A spindle-independent cleavage pathway controls germ cell formation in Drosophila

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The primordial germ cells (PGCs) are the first cells to form during Drosophila melanogaster embryogenesis. Whereas the process of somatic cell formation has been studied in detail, the mechanics of PGC formation are poorly understood. Here, using four-dimensional multi-photon imaging combined with genetic and pharmacological manipulations, we find that PGC formation requires an anaphase spindle-independent cleavage pathway. In addition to using core regulators of cleavage, including the small GTPase RhoA (Drosophila rho1) and the Rho-associated kinase, ROCK (Drosophila drok), we show that this pathway requires Germ cell-less (GCL), a conserved BTB-domain protein not previously implicated in cleavage mechanics. This alternative form of cell formation suggests that organisms have evolved multiple molecular strategies for regulating the cytoskeleton during cleavage.

Although insects of the order Diptera initially develop as large multinucleated cells, extensive cytoskeletal remodelling transforms the syncytial cell into a multicellular embryo by the start of gastrulation. This process of cellularization has been best studied in Drosophila melanogaster1–3. Intriguingly, somatic cells and PGCs require distinct genetic programs to cellularize. For example, somatic cell formation requires several zygotically transcribed gene products4–12. Together, these factors contribute to an essential feature of somatic cellularization—the synchronous progression of newly synthesized membrane between, and then around, individual nuclei4. In contrast, PGC formation is strictly controlled by maternal gene products collectively known as the germ plasm13,14. As PGC formation has not been described in detail, the contribution of individual germ plasm components to this process is not understood and the defining feature(s) of this mode of cell formation remain unknown.

Several lines of evidence suggest that PGC formation may be similar to the process of animal cytokinesis15. For example, PGC formation, like cytokinesis, proceeds during mitosis and requires the contractile ring components, Anillin and Diaphanous16–18. However, the strict dependence on germ plasm components for PGC formation suggests that cytokinesis alone is not sufficient to account for all aspects of this process. In particular, mutations in the germ plasm component, gcl, disrupt PGC formation but do not impact cytokinesis in other tissues19.

To determine the mechanism of PGC formation, we began our studies by analysing the events leading to PGC formation. When nuclei reach the embryonic cortex at the tenth nuclear cycle, they induce membrane and cytoplasmic protrusions, called buds4,20 (Supplementary Fig. S1a). Although most of these buds collapse shortly after their nuclei enter mitosis, the small fraction of buds that form within the germ plasm reorganize into PGCs. To capture the transformation of buds into cells, we developed a four-dimensional (4D) imaging assay (Fig. 1a) that revealed and quantified the localization of green fluorescent protein (GFP), fused to either of two known cleavage furrow components, Myosin-II regulatory light chain–GFP (MRLC, Drosophila sah; now called Myosin–GFP) and Anillin–GFP (Drosophila scraps), along with a kusabira-orange-fused germ plasm marker, Vasa–KO (refs 21–23; Fig. 1b,c). We found that both Anillin–GFP and Myosin–GFP were enriched at the neck of posterior buds (hereafter termed the bud furrow, BF; Fig. 1d,e and Supplementary Fig. S1e). When nuclei within these buds entered mitosis, the BF constricted beneath the chromosomes, in a plane parallel to the mitotic spindle. During anaphase, we found that a second cleavage furrow (hereafter termed the anaphase furrow, AF) assembled orthogonally to both the mitotic spindle and BF (Fig. 1b,c and Supplementary Fig. S1b,f and Videos S1 and S2). Although the AF ingressed asymmetrically, it divided the bud into two daughter cells in a manner similar to a cytokinetic furrow (Fig. 1c and Supplementary Fig. S1f). In contrast, BF cleavage separated the bud from the embryo, asymmetrically partitioning the germ plasm, marked by Vasa–KO, into the PGCs (Fig. 1c and Supplementary Videos S2). Following their constriction, these paired furrows (AF–BF) resolved into a tripartite midbody-like structure that attached the newly formed cells to the...
Figure 1 Anillin–GFP and Myosin–GFP localize to paired cleavage furrows during Drosophila PGC formation. (a) Diagram of 4D imaging strategy used to capture PGC formation at the posterior of the Drosophila embryo. The embryo is positioned with its dorsal surface closest to the coverslip. Forty Z slices (red line) spaced 1 μm apart along the dorsal–ventral axis were acquired per time point at the apex of the posterior pole. A, anterior; P, posterior; D, dorsal; V, ventral. (b) Micrographs of time-lapse maximum-intensity projections of paired furrows during PGC formation revealed with Anillin–GFP (see Supplementary Video S1). (c) Time-lapse micrographs (single optical sections) of a single bud during PGC formation revealed by Myosin–GFP and Vasa–KO (see Supplementary Video S2). (b,c) Arrows and arrowheads mark the anaphase furrow (AF) and bud furrow (BF) respectively. (d,e) Kymographs showing the localization and quantification of Anillin–GFP (d) and Myosin–GFP (e) at a single paired furrow during PGC formation. Biphasic enrichment of Myosin–GFP at the BF was observed consistently, suggesting regulation by the cell cycle as previously reported \(^{11}\). Data represent the mean of quantifications carried out in 4 embryos, with 3 buds measured in each embryo. Error bars: s.d. (f) Schematic depiction of PGC formation showing the remodelling of one bud into two cells. Scale bars, 5 μm.

embryonic cortex (Supplementary Fig. S1c,d). We conclude that the constriction of two orthogonally paired cleavage furrows remodels one bud into two PGCs (Fig. 1f).

The small GTPase RhoA (Drosophila Rhol) is a major regulator of cellular contractility and functions upstream of Anillin and Diaphanous during cytokinesis \(^{24,25}\). To determine whether PGC formation requires RhoA activity, we injected the RhoA inhibitor, C3 peptide \(^{26,27}\), into embryos shortly after bud formation. We found that injection of the C3 peptide, but not vehicle, blocked PGC formation (number of embryos with PGCs, vehicle-injected D15/15, C3-injected D0/12; Fig. 2a).

In Drosophila S2 cells, RhoA targets Anillin to the cleavage furrow during cytokinesis \(^{25}\). Therefore, we investigated whether targeting of Anillin–GFP to the BF was dependent on RhoA activity. Using our live imaging assay, we monitored Anillin–GFP at the BF following RhoA inhibition. In contrast to vehicle controls, C3-peptide-injected embryos exhibited a 2.5-fold reduction in the Anillin–GFP level at the BF shortly after injection (Fig. 2b and Supplementary Videos S3 and S4). These data demonstrate that PGC formation requires RhoA and suggest that a common RhoA signalling cascade regulates Anillin localization during both PGC formation and cytokinesis.
Figure 2 A spindle-independent cleavage pathway directs BF cleavage. (a) Micrographs of vehicle (dimethylsulphoxide; DMSO)- and C3-peptide-injected embryos. Arrows mark the PGCs in vehicle-injected embryos. Total number of embryos injected and scored: vehicle-injected = 15, C3-peptide-injected = 12. (b) Time-lapse micrographs (top) and quantification (below) of Anillin–GFP at the BF in vehicle- and C3-peptide-injected embryos (see Supplementary Videos S3 and S4). Arrowheads mark the BF. Quantification shows the mean of 4 embryos, with 3 buds measured in each embryo. Error bars: s.d. (c) Micrographs of vehicle- and Y27632-injected embryos. Arrows mark the PGCs in vehicle-injected embryos. Total number of embryos injected and scored: vehicle-injected = 21, Y27632-injected = 26. (d) Maximum-intensity projection micrographs of single PGCs from vehicle- and colcemid-injected embryos. Total number of embryos injected and scored: vehicle-injected = 10, colcemid-injected = 18. (e) Time-lapse micrographs (single optical sections) of a bud from a colcemid-injected Anillin–GFP-expressing embryo showing complete constriction of BF (see Supplementary Videos S5). Arrowhead marks the BF. Note the absence of the AF in the time series. Total number of embryos observed is 4. Scale bars, 5 μm.

A major target of RhoA signalling during cytokinesis is the serine/threonine kinase, Rho-associated protein kinase (ROCK, *Drosophila drok*). In *Drosophila*, ROCK promotes constriction of the cleavage furrow by phosphorylating serine and threonine residues in MRLC (refs 28,29). To determine whether ROCK acts downstream of RhoA during PGC formation, we injected embryos with a ROCK inhibitor, Y-27632, shortly after bud formation30,31. We found that injection of Y-27632, but not vehicle, blocked PGC formation in most embryos assayed (number of embryos with PGCs, vehicle-injected = 21/21, Y-27632-injected = 4/26; Fig. 2c). Thus, we conclude that ROCK acts downstream of RhoA during PGC formation. As MRLC is the main target of ROCK in *Drosophila*, these data suggest that myosin II activity is probably essential for both AF and BF cleavage.

During cytokinesis, the anaphase spindle signals to RhoA at the cell cortex to direct assembly and ingestion of the cleavage furrow32,33. By coupling furrow assembly to the anaphase spindle, this mechanism ensures cleavage only after sister chromatid separation has begun. As PGC formation, like cytokinesis, occurs during mitosis, we reasoned that signalling from the anaphase spindle may activate RhoA to regulate paired furrow cleavage. To determine whether the anaphase spindle controls paired furrow assembly and/or constriction, we inhibited spindle assembly by injecting embryos with colcemid, which depolymerizes microtubules and arrests mitotic nuclei in metaphase. If paired furrow activity required anaphase spindle assembly, we reasoned that colcemid injection would prevent PGC formation. Surprisingly, we found that embryos injected with colcemid, but not vehicle, formed large, metaphase-arrested PGC-like cells (Fig. 2d; number of
Figure 3 Germ cell-less is required for BF constriction. (a) Time-lapse micrographs (single optical sections) of a single WT and gcl bud during PGC formation revealed by Anillin-GFP. Red arrow marks the AF; red dot marks the BF. Note that BF constriction fails in the gcl mutant buds although the AF forms and constricts. (b) Maximum-intensity projection micrographs and quantification of the BF diameter shortly after AF formation in WT and gcl buds. Red arrows mark the AF and red dots mark the BF. Each paired furrow is rotated around the x axis by 40° and 80° to better reveal the BF. BF diameter was measured at t = AF formation in WT and gcl mutants. The total number of embryos filmed: WT = 4 and gcl = 7, where 1–3 BF diameters were measured per embryo. Total number of buds measured: WT = 9 and gcl = 20. The black bar represents the mean BF diameter. ** represents a two-tailed t-test with P < 0.001. Scale bars, 5 μm.

embryos with large, metaphase-arrested PGCs, vehicle-injected = 0/10, colcemid-injected = 16/18). To further investigate these findings, we injected embryos with colcemid and captured paired furrow behaviour live using our 4D imaging assay. Using Anillin–GFP to mark the furrows, we found that colcemid treatment blocked AF assembly in posterior buds, suggesting that the anaphase spindle may instruct AF assembly and constriction (Fig. 2e and Supplementary Fig. S2 and Video S5). In contrast, BF constriction proceeded in colcemid-injected embryos, resulting in the formation of the large PGC-like cells (Fig. 2e and Supplementary Fig. S2 and Video S5). Therefore, we conclude that BF cleavage is regulated in an anaphase spindle-independent manner. Moreover, these data illustrate that at least two distinct signalling pathways govern paired furrow activity.

Although syncytial nuclei reach the cortex of the embryo synchronously and form buds, only those nuclei surrounded by germ plasm become PGCs. Thus, we reasoned that components of the germ plasm probably played an instructive role in this mechanism of cellularization. Among known gene products localized to the germ plasm, gcl is an attractive candidate, because embryos that lack maternally deposited gcl (hereafter referred to as gcl mutant embryos) show specific defects in PGC formation\textsuperscript{19,34}. The exact role of GCL in this process, however, is unknown. We therefore analysed AF and BF cleavage in GCL mutants using our live imaging assay. Mutant and control embryos exhibited an enrichment of Anillin–GFP at the BF, suggesting that Anillin is targeted to the BF independently of GCL (Fig. 3a). However, despite AF assembly and cleavage, we found that BF cleavage failed in mutant embryos, preventing PGC formation (Fig. 3a and Supplementary Video S6). We quantified the BF diameter shortly after AF assembly in both control and mutant embryos and determined that mutants exhibited a threefold greater BF diameter (Fig. 3b). We conclude that BF, but not AF, cleavage requires GCL and thus identify GCL as the first unique regulator of spindle-independent cleavage.

GCL is a BTB domain-containing protein that resides in the germ plasm and becomes enriched at the nuclear membrane of posterior buds before PGC formation\textsuperscript{35}. Previous work suggested that GCL represses transcription during PGC formation\textsuperscript{34}. Our results suggest that GCL may transcriptionally repress one or more negative regulators of BF cleavage. We tested this model by globally inhibiting Pol-II-dependent transcription, shortly after fertilization, by injecting α-amanitin and then assaying for PGC formation in control and gcl mutant embryos. We found that α-amanitin had no effect on PGC formation in control embryos (n = 15/15 embryos with >15 pole cells), confirming that PGCs form in a transcription-independent manner as reported previously\textsuperscript{36}. Surprisingly, PGC formation was not rescued in
α-amanitin-injected gcl embryos (n = 0/27 embryos with >15 pole cells; Supplementary Fig. S3). In these experiments, somatic cellularization was inhibited in all injected embryos, indicating that our injection assay efficiently inhibited Pol-II-dependent transcription3 (Supplementary Fig. S3). Thus, we conclude that GCL does not inhibit Pol-II-dependent transcription during PGC formation.

We next considered whether GCL counteracts the forces generated by the anaphase spindle during sister chromatid separation3. Our fixed tissue and live observations show that constriction of the BF coincides with the segregation of chromosomes towards opposing spindle poles (Supplementary Fig. S1f). Therefore, GCL may function to stabilize the BF during this phase of mitosis. We reasoned that inhibiting assembly of the anaphase spindle might rescue BF cleavage in the gcl mutants. However, we found that gcl mutants injected with colcemid, unlike controls, failed to form PGC-like cells (number of embryos with PGC-like cells = 0/11; Supplementary Fig. S4 and Videos S7 and S8). Therefore, we conclude that GCL does not act to stabilize the BF during PGC formation.

As GCL overexpression within the germ plasm directs additional posterior buds to undergo PGC formation16,19, we considered two final models, in which GCL acts as either a permissive or instructive signal for BF cleavage. To distinguish between these two models, we filmed BF cleavage in control, gcl mutant and GCL-overexpressing (ep-gcl) embryos using our 4D imaging assay (Supplementary Videos S9, S10 and S11). We measured the BF diameter at several time points and calculated the rate of furrow constriction (Fig. 4a,b). If GCL acts as a permissive cue, we would expect equal rates of constriction in control and GCL-overexpressing embryos. However, we found that the rate of constriction was proportional to the amount of GCL present within the germ plasm (Fig. 4c). Indeed, even in control embryos, where the highest concentration of GCL protein is found in the most posterior buds, we found a direct correlation between the position of the bud and the degree of BF constriction (Supplementary Fig. S5). Thus, we conclude that GCL activity instructs BF cleavage by regulating the rate of furrow constriction.

Although increased GCL levels in the germ plasm result in supernumerary PGCs, mis-expression of GCL alone is not sufficient to cause ectopic cell formation16. However, ectopic expression of GCL at the anterior pole (gcl–bcd) causes defects in somatic celluarization16. To determine how GCL activity disrupts somatic cell formation, we immunostained control and GCL mis-expressing embryos using an antibody against a somatic contractile ring component, Anillin. Whereas constriction of the somatic contractile ring normally occurs only after membranes have surrounded the nuclei, we found that this Anillin-stained structure prematurely contracted in gcl–bcd embryos (Fig. 5a). Premature constriction of the contractile ring displaced somatic nuclei from the embryonic cortex and caused disruption of somatic cell organization. Thus, we conclude that GCL is sufficient to promote constriction of the contractile ring independently of other germ plasm components. As GCL can influence the constriction behaviour of both the BF and somatic contractile ring, we suggest that GCL activity directly or indirectly regulates a core contractile component during cleavage.

As GCL and Anillin are both required for PGC formation16,19 and because GCL is not required for Anillin localization to the BF (Fig. 3a), we reasoned that they may collaborate to promote BF cleavage. To test this model, we mis-expressed GCL together with Anillin–GFP and scored ectopic cell formation at the anterior of the embryo. Although neither protein alone was sufficient to direct BF cleavage of anterior buds, we found that embryos mis-expressing both GCL and Anillin–GFP induced some PGC-like cells in several embryos (number

**Figure 4** Germ cell-less is a rate-limiting component of BF constriction. (a) Time-lapse micrographs (single optical sections) showing the BF diameter revealed with Anillin–GFP in WT, gcl mutant and gcl-overexpressing embryos (ep-gcl) between t = −322 s and t = −196 s before AF formation (see Supplementary Videos S6, S7 and S8). (b) Quantification of the BF diameter between t = −322 s and t = −196 s in WT, gcl mutant and ep-gcl embryos. (c) BF rate of constriction between t = −322 s and t = −196 s in WT, gcl mutant and ep-gcl buds calculated from the slopes of the lines shown in **b.** Total number of embryos analysed for **b,c:** WT = 7, gcl = 6, ep-gcl = 8, where 1 or 2 buds were measured in each embryo. One-way analysis of variance: P < 0.05 for WT versus gcl and P < 0.001 for WT versus ep-gcl.
Figure 5 Mis-expression of Germ cell-less with Anillin is sufficient for ectopic cell formation. (a) Surface micrographs of WT and GCL-mis-expressing embryos (gcl–bcd) during somatic cellularization. Note that mis-expression disrupts somatic cell formation by inducing the premature constriction of the cellularization furrow. Insets are representative micrographs depicting lateral views of the cellularization furrow in WT and GCL-mis-expressing embryos. (b) Micrographs of the anterior pole of Oskar mis-expressing (osk–bcd), gcl–bcd and gcl–bcd, anillin–GFP embryos (Vasa, germ plasm marker. Note Oskar is sufficient to ectopically recruit germ plasm (Vasa) and instruct cell formation, whereas gcl–bcd, anillin–GFP embryos instruct cell formation but do not recruit germ plasm. Arrows mark ectopic cells in gcl–bcd, anillin–GFP embryos before somatic cell formation. (c) Micrographs of the anterior pole of embryos from females expressing gcl–bcd, anillin–GFP (green), GCL–Actin (red) and DNA (blue)). Arrows mark ectopic cells in gcl–bcd, anillin–GFP embryos at the start of somatic cell formation. Scale bars, 5 μm.

In summary, we identify an alternative cleavage pathway that acts during the process of PGC formation and suggest that similar mechanisms may be used more broadly in biology. For example, the Drosophila neuroblast uses both spindle-dependent and -independent pathways to instruct cleavage during development. Similarly to Drosophila PGCs and neuroblasts, polar lobe formation in gastropods also seems to require spindle-independent cleavage. In all three systems, the spindle-independent furrow partitions cytoplasmic determinants asymmetrically to instruct cell fates. One possibility is that these cleavage pathways initially co-evolved with mechanisms of cytoplasmic determination. In such a scenario, regulators of these alternative cleavage pathways, much like GCL, are likely to reside within the determinate-rich cytoplasm of the differentiating daughter cell.

GCL activity defines the only known molecular asymmetry between spindle-dependent and -independent cleavage pathways. Although mammalian GCL can act as a transcriptional regulator, our results reveal a transcription-independent role for Drosophila GCL during BF cleavage. Regardless of its precise molecular function, mammalian GCL has maintained an ability to instruct spindle-independent cleavage during the course of evolution as the mouse homologue can rescue PGC formation in Drosophila mutants. In mammals, GCL is required for spermatogenesis. GCL-mutant mice have defective sperm, with multinucleated heads and bent necks. Reduced human gel expression, along with defective sperm motility, has also been reported in azoospermic men with normal karyotypes and intact Y-chromosomes. Thus, GCL may have a conserved role in regulating cytoskeletal or contractile behaviour during germline development in diverse animals.

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.
ACKNOWLEDGEMENTS

We thank all members of the Lehmann laboratory for discussions and reagents. We thank A. Blum, S. Burden, T. Hurd, M. Sladina, F. Teixeira and A. Zamparini for critical reading of the manuscript. We also thank S. Burden, H. Knaut, J. Nance, G. Schübler, S. Small, J. Treisman and E. Wieschaus for discussions during the course of this work. We thank the Drosophila Bloomington Stock Center, T. Jongens, C. Fields, H. Sano, and A. Wilde for reagents. This work was supported by a NSF Predoctoral Fellowship to R.M.C. R.L. is a Howard Hughes Medical Institute Investigator. We dedicate this manuscript to the memory of G. Schubiger whose work in the early Drosophila embryo largely inspired the experiments presented here.

AUTHOR CONTRIBUTIONS

R.M.C and R.L. conceived the project. R.M.C carried out the experiments and analysed the data. R.M.C and R.L. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at www.nature.com/doidfinder/10.1038/ncb2761

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**METHODS**

**Fly strains and genetics.** w1118 was used as the wild-type strain. usa-dgrip75-gfp, usa-anillin-gfp (ref. 45), mkc-gfp (ref. 31) and usa-vasa-ko (ref. 46) were described previously. anillin-gfp was driven maternally by the nanos-gal4vp16 insertion on the third chromosome ref. 9. Dgel was used as the gel allele in all experiments. The insertion y w 23C11; P[EP152]gcl 23C11 was used in combination with the nanos-gal4vp16 insertion on the third chromosome to overexpress gcl maternally (ep-gcl). gcl-bcl (hbg1882) and osk-bcl were used to mis-express gcl and osk through the bcl 3'UTR at the anterior pole. 23, 25

**Embryo microinjection.** Embryos were injected through the anterior pole with chemical and peptide inhibitors as described previously. Briefly, embryos were collected on agar plates containing apple or grape juice and a drop of yeast paste. Embryos were then dechorinated in a freshly prepared bleach solution (50% Cloroxy/50% water) for 1.5 min with constant agitation. Embryos were then aligned on agar blocks with their dorsal surfaces up. A 20 mm  10 mm glass coverslip was then prepared by painting a thin line of heptane-solubilized glue along its long edge. This glue was then allowed to dry before gently touching the dorsal surface of the embryo with the glue-coated coverslip. Embryos were then dehydrated for 10–15 min depending on the desiccant used. Following desiccation, the embryos were covered with Halocarbon oil and placed on a microscope stage equipped with an injection rig. Needles for injection were pulled mechanically, backfilled with injection buffer and then broken by gently scraping the tip against the edge. This glue was then allowed to dry briefly before they were transferred to a glass slide, and covered with a coverslip.

**Histology.** To visualize Anillin, Vasa, GCL and Actin, embryos were fixed in formaldehyde-saturated heptane and immunostained with a rabbit anti-Anillin (a gift from C. Field, Harvard Medical School, Boston, USA; 1:1,000), rabbit anti-Vasa (Lehmann laboratory 1:5,000), rabbit anti-GCL (a gift from T. Jongens, University of Pennsylvania, Philadelphia, USA; 1:1,000) and Phalloidin (Molecular Probes, Invitrogen; R415) as previously described. Briefly, embryos were collected on agar plates containing apple or grape juice and a drop of yeast paste. Embryos were then dechorinated in a freshly prepared bleach solution (50% Cloroxy/50% water) for 1.5 min with constant agitation. Embryos were collected on a plastic mesh, washed with water (or 0.05% Triton/0.07% NaCl) and then dried on paper towels. To avoid axonic conditions, embryos were collected on meshes with enough surface area such that embryos were arranged as a sparsely populated monolayer. Once the embryos dried, they were added to a scintillation vial containing 5 ml of formaldehyde-saturated heptane (made by shaking 50/50 formaldehyde/heptane solution overnight, at room temperature, in the dark) for 40 min to 1 h with gentle agitation. Embryos were then removed using a glass Pasteur pipette and pipetted onto a plastic mesh sitting atop a stack of paper towels. Embryos were allowed to dry briefly before they were painted onto double-sided tape. The tape was then transferred to a Petri dish and embryos were covered with 0.1% Triton in PBS (PBST). Using a sharp 21-gauge needle, vitelline membranes were manually removed by gently scraping the sides of the embryo with the needle. Embryos were then washed in PBST 3 times on a Nutator before being stored at 4°C overnight to allow the detergent to fully penetrate the embryo. The next day, embryos were incubated with primary antibodies in PBST on a Nutator overnight at 4°C, washed in PBST on a Nutator for several hours, and then incubated with secondary antibodies in PBST on a Nutator overnight. Embryos were then washed in PBST on a Nutator for several hours, incubated with DAPI (1 µg ml–1) on a Nutator for 10 min, and then washed with PBS 2× before adding Vectashield. Embryos were allowed to equilibrate for several hours before they were transferred to a glass slide, and covered with a coverslip.

**Imaging.** All fixed tissue imaging was performed using a Zeiss 510 Meta confocal microscope, using 40 × 63 oil and glycerol objectives, respectively. Pinholes were set such that confocality was achieved for each image acquisition. All post-acquisition analysis was performed using ImageJ (NIH; http://rsb.info.nih.gov/ij/). Most micrographs are represented as maximum-intensity projections unless otherwise stated.

**Measurements and statistics.** All post-acquisition measurements were performed using ImageJ and the plugin multiple kymographs. To measure the diameter of the BF in Fig. 3, the BF was rotated 90° and then a line was drawn and measured as depicted in the figure. Slopes were calculated automatically by PRISM (www.graphpad.com) and excluded if the R2 value was less than 0.9. PRISM calculated several different statistics depending on the nature of the data. A two-tailed t-test was applied to data presented in Fig. 3b, and a one-way analysis of variance (P < 0.001) was used to analyse the data in Fig. 4c. These same data were subjected to a Bartlett’s test for equal variances (P < 0.2005) and (*) on the graph in Fig. 4c represent the P values generated from a Dunnett’s multiple comparison test.
Figure S1. Paired furrows remodel buds into PGCs. (a) Time-lapse micrographs (single optical sections) of a single bud during bud formation revealed by Myosin-GFP and Dgrip-GFP to mark the membrane and centrosomes, respectively. Note that the cortical Myosin-GFP becomes enriched at the ‘feet’ of the bud. Boxes indicate regions of image measured in accompanying histograms. Total number of embryos observed = 2. (b) Micrographs of maximum intensity projections (MIP) of a single paired furrow during PGC formation (Anillin (green), Actin (red) and DNA (blue)). The paired furrow is rotated around the X-axis by 20° intervals to better reveal the BF. (c) MIP of paired furrows during PGC formation (Anillin (green), Vasa (red) and DNA (blue)). The arrowhead denotes a lateral view of an AF, while the arrow highlights a top-down view of a BF. (d) MIP micrograph of the posterior pole of the embryo following PGC formation (Anillin (green), Actin (red) and DNA (blue)). Note Anillin-labelled structures appear like tripartite midbodies, connecting sister cells to each other and to the embryonic cortex. Scale bar = 5 μm. (e) Micrographs of average intensity projections (AIP) and quantification of Anillin-GFP and Myosin-GFP at single paired furrows shortly after AF formation. Note that there is 3-fold enrichment of both Anillin-GFP and Myosin-GFP at the BF compared to the AF. (f) Time-lapse micrographs (single optical sections) of a bud from a Myosin-GFP, Dgrip-GFP expressing embryo. Arrowhead marks the mitotic spindle, while the arrow denotes the AF. Scale bars = 5 μm.
Figure S2 A spindle-independent cleavage pathway directs bud furrow cleavage. Time-lapse micrographs (single optical sections) of a bud from a colcemid-injected Anillin-GFP expressing embryo. Note the absence of the AF in the time series. The arrowheads mark the BF in the time series. Total number of embryos observed = 4. Scale bar = 5 μm.
Figure S3 Inhibition of Pol II dependent transcription fails to rescue PGC formation in \( gcl \) mutants. Representative micrographs of \( wt \) and \( gcl \) embryos that were injected with \( \alpha \)-amanitin shortly after fertilization and then allowed to develop just prior to gastrulation before being fixed and stained with Vasa (green), Actin (red) and DNA (blue). Variability of PGC formation in \( gcl \) mutants is similar in untreated and \( \alpha \)-amanitin injected embryos.
Figure S4 Inhibition of spindle assembly fails to rescue BF cleavage in gcl mutants. Time-lapse micrographs (single optical sections) of colcemid-injected wt and gcl embryos. Note colcemid was injected shortly after bud formation. Arrowheads mark the BF. Total number of embryos observed: wt = 4, gcl = 3. Scale bar = 5 μm.
Figure S5 Gcl levels and degree of BF constriction correlate in wild-type embryos. (a) Micrograph of a MIP of buds prior to PGC formation (Gcl (green), Actin (red) and DNA (blue)). (b) Micrographs of AIP and quantification of Gcl and Actin in 4 buds. Note highest levels of Gcl are found in the 2 buds at the apex of the posterior pole where the germ plasm is most enriched. (c) Micrograph of a MIP of BFs during PGC formation (Anillin (green), Actin (red) and DNA (blue)). The diameter of the BF marked by Anillin is smallest at the apex of the posterior pole. Note that all buds are initially of similar diameter. Scale bars = 5 μm.
Supplementary Videos

Supplementary Video S1 PGC formation in an Anillin-GFP expressing embryo. This video shows 4-paired furrows forming and then constricting.

Supplementary Video S2 Paired furrow constriction in a Myosin-GFP and Vasa-KO embryo. This video shows a single paired furrow constricting. Myosin-GFP (green) and Vasa-KO (red).

Supplementary Video S3 Anillin-GFP expression at the BF following DMSO injection. DMSO injection does not disrupt the localization of Anillin at the BF.

Supplementary Video S4 Anillin-GFP expression at the BF following C3 peptide injection. C3 peptide injection does disrupt the localization of Anillin at the BF.

Supplementary Video S5 BF constriction following colcemid injection in an Anillin-GFP expressing embryo. While the BF constricts in this video, note that the AF does not form in this video.

Supplementary Video S6. PGC formation in a gcl mutant; Anillin-GFP expressing embryo. This video shows that the BF fails to constrict in gcl mutants.

Supplementary Video S7 BF constriction following colcemid injection in an Anillin-GFP expressing embryo. While the BF constricts in this video, note that the AF does not form in this video

Supplementary Video S8 BF constriction following colcemid injection in a gcl mutant; Anillin-GFP expressing embryo. Note that the BF fails to constrict in this video.

Supplementary Video S9 BF constriction following in an Anillin-GFP expressing embryo.

Supplementary Video S10 BF constriction in a gcl mutant; Anillin-GFP expressing embryo.

Supplementary Video S11 BF constriction following colcemid injection in a Gcl over-expressing, Anillin-GFP expressing embryo.