Mouse oocytes develop in cysts with the help of nurse cells

Graphical abstract

Highlights

- A comprehensive model of the nurse cell development in mice is presented
- Mouse nurse cells die by a programmed cell death pathway like Drosophila nurse cells
- Genetic changes associated with nurse cell activation are identified
- Centrosome transfer and consolidation generate a large microtubule aster

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In brief
By providing a detailed investigation of nurse cells involved in mammalian oocyte development, this manuscript identifies evolutionarily conserved mechanisms of oocyte fate determination and development and addressed the question of why so many germ cells are initially made but only a subset eventually become oocytes and make up the ovarian reserve.
Mouse oocytes develop in cysts with the help of nurse cells

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SUMMARY

Mouse germline cysts, on average, develop into six oocytes supported by 24 nurse cells that transfer cytoplasm and organelles to generate a Balbiani body. We showed that between E14.5 and P5, cysts periodically activate some nurse cells to begin cytoplasmic transfer, which causes them to shrink and turnover within 2 days. Nurse cells die by a programmed cell death (PCD) pathway involving acidification, similar to Drosophila nurse cells, and only infrequently by apoptosis. Prior to initiating transfer, nurse cells co-cluster by scRNA-seq with their pro-oocyte sisters, but during their final 2 days, they cluster separately. The genes promoting oocyte development and nurse cell PCD are upregulated, whereas the genes that repress transfer, such as Tex14, and oocyte factors, such as Nobox and Lhx8, are under-expressed. The transferred nurse cell centrosomes build a cytocentrum that establishes a large microtubule aster in the primordial oocyte that organizes the Balbiani body, defining the earliest oocyte polarity.

INTRODUCTION

Mouse female germ cells begin developing immediately after reaching the embryonic ovary, rapidly undergo key meiotic events, and form into ovarian follicles by just 5 days after birth (Edson et al., 2009; Richards and Pangas, 2010; Lin and Capel, 2015; Ikami et al., 2017; Wu and Dean, 2020). Most of prefolicular development takes place in germ cell groups known as cysts, structures that initiate both male and female gametogenesis prior to meiosis in diverse animals (King, 1970; Gondos, 1973; deCuevas et al., 1997; Pepling and Spradling, 1998; Matova and Cooley, 2001; Greenbaum et al., 2011; Haglund et al., 2011). Single primordial germ cell lineage-labeling experiments revealed the general characteristics of mouse female cysts, which start with an average of 30 cells interconnected by intercellular bridges (also known as ring canals) when division ceases at embryonic day E14.5 (Lei and Spradling, 2013). Over the next 10 days, the 24–25 nonoocyte cyst germ cells are sequentially activated for turnover, until by P5 only 6 single primordial oocytes remain (Pepling et al., 2007; Lei and Spradling, 2013). Figure 1A shows a model consistent with observed cyst parameters. The initial cyst of 30 cells quickly fragments on average into six smaller cysts of about five cells, suggesting that each oocyte develops from a cyst containing four nonoocytes (Figure 1B). However, many features of primordial oocyte production, including the exact starting structures of initial cysts, their fragmentation pattern, how pro-oocytes and nonoocytes are selected, how the genetic activity of these two cell types differs, and the ultimate function of cyst-based oocyte production remain imperfectly understood.

Previously, most mouse cyst cells that do not become oocytes were shown to be “nurse cells” since they transfer their cytoplasm and organelles in a nonsynchronized manner into the pro-oocytes, although their nuclei are excluded (Lei and Spradling, 2016; see Figure 1B). As predicted, if nearly all nonoocyte cyst cells are nurse cells, oocytes by P5 contained about 5 times as many centrosomes, Golgi material, and mitochondria and about 4 times as much cytoplasmic volume as E14.5 germ cells (Lei and Spradling, 2016). Nurse cell transfer correlates with the appearance only in the oocyte cytoplasm of a Balbiani body, a juxtanuclear aggregate of organelles that has been reported in newly formed follicles of many vertebrates and invertebrates (Hertig and Adams, 1967; Heasman et al., 1984; Cox and Spradling, 2003; Pepling et al., 2007; Elkouby and Mullins, 2017).

Initially, genetic studies of mice lacking the intercellular bridge (IB) protein Tex14 found that females lacked normal IBs but remain fertile (although less fecund), suggesting that cysts are not essential for oocyte production (Greenbaum et al., 2006, 2009). More detailed studies confirmed that Tex14 mutation disrupts normal IB formation but found that some female cysts form and polarize. Their cyst cells compensate for the defective bridges and transfer organelles from nurse cells to oocytes through broken membranes on an accelerated schedule, suggesting that Tex14 is a transfer inhibitor (Ikami et al., 2021).

Cysts play two roles in Drosophila and other animals, where cyst function has been extensively investigated using developmental genetic and comparative studies (Spradling, 1993;
First, cyst production establishes an asymmetric microtubule cytoskeletal polarity based on its formative divisions (Koch and King, 1966; Lin et al., 1994; de Cuevas and Spradling, 1998; Röper and Brown, 2004; Huynh and Johnston, 2004). This polarity underlies specification of distinct nurse cell and oocyte fates and blueprints the earliest polarity of the oocyte itself. Beginning around pachytene and continuing over several days, centrioles, mitochondria, Golgi, ER, cytoplasm, and specific mRNAs move through the cyst’s nurse cells until they reach the oocyte (Mahowald and Strassheim, 1970; Grieder et al., 2000; Bolivar et al., 2001). As a follicle forms, organelles enter the oocyte en masse and generate a Balbiani body, whose centrioles and some mRNAs relocate to the cortex opposite the entry site to define

**Figure 1. Germline cyst nurse cells shrink in size before turning over and fail to express key oocyte genes**

(A) A primordial germ cell (PGC) generates a 30-cell clone containing germline cysts interconnected by bridges (gray bars between cells). Due to nurse cell cytoplasmic transfer (indicated by darker gray shading) and cell turnover (presaged by cells with reduced size), six cysts eventually arise that each produce one oocyte by P5.

(B) A lineage-labeled cyst (blue) of four cells at E18.5, showing the pro-oocyte (arrowhead) enriched in GM130-marked Golgi elements (red) and AIF-labeled mitochondria (green), and three attached nurse cells, one (*) with few and one (**) with no remaining organelles (from [Lei and Spradling, 2016] with permission from the American Association for the Advancement of Science [AAAS]).

(C–F) Analysis of 134 lineage-marked cysts to document cyst properties at each age, and cyst cell size. (C) E16.5, E18.5, and P1 cysts lineage-labeled with YFP (green) and outlined in yellow dashes. Clonal cell(s) of reduced size (small cells, SC) are indicated by yellow arrowheads. (D) Mean clone size versus developmental age reveals the average number of cyst cells lost every 2 days. (E) Summary plots of cyst sizes (red) and small cells (SC, blue) at indicated ages. (F) Comparison of the percentage of cells lost in next 2 days, and percentage of small cells.

(G) Reduced expression of oocyte genes (green) in cyst nurse cells at indicated times. Cyst (yellow dashes), oocyte indicated by the presence of accumulated mitochondria (ATP5a, red).
the oocyte posterior (Mahowald and Strassheim, 1970; Grieder et al., 2000; Cox and Spradling, 2003). Near the end of oogenesis, cyst nurse cells dump their remaining cytoplasmic contents into the oocyte and undergo a distinctive pathway of programmed cell death (PCD) (Mondragon et al., 2019; reviewed by Lebo and McCall, 2021).

RESULTS

Nurse cell cytoplasmic transfer is associated with a reduction in cell size

Previous studies of mouse cyst development (Lei and Spradling, 2016) reported the presence within cysts of small germ cells containing few organelles (Figure 1B) but did not investigate them quantitatively. We considered that such small, organelle-poor cells likely arise as a result of ongoing nurse cell cytoplasmic transfer and that analyzing small germ cells might be useful in gaining greater insight into cyst breakdown. Consequently, we systematically studied germ cell clones lineage-labeled with YFP at E10.5 and analyzed at five subsequent developmental times. We measured the size of 134 cysts and counted how many small germ cells are recognizable by microscopy each contained (Figures 1C–1E, and S1F). The average clone size at each time point (Figure 1D) is shown in Figure 1F line 1. The difference between the average clone size every 2 days (Figure 1D) represents the number of germ cells that turn over during the next 2 days in the cohort of cysts derived from single E10.5 progenitors (Figure 1F; line 2). The number of small cells per cohort is shown in Figure 1F line 3, and nearly, all of these cells had reduced levels of Golgi and/or mitochondria (Figure S1A). This demonstrates that nurse cell turnover involves a terminal stage about 2 days in length during part of which cells become depleted of organelles and cytoplasm. In total, 61%–84% of these nurse cells that turn over during any given 2-day interval can be recognized microscopically as small cells, suggesting that shrinking nurse cells become recognizable small during much of this period.

We also scored the volume of these cysts and quantitated the Golgi and mitochondrial marker levels (Figures S1A–S1D). We measured the small size of the remnant cells compared with oocytes (Figure S1B) and validated by marker staining that mitochondrial and Golgi material per cell increases about 5-fold between E14.5 and P5 (Figures S1C and S1D). This increase is close to the ratio of nurse cells to oocytes in E14.5 cysts (30.5/6 = 5.1) and further supports previous studies that most or all nonoocyte cells in mouse cysts are nurse cells.

Finally, to further validate that the small cells seen in cysts are nurse cells, we immunostained ovaries throughout cyst development with markers for five well-known genes that are functionally important for oocyte development—Nobox, Lhx8, Nirp5, Filia, and Tie6 (Figure 1G). Nobox expression was specific for the pro-oocyte (indicated by the large accumulation of mitochondria), even at E18.5. Labeling was not observed or was extremely weak in both smaller and full-sized nurse cells present in the cyst. The same high level of specificity was observed for the other genes at P0 or P1. Full-time series at six points spanning cyst development are shown in Figures S1G–S1I for Nobox, NLRP5, and Filia. Taken together with previous studies, these experiments show that the great majority if not all cyst cells that fail to become oocytes serve as nurse cells and that nurse cells become smaller in size and differentially express genes relative to pro-oocytes during a majority of their last 2 days before turnover.

Mouse nurse cells undergo programmed cell death like Drosophila nurse cells

The turnover of mammalian germ cells during fetal and perinatal development has historically been described as apoptosis, but multiple pathways were identified more recently (reviewed in Tilly, 2001; Grive, 2020). However, nurse cells were recently shown to undergo a novel type of PCD in the Drosophila ovary that is dependent on adjacent somatic cells (Mondragon et al., 2019; reviewed in Lebo and McCall, 2021). A major indicator of this PCD pathway is nurse cell acidification that is driven by fusion with acidic vesicles generated within surrounding somatic follicle cells (Figure 2A). Subsequently, the follicle cells express and transfer to the nurse cells cathepsins and DNAases that digest the nurse cells. In contrast, apoptotic cell death (Figure 2B) can be detected using antibodies to cleaved caspase3, a downstream effector caspase. Moreover, in apoptotic cell death, but not in PCD, nuclear lamin B1 protein is degraded. Consequently, we set out to characterize mouse germ cell turn over using these markers of the two pathways.

Germline cysts in perinatal mouse ovaries break down normally in culture, which allowed us to observe the process after 12 h of culture in the presence of the acidophilic dye Lysotracker.
Many germ cells whose small nuclei identified them as nurse cells became Lysotracker positive from E16.5–P1 (Figure 2C). The small nurse cells undergoing acidification are surrounded by Lgr5-positive cells, a marker for pregranulosa cells (Figure 2D). In Drosophila, somatic cell V-ATPases generate a pH gradient across the somatic cell membrane in the nurse cell. Exocytosis machinery then transfers acid vesicles and cathepsins (acid-activatable proteases) to drive acidification and proteolysis nonautonomously. The pregranulosa cells surrounding acidified germ cell cells contained acidic cytoplasmic vesicles (Figure 2E). Multiple mouse V-ATPases as well as multiple cathepsins are highly expressed in these pregranulosa cells based on previous scRNA-seq analyses (Niu and Spradling, 2020; Figures S2A and S2B). The most highly expressed cathepsin in mouse pregranulosa cells, Ctsl, is the ortholog of the Drosophila cathepsin Cp1 that is genetically required for nurse cell turnover (Mondragon et al., 2019), whereas expression of the second most highly expressed cathepsin (Ctsb) is elevated in pregranulosa cells surrounding remnant nurse cells (Figure 2F). This expression, like nurse cell turnover, is gone by P4 (Figure S2C).

To investigate if acidification is required for mouse nurse cell turnover, we cultured ovaries in vitro starting at E17.5 either in the presence or absence of the vacuolar ATPase inhibitor Bafilomycin A1 (BafA1). Without the inhibitor, oocyte maturation into primordial follicles is largely complete after 6 days in culture as indicated by the presence of GM130-positive Balbiani bodies (Bb) (Figure 2G, arrow). In contrast, in the BafA1-treated cultures, Lysotracker vesicles are no longer seen in somatic cells (Figure S2D), and many nurse cell nuclear remnants persist (Figure 2E). Multiple mouse V-ATPases as well as multiple cathepsins are highly expressed in these pregranulosa cells based on previous scRNA-seq analyses (Niu and Spradling, 2020; Figures S2A and S2B). The most highly expressed cathepsin in mouse pregranulosa cells, Ctsl, is the ortholog of the Drosophila cathepsin Cp1 that is genetically required for nurse cell turnover (Mondragon et al., 2019), whereas expression of the second most highly expressed cathepsin (Ctsb) is elevated in pregranulosa cells surrounding remnant nurse cells (Figure 2F). This expression, like nurse cell turnover, is gone by P4 (Figure S2C).

We compared the relative frequency of germ cell PCD and apoptosis from E14.5 with P5 using Lysotracker to mark PCD and TUNEL positivity or cleaved caspase 3 to indicate apoptosis. Many more cells were stained with Lysotracker, than with either apoptosis marker (Figure 2I). Between 2 and 6 times, as many germ cells (Figure 2J) were observed turning over using PCD than apoptosis, indicating that as in Drosophila, mouse nurse cells mostly turn over by PCD. This conclusion was further supported by staining ovarian tissue for lamin B1 (Figure 2K) and observing that the lamin B1-rich subnuclear layer was degraded in very few dying nurse cells (Figure 2L). Turnover by apoptosis may be used by a separate small subclass of germ cells, such as those that have experienced developmental arrest, rather than normally developing cyst cells. Overall, these experiments illustrate striking similarities in nurse cell turnover in mouse and Drosophila (Figure S2E).

**Determining the gene expression program of mouse nurse cells using scRNA-seq**

Having measured the fraction of nurse cells turning over at 2-day intervals and shown that most eventually shrink in size (Figure 1F), we investigated whether these cells can be recognized by scRNA-seq. We used data from a large scRNA-seq analysis of mouse ovaries comprising seven time points beginning with cyst formation (E11.5, E12.5) and following at 2-day intervals (E14.5, E16.5, E18.5, and P1) spanning nurse cell dumping and turnover until the completion of cyst breakdown and primordial follicle formation by P5 (Niu and Spradling, 2020). Using Seurat 2, we reclustered nearly 1,800 germ cells, identifying 22 separate groups (Figure 3A; Table S1). Fifteen clusters comprising 74.5% of total cells had moderate to high levels of UMI/cell (Figure 3B, above dashed line), a measure of mRNA content, and localized on an outer ring of the tSNE plot (Figure 3A, outer arrows). The individual clusters in the outer ring recapitulate developmental time and meiotic stage, which increased uniformly in a clockwise direction (Figure S3). Clusters starting with “0” are mitotic cystocytes found mostly at E12.5, “1” are leptotene or zygote cells found mostly at E14.5, “2” are pachytene cells found mostly at E16.5, “3” are diplotene cells found mostly at E18.5, and “4” are dictyate cells found mostly at P1 (4a) or P5 (4b). Substages indicated by “a,” “b,” etc. represent finer subdivisions of the corresponding meiotic stages. This information is also shown in the form of labeled arrows on Figure 3A. These outer ring clusters contain pro-oocytes based on meiotic gene expression, but they must also include many nurse cells with similar expression because pro-oocytes only make up 20% of germ cells at this time (6 pro-oocytes per 30.5 starting cysts/clones), suggesting that 54.5% of main sequence cells at E14.5 are young nurse cells that still express the same genes as their pro-oocyte sisters.

Six of the remaining seven clusters (1N, 1Nr, 2Nr, 3N, 4N, and 4Nr) contain 22.1% of cells and likely comprise nurse cells at each developmental stage that have recently changed their gene expression enough to diverge from the main ring. This change in gene expression likely occurs when cyst nurse cells are “activated” by a local signal within their cyst to begin transferring their cytoplasm about 2 days before they turn over. Four observations strongly support this conclusion: (1) The stage-specific nurse cell clusters are mostly located very close on the tSNE plot to the main sequence clusters from the same developmental age, consistent with the fairly small genetic divergence expected after a recent change in gene expression. (2) There is a very close correspondence between the normalized number of activated nurse cells at each time point (Figure 3E, “nurse cell, activated”) and the normalized number of cells that will turn over in the next 2 days (Figure 1F, line 2). For example, at E14.5, the values are 6 versus 5.7 cells, at E16.5–6.2 versus 7.0 cells, at E18.5–4.8 versus 7.1 cells, and at P1–4.5 versus 4.7 cells. (3) The turnover of these cells in little more than 2 days is independently validated because each nurse cell cluster was found at mainly one time point (Table S1). Thus, at E14.5, 128 cells are found in the 1N and 1Nr clusters, but only 1 cell is found in these cluster at E16.5, and none thereafter. At E16.5, 70 cells are found in cluster 2Nr, but only 3 cells are found at E18.5, and 1 at P1, etc. (4) Finally, these clusters contain almost all the small cells based on low UMI/cell, and small cells were microscopically observed to consist of nurse cells in various stages of organelle and cytoplasmic transfer (Figures 1B, 1G, S1A, and S1B) and programmed cell death (Figure 2).

Cluster 5 cells (3.3% of germ cells) were found at low frequency at all time points and might represent apoptotic cells.
Figure 3. Identification of oocyte and nurse cell scRNA-seq clusters in fetal mouse ovaries

(A) tSNE diagram showing 22 highly stage-specific germ cell clusters from scRNA-seq of mouse ovaries from seven times E11.5-P5. “Main sequence” clusters distribute in developmental order from mitotic germ cells (0a) to dictyate oocytes (4a, b) in an outer ring as shown (arrows). The narrow stage and developmental time window when particular clusters are observed is given near arrows. Stage-specific clusters of nurse cells (NC) (1N = nurse cells from E14.5, etc.) occupy the central area (dashed circle).

(B) The UMI/cell distributions of indicated clusters; NC clusters fall mostly below the dashed line.

(C) Total nUMI/cell for main sequence cells at E11.5-P5.

(D) Average germ cell volume at E14.5-P4 (from [Lei and Spradling, 2016] with permission from AAAS) for comparison with (C).

(E) The calculated number of the indicated cell types in an average E10.5 PGC clone at E14.5-P5.

(F) Plot showing data in (E).

(G) Diagram showing average cyst structures of an E10.5 PGC clone E14.5-P5, with the cells color coded as in (E) and (F).
A. Mitotic, Lepto, Zygo, Pachy, Diplo, Dic, Low UMI

B. GC: 2b vs 1b, 1c

C. GC: 3b vs 2b

D. GC: 4a vs 3b

E. Up-regulated genes in nurse cells

E14.5 (1Nr>1.5; FDR<0.05)

| Term               | Description                                           | LogP   | Log(q-value) | InTerm | n_Len
|--------------------|--------------------------------------------------------|---------|--------------|--------|-------
| GO:0001890         | placenta development                                   | -6.46516| -2.199       | 10/174 | 1
| GO:0048608         | reproductive structure development                     | -5.41075| -1.719       | 14/458 | 1
| GO:0014548         | reproductive system development                        | -5.43752| -1.719       | 14/462 | 1
| GO:0006074         | placenta blood vessel development                      | -5.32047| -1.719       | 5/39   | 1
| GO:0103942         | positive regulation of cell death                     | -5.29422| -1.719       | 17/689 | 1
| GO:0043066         | positive regulation of apoptotic process               | -4.73862| -1.426       | 15/506 | 1
| GO:0043068         | positive regulation of programmed cell death          | -4.64788| -1.381       | 15/617 | 1

E16.5 (2Nr>1.5; FDR<0.05)

| Term               | Description                                           | LogP   | Log(q-value) | InTerm | n_Len
|--------------------|--------------------------------------------------------|---------|--------------|--------|-------
| WP3655             | dysregulated miRNA targeting in muscle/PI3K-akt signaling | -6.8809 | -2.413       | 5/26   | 1
| GO:0030336         | positive regulation of cell migration                 | -5.75517| -1.888       | 14/577 | 1

E18.5 (3N>1.5; FDR<0.05)

| Term               | Description                                           | LogP   | Log(q-value) | InTerm | n_Len
|--------------------|--------------------------------------------------------|---------|--------------|--------|-------
| GO:0000226         | microtubule cytoskeleton organization                 | -5.20465| -1.407       | 11/521 | 1
| GO:0045705         | regulation of angiogenesis                            | -5.10861| -1.407       | 9/345  | 1
| GO:0098036         | synaptic signaling                                     | -5.07955| -1.407       | 10/442 | 1

F. Down-regulated genes in nurse cells

E18.5 (3N<0.3; FDR<0.05)

| Term               | Description                                           | LogP   | Log(q-value) | InTerm | n_Len
|--------------------|--------------------------------------------------------|---------|--------------|--------|-------
| GO:0006325         | chromatin organization                                | -11.1397| -8.797       | 23/358 | 1
| WP310              | mRNA processing                                       | -10.8444| -6.679       | 21/453 | 1
| GO:0016570         | histone modification                                  | -9.77283| -5.983       | 18/365 | 1
| GO:0010113         | regulation of mRNA catabolic process                  | -8.3325 | -4.687       | 12/172 | 1
| GO:0031066         | regulation of histone methylation                    | -7.5279 | -4.106       | 9/90   | 1

P1 (4N<0.2; FDR<0.05)

| Term               | Description                                           | LogP   | Log(q-value) | InTerm | n_Len
|--------------------|--------------------------------------------------------|---------|--------------|--------|-------
| GO:0019827         | stem cell population maintenance                      | -10.5376| -8.416       | 13/141 | 1
| GO:0043484         | regulation of RNA splicing                            | -7.56036| -3.771       | 11/163 | 1
| GO:0006325         | chromatin organization                                | -7.18544| -3.618       | 18/538 | 1
| GO:0013668         | posttranscriptional regulation of gene                | -8.9609 | -3.472       | 18/557 | 1
| R-MML-1540         | Cell Cycler                                          | -5.35281| -2.341       | 16/570 | 1
| GO:1905616         | regulation of mRNA-mediated gene silencing by inhibition of translation | -4.90347| -2.052       | 3/7    | 1
| WP310              | mRNA processing                                      | -4.60655| -1.871       | 13/453 | 1
| R-MML-2470846      | Cohesin Loading onto Chromatin                        | -4.52788| -1.818       | 3/9    | 1

(legend on next page)
Cluster 5 cells displayed low UMI, but enrichment of apoptotic genes was not observed. They are included in Figures 3E and 3F as potential “apoptotic cells.” These findings allow us to estimate the fraction of oocytes versus nurse cells present in fetal and perinatal germ cells throughout cyst development (Figures 3E and 3F). For example, at E14.5, we know that the percentage of oocytes is 6/30 (or 20%). Activated nurse cells make up another 6% (20%) and cluster 5 cells comprise 4% (28/688 E14.5 cells, Table S1). That implicates the remaining 56% of cells as nurse cells that reside in the main ring clusters. These nurse cells have not yet become “activated” to alter their gene expression, to open membrane gaps, and to begin transferring cytoplasm and organelles within the cyst. Figure 3F displays in graphic form the relative number of nurse cells and oocytes in mouse fetal and perinatal germ cells. Finally, Figure 3G models the behavior of a single PGC-derived clone of germ cells that matches the average cyst parameters in our experiments and with the different types of cells indicated by color.

**Identification of the genetic changes associated with nurse cell activation**

Since all the cells in a female germline cyst derive from a common progenitor and since virtually all enter meiosis together by E14.5, it is not surprising that the main sequence clusters that comprise pro-oocytes and unactivated nurse cells express well-characterized meiotic-stage-enriched transcripts (Figure 4A, left columns). Interestingly, even nurse cells that have begun to transfer cytoplasm (Figure 4A, right columns) largely follow the meiotic expression, more weakly, of normal germ cells of the same age (Figure 4A, left columns). This is not surprising, given that these cells became activated to diverge from the main sequence expression program less than 2 days earlier, providing little time to accumulate a drastically different transcriptome. We confirmed these conclusions by comparing upregulated and downregulated genes generally during corresponding time points of the indicated main sequence clusters (Figures 4B–4D) with upregulated and downregulated genes in activated nurse cells from the same developmental stages (Figures 4B–4D*). The highly expressed changes in either direction are the largely same in normal meiotic cells and in transferring nurse cells.

We searched for changes in less abundant genes potentially downstream from a transfer signal and responsible for the divergence of nurse cells on the tSNE plot using Metascape gene enrichment analysis (Zhou et al., 2019). When we analyzed genes upregulated 1.5x or more in E14.5 cluster 1Nr nurse cells relative with their average expression in main sequence E14.5 germ cells, we observed several potentially significant GO categories that were enriched with an false discovery rate (FDR) <0.05 (Figure 4E). The highest enrichment was GO:0001890 placenta development. This was particularly interesting, given that both nurse cells and placental cells provide support during early oogenesis or fetal development, respectively, to other developing cells and suggests that some upregulated nurse cell genes aid the oocyte. The second highest enrichment was GO:0010942—positive regulation of cell death, which included GO:0043068—positive regulation of PCD and GO:0043065—positive regulation of apoptosis. Considering that 1Nr nurse cells are likely to undergo cell turnover within 2 days or less, some upregulated nurse cell genes likely promote their own turnover. Similar searches for genes enriched in E16.5 and E18.5 nurse cells (Figure 4E) yielded evidence of gene expression changes relevant to cell migration and microtubule cytoskeletal organization. At E18.5 and P1, downregulated GO categories were also observed with high FDR scores involving chromatin organization, suggesting that the transfer signal may modulate pro-oocyte nuclear development during later cyst stages.

We further investigated genes downregulated in nurse cells compared with closely related main sequence germ cells (Figure S4). We validated that the oocyte genes studied in Figure 1G are downregulated in nurse cells at the level of nurse cell gene expression by scRNA-seq (Figure S4A). Expression of these genes in nurse cells was 22%–40% of that in main stream germ cells. This differential appeared smaller than the difference we observed previously at the protein level, suggesting that translational regulation contributes to the oocyte specific expression.

Additional genes downregulated in nurse cells were also identified (Figure S4B). One of the most interesting was Tex14. Downregulation of Tex14 expression as nurse cells become activated might serve to derepress and activate cytoplasmic transfer. Thus, genetic changes in nurse cells following activation serve to support nurse cell function, including cytoplasmic transfer, as well as promote prooocyte differentiation.

Female germ cell gene expression during E11.5–P5 has been extensively studied using both developmental genetic (Wang et al., 2020) and high throughput studies (Soh et al., 2015; Miyauuchi et al., 2017; Nagaoka et al., 2020; Niu and Spradling, 2020). Figla (Joshi et al., 2007; Wang et al., 2020) and Taf4B (Grive et al., 2014, 2016) are required for normal meiotic gene expression, and Figla also promotes oocyte growth and development, acting in part by regulating transcription factors such as Nobox, Lhx8, Sohlh1, and Sohlh2 (Liang et al., 1997; Joshi et al., 2007; Wang et al., 2020). We identified nearly 200 genes that are upregulated in dictyate oocytes, including many target genes in this cascade (Figure S6; Table S3). Only 13% of these genes were expressed in P1 nurse cells at even 50% of their level in oocytes, whereas a majority were expressed at less than 10% of oocyte levels. This is the same range of differential RNA expression

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**Figure 4. Nurse cell and pro-oocyte gene expression is initially similar but diverges after nurse cells initiate cytoplasmic transfer**

(A) The relative expression (yellow is high, purple is low) of the genes indicated at right for main sequence (left) and nurse cell (right) scRNA-seq clusters (indicated at bottom); cluster numbers denote meiotic stage. (B–D) Volcano plots showing gene expression changes between successive stages (indicated at top) for main sequence clusters (GC, red) (B, C, and D) and nurse cell clusters (NC, blue) (B’, C’, and D’). (E and F) Top significant GO terms (Metascape) of indicated upregulated (green) or downregulated (pink) gene groups selected using criteria at top. Log10 probabilities are given for LogP and false discovery rates (Log q-value).
we observed for the genes analyzed in Figure 1G (Figure S4A) and suggests that the expression of many additional oocyte genes are repressed in nurse cells.

**Somatic pregranulosa cells and the cyst cytoskeleton contribute to oocyte development**

The strong similarity in gene expression between meiotic germ cells and nurse cells suggested that nurse cells initially develop as normal meiotic cells, but at varying times, during meiosis, receive a signal initiating cytoplasm and organelle transport. One possible signal source are the somatic cells that surround developing cyst cells (Figure 5i). To investigate the role of epithelial pregranulosa (EPG) cells associated with developing cortical follicles, we ablated EPGs as described previously using Lgr5-DTR-EGFP mice and diphtheria toxin injection at E14.5 (Figure SSA; see also Niu and Spradling, 2020). Ablation was highly effective, as the number of Lgr5+ cells, which normally surround developing germ cells was greatly reduced (Figures 5A and S5B). Germ cells continued to develop, although their numbers decreased after E18.5 (Figure SSC). Golgi elements decreased in P1 and P4 germ cells (Figures 5D and S5D) but mitochondria/per cell (Figures 5E and S5E) and Pericentrin (centrosomes)/per cell (Figures 5F and S5F) decreased. Thus, germ cells lacking interactions with EPGs did not undergo the normal, large increase in organelle numbers associated with nurse cell transfer after E18.5 and did not form Balbiani bodies.

Another well-established contributor to oocyte development in invertebrates is the cyst cytoskeleton (Figure 5i). We labeled cyst microtubules with an antialpha-tubulin antibody and observed synchronous cyst mitoses up to E14.5 (Figure 5G, left). Cysts that had completed mitotic divisions retained abundant cytoplasmic microtubules (Figure 5G, middle). Cysts at E15.5 displayed a microtubule cytoskeleton whose density varied within different cyst regions and that associated with centrosomes in the process of transport (Figure 5G, right). Organelle transport along a polarized microtubule cytoskeleton may explain why treatment of cultured ovaries with low levels of anti-microtubule drugs impairs the formation of primordial oocytes and Balbiani bodies (Koch and Spitzer, 1983; Lei and Spradling, 2016).

The actin cytoskeleton plays critical roles during *Drosophila* cyst development. Bundled actin fibers form in stage 10A that attach to nurse cell nuclei just before bulk cytoplasmic transfer at stage 10B. If these fibers are disrupted by a mutation in profilin or a variety of actin bundling proteins, nuclei move toward the oocyte and block transfer (reviewed in Hudson and Cooley, 2002). We looked for the presence of actin fibers in mouse cysts before and during transfer. Strong focal accumulations of actin were observed surrounding nurse cell remnant nuclei in cells that were transferring cytoplasm (Figures 5H and S5G). This suggests that actin fibers tether mouse nurse cell nuclei and prevent their entry into the oocyte.

**The mechanism of Balbiani body formation**

One of the earliest events of primordial oocyte development is Balbiani body (Bb) production (Figure 6A). The Bb in humans contains a large central region known as the cytocentrum that is thought to be a centrosome-like structure (Hertig and Adams, 1967), and a similar region is seen in the mouse Bb (Pepling et al., 2007). The migration and accumulation of multiple centrosomes in the oocyte perinuclear region occurs concomitantly with Bb formation and were proposed to help generate this structure (Lei and Spradling, 2016). We further studied the migration of centrosomes, often with an associated Golgi element, and confirmed that they accumulate initially as separate structures in E18.5 to P1 oocytes (Figure 6B). Subsequently, the dispersed centrosome/Golgi pairs assemble into a larger structure by P5 (Figure 6B). The dispersed centrosomes (Figure 6C, left) interact with each other, aggregate, and fuse into a large cytocentrum, which then nucleates microtubules and organizes oocyte organelles into a Bb (Figure 6C, right). Staining for alpha-Tubulin revealed that the consolidated centrosomes nucleate a very large microtubule aster in primordial oocytes that is many times larger than the aster typically seen associated with the single centrosome of younger female germ cells (Figure 6D).

In order to identify genes involved in cytocentrum formation, we investigated the role of two tubulin genes, Tuba1c and Tubb2b, that our data showed turn on selectively in oocytes beginning in P1 (Figure 6E). In mice whose germlines lack Tuba1c, the final assembly of the Golgi elements in the P1 oocyte into a mature Balbiani body by P4 did not take place (Figure 6F) in about 40% of oocytes (Figure 6H). This same defect became even more frequent (55%) in the double mutant of Tuba1c and Tubb2b (Figures 6G and 6H). Thus, Tuba1c and Tubb2b represent genes that contribute to cytocentrum consolidation and Balbiani body formation.

**DISCUSSION**

**Mouse oocytes develop with the assistance of nurse cells in germline cysts**

These studies provide a much clearer picture at both the cellular and molecular levels of how oocytes develop in germline cysts. Unactivated nurse cells strongly resemble pro-oocytes and follow a very similar program of gene expression. However, once an individual nurse cell receives a local signal from within its cyst, its gene expression changes quickly, and it embarks on a program of organelle and cytoplasmic transfer that shrinks its size and primes it to turn over by a special PCD pathway involving acidification mediated by surrounding pregranulosa cells.

Activated E14.5 nurse cells upregulate genes that likely promote the oocyte’s development, as suggested by their association with the GO term “placental development.” The most highly upregulated 1Nr gene (4.0 fold) in this GO class is *follistatin* (*Fst*), a repressor of activin signaling, which is required for uterine decidu- alization (Fullerton et al., 2017). Three other genes in this class that are all upregulated more than 3-fold are *Cited2*, which is needed for placental trophoblast production (Withington et al., 2006), *Plei- otropin* (*Ptn*), and *Wilm’s tumore 1* (*WT1*), which are both also required for deciduialization (Yu et al., 2018; Tamura et al., 2021). These observations suggest that pre-existing nurse cell genes may have been repurposed during the evolution of placental mammals. Later, nurse cells modulate genes involved in chromatin organization, which may promote prefollicular changes in oocyte
Figure 5. Somatic cell interactions and the cyst cytoskeleton guide nurse cell selection and activation

(A–F) Effect of pregranulosa cell ablation using Lgr5-DTR-EGFP (see Figure S5A) on germ cell organelles. (A) Pregranulosa cells (LGR5-GFP, green) surround cortical germ cells (DDX4, red) at E18.5 (control) but are gone in ablated ovary (dashed line). (B and C) Ablation greatly reduces the size and structure of Bb-associated Golgi (control, arrows; ablated, arrowheads) at P1 (B) and P4 (C). (D–F) Bar graphs showing that ablation significantly reduces normal increases in Golgi/cell (D), mitochondria/cell (E), and Pericentrin (centrosomes)/cell (F) at P1–P4.

(G) Germ cell alpha-Tubulin at E14.5 labels synchronous cyst metaphases (left panel, arrowhead), the extensive cyst microtubule cytoskeleton (middle panel, arrowhead), and microtubule-associated Golgi/centrosome pairs at E15.5 (right panel, arrowheads). Lower images: tubulin channel alone.

(H) E18.5 and P1 germ cell cysts stained with phalloidin to mark actin fibers. Large aggregations of actin (magenta) associate with nurse cell remnant nuclei (yellow arrowheads); full-sized germ cell or oocyte nuclei (arrow) lack similar actin. Lower images: actin channel alone.

(I) Model depicting a five-cell cyst containing a central pro-oocyte (Pro-O) with 3 intercellular bridges (IBs), 3 smaller-sized nurse cells (Nc), and 1 nurse remnant (Nr), each with 2 or 1 IBs, and surrounded by somatic cells (tan). The location of IBs (pink circles), microtubules (green lines), actin fibers (purple lines), Golgi elements (long red ovals), mitochondria (green ovals), and centrosomes (orange small circles) are shown. Size bars are indicated. Data are shown as mean ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001.

Proposed mechanisms of cyst regulation:
1. Timing signals from cyst somatic cells to control transfer;
2. Internal cytoskeletal polarity to direct organelle movement;
3. Ring canal repressive signal to select individual nurse cells for activation.
Proposed cyst functions:
1. Centrosome transfer to establish oocyte polarity center;
2. Transfer of other organelles to form Bb;
3. Transfer of nutrients to jumpstart growth;
4. Transfer of small RNAs to protect oocyte (?)

Figure 6. Centrosome transfer and consolidation generates a large microtubule aster that organizes the Balbiani body and establishes initial oocyte polarity

(A) A P4 oocyte showing a Balbiani body (Bb, arrow) with typical Golgi elements (GM130, red) and clustered mitochondria (ATP5a, green). (DAPI, blue).
(B) Pericentrin(green)-labeled centrosomes and associated Golgi(red) transferred from nurse cells consolidate into a Bb-organizing cytocentrum E18.5-P4.
heterochromatin (Lei and Spradling, 2016), suggesting also that
nurse cells activate stage-specific expression programs, rather
than a single stereotyped response.

Nurse cells also downregulate multiple genes that may promote
their function. One of the most interesting is Tex14. This com-
ponent of IBs was shown recently to have profound repressive ef-
fects on the kinetics of nurse cell intercellular organelle transporte.
In Tex14 −/− mutants, centrosome transport and Balbiani body for-
mation is accelerated such that many E14.5 cysts already contain a Bb (Ikami et al., 2021). Tex14 may also control bridge stability. Reduced Tex14 expression in activated nurse cells might destabilize bridges and open up gaps in the plasma membranes between cyst cells that are the main route for cytoplasmic trans-
fer. The resulting reduction in repression might initiate transport in
nurse cells that have received an activation signal.

**Programmed cell death of mouse nurse cells resembles that in Drosophila**

We found that mouse nurse cells turn over almost exclusively by a
pathway that strongly resembles the non-cell autonomous
PCD pathway that has been extensively characterized in
Drosophila nurse cells (Mondragon et al., 2019; Lebo and
McCall, 2021). Acidic vesicles and cathepsins are produced in
pregranulosa cells that surround nurse cell remnants. These ves-
icles are likely transported to nurse cells since inhibiting
V-ATPase activity prevented vesicle production in the pregrani-
losa cells and nurse cell turnover.

A much lower frequency of germ cells was positive for several
markers of apoptosis. These cells did not seem to be the main
pathway of nurse cell turnover, since most small nurse cells were
seen to associate with pregranulosa cells and undergo acidification. Apoptotic cells may have undergone develop-
mental arrest, but we never saw entire cysts of germ cells under-
ginging synchronous apoptosis, which is observed in Drosophila
under certain circumstances (Lebo and McCall, 2021).

Our observations help resolve the long-standing debate about
why large numbers of female germ cells die during perinatal
stages even in healthy, well-fed females. Although a small frac-
tion of dying germ cells may be due to the culling of defective oo-
cytes with chromosome abnormalities (Tilly, 2001), mitochon-
drial dysfunction (Palozzi et al., 2018), or LINE element
activation (Tharp et al., 2020), the great majority of germ cell turn-
over represents nurse cells programmed to die as part of a de-
velopmental program that contributes to oocyte production.

**Three mechanisms help the cyst specify oocyte and nurse-cell fates and time nurse-cell activation**

How oocytes are selected among cyst cells has remained only
partially understood despite decades of research in Drosophila,
mice, and other animals. The relatively simple, invariant cysts
found in Drosophila contain 16 cells interconnected by a polar-
ized microtubule-based cytoskeleton whose minus ends all
focus on the initial cell. The initial cell eventually becomes the
only oocyte, after organelles and unknown “determinants” are
transported into it from the remaining 15 cells that develop as
nurse cells (deCuevas and Spradling, 1998; Matova and Cooley,
2001; Roper and Brown, 2004; Huynh and Johnston, 2004;
Nashchekin et al., 2021). Multiple mouse oocytes are chosen
within even larger cysts that face the added complexity of vari-
able interconnections and fragmentation. However, our studies
suggest that similar underlying mechanisms specify oocytes
and nurse cells and control cyst breakdown in both mouse and
Drosophila.

First, we observed that the mouse somatic cells that continu-
ously surround developing cysts are essential for full organelle
transfer. Pregranulosa cells surround the cysts, and thin mem-
brane extensions of these cells largely separate even individual
cyst germ cells from each other, except at the ring canal and later
after membranes break down. These cells likely control the over-
all timing of cyst maturation by controlling the pace at which new
nurse cells are activated, possibly by increasing the level of their
positive signal as cyst development proceeds. Individual nurse
cells are probably activated when these positive signals outweigh local inhibitory signals associated with IBs. Such a sys-
tem can explain the tendency of oocytes to develop from cells
with the most bridges. However, the ultimate fate of cells is prob-
ably preordained by cytoskeletal asymmetries such as the cyst
microtubule network we visualized associated with organelles
in transit. Such polarity will ensure that transferred materials
move toward a pro-oocyte as their ultimate destination.

**Why are cysts and nurse cells used to generate oocytes?**

The study of insect nurse cells that persist throughout the entire
period of oocyte growth led to the view that nurse cells exist to
help the oocyte achieve great size, often the species’ largest
cell type. However, there are several additional reasons that fe-
male gametes might benefit from sharing cytoplasm with their
sister germ cells. Cysts are thought to protect male gametes
from selfish meiotic drive elements and other parasites by
sharing cytoplasm between potential gametes. Although unlike
male gametes, oocytes remain diploid, receiving cytoplasm con-
taining small RNA from multiple nurse cells still might assist in
controlling parasites and transposable elements.

Our work suggests a third potential advantage of cysts, and
cytoplasmic transfer is their role in generating the Balbiani
body. Although the term “Balbiani body” has been applied to a
disparate set of oocyte structures, we propose that organelles
transferred from nurse cells or otherwise organized at the oocyte

(C) Model of centrosome consolidation. oocyte nucleus (light blue); MT microtubules, others as labeled.
(D) P8 primordial oocyte (left) showing the large microtubule aster organized by the cytocentrum (Bb-MTOC). The microtubule aster of an E12.5 interphase germ
(cell (right, arrowhead) is much smaller. Insets: Pericentrin(green)-stained clustered centrosomes from the P4 primordial oocyte in (B), and a different cyst germ cell presented
at similar scale to show large size difference.
(E) scRNA-seq expression plot showing Tuba1c and Tubb2b induction in dictyate oocytes.
(F and G) P4 oocytes of Tuba1c +/− (F, left), or Tuba1c (+/−);Tubb2b (+/−) (G, left) with normal Bbs (arrow), contrasted with unconsolidated, discrete Golgi
elements in P4 oocytes of Tuba1c −/− (F, right), or Tuba1c (−/−);Tubb2b (−/−) (G, right) (arrowheads).
(H) Quantitation of Bb consolidation defects seen in indicated genotypes.
cytocenter at the time of follicle formation represent true Balbiani bodies that fundamentally promote oocyte development. In mouse, the multiple centrosomes acquired by transfer from nurse cells aggregate into a single structure known as the cytocentrum in dictyate oocytes, which nucleates a large microtubule bundle that assembles organelles into an aggregate. Bb assembly is assisted by Tuba1c and Tubb2b. Tubb2b, shown previously to be developmentally regulated, and its deletion causes a mild cortical structural defect (Bittermann et al., 2019). Collecting centrosomes from interconnected sister cells may be an ancient mechanism of oocyte polarization that has been retained in many species, including mammals.

In Drosophila, centrosomes transferred from nurse cells leave the Balbiani body as the follicle is forming and migrate to the oocyte posterior along with oskar, CPEB, and cup mRNAs (Mahowald and Strassheim, 1970; Cox and Spradling, 2003). This process establishes a major microtubule organizing center (MTOC) that defines the oocyte posterior and sends important signals needed for subsequent patterning steps that involve microtubule reorientation. Although similar initial steps take place in mouse oocytes, mRNAs associated with the Bb remain to be been identified.

Limitations of the study
These studies delineate the average structure and development of mouse female germline cysts. However, the range of exact starting structures of individual cysts and their particular fragmentation patterns that give rise to this average remain to be determined. An initial cytoskeletal polarity was established, although cysts form mitotically likely guides transferred nurse cell contents to pro-oocytes, but the nature of this polarity remains to be established. The lineage-labeling method does not track remnant nurse cells after they separate from the cyst, become surrounded by somatic cells, and undergo PCD because EYFP levels after nurse cells leave the cyst have fallen too low for detection.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Labeling and tracing experiments
  - Diphertheria Toxin injection
  - Immunofluorescence
  - Whole-mount staining of mouse fetal ovaries
  - Ovary in-vitro culture
  - LysoTracker staining of mouse fetal ovaries
  - TUNEL staining of mouse fetal ovaries
  - Actin and tubulin cytoskeleton staining
  - Cell identification and clustering analysis
- Gene enrichment analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Quantifying germ cell number, follicle number, small cell number
  - Whole nurse remnant volume and whole oocyte volume measurement
  - Golgi, mitochondria, and centrosome quantification
  - Statistical analysis

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.cell.2022.05.001.

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The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| anti-Ddx4           | Abcam  | Cat#Ab13840; Lot#GR294410-1 |
| anti-Ddx4           | Abcam  | Cat#Ab27591; Lot#GR290112-3 |
| anti-YFP            | Aves Labs | Cat#GFP-1020; Lot#GFP697986 |
| anti-GM130          | Fisher Scientific | Cat#BD610822 |
| anti-ATP5a          | Abcam  | Cat#Ab14748; Lot#GR3306993 |
| anti-Pericentrin    | Abcam  | Cat#Ab4448; Lot#GR3200989 |
| anti-alpha Tubulin   | Novus Biologicals | Cat#NB100-690SS |
| anti-alpha Tubulin   | Novus Biologicals | Cat#NB800-506; Lot#E-4 |
| anti-Cathepsin B     | Abcam  | Cat#Ab214428; Lot#GR3241171 |
| anti-Cleaved Caspase-3 | Cell Signaling Technology | Cat#9664T; Lot#21 |
| anti-NLRP5          | Dr. Jurrien Dean lab | Li et al., 2008 |
| anti-Filia          | Dr. Jurrien Dean lab | Ohsugi et al., 2008 |
| anti-TLE6           | Dr. Jurrien Dean lab | Li et al., 2008 |
| anti-Nobox          | Dr. Aleksandar Rajkovic lab | Shin et al., 2017 |
| anti-Lhx8           | Dr. Aleksandar Rajkovic lab | Shin et al., 2017 |
| anti-Lamin B1       | Dr. Yixian Zheng lab | Tran et al., 2021 |
| Alexa 488-Phalloidin| Invitrogen | Cat#A-12379 |
| Alexa Fluor488 donkey anti chicken IgY | Jackson ImmunoResearch Lab | Cat#703-545-155 |
| Cy5 Affinipure donkey anti rabbit IgG | Jackson ImmunoResearch Lab | Cat#711-175-152 |
| Alexa Fluor568 donkey anti goat IgG | Invitrogen | Cat#A-11057; Lot#1871957 |
| Alexa Fluor568 donkey anti mouse IgG | Invitrogen | Cat#A-10037; Lot#1141878 |
| Alexa Fluor488 donkey anti mouse IgG | Invitrogen | Cat#A-21202; Lot#1796361 |
| Alexa Fluor488 donkey anti rabbit IgG | Invitrogen | Cat#A-21206; Lot#17323019 |
| Alexa Fluor568 donkey anti rabbit IgG | Invitrogen | Cat#A-10042; Lot#1826664 |
| Alexa Fluor488 donkey anti rat IgG | Invitrogen | Cat#A-21208; Lot#50046A |

| Chemicals, peptides, and recombinant proteins | | |
|-----------------------------------------------|--------|------------|
| Paraformaldehyde                             | Electron Microscopy Sciences | Cat#15714 |
| DMEM/F-12, HEPES                              | Fisher | Cat#11330032 |
| Penicillin-Streptomycin-Glutamine             | Fisher | Cat#10378016 |
| Tween-20                                      | Sigma-Aldrich | Cat#P1379-100ML |
| Triton X-100                                  | Sigma-Aldrich | Cat#K100-100ML |
| IHC Antigen Retrieval Solution                | Invitrogen | Cat#00-4955-58; Lot#2085704 |
| Trypsin-EDTA (0.25%)                          | Fisher | Cat#25-200-056 |
| DAPI                                          | Sigma-Aldrich | Cat#D9542 |
| Diphtheria Toxin from Corynebacterium diphtheriae | Sigma-Aldrich | Cat#D0564-1MG |
| Vectashield, Mounting medium                  | Vector Labs Inc. | Cat#H-1000 |
| Tamoxifen                                     | Sigma-Aldrich | Cat#T5648-5G; Lot#WXBC1801V |

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Allan C. Spradling (spradling@carnegiescience.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
Raw sequencing data has been deposited at deposited at Gene Expression Omnibus (GEO) database (accession: GSE136441; ID: 200136441; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE136441). All original code has been deposited at GitHub (https://github.com/wanbaoniu8314/scRNAseq_mouse-nurse-cells). Any additional information required to reanalyze the data reported in this paper is available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

C57BL/6J, CAG-CreER (004682), Lgr5-CreERT2 mice (008875), and R26R-EYFP reporter mice (006148) were acquired from the Jackson Laboratory. Tuba1cem1(IMPC)J/Mmjax mice (051205-JAX), and Tubb2bem1(IMPC)J/Mmjax mice (046114-JAX), were acquired from Mutant Mouse Resource & Research Centers (MMRRC). Lgr5-DTR-EGFP mice were obtained from Genentech (South San Francisco, CA). For all experiments, to set a breeding pair, two females (8 weeks or older) and one male (8 weeks or older) are housed in one cage. Vaginal plugs are checked each morning after setting up mating. Only female embryos (E12.5–E19.5) and pups...
(P0-P8) are collected for sample analysis. Mouse experiments in this study were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Carnegie Institution of Washington.

**METHOD DETAILS**

**Labeling and tracing experiments**
The R26R-EYFP females were crossed with the CAG-CreER males, those with a vaginal plug were considered as E0.5. The pregnant females at E10.5 were given a single intraperitoneal injection of tamoxifen (Tmx; 10 mg/ml in corn oil (Sigma)) at 0.2 mg per 40 g body weight.

**Diphtheria Toxin injection**
Pregnant mice (E14.5) were injected i.p. with 10 μg/kg of diphtheria-toxin solution (D0564, Sigma) in PBS.

**Immunofluorescence**
Ovaries were fixed in cold 4% Paraformaldehyde overnight, incubated sequentially in 10% and 20% sucrose in PBS overnight, embedded in OCT, and stored at -80°C until cryosectioning. After high-temperature antigen retrieval with 0.01% sodium citrate buffer (pH 6.0), the frozen sections (10 μm) were blocked with 10% normal donkey serum for 30 mins, and then incubated with primary antibodies overnight at 4°C. The primary antibodies used are presented in the key resources table. The sections were washed with wash buffer and incubated with the appropriate Alexa-Fluor-conjugated secondary antibodies (see key resources table; 1:200) at room temperature for 2 hs. After staining with DAPI, samples were analyzed using confocal microscopy (Leica SP5).

**Whole-mount staining of mouse fetal ovaries**
Ovaries were fixed in cold 4% PFA for 4 hours. After washing in PBST2 (PBS with 0.1% Tween-20 and 0.5% Triton X-100) for 90 minutes, ovaries were then incubated with primary antibodies for 48 hours at 4°C. After washing in PBST2 for 90 minutes, ovaries were incubated with secondary antibodies (see key resources table; 1:200) overnight at 4°C. After washing in PBST2 (90 minutes) and DAPI staining (1 hour), samples were analyzed using confocal microscopy (Leica SP5).

**Ovary in-vitro culture**
Ovaries were dissected in cold PBS, and then cultured in 1 mL DMEM/F12 medium (ThermoFisher) at 37°C in an atmosphere of 5% CO2. The medium was supplemented with antibiotics penicillin and streptomycin (Fisher) to prevent bacterial contamination. Cultured ovaries were carefully placed on a membrane insert (0.4 μm, Millipore) and treated with Bafilomycin A1 (BafA1, 100nM, Abcam), which is a selective (V)-ATPase inhibitor. After 2 or 6 days in culture, ovaries were fixed in 4% PFA for further analysis.

**LysoTracker staining of mouse fetal ovaries**
LysoTracker red (L7528, Thermofisher) was diluted 1:10,000 in DMEM/F12 medium. Ovaries were carefully dissected and cultured in-vitro in 1 mL Lysotracker red added medium at 37°C for 12 hours. Ovaries were then harvested and fixed in 4% PFA for further analysis.

**TUNEL staining of mouse fetal ovaries**
Fix frozen sections in 4% PFA for 15 min at 37°C. After washing in PBS for 5 min, add sufficient volume of Proteinase K to completely cover the section and incubate the sample for 15 min. After washing, section was fixed in 4% PFA for 5 min at 37°C. TdT reaction and Click-iT Plus reaction was performed according to the manufacturer’s instructions. After staining with DAPI, mount slides using anti-fade mounting medium and store in the dark for analysis.

**Actin and tubulin cytoskeleton staining**
Fix ovaries in 4% PFA for 2 hours on ice. Permeabilize ovaries in PBST2 for 24-48 hours on a rotator at room temperature. For tubulin cytoskeleton staining, after blocking in donkey serum (10% in PBST2) for 2 hours for decreasing background fluorescence, ovaries were then incubated with anti-alpha-tubulin antibody for 2-3 days on a rotator at room temperature. After washing in PBST2 for 90 minutes, ovaries were incubated with donkey secondary antibody for 24 hours on a rotator at room temperature. After PBST2 washing (90 minutes) and DAPI staining (1 hour), samples were analyzed using confocal microscopy (Leica SP5). For actin filament staining, ovaries were incubated with Alexa 488-Phalloidin (1 μg/mL) for 1 hour on a rotator at room temperature. After washing in PBST2 for 30 minutes and DAPI staining for 1 hour, samples were analyzed using confocal microscopy (Leica SP5).

**Cell identification and clustering analysis**
Package “Seurat” v2.3.4 was used to analyze the scRNAseq data (Satija et al., 2015). Nearly 1,800 germ cells were isolated from the large integrated scRNAseq datasets spanning from E11.5 to P5 (Niu and Spradling, 2020). We re-clustered these cells to 22 clusters using FindClusters function. To carry out the analysis in a deeper resolution, we set “force.recalc” to “TRUE” for building the new SNN algorithm parameters (reduction.type = “pca”, resolution = 2.8, dims.use = 1:12). To make our dataset more accessible and
usable, the data slot (seurat.object@data), which stores normalized and log-transformed single-cell expression, was extracted and converted to Table S2. The values represent the mean milliUMI (mUMI) per cell after row sum and average of the data slot in each cluster (object@data sums and divided by cell numbers). DoHeatmap function was used to compare different meiosis genes in GC clusters with that in NC clusters (Figure 4A). To search for genes that are highly enriched in pro-oocyte and nurse cell unactivated, but not in nurse cell activated, “FindMarkers” function was used to compare clusters as following: cluster 1a-1e vs 1N, 1Nr; 2a-2c vs 2Nr; 3a-3c vs 3N; 4a, 4b vs 4N, 4N. Each pair of comparison yield a gene list, then “intersect” function was used to find common genes between these four gene lists, and 175 genes were generated in this method (Figure S4B). In addition, we also used “FindMarkers” function to compare dictyate GC clusters (4a, 4b) to other earlier GC clusters (1a-1e, 2a-2c, and 3a-3c) (avg_logFC>0.25; p_val_adj<E-25), and identified nearly 200 “oocyte genes/Dictyate genes/late genes” (Figure S6A; Table S3).

Gene enrichment analysis
Gene ontology (GO) functional enrichment analysis of the up-regulated gene sets and down-regulated gene sets in nurse cells was performed using Metascape (http://metascape.org).

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantifying germ cell number, follicle number, small cell number
For germ cell or follicle counts, collected ovaries were fixed in 4% PFA, incubated in sucrose, embedded in OCT, and sectioned to a thickness of 10 μm. The sections were stained with DDX4 antibody and every fifth section was analyzed for the presence of germ cells. The cumulative germ cell or follicle counts were multiplied by five. For small cell counts, single germ cell was labeled at E10.5 through low TMX injection (see labeling and tracing experiments for details), and ovaries were harvested at different timepoints for analysis. Collected ovaries were fixed in 4% PFA and stained with DDX4 and EGFP antibodies for whole-mount staining. Small cells (nurse cells) can be recognized microscopically because of smaller cell volume compared to nurse cells unactivated and pro-oocytes.

Whole nurse remnant volume and whole oocyte volume measurement
Single germ cell lineage labeled ovaries (see labeling and tracing experiments) were carefully dissected in cold PBS, and fixed in 4% PFA overnight. After 60 minutes PBST2 washing, samples were incubated with anti-YFP (clone marker) and anti-GM130 (golgi marker) antibodies overnight at 4°C. After secondary antibodies incubation and DAPI staining, samples were mounted for confocal analysis. The cyst cell that only contain little more than a nucleus and a tiny rim of cytoplasm was identified as a nurse remnant. For E18.5 and P1 ovaries, only Bb forming pro-oocytes were measured. Z-stack images were acquired through SP5 confocal microscope with a 63X oil lens. 3D images were reconstructed using Bitplane IMARIS. Nurse remnant volume and pro-oocytes volume were calculated by IMARIS in the surface statistics.

Golgi, mitochondria, and centrosome quantification
For quantifying golgi in single-germ cell lineage tracing, GM130 immunostaining was performed to indicate golgi. For cysts in E18.5, P1 and P5 ovaries, only pro-oocytes with Bb (a large Golgi sphere) or a forming Bb were analyzed. Golgi were modeled as ellipsoids to calculate the approximate surface area of Golgi membranes as described previously (Lei and Spradling, 2016). Excessive mitochondria structures were clustered together in germ cells, and high background signal results in inaccurate measures of mitochondrial number. To decrease the amount of background mitochondria signal of whole-mount staining, the single germ cell lineage labeled ovaries were fixed and embedded in OCT. Series of 6 μm frozen section were collected for YFP (clone marker), GM130 (to indicate Bb sphere) and ATP5a (mitochondria marker) staining. Generally a cyst can be fully harvested in 3-6 successive sections. Individual mitochondria structure is able to be recognized in confocal z-stack analysis (0.42 μm interval) in these 6 μm sections. To quantify centrosome, frozen ovary sections were stained with anti-Pericentrin antibody, and the surface area of each centrosome foci was calculated and summed.

Statistical analysis
Data are presented as mean±SD. Data were analyzed by t-test. P-value less than 0.05 (significant) is flagged with one star. P-value less than 0.01 (very significant) is flagged with two stars. P-value less than 0.001 (extremely significant) is flagged with three stars.
Supplemental figures

A. ATP8b GM130 YFP

B. Bar chart showing average volume per cell.

C. Mitochondria per cell.

D. Golgi per cell.

E. Nobox GM130 YFP

F. Table showing clone size and cyst size.

| Clone Size | Cyst size (#SC in cyst) | Clone Size | Cyst size (#SC in cyst) | Clone Size | Cyst size (#SC in cyst) | Clone Size | Cyst size (#SC in cyst) | Clone Size | Cyst size (#SC in cyst) |
|------------|-------------------------|------------|-------------------------|------------|-------------------------|------------|-------------------------|------------|-------------------------|
| 26         | 7(1); 9(2); 10(0)       | 13         | 5(1); 2(1); 6(1)        | 17         | 1(0); 2(1); 4(1); 5(1) | 8          | 3(1); 5(1); 10(0)      | 6          | 1(0); 0(0); 10(0)      |
| 31         | 14(2); 5(0); 4(0); 6(2) | 25         | 4(0); 6(1); 8(1); 5(1)  | 22         | 5(0); 6(1); 8(1); 5(1) | 13         | 4(1); 3(1); 10(0)      | 6          | 1(0); 10(0); 10(1)     |
| 32         | 18(4); 14(1)            | 35         | 4(0); 8(2); 5(1); 6(1)  | 17         | 4(1); 3(1); 8(1); 5(1) | 9          | 0(0); 3(2); 10(0); 10(1) | 9          | 1(0); 3(2); 10(0); 10(1) |
| 32         | 14(3); 6(0); 7(0); 5(1) | 28         | 13(3); 3(0); 9(2); 1(1) | 13         | 6(2); 4(2); 3(1)       | 9          | 1(0); 4(2); 10(0); 10(1) | 10         | 1(0); 4(2); 10(0); 10(1) |
| 36         | 12(1); 13(3); 11(2)     | 27         | 19(3); 6(0); 8(1); 4(0) | 18         | 3(0); 4(1); 5(1); 3(1) | 11         | 3(1); 10(0); 10(0); 2(1) | 10         | 3(1); 10(0); 10(0); 2(1) |
| 31         | 13(2); 4(1); 14(1)      | 21         | 7(1); 4(2); 6(1); 4(0)  | 20         | 4(0); 2(1); 7(1); 7(2) | 14         | 3(1); 10(0); 4(2); 10(0); 3(1); 10(0); 1(0) |

Ave. 30.5 8.8 (1.1) 24.8 5.3 (0.9) 17.8 3.5 (0.9) 10.7 1.9 (0.7) 6.0 1.0 (0.0)

% SC 12.5 17.0 25.7 36.8 0

G. Nobox ATP8b-DDX4

H. NLRP5-ATP8b-DAPI

I. NLRP5-ATP8b-DDX4

(legend on next page)
Figure S1. Data from 134 lineage-labeled clones, showing clone sizes, frequency of small cells (SC) and organelle content, related to Figure 1
(A) Examples of lineage-marked (YFP, blue) cysts as described in Figures 1C–1F, at E18.5 and P1, times when loss (nurse cells, Nc) or gain (oocytes, O) of
organelles (mitochondria, green; Golgi, red) often clarifies cyst structure.
(B–D) Quantitation among the cysts of oocyte and remnant nurse cell volume (B), mitochondria/cell (C), and Golgi surface area, \( \mu \text{m}^2 \) (D).
(E) Oocyte-enriched expression of Nobox in background of lineage-labeled cyst (YFP, blue) E17.5–P2.
(F) Individual cyst data from the experiment, which was reported in Figures 1E and 1F, and used to calculate the average number of cells per single PCG clone, of
cell loss and small cells (Figure 1F).
(G–I) Full-time sequences (E14.5–P6) for three of the oocyte genes (green) in Figure 1G, Nobox (G), NLRP5 (H), and Filia (I). Data are shown as mean ± SD. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).
Figure S2. Supplementary analysis of nurse cell turnover, related to Figure 2
(A and B) Robust expression of multiple V-ATPases (A) and cathepsin genes (B) in EPGs during E14.5-P5 (data from Niu and Spradling, 2020).
(C) Cathepsin B expression is no longer detected in P4 ovarian somatic cells.
(D) Lysotracker positive vesicles are seen in EPG cytoplasm prior to acidification in control culture incubated at E17.5 with Lysotracker for 2 days, but is absent for identical cultures that also received BafA1.
(E) (Left upper panel) clustering of 11–15 remnant nurse cell nuclei (arrow) in S12-13 Drosophila follicle; (left lower panels) an apparent cluster of 4 mouse remnant nurse cell nuclei. (Right panel) model drawing comparing nurse cell turnover by acidification in an anterior stage 11 Drosophila follicle with turnover in mouse nurse cells.
Figure S3. Developmental timing of scRNA-seq clusters, related to Figure 3

(A) tSNE plot identical to that shown in Figure 3A, but color coded to reveal the developmental time origin of all the component cells (see color key).

(B) Figure 3A is reproduced to facilitate comparison with (A). Below is summarized the development time key used to name the different germ cell clusters based on their time of origin.

0 = E11.5-E12.5
1 = E14.5
2 = E16.5
3 = E18.5
4a = P1
4b = P5
Figure S4. Analysis of genes preferentially expressed in meiotic cells versus nurse cells, related to Figure 4
(A) Expression of the genes shown in Figure 1G, using the scRNA-seq data. Expression is enriched in main sequence germ cells (GC) compared with nurse cell clusters of corresponding time points.
(B) Expression plots of 12 specific genes, including Tex14, from among the class that is under-expressed in nurse cell clusters compared with GC of the same age. Expression is shown cell by cell using mini tSNE plots. Darker colors indicate higher expression levels. Cells in the main sequence (red dashed line) generally express these genes more strongly than activated nurse cells (blue dashed line). Consult Figures 3A or S3A and S3B to identify location of particular cell groups on these plots.
Figure S5. Temporal effects of somatic cell ablation on organelle transfer, related to Figure 5
(A) Scheme for ablation of epithelial pre-granulosa (EPG) cells using Lgr5-DTR-EGFP (diptheria toxin receptor) and DT (diptheria toxin) injection at E14.5.
(B) Quantitation of Lgr5+ cells in control and EPG-ablated ovaries.
(C) Quantitation of germ cell numbers in control and EPG-ablated cultures.
(D–F) Examples of germ cells stained to reveal Golgi elements (D), mitochondria (E), or centrosomes (F) at E15.5, E16.5, E18.5, P1 and P4. G. E14.5 ovarian cysts (upper) and P4 primordial follicle (lower) stained with phalloidin to reveal actin fibers in germ cells. Aggregations of actin are seen associated with small nurse cell nuclei (nurse remnants, yellow arrowheads), but similar fibers are not seen at P4. Size bars are indicated. Data are shown as mean ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure S6. Expression of selected “oocyte” genes, related to Figure 6

(A and B) Expression profiles of selected genes strongly upregulated in late-diplotene and dictyate oocytes that peak at P5 (A) or P1 (B).

(C) GO analysis of these genes.

(D and E) Violin plots showing the relative expression of maternal effect genes (a class of “oocyte genes”) in cell clusters. Genes Tle6, Nlrp5, Khdc3, and Ooep make up the subcortical maternal complex (SCMC).

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(F–H) Effect of the indicated Tuba1c and Tubb2b genotypes in P4 oocytes on (F) the amount of Golgi area, (G) the number of mitochondria (green), or (H) the Pericentrin area/cell. In each case indicated control Tuba1c and Tubb2b genotypes (upper panels) are compared with mutant genotypes (lower panels).

(I–M) Note: the right panels are enlargements of the boxed region in the left panels (I–M). (I) Quantitation of the number of primordial follicles and primary follicles at P4. (J) Quantitation of cell volume. (K) Quantitation of Golgi area/cell from (F). (L) Quantitation of mitochondrial number/cell from (G). (M) Quantitation of Pericentrin/cell from (H). Size bars are indicated. Data are shown as mean ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001.