisata, K., Raabe, T. & Matsuzaki, F. Drosophila Pins-binding protein Mud regulates spindle-polarity coupling and centrosome organization. Nat. Cell Biol. 8, 586–593 (2006).
10. Guilgur, L. G., Prudencio, P., Ferreira, T., Pimenta-Marques, A. R. & Martinho, R. G. Drosophila aPKC is required for mitotic spindle orientation during symmetric division of epithelial cells. Development 139, 503–513 (2012).
11. Nakajima, Y.-I., Meyer, E. J., Kroesen, A., Mcrinney, S. A. & Gibson, M. C. Epithelial junctions maintain tissue architecture by directing planar spindle orientation. Nature 500, 359–362 (2013).
12. Packard, A. et al. Luminal mitosis drives epithelial cell dispersal within the branching ureteric bud. Dev. Cell 27, 319–330 (2013).
13. Ciruna, B., Jenin, A., Lee, D., Mlodzik, M. & Schier, A. F. Planar cell polarity signalling couples cell division and morphogenesis during neurulation. Nature 439, 220–224 (2006).
14. Egger, B., Boone, J. Q., Stevens, N. R., Brand, A. H. & Doe, C. Q. Regulation of spindle orientation and neural stem cell fate in the Drosophila optic lobe. Neural Dev. 2, 1 (2007).
15. Ruijano, M. A., Sanchez-Pulido, L., Pennettier, C., Ide, D. & Basto, R. The microcephaly protein Asp regulates neuroepithelium morphogenesis by controlling the spatial distribution of myosin II. Nat. Cell Biol. 15, 1294–1306 (2013).
16. Herszberg, S., Latif, M., Boxeld, F., Martin, C. & Belläiche, Y. Interplay between the dividing cell and its neighbors regulates adherens junction formation during cytokinesis in epithelial tissue. Dev. Cell 24, 256–270 (2013).
17. Moreau-de-Sâ, E. & Sunkel, C. Adherens junctions determine the apical position of the midbody during follicular epithelial cell division. EMBO Rep. 14, 696–703 (2013).
18. Tanentzapf, G., Smith, J. & Tepass, U. Apical, lateral, and basal polarization cues contribute to the development of the follicular epithelium during Drosophila oogenesis. J. Cell Biol. 151, 891–904 (2000).
19. Bieber, A. J. et al. Drosophila neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebral neural adhesion molecule L1. Cell 59, 447–460 (1989).
20. Grenningloh, G., Rehm, E. J. & Goodman, C. S. Genetic analysis of growth cone guidance in Drosophila: fasciclin II functions as a neuronal recognition molecule. Cell 72, 45–57 (1993).
21. Szafranski, P. & Goode, S. A Fasciclin 2 morphogenetic switch organizes epithelial cell cluster polarity and motility. Development 131, 2023–2036 (2004).
22. Wei, J., Hortsch, M. & Goode, S. Neuroglian stabilizes epithelial structure during Drosophila oogenesis. Dev. Dyn. 230, 800–808 (2004).
23. Szafranski, P. & Goode, S. Basal regulation is sufficient to suppress epithelial invasion during Drosophila oogenesis. Dev. Dyn. 236, 364–373 (2007).
24. Bilder, D. Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. Genes Dev. 18, 1909–1929 (2004).
25. Lecuit, T. & Lenne, P.-F. Cell surface mechanics and the control of cell shape, tissue patterns and morphogenesis. Nat. Rev. Mol. Cell Biol. 8, 633–644 (2007).
26. Genova, J. L. & Fehon, R. G. Neuroglian, Gliotactin, and the Na+/K+ ATPase are essential for sequestration function in Drosophila. J. Cell Biol. 161, 979–989 (2003).
METHODS

Mutant alleles. The following Drosophila melanogaster mutant alleles have been described previously and can be found on FlyBase.org: armadillo, mud, and mud'. Fas2 was obtained from the Bloomington Drosophila Stock Center. w1118 was used as a control line.

Fluorescent marker stocks. Neuroglian::YFP, Zipper::YFP and Basigin::YFP are Cambridge Protein Trap Insertion lines. Jupiter::Cherry was derived by N. Lowe from the Jupiter::YFP CPT1 line using a P-element exchange. Bazooka::GFP was generated by the Carnegie Protein Trap Project. The following markers have been described previously: Viking-GFP (ref. 27), Arm-GFP (ref. 28), pUbq-α-tub-RFP (ref. 29), Sdq-mCherry (ref. 30), and Histone 28-RFP (ref. 31).

GAL4 drivers. Ectopic protein expression was accomplished using the UAS–GAL4 system. Expression in follicle cells was induced by any of the following drivers: T155–GAL4, Gr1–GAL4, Traffic Jam–GAL4 or actin-5c–FPlout–GAL4 (inducible by FRT/FLP-mediated removal of a stop codon). Expression in the larval optic lobe was driven using c855a–GAL4. Expression in the embryo was driven with mas-t4–GAL4:VP16.

UAS lines. The UAS–Neuroglian-shRNA line TRIPHM301638 was generated by the Transgenic RNAi Project Research. UAS–Inscutable and UAS–p35 (ref. 32) have been described previously.

Reagents. The following antibodies were used in this study: mouse anti-Armadillo (N2 7A1), anti-Fas2 (1D4), and anti-Discs large (4F3). Developmental Hybridoma Bank, rabbit anti-aPKCs (sc2166) (Santa Cruz Biotechnology), mouse anti-α-tubulin (F2168) (Sigma), rabbit anti-cleaved Caspase-3 (8G10) No. 9665 (Cell Signaling), rabbit anti-Inscutable and rabbit anti-Par6 (ref. 33; gifts from J. Knoblich, Institute of Molecular Biotechnology, Austria), rabbit anti-Scribble and rat anti-Deadpan (gifts from C. Q. Doe, HHMI and University of Oregon, USA), di-phospho Sqh (ref. 35; gift from R. Ward, University of Kansas, USA), rat anti-DE-cadherin (gift from H. Oda, JT Biohistory Research Hall, Japan), and rabbit anti-Bazooka (gift from A. Wodarz, University of Cologne, Germany). Rhodamine–Phalloidin was purchased from Invitrogen. Vectashield with DAPI was purchased from Vector Labs. Conjugated secondary antibodies were purchased from Jackson Immunoresearch. CellMask was purchased from Life Technologies.

Immunostaining. Ovaries were fixed for 10 min in 10% paraformaldehyde and 2% Tween in phosphate-buffered saline. Ovaries were then incubated in 10% bovine serum albumin in PBS to block for one hour at room temperature. Embryos were dechorionated with 50% bleach for 3–5 min, and then washed three times with water. Embryos were fixed for 20 min in 6% paraformaldehyde in PBS overlaid with heptane (1:1 fixative/heptane). To divitellinize the embryos, the lower phase was removed and replaced with methanol. After vigorously shaking the vial for 30 s, divitellinized embryos sank down from the interphase into the lower phase. These embryos were washed three times in PBS with 0.2% Tween. For both ovaries and embryos the primary and secondary immunostainings lasted at least 3 h in PBS with 0.2% Tween. Three washes (approximately 10 min each) in PBS–0.2% Tween were carried out between stainings and after the secondary staining. Primary antibodies were diluted 1:150. Secondary antibodies and phalloidin were diluted 1:1,000.

Imaging. Fixed-cell imaging was performed using an Olympus IX81 (40×/1.3 UPlan FLN Oil or 60×/1.35 UPlanSapio Oil). Live imaging was performed using a Leica SPS (63×/1.4 HCX PL Apo CS Oil) or Olympus IX81 (40×/1.3 UPlan FLN Oil or 60×/1.35 UPlanSapio Oil) inverted, used with or without a Yokogawa CSU22 spinning-disc imaging system. Ovaries were dissected and imaged in Schneider’s medium (Sigma) with 10 μg ml−1 insulin (Sigma) and 0.2% agarose or in 105 Voltatef Oil (VWR). Embryos were imaged in 105 Voltatef. Optic lobes were imaged in Schneider’s with insulin and agarose. Images were collected with Olympus Fluoview Ver 3.1, MetaMorph software, or Leica LAS AF and processed (Gaussian blur) using ImageJ. In Fig. 2c, each time point represents a Z-projection of three slices spaced 1 μm apart. In Fig. 2d, each time point represents a Z-projection of five slices spaced 2 μm apart.

Spindle angle measurements. Spindle angles were measured using ImageJ. The angle was measured between one line drawn connecting the apical corners of the mitotic cell and another line drawn along the spindle.

Statistical analyses. The Kolmogorov–Smirnov test was used to determine significance between metaphase spindle angles. An unpaired, two-tailed Student’s t-test with Welch’s correction was used to determine significance when comparing the number of misplaced cells in an egg chamber. No statistical method was used to pre-determine sample size, the experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

Box plots. Box plots were generated using BoxPlotR. The position of whiskers was determined by the Tukey method.

Misplaced cell counting. Ten stage 6 egg chambers from six flies were counted in each condition. The phenotype of Fas2 mutant egg chambers was measured in only those egg chambers that were at least 50% mutant.

Drosophila genetics. Follicle cell clones of pmo2, Fas2 and armadillo were induced by incubating larvae or pupae at 37° for two out of every twelve hours over a period of at least two days. Adult females were dissected at least two days after the last heat shock. In two independent armadillo mutant clones, we observed one cell apical to the layer. These cells had condensed nuclei, indicating that they were newly born and had yet to reintegrate.

Reproducibility of experiments. Images represent the number of independent experiments as follows: Fig. 1a (2) (b) (5), (c) (20), (e) (103), (f) (7); the images in Fig. 2a,b,d are representative of the 60 live divisions quantified in 2c; Fig. 3b (34), c (2), d (2 of 3); Fig. 4a (60), b (20), c (3), d (2), e (15); Fig. 5a (16), b (18), c (12), d (15), e (17), f (10), g (4); Supplementary Fig. 1A (83), B (7), C (6), D (4), F (23), G (12); Supplementary Fig. 2A (5), B (11), C (14); Supplementary Fig. 3A (2), D (3); Supplementary Fig. 4A (9), B (7), C (3), D (2); Supplementary Fig. 5A (5), B (8), C (9), D (5), E (5), F (6), G (4), H (6), I (5), J (3). Figure 3a, Supplementary Figs 3C and 1E are representative of previously published work and included in this manuscript for confirmation and comparison.

27. Morin, X., Daneman, R., Zavortink, M. & Chia, W. A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in Drosophila. Proc. Natl Acad. Sci. USA 98, 15050–15055 (2001).
28. McCartney, B. M. et al. Drosophila APC2 and Armadillo participate in tethering mitotic spindles to cortical actin. Nat. Cell Biol. 3, 933–938 (2001).
29. Basto, R. et al. Centrosome amplification can initiate tumorigenesis in flies. Cell 133, 1032–1042 (2008).
30. Martin, A. C., Kaschube, M. & Wieschaus, E. F. Pulsed contractions of an actin-myosin network drive apical constriction. Nature 457, 495–499 (2009).
31. Pandey, R., Heidmann, S. & Lehner, C. F. Epithelial re-organization and dynamics of progression through mitosis in Drosophila separase complex mutants. J. Cell Sci. 118, 733–742 (2005).
32. Zhou, L. et al. Cooperative functions of the reaper and head involution defective genes in the programmed cell death of Drosophila central nervous system midline cells. Proc. Natl Acad. Sci. USA 94, 5131–5136 (1997).
33. Petronczki, M. & Knoblich, J. A. DmPar-6 directs epithelial polarity and asymmetric cell division of neuroblasts in Drosophila. Nat. Cell Biol. 3, 43–49 (2001).
34. Albertson, R. & Doe, C. Q. Dig. Scrib and Lgl regulate neuroblast cell size and mitotic spindle asymmetry. Nat. Cell Biol. 5, 166–170 (2003).
35. Lee, C. Y., Robinson, K. J. & Doe, C. Q. Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. Nature 439, 594–598 (2006).
36. Zhang, L. & Ward, R. E. Distinct tissue distributions and subcellular localizations of differently phosphorylated forms of the myosin regulatory light chain in Drosophila. Gene Expr. Patterns 11, 93–104 (2011).
37. Oda, H., Uemura, T., Hara, Y. Iwai, Y. & Takeichi, M. A Drosophila homolog of cadherin associated with armadillo and essential for embryonic cell-cell adhesion. Dev. Biol. 165, 716–726 (1994).
38. Wodarz, A., Ramnath, A., Kuchinke, U. & Krust, E. Bazooka provides an apical cue for Insactivated localization in Drosophila neuroblasts. Nature 402, 544–547 (1999).
39. Besse, F. & Prét, A.-M. Apoptosis-mediated cell death within the ovarian polar cell lineage of Drosophila melanogaster. Development 130, 1017–1027 (2003).
Supplementary Figure 1  Neither mutation of Pins nor overexpression of Inscuteable cause disorganization of the follicle epithelium. A) Loss of Pins function does not affect the organization of the follicle cell monolayer. *pins<sup>p62</sup>* mutant clones are marked by the absence of GFP. This is one of 83 ovarioles imaged. B) Inscuteable expression promotes reorientation of cell division. The circled division is in early telophase, with the two daughters connected by a thick, central midbody. This is one of seven completely reoriented divisions imaged. C) UAS-GFP is a reliable marker for Inscuteable expression. For the experiment in Fig. 1E we used the FLPout system to express UAS-Inscuteable and UAS-GFP in large clonal populations. The sample in Fig. 1E was also stained for Inscuteable, which is shown here in red. GFP is not always a reliable marker in the follicle epithelium as it can leak between sister follicle cells through somatic ring canals. However, immunoreactivity with the anti-Inscuteable antibodies overlaps substantially with the expression of UAS-GFP. This result was confirmed in six ovarioles imaged. This result show that UAS-GFP is a reliable marker for Inscuteable expression in Fig. 1F and Supplementary Video 1. D) Inscuteable expressing follicle cells do not express the neuroblast marker Deadpan. Traffic-Jam Gal4 was used to drive both UAS-Inscuteable and UAS-myr.RFP in the follicle epithelium. The Deadpan antisera gives a non-specific background signal (D') that is not due to Deadpan protein as it is extends into the germline, is not nuclear, and does not correlate with myr.RFP intensity (D). E) Neither product of a misoriented division is apoptotic. This is one image representative of 13. A supernumerary polar cell provides a positive control for caspase-3 immunoreactivity. This was shown previously<sup>27</sup>. F) Co-expression of p35 with UAS-Inscuteable does not cause tumor formation. This image is representative of 23 Stage 4-6 egg chambers. G) Expression of p35 in *pins<sup>p62</sup>* mutant clones (cells lacking GFP) does not cause tumor formation. This image is representative of 12 clones < 5 cells in Stage 4-6 egg chambers. Scale bars in this figure represent 10μM.
**Supplementary Figure 2** Follicle cells move relative to the layer during division. **A** An early stage egg chamber, imaged live, has an uneven appearance. The plasma membrane is stained with Cell Mask. This uneveness was consistently observed in over two hundred live imaging experiments using a variety of markers. This particular staining (Cell Mask alone) was performed five times. **B** Mitotic cell rounding is accompanied by an increase in di-phosphorylated (active) myosin regulatory light chain (Spaghetti Squash). This is one image representative of 11. **C** Detachment of a new daughter cell from the basement membrane (in box) is confirmed by three dimensional imaging. The lateral marker Discs large extends fully around the basal cortex of the daughter cell. Images are 20 planes spaced .5 μm apart, collapsed to show the full diameter of the cell in all dimensions. This is one image representative of 14. Scale bars in this figure represent 10μM.
Supplementary Figure 3  Reintegration occurs in the embryo and optic lobe. A) Reintegration in the neuroepithelium of the optic lobe. These data are also presented in Fig. 3C with false coloring to indicate division products. This is one of two complete reintegrations imaged. B) Quantification of Domain 11 spindle angles in wild type (n = 33 cells) or embryos expressing Inscuteable (n = 34 cells). The distribution of angles differs significantly, with a p value of 1.618x10^{-12} as determined by the Kolmogorov-Smirnov test. Spindle angles are presented as a cumulative data plot. C) A transverse image of an embryo at approximately Stage 8 of development shows exogenously expressed Inscuteable (in red) localized apically. A dividing epithelial cell at telophase (within the white outline) is oriented along the apical-basal axis. This is representative of previous work published by Kraut et al. D) The basal product of a misoriented division in the early embryo reintegrates apically into the layer. The cell is followed in both XY and YZ planes. The dashed grey line in the first image of the YZ plane (top left) indicates the plane of focus in the XY images below. The white arrows in the YZ planes (top) point to the basal daughter cell. The arrow in the XY plane at 3' points to the first appearance of the reintegrating cell in the plane of focus. This “hole” in the layer does not become obvious again until 6', when the nucleus of the reintegrating cell moves into the focal plane. Scale bars in this figure represent 10μM.
**Supplementary Figure 4** Cell reintegration does not depend on myosin or adherens junctions.  

**A)** Zp::YFP does not show localized cortical enrichment apically or basally in a dividing cell detached from the basement membrane. The arrow points to enrichment at the contractile ring. The egg chamber was imaged live. This is one of nine divisions imaged with these markers.  

**B)** A reintegrating cell can demonstrate expansion at its apical cortex. The egg chamber was imaged live using Basigin::YFP to mark cell outlines. This is one of seven such expansions observed.  

**C)** The apical daughter cell of an Inscuteable-induced perpendicular division inherits the adherens junction belt (large arrows). A new adherens junction is also made between sister cells (small arrow). Gal4 activity marked with UAS-myristoylated.RFP. This is one image representative of three.  

**D)** Live imaging shows that an arm³ mutant cell can reintegrate. Mutant cells are marked by the absence of GFP. Cell outlines were marked with CellMask. This is one of two complete reintegrations imaged. Scale bars in this figure represent 10μM.
Supplementary Figure 5 Neuroglian and Fas2 are required for reintegation but not polarity. **A)** Neuroglian is expressed at lateral cell-cell contacting surfaces in the embryonic ectoderm at approximately Stage 8 of development. A’ is a Z-reconstruction of images taken 0.5μM apart. This is one image representative of 5. **B)** Neuroglian is expressed along the lateral cortex of cells in the developing neuroepithelium. This is one image representative of eight. **C)** FasII G0336 clones are Fas II protein null as measured by antibody staining. This is one image representative of nine. **D and E)** Epithelial cell polarity is unaffected by the FasII mutation (D) or Nrg-RNAi (E), as revealed by staining for the polarity markers aPKC (apical - red) and Discs large (lateral - green). Wild type cells in (D) are marked by the absence of RFP (in gray). These images represent one of seven (D) or five (E). **F-I)** Loss of Nrg does not alter that expression of factors that influence adhesion at the level of adherens junctions. Actin-Gal4 drives clonal expression of GFP and Nrg shRNAi. Egg chambers were stained for **F)** DE-Cadherin, **G)** Armadillo, **H)** Par-6, and **I)** Bazooka. These images represent one of six (F), four (G), six (H), and five (I). **J)** A series of still images showing the failure of a cell expressing both Nrg-shRNA and Inscuteable to reintegrate in follicle epithelium. The cell was observed for 80 minutes total. Cell outlines were marked with CellMask. This is one of three such failed reintegrations imaged. Scale bars in this figure represent 10μM.
Supplementary Video 1 Reintegration of a follicle cell after a misoriented division. GFP marks cells expressing Inscuteable. Tubulin-RFP marks the mitotic spindle. Frames taken one minute apart. The frame rate is seven per second. Each frame is a merge of 4 planes spaced 1μM apart. The scale bar represents 10μM.

Supplementary Video 2 Reintegration in wild type follicle epithelium. Cells marked with Basigin::YFP to reveal cell outlines and Jupiter-Cherry to reveal the mitotic spindle. Each frame is a merge of four planes spaced 1μM apart. Frames taken one minute apart. The frame rate is seven per second. The scale bar represents 10μM.

Supplementary Video 3 Reintegration in the neuroepithelium of the optic lobe. Inscuteable expression was induced to misorient spindles and divisions. Cell outlines were marked with Basigin::YFP. Each frame represents a Z-projection of three slices spaced one μm apart. The apical division product is marked with a red asterisk. After reaching the bottom of the tissue the cell again moves up and divides. Frames are two minutes apart. The frame rate is five per second. The scale bar represents 10μM.

Supplementary Video 4 A wild type follicle cell expands its apical surface as it reintegrates into the monolayer. The other division product is not visible in this plane of focus. Cell outlines marked with CellMask. Frames taken one minute apart. The frame rate is seven per second. The scale bar represents 10μM.

Supplementary Video 5 Myosin Regulatory Light Chain is not asymmetrically localized in a wild type reintegrating follicle cell. Myosin is labelled with Sqh-RFP. Cell outlines are marked with Basigin-YFP. Frames taken one minute apart. The frame rate is seven per second. The scale bar represents 10μM.

Supplementary Video 6 A transient adherens junction is made by the basal daughter cell of a misoriented division. UAS-Inscuteable expression was driven with Traffic-Jam Gal4. Frames were taken one minute apart. The frame rate is seven per second. Note that the apical daughter cell moves backwards out of the plane of focus as it reintegrates. The scale bar represents 10μM.

Supplementary Video 7 Nrg localizes along the cortex during reintegration. Nrg-YFP remains localized along the cortex during reintegration. Frames taken one minute apart. The frame rate is seven per second. This reintegration takes place in wild type tissue. The scale bar represents 10μM.

Supplementary Video 8 A Fas2G0336 mutant follicle cell expressing Inscuteable fails to reintegrate. Mutant cells are marked by the absence of RFP in green. Cell outlines marked with CellMask. UAS-Inscuteable expression was driven with Traffic-Jam Gal4. Frames taken one minute apart. The frame rate is seven per second. The scale bar represents 10μM.