BIOLOGY OF REPRODUCTION **80,** 449–457 (2009)
Published online before print 19 November 2008.
DOI 10.1095/biolreprod.108.070649

Mouse TEX14 Is Required for Embryonic Germ Cell Intercellular Bridges but Not Female Fertility[1]

Michael P. Greenbaum,[3,4] Naoki Iwamori,[3] Julio E. Agno,[3] and Martin M. Matzuk[2,3,4,5]

Departments of Pathology,[3] Molecular and Human Genetics,[4] and Molecular and Cellular Biology,[5] Baylor College of Medicine, Houston, Texas

ABSTRACT

A conserved feature of germ cell cytokinesis is the formation of stable intercellular bridges between daughter cells. These intercellular bridges are seen in diverse species from *Drosophila melanogaster* to *Homo sapiens* and have been shown to have roles in communication of large numbers of germ cells. In testis expressed gene 14 (*Tex14*) knockout mice, intercellular bridges do not form during spermatogenesis, and male mice are sterile, demonstrating an essential role for intercellular bridges in postnatal spermatogenesis in mammals. Intercellular bridges also form between dividing germ cells in both male and female embryos. However, little is known about the formation or role of the embryonic intercellular bridges in mammals. In females, embryonic intercellular bridges have been proposed to have a role in development of the presumptive oocyte. Herein, we show that TEX14 is an essential component of male and female embryonic intercellular bridges. In addition, we demonstrate that mitotic kinesin-like protein 1 (MKLP1, official symbol KIF23), which we have discovered is a component of intercellular bridges during spermatogenesis, is also a component of male and female embryonic intercellular bridges. Germ cell intercellular bridges are readily identified by KIF23 immunofluorescence between the gonocytes and oogonia of control mice but are absent between germ cells of *Tex14*-null mice. Furthermore, by electron microscopy, intercellular bridges are present in all control newborn ovaries but are absent in the *Tex14* knockout ovaries. Despite the absence of embryonic intercellular bridges in the *Tex14*-null mice, male mice initiate spermatogenesis, and female mice are fertile. Although fewer oocytes were present in *Tex14*-null neonatal ovaries, folliculogenesis was still active at 1 yr of age. Thus, while TEX14 and intercellular bridges have an essential role in postnatal spermatogenesis, they are not required in the embryo.

follicle, gamete biology, gametogenesis, oocyte development, ovary

INTRODUCTION

Intercellular bridges are large cytoplasmic channels that connect germ cells in syncytia. The intercellular bridge is a highly conserved structure from insects [1–4] to mammals [5–12]. This high level of conservation strongly suggests that germ cell intercellular bridges are essential for fertility. In insects, genetic studies [1–4] have demonstrated that intercellular bridges are essential for both male and female fertility. "Incomplete" cytokinesis during these divisions in the female fruit fly results in the formation of a 16-cell cyst [13]. The cyst migrates posteriorly in the ovary as it matures, and one germ cell is selected as the oocyte and supported by the other 15 "nurse" cells [13]. In female *Drosophila*, intercellular bridges transfer several cytoplasmic components from the nurse cells to the oocyte, increasing its size. For example, oocyte-specific proteins and mRNAs (e.g., Bicoid D and oskar [13]), are concentrated in the oocyte. The centrosomes from the nurse cells are inactivated and transferred to the oocyte [14], and mitochondria are also concentrated in the oocyte [15].

TEX14 and the midbody proteins KIF23 and RACGAP1 (MgcRacGap) are expressed in both mouse and human testis [16]. As spermatogonia divide and differentiate, these proteins form intercellular bridges, a stable structure formed by mitotic and meiotic divisions and maintained until formation of spermatozoa. These structures are so stable that we were able to identify components of the intercellular bridge through a simple biochemical enrichment protocol and proteomic analysis [16]. Using embryonic stem cell/knockout mouse technology, we determined that TEX14 has an essential role in intercellular bridge formation in the mammalian testis [17]. We found that TEX14 is required to convert midbodies into stable intercellular bridges [16]. In *Tex14* knockout males, germ cells continue to form midbodies during telophase of cytokinesis. However, in the absence of TEX14, the midbody is transient, as in somatic cells, and no intercellular bridge is formed [16]. *Tex14* knockout mice completely lack intercellular bridges, and spermatogenesis fails before the first meiotic division [17], resulting in sterility. Thus, postnatal bridges seem to be functionally required in males.

Dividing embryonic male [18, 19] and female [20–23] germ cells are also connected by intercellular bridges. Mitotic division of germ cells occurs for a finite period during embryogenesis in mammals. In female mice, germ cells enter meiosis around Embryonic Day (E) 14.5 [22, 24]. No divisions occur beyond meiosis, so no new intercellular bridges are formed beyond embryogenesis. As a result, the germ cell cysts seen in newborn females are the result of stable intercellular bridges that were formed before approximately E14.5.

In male mice, mitosis and intercellular bridge formation resume after embryogenesis, although not until 3 days after birth. In newborn males, gonocytes migrate and arrive at the seminiferous tubule basement membrane within the first few postnatal days [25]. Mitosis resumes at Postnatal Day (P) 3,

[1]Supported in part by the National Institutes of Health Specialized Cooperative Centers Program in Reproduction and Infertility Research (HD07495 to M.M.M.). M.P.G was a student in the Medical Scientist Training Program (T32GM07330) and was supported by National Institute of Mental Health National Research Service Awards grant F30MH066542, a Hudson Scholarship, and the BRASS Foundation.
[2]Correspondence: Martin M. Matzuk, Department of Pathology, Baylor College of Medicine, Houston, TX 77030. FAX: 713 798 5838; e-mail: mmatzuk@bcm.tmc.edu

Received: 15 May 2008.
First decision: 6 June 2008.
Accepted: 23 October 2008.

eISSN: 1259-7268 http://www.biolreprod.org
ISSN: 0006-3363

when mitotic figures are seen in 10% of the gonocytes before reaching the basement membrane [25, 26].

Except for spermatogonial stem cells [27], all germ cell divisions in both genders are thought to result in the formation of stable intercellular bridges. This process leads to the formation of germ cell cysts in both male and female embryonic gonads [5–12, 20–23]. In females, it has been proposed that one germ cell in the cyst is selected to become an oocyte in a primordial follicle, while the other germ cells in the cyst are eliminated by apoptosis [23, 28]. As few as 2% of the embryonic germ cells survive to become oocytes [23]. It is unknown if the presumptive oocyte is chosen randomly or by a selective mechanism. Mitochondria seem to accumulate preferentially in one germ cell in the cyst, suggesting that the intercellular bridges may serve to help prepare and select the presumptive oocyte by the transfer of materials and/or signals [23]. An analogous syncytial organization occurs in male embryonic germ cell cords, with 30%–75% of the gonocytes degenerating [29].

MKLP1/Pavarotti (KIF23) was first identified as a component of both male [30] and female [31] *Drosophila melanogaster* intercellular bridges. By a molecular analysis of intercellular bridge formation in mammalian spermatogenesis, we have shown that KIF23 is a midbody component that becomes part of the intercellular bridge in a TEX14-dependent process [16]. Thus, KIF23 is the only protein known to be conserved in all intercellular bridges from fruit flies to humans [16]. No components of the embryonic intercellular bridge have been described to date in mammalian males or females, and the function of embryonic intercellular bridges is unknown. Herein, we used KIF23 and TEX14 as molecular markers to analyze the formation and essential requirements of embryonic intercellular bridges.

MATERIALS AND METHODS

Tex14 *Knockout Mice*

The generation of *Tex14*$^{-/-}$ mice has been described elsewhere [16]. *Tex14*$^{-/-}$ mice were previously demonstrated to lack *Tex14* mRNA by Northern blot analysis and are therefore null for *Tex14* [16]. Knockout mice were generated in our laboratory and were maintained at the Baylor College of Medicine Transgenic Mouse Facility in accordance with the facility's rules and animal handling protocols. All experimental animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Tissue Preparation

Mouse ovaries and embryonic testes and ovaries were fixed overnight at 4°C in 4% paraformaldehyde in Tris-buffered saline (TBS) (100 mM Tris-Cl, pH 7.5; 0.9%/150 mM NaCl). Tissues were washed three times in 70% EtoH and then overnight at 4°C in 70% EtoH. They were then embedded in paraffin for sectioning. Five-micrometer sections were placed on Superfrost/Plus slides (Fisher Scientific, Houston, TX) and dried overnight on a slide warmer (Clinical Scientific Equipment, Melrose Park, IL).

Immunofluorescence

Steps for rehydration and antigen retrieval for immunostaining have been described previously [17]. Tissue samples were circled with a PAP pen (Zymed, South San Francisco, CA) and blocked in TBS plus 3% bovine serum albumin (BSA) for 1 h at room temperature. Antibodies were diluted in TBS plus 3% BSA and used for overnight incubation at 4°C at the following dilutions: goat anti-TEX14 [16] 1:300, rabbit anti-KIF23 1:300, mouse anti-GCNA1 1:1, rabbit anti-STAT3 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-NOBOX 1:300, and guinea pig anti-ASZ1 1:300. Tris-buffered saline plus 6% BSA was used to dilute anti-GCNA1. Alexa 488, 555, and 647 secondary antibodies were purchased from Molecular Probes (Eugene, OR). Samples were mounted using Prolong Gold mounting media with 4′,6′-diamidino-2-phenylindole (DAPI) (Invitrogen, San Diego, CA) and examined on a Zeiss (Carl Zeiss MicroImaging, Thornwood, NY) Axiovert s100 2TV. When required, deconvolution was performed using softWoRx version 3.3.6 (Applied Precision, Issaquah, WA).

Electron Microscopy

Tissue was surgically removed from newborn animals and then drop fixed in a modified Karnovsky mixture of 2.5% glutaraldehyde, 2% paraformaldehyde plus 2 mM $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.4) overnight at 4°C. After primary fixation, the tissue was given three rinses of 5 min each in 0.1 M cacodylate buffer (pH 7.4). The tissue was then postfixed in 1% Oso_4 in 0.1 M cacodylate buffer for 3 h and rinsed three times for 5 min each in 0.1 M cacodylate buffer (pH 7.4). The tissue was dehydrated in a gradient series from 30% to 100% ethanol. After ethanol dehydration, the tissue was given three changes of fresh propylene oxide (PO) for 20 min each. It was then infiltrated up to 1:1 PO plus Spurr low-viscosity embedding resin, where it was held overnight. The next day, the tissue was further infiltrated with a 2:1 resin:PO mixture for 1 h, followed by three changes of 2 h each of pure Spurr low-viscosity embedding resin. The tissue was placed in individual 00 BEEM capsules and polymerized at 70°C overnight.

Thick sections were cut using a Histo knife (Diatome U.S., Fort Washington, PA) on an RCM (Tucson, AZ) MT-6000XL ultramicrotome, stained with toluidine blue, and examined on a light microscope for orientation. Thin sections were cut at 8 nm using an Ultra knife (Diatome U.S.) on the same ultramicrotome and picked up on 200-mesh copper grids. Thin sections were stained for 15 min in a saturated aqueous solution of uranyl acetate, counterstained for 6 min with Reynold lead citrate, and examined on a Hitachi H7500 transmission electron microscope (Hitachi High Technologies America, Inc., Schaumburg, IL). Digital images were captured using Gatan Digital Micrograph software and a Gatan US1000 camera (Gaton Inc., Pleasonton, CA).

Oocyte Counting

Female littermates were euthanized at Postnatal Day 2.5 (P2.5). The ovaries were removed and fixed for 2 h at room temperature in Bouin fixative. They were then prepared for paraffin embedding as already described. Paraffin-embedded oocytes were cut in 8-μm sections, and every fifth section was counted. To aid in counting, oocyte nuclei were labeled by immunofluorescence using an anti-NOBOX antibody. Counting was further assisted with ImageJ (National Institutes of Health, Bethesda, MD) and was checked manually. A correction factor of 5 was applied to the absolute counts according to the method described by Tilly [32]. For Embryonic Day 11.5 (E11.5) and Embryonic Day 18.5 (E18.5) embryos, 7-μm sections were cut, and every section was counted. An OCT4 antibody, ab19857 (Abcam, Cambridge, MA), and a GCNA1 rat monoclonal antibody were used to visualize germ cell nuclei at E11.5 and E18.5, respectively. Significance was determined by two-tailed Student *t*-test.

RESULTS

Subcellular Localization of TEX14

As already mentioned, TEX14 is an essential component of the intercellular bridge in postnatal testes. To determine whether TEX14 is also present in embryonic gonads, we used immunohistochemical analysis of embryos from timed matings. Similar to postnatal testes, TEX14 was found to localize to the intercellular bridges of E14.5 male gonocytes by immunohistochemistry (Fig. 1A). TEX14 also localized to the intercellular bridges of E14.5 oogonia (Fig. 1B). A germ cell membrane-specific marker, signal transducer and activator of transcription 3 (STAT3), was used to localize TEX14-containing intercellular bridges between gonocytes in newborn testis (Fig. 1C). TEX14-containing bridges were also found between oogonia in newborn germ cell cysts labeled with germ cell-specific protein containing four ankyrin repeats, a sterile-α motif, and a basic leucine zipper (ASZ1, also known as GASZ) (Fig. 1D). To prove that the antibody labeling of intercellular bridges was specific for TEX14, we compared control and knockout newborn ovaries (Fig. 1, E and F). While a low level of nonspecific staining was identified in oocyte cytoplasm in both groups, only the control had specific staining for TEX14

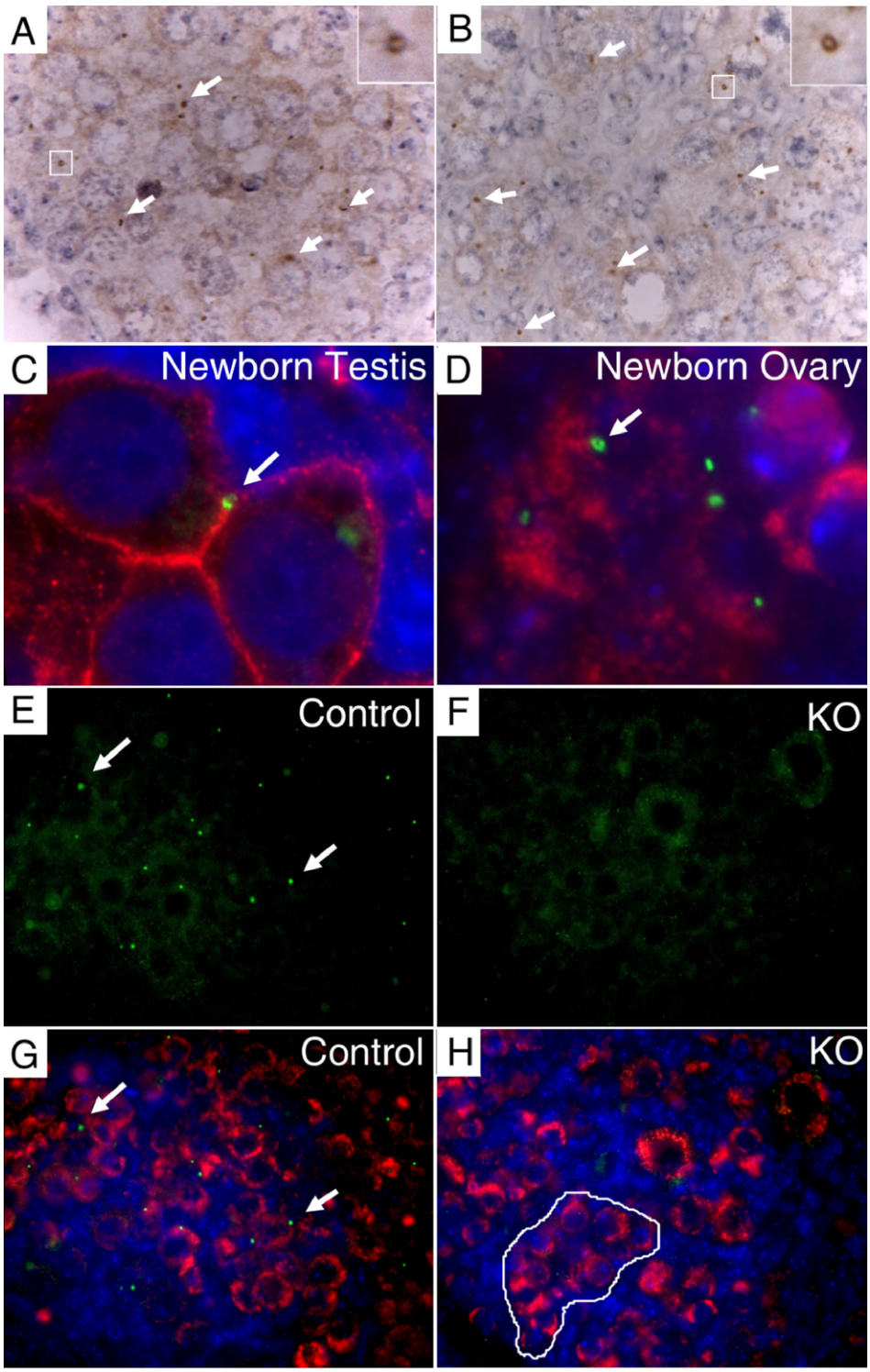

FIG. 1. TEX14 localization to intercellular bridges in embryonic and newborn gonocytes and oogonia. TEX14 is immunohistochemically stained with diaminobenzidine, and nuclei are counterstained with hematoxylin. TEX14 localizes to the intercellular bridge in E14.5 testis (**A**) and E14.5 ovary (**B**) between germ cells (arrows). **C**) TEX14 (green) is present between gonocytes labeled with STAT3 (red) in newborn testis. **D**) TEX14 (green) is present between ASZ1-positive (red) oocytes in germ cell cysts. Comparison of control (**E**) and *Tex14*-null (**F**) newborn ovaries shows that labeling of TEX14 (green) in the intercellular bridge is specific. **F**) A low level of oocyte cytoplasmic staining is a nonspecific cross-reaction of the antibody. **G**) Germ cell cysts (ASZ1, red) with intercellular bridges (TEX14, green) are abundant in control newborn ovaries. **H**) *Tex14* knockout (KO) ovaries also seem to have germ cell cysts (white outline) but fewer oocytes overall by comparison. Arrows indicate intercellular bridges. Original magnification ×400 (**A**, **B**, and **E**–**H**) and ×1000 (**C** and **D**); insets in **A** and **B** are 4 μm wide.

in intercellular bridges (Fig. 1, E and G, arrows). Although germ cell cysts were present in both control and null ovaries (Fig. 1, D, G, and H), no TEX14-positive bridges were present in the *Tex14* knockout cysts.

Colocalization of KIF23 and TEX14 in Embryo Bridges

Because intercellular bridges are retained through early postnatal life based on ultrastructural and other analyses [22, 23], we examined newborn testes and ovaries for the presence of markers. DAPI weakly stained the germ cell nuclei of newborn testis (Fig. 2A, asterisks) compared with somatic nuclei. Germ cell nuclear antigen 1 (GCNA1) [33] labeling was used to confirm the identity of male germ cells by immunofluorescence (Fig. 2, B–E). KIF23 colocalizes with TEX14 in the intercellular bridges located between germ cells (Fig. 2, D–F, large arrows) similar to postnatal testes [16]. Thus, both KIF23 and TEX14 are markers for the embryonic testis germ cell intercellular bridge. KIF23 is also normally present in the midbody between all cells, both germ cells and somatic cells, at the end of cytokinesis [34]. TEX14 is only present in germ cells [17]. Accordingly, KIF23, but not

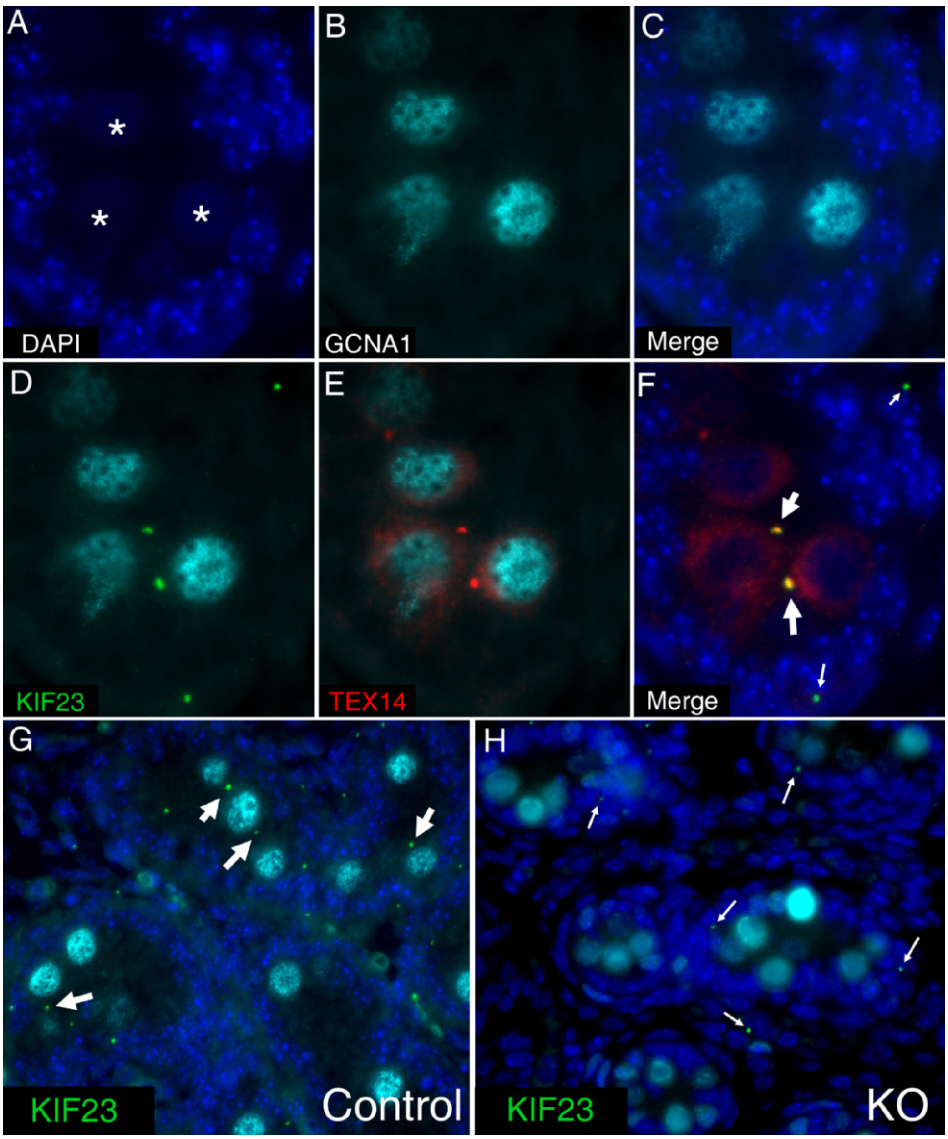

FIG. 2. TEX14 is required for intercellular bridges between male gonocytes. **A**–**F**) Germ cells (**B**–**E**; GCNA1, light blue) are organized in cords in the newborn testis. Asterisks indicate that surrounding somatic cells stain strongly with DAPI (**A**, **C**, and **F**; dark blue) compared with weakly stained germ cells (**A** and **F**). KIF23 (**D**, green) and TEX14 (**E**, red) colocalize (**F**) in the intercellular bridges between germ cells (large arrows). Only KIF23 (**D** and **F**) localizes to the transient midbody between dividing somatic cells (**F**, small arrows). Low-power views of newborn testes show that control (**G**) and *Tex14* knockout (KO, **H**) testes both have KIF23 localized to the transient midbody between somatic cells (**H**, arrows) but that only control mice have stable intercellular bridges between germ cells (**G**, arrows). Original magnification ×1000 (**A**–**F**) and ×400 (**G** and **H**).

TEX14, is occasionally observed between dividing somatic cells (Fig. 2, F and H, small arrows).

Similar results were seen in newborn female ovaries. Germ cell nuclei are again labeled with GCNA1. KIF23 colocalizes with TEX14 in the intercellular bridges between germ cells (Fig. 3, A–C). Closer examination (Fig. 3, A–C, insets) shows that the KIF23 ring is slightly larger than the TEX14 ring. While they overlap significantly, some area of KIF23 is completely outside TEX14, and some area of TEX14 is completely inside KIF23. These findings are consistent with our model for how mammalian intercellular bridges form [16].

Embryonic Intercellular Bridges Are Absent in Male and Female Tex14 *Knockout Mice*

To define the requirement for TEX14 in formation of the intercellular bridges in embryonic gonads, we examined male and female *Tex14*-null and control gonads. At low magnification (40×), GCNA1-labeled germ cells are seen in clusters surrounded by somatic cells (DAPI, blue) in both control (Fig. 2G) and *Tex14*-null (Fig. 2H) newborn testis. KIF23 is frequently found in intercellular bridges between GCNA1-positive germ cells in testis cords (Fig. 2G, large arrows), while KIF23 is never seen between germ cells in the testis cords of *Tex14* knockout mice (Fig. 2H). KIF23 is still seen in the midbody between dividing somatic cells in *Tex14* knockout mice (Fig. 2H, small arrows). KIF23 also labels many intercellular bridges between GCNA1-positive cells in control newborn ovary (Fig. 3D), but no germ cell intercellular bridges are labeled in *Tex14* knockout ovaries (Fig. 3E).

To further evaluate embryonic intercellular bridges, we examined newborn ovaries by electron microscopy. Three control and three *Tex14*-null ovaries were studied. The ovaries were oriented, and a section close to the top surface was examined from each ovary. A section near the middle of each ovary was also examined. For each of the null ovaries, a third section obtained near the bottom of each ovary was also examined. Intercellular bridges were found in all three of the control animals (Fig. 4, A–D). No intercellular bridges were observed in the *Tex14* knockout ovaries. While oocytes in the knockout ovaries were occasionally adjacent, as in the germ cell cysts of control ovaries (Fig. 4A), they always remained separated by cell membranes.

Neonatal Tex14 *Knockout Mice Have Fewer Oocytes*

It has been proposed that one "dominant" oocyte arises from each cyst, while the remainder die or are sacrificed to

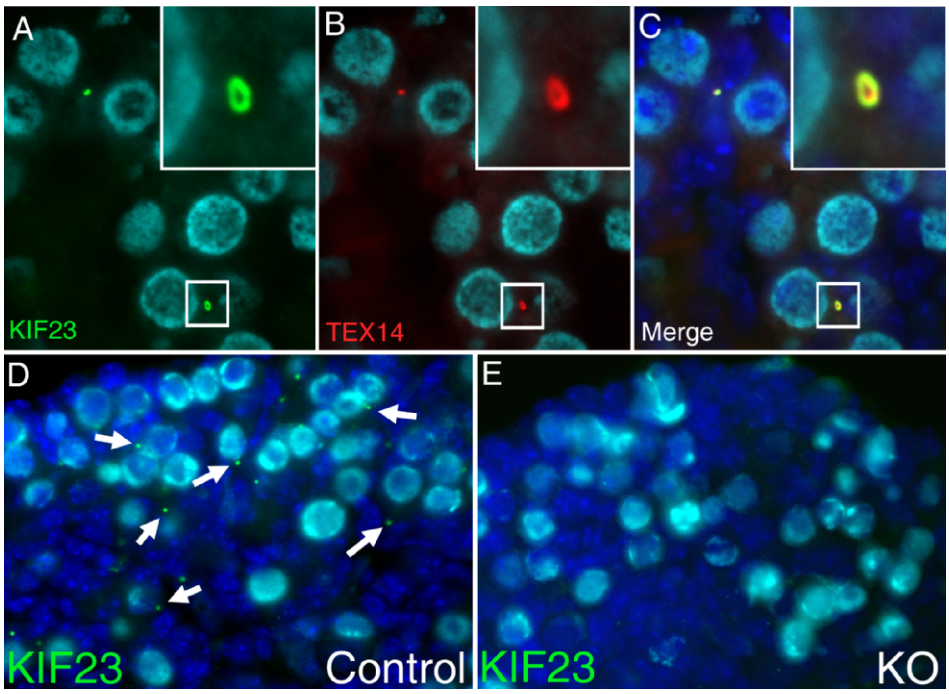

FIG. 3. TEX14 is required for intercellular bridges between female oogonia. KIF23 (**A**, green) and TEX14 (**B**, red) localize to the stable intercellular bridges between germ cells (**A**–**E**; GCNA1, light blue). **A**–**C**) Insets show that KIF23 (**A**) and TEX14 (**B**) colocalize to the intercellular bridge. The outer KIF23 ring mostly, but not completely, colocalizes with the inner TEX14 ring. **D** and **E**) KIF23 labeling of control (**D**) and *Tex14* knockout (KO, **E**) newborn ovaries shows that intercellular bridges are present between the germ cells of control (**D**, arrows) but not *Tex14* KO (**E**) ovaries. Original magnification ×630 (**A**–**C**) and ×400 (**D** and **E**); insets in **A**–**C** are 6 μm wide.

support it. Intercellular bridges are proposed to have an essential role in this process [23]. Cysts have been reported to completely break down by P2.5. This process is responsible for most of the perinatal reduction in germ cell numbers [23]. Using TEX14 staining, we confirmed that intercellular bridges are no longer present at P2.5. Therefore, we chose this point to determine the maximal influence of intercellular bridges on germ cell number. Three control ovaries were compared with three knockout ovaries. NOBOX staining was performed to ensure that all germ cells were visualized [35]. With the staining, it seemed that knockout ovaries had fewer oocytes than controls (Fig. 5, A and B). Every fifth section was counted, and a correction factor of 5 was used as described by Tilly [32]. Counting confirmed that knockouts had roughly half the initial oocyte pool compared with controls (mean ± SEM, 5708 ± 1027 for knockout and 11 040 ± 886 for control; $P = 0.017$) (Fig. 5C).

To determine if the differences in oocyte number occurred after birth (during cyst breakdown) or during embryogenesis, we first looked at primordial germ cell migration by counting the number of cells in the genital ridge at E11.5. There seemed to be similar numbers of germ cells in the E11.5 genital ridge for *Tex14* knockout and control females (Fig. 5, D and E). Because of the smaller size of the gonads at this stage, we counted the germ cells in every section. A POU domain, class 5 transcription factor 1 (POU5F1, also known as OCT4) antibody was used to ensure that every germ cell was counted. There was no significant difference in the number of germ cells at E11.5 (mean ± SEM, 871 ± 225 for knockout and 1193 ± 254 for control; $P = 0.177$) (Fig. 5F).

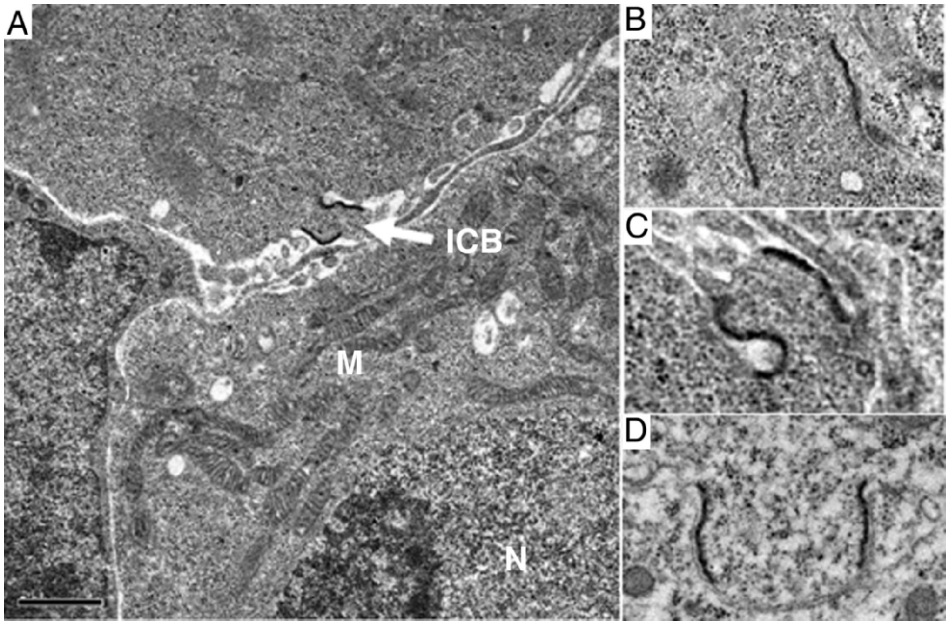

FIG. 4. Intercellular bridges were identified in control newborn ovaries. **A**) Two oocytes in a germ cell cyst connected by an intercellular bridge. **B**–**D**) Examples of intercellular bridges found in control newborn ovaries. No intercellular bridges were found in knockout ovaries. ICB, intercellular bridge; M, mitochondria; N, nucleus. Bar = 2 μm. Panels **B**–**D** are 2 μm wide.

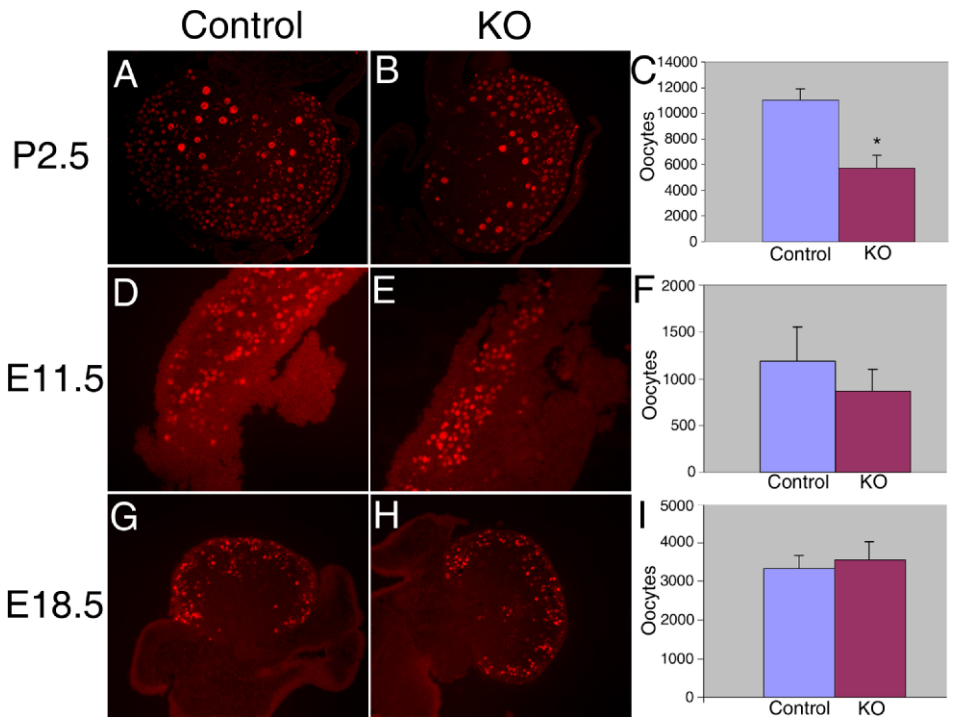

FIG. 5. Fewer oocytes are present in *Tex14* knockout (KO) ovaries compared with controls. **A** and **B** compare representative examples of control and KO P2.5 ovaries. The oocyte nuclei are labeled with a germ cell-specific anti-NOBOX antibody. **C**) Quantification of the oocytes shows that approximately twice as many oocytes are present in the control ovaries. *$P = 0.017$. **D**–**F**) Similar numbers of oocytes have migrated to the genital ridge by E11.5. **G**–**I**) Similar numbers of germ cells are present after all divisions are complete and just before birth on E18.5. Note that the number of oocytes in **C** cannot be compared with **F** and **I** because of the different counting method used for the larger P2.5 ovaries. Error bars in graphs are SEM. Original magnification ×200 (**A** and **B**) and ×100 (**D**, **E**, **G**, and **H**).

We next counted the number of germ cells present at E18.5, a time point after all embryonic germ cell expansion was complete but just before germ cell cyst breakdown. Again, we saw similar numbers of germ cells in knockouts and controls (Fig. 5, G and H). Every section was counted using an antibody to GCNA1. No significant difference in germ cell numbers was found (mean ± SEM, 3359 ± 326 for knockout and 3346 ± 326 for control; $P = 0.562$) (Fig. 5I).

Female Tex14 *Knockout Mice Complete Oogenesis*

Histologic analysis was performed to determine if oogonia that developed in the absence of intercellular bridges could go through all stages of folliculogenesis (Fig. 6). At 10 days, both wild-type control and *Tex14*-null ovaries had primordial follicles, primary follicles, and secondary follicles (Fig. 6, A and B). By 25 days, the secondary follicles had matured into antral follicles in both the control and *Tex14*-null ovaries (Fig. 6, C and D). Normal-appearing viable oocytes and follicles at every stage of folliculogenesis (primordial follicles, primary follicles, secondary follicles, antral follicles, and corpus lutea) were found in control and knockout ovaries at 8 wk (Fig. 6, E and F). At 1 yr, the knockout ovaries continued to maintain all stages of folliculogenesis (Fig. 6H). Thus, *Tex14* is not essential for postnatal oocyte growth and ovarian folliculogenesis.

Female Tex14 *Knockout Mice Are Fertile*

Because *Tex14*-null ovaries had oocytes and completed all stages of folliculogenesis, we tested whether female fertility was altered. Homozygous ($Tex14^{-/-}$) female mice were fertile during 6 mo of breeding beginning at 42 days of age to heterozygous ($Tex14^{+/-}$) males (mean ± SEM, 6.62 ± 0.30 pups/litter and 0.97 ± 0.05 litters/mo; n = 10 mating pairs). This was not a significant change in number of progeny compared with control heterozygous ($Tex14^{+/-}$) females bred to heterozygous ($Tex14^{+/-}$) males (mean ± SEM, 7.27 ± 0.22 pups/litter and 1.11 ± 0.08 litters/mo; n = 19 mating pairs) as determined by two-tailed Student *t*-test ($P = 0.34$). The slight decrease in litter number was statistically significant by Student *t*-test ($P = 0.026$).

DISCUSSION

In mammals, both male and female embryonic intercellular bridges require TEX14. Thus, a common protein is essential for all mammalian germ cell intercellular bridge formation from embryo (males and females) through adulthood (males) [16, 17]. KIF23, a protein essential for cytokinesis, is also present in all mammalian intercellular bridges. KIF23 localizes to an outer ring, with TEX14 forming an inner ring in the embryonic intercellular bridge. An identical localization pattern for these proteins was observed in developing mammalian intercellular bridges during spermatogenesis [16]. Before this study, it was uncertain whether *Tex14* would be essential in both male and female intercellular bridges. In *Drosophila*, male and female intercellular bridges are formed with different molecular requirements. For example, female *Drosophila* bridges contain proteins such as Kelch [36], Hu-li tai shao (Hts) [37], and Btk29A (Tec29) [38], which are not components of the male *Drosophila* bridge, while male bridges contain the septins Peanut, SEP1, and SEP2 [4], which are absent from female bridges. In addition, genes required for male fruit fly intercellular bridge formation such as the phospholipid kinase four-wheel drive (fwd) are not required in the female [3], while the Src kinase homologue Src64 is required for the female fruit fly intercellular bridge but not the male [39].

While TEX14 is required for all mammalian germ cell intercellular bridges, its exact mechanism of action remains unknown. Somehow, TEX14 localizes to the germ cell midbody, stabilizes it, and converts it into an intercellular bridge [16]. Proteins such as KIF23 are normally components of the midbody matrix in late telophase [40–42]. TEX14 forms an inner ring, possibly disrupting the midbody matrix by excluding proteins such as KIF23 with which it can directly interact [16]. Formation of the midbody matrix requires KIF23 and is an essential step for completion of cytokinesis [42].

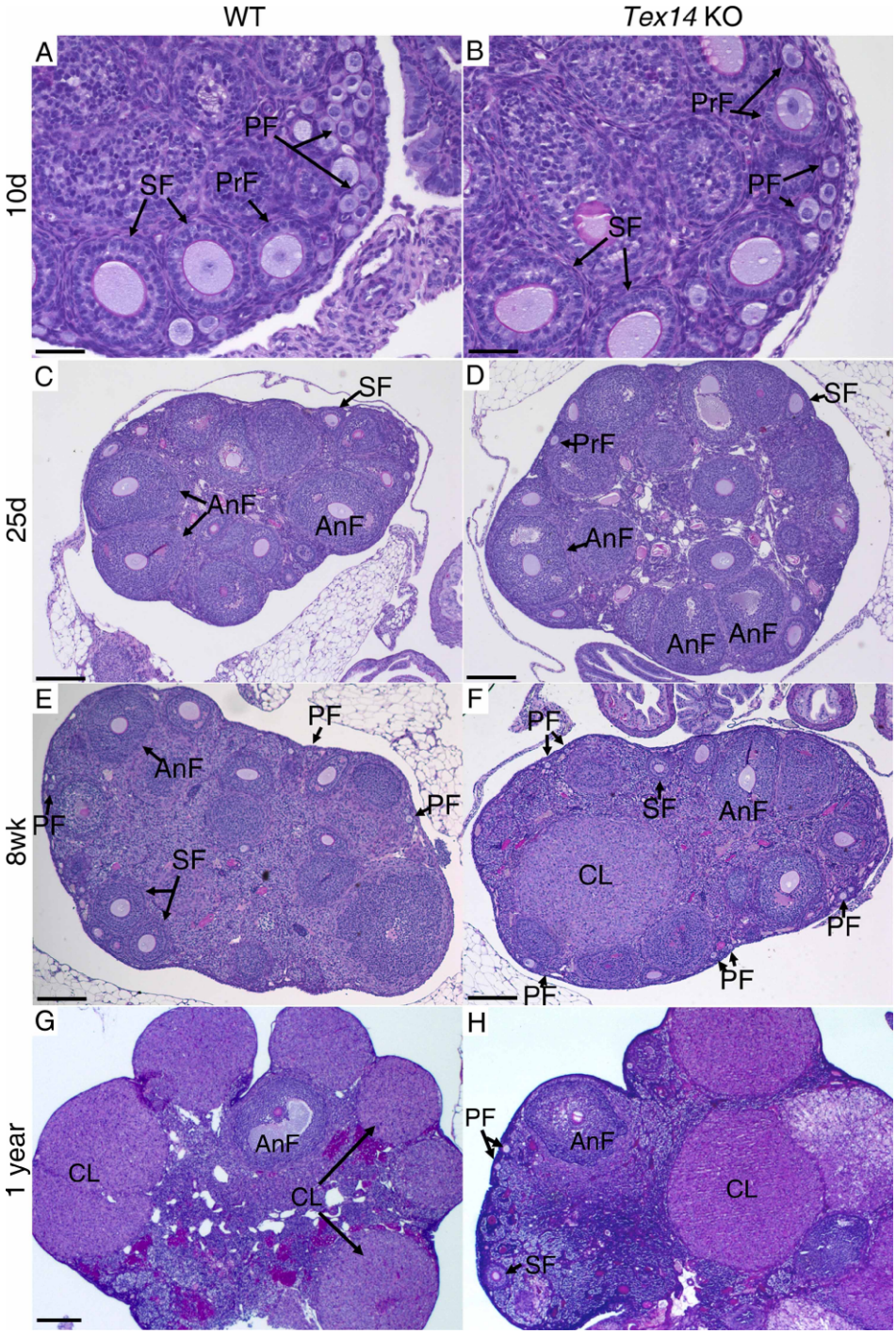

FIG. 6. Histologic analysis of *Tex14* knockout (KO) ovaries. Wild-type (WT) control (**A**, **C**, and **E**) and *Tex14* KO (**B**, **D**, and **F**) ovaries were examined. Similar stages of folliculogenesis were observed in both WT and *Tex14* KO ovaries at 10 days (**A** and **B**), 25 days (**C** and **D**), 8 wk (**E** and **F**), and 1 yr (**G** and **H**). AnF, antral follicle; CL, corpus luteum; PF, primordial follicle; PrF, primary follicle; SF, secondary follicle. Bar = 50 μm (**A** and **B**), 100 μm (**C**–**F**), and 200 μm (**G** and **H**).

Thus, the direct interaction of an "inner" TEX14 ring with the "outer" KIF23 ring may disrupt the midbody matrix and partly explain intercellular bridge formation. A similar process of inner and outer ring formation is seen in female *Drosophila* bridges [1]. Although TEX14 is a "dead" kinase with no activity [17], both Btk29A [43] and Src64 [39] have kinase domains that align to the TEX14 kinase-like domain better than any other kinase in the *Drosophila* genome. TEX14 localizes in mouse intercellular bridges in a pattern similar to that of *Drosophila* Tec29 [43]. Src64 is not a component of the *Drosophila* intercellular bridge [39]. This suggests that TEX14 may have a role analogous to that of Btk29A in mammalian intercellular bridges. The only known function for Btk29A kinase activity in the *Drosophila* intercellular bridge is to create phosphotyrosine binding sites for its own SH2 domains [43] on unidentified substrates. In fact, a hallmark of the *Drosophila* intercellular bridge is the phosphorylation of tyrosine residues in the intercellular bridge, and *Drosophila* intercellular bridges are easily identified with anti-phosphotyrosine antibodies [1]. This is not the case for mammalian intercellular bridge formation (Greenbaum and Matzuk, unpublished results). As an inactive kinase, TEX14 does not need to phosphorylate substrates to localize to the intercellular bridge, nor are any germ cell-specific kinases required for localization of TEX14 to the midbody [16]. It is possible that, as a germ cell-specific protein [17], TEX14 localization is regulated by its expression rather than by phosphorylation of targets, unlike Btk29A, which is broadly expressed [38]. TEX14 was found to self-

interact by targeted yeast two-hybrid [16], suggesting that, once localized, TEX14 may interact with itself to form the inner ring.

It is unexpected that a structure that has been maintained for more than 1 billion years of evolution [44] from invertebrates to man is not required for female fertility. Clearly, the idea that female embryonic bridges are required for a "synchronizing signal" necessary to enter meiosis does not apply to mammals [45], nor does the linkage to sex determination observed in *Drosophila* [46]. Important signals may, of course, still be transmitted between cells through small molecules independent of intercellular bridges. However, possible roles of the intercellular bridge in facilitating organelle biogenesis [21] or distribution [23] are evidently not essential for fertility of mammalian females. The benefit of transferring mitochondria or other cellular components is limited. At best, this mechanism doubles the initial oocyte pool in mice. This was not a major factor in the fertility of the mice in this study.

While loss of intercellular bridges did not decrease fertility, it is important to note that it also did not improve fertility. Because so many germ cells are lost during cyst breakdown, one could have proposed a model in which more germ cells survive if they do not sacrifice themselves for dominant oocytes. The results of this study show that if germ cells die for dominant oocytes, it is not because of organelles or information passed through intercellular bridges.

The data presented herein show statistically identical fertility during the first 6 mo of mating between *Tex14* knockout and control females. All stages of folliculogenesis were still present at least until 1 yr of age. Given the fertility of *Tex14* knockout females, it seems possible that the female intercellular bridge is a vestigial structure, possibly remaining only because of the essential role of intercellular bridges in spermatogenesis [17]. In the fruit fly ovary, intercellular bridges interconnect nurse cells and a single oocyte that forms, and this connection is necessary for nourishing the oocyte, which becomes larger at the expense of the nurse cells [13]. A similar physiologic change in the size of one oocyte vs. its neighbor in the germ cell cyst is not observed in mammals, further suggesting that the intercellular bridge is a vestigial structure and that its functional role in female fruit flies and mammals is not conserved.

ACKNOWLEDGMENTS

We thank Ms. Shirley Baker for her expert assistance in manuscript formatting; Drs. Stephanie Pangas and Aleksandar Rajkovic for review of the manuscript; and Dr. G. Enders (University of Kansas Medical Center, Kansas City, KS) for the anti-GCNA1 antibody, Dr. A. Rajkovic (Baylor College of Medicine, Houston, TX) for the anti-NOBOX antibody, and Dr. R. Kuriyama (University of Minnesota, Minneapolis, MN) for the anti-KIF23 antibodies.

REFERENCES

1. Robinson DN, Cooley L. Stable intercellular bridges in development: the cytoskeleton lining the tunnel. Trends Cell Biol 1996; 6:474–479.
2. Robinson DN, Cant K, Cooley L. Morphogenesis of *Drosophila* ovarian ring canals. Development 1994; 120:2015–2025.
3. Brill JA, Hime GR, Scharer-Schuksz M, Fuller MT. A phospholipid kinase regulates actin organization and intercellular bridge formation during germline cytokinesis. Development 2000; 127:3855–3864.
4. Hime GR, Brill JA, Fuller MT. Assembly of ring canals in the male germ line from structural components of the contractile ring. J Cell Sci 1996; 109(pt 12):2779–2788.
5. Huckins C, Oakberg EF. Morphological and quantitative analysis of spermatogonia in mouse testes using whole mounted seminiferous tubules, I: the normal testes. Anat Rec 1978; 192:519–528.
6. Burgos MH, Fawcett DW. Studies on the fine structure of the mammalian testis, I: differentiation of the spermatids in the cat (*Felis domestica*). J Biophys Biochem Cytol 1955; 1:287–300.
7. Fawcett DW, Ito S, Slautterback D. The occurrence of intercellular bridges in groups of cells exhibiting synchronous differentiation. J Biophys Biochem Cytol 1959; 5:453–460.
8. Dym M, Fawcett DW. Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. Biol Reprod 1971; 4:195–215.
9. Ventela S, Toppari J, Parvinen M. Intercellular organelle traffic through cytoplasmic bridges in early spermatids of the rat: mechanisms of haploid gene product sharing. Mol Biol Cell 2003; 14:2768–2780.
10. Weber JE, Russell LD. A study of intercellular bridges during spermatogenesis in the rat. Am J Anat 1987; 180:1–24.
11. Moens PB, Hugenholtz AD. The arrangement of germ cells in the rat seminiferous tubule: an electron-microscope study. J Cell Sci 1975; 19: 487–507.
12. Ren HP, Russell LD. Clonal development of interconnected germ cells in the rat and its relationship to the segmental and subsegmental organization of spermatogenesis. Am J Anat 1991; 192:121–128.
13. Huynh JR, St Johnston D. The origin of asymmetry: early polarisation of the *Drosophila* germline cyst and oocyte. Curr Biol 2004; 14:R438–R449.
14. Bolivar J, Huynh JR, Lopez-Schier H, Gonzalez C, St Johnston D, Gonzalez-Reyes A. Centrosome migration into the *Drosophila* oocyte is independent of BicD and egl, and of the organisation of the microtubule cytoskeleton. Development 2001; 128:1889–1897.
15. Cox RT, Spradling AC. A Balbiani body and the fusome mediate mitochondrial inheritance during *Drosophila* oogenesis. Development 2003; 130:1579–1590.
16. Greenbaum MP, Ma L, Matzuk MM. Conversion of midbodies into germ cell intercellular bridges. Dev Biol 2007; 305:389–396.
17. Greenbaum MP, Yan W, Wu MH, Lin YN, Agno JE, Sharma M, Braun RE, Rajkovic A, Matzuk MM. TEX14 is essential for intercellular bridges and fertility in male mice. Proc Natl Acad Sci U S A 2006; 103:4982–4987.
18. Fukuda T, Hedinger C, Groscurth P. Ultrastructure of developing germ cells in the fetal human testis. Cell Tissue Res 1975; 161:55–70.
19. Gondos B, Hobel CJ. Ultrastructure of germ cell development in the human fetal testis. Z Zellforsch Mikrosk Anat 1971; 119:1–20.
20. Gondos B, Bhiraleus P, Hobel CJ. Ultrastructural observations on germ cells in human fetal ovaries. Am J Obstet Gynecol 1971; 110:644–652.
21. Pepling ME, de Cuevas M, Spradling AC. Germline cysts: a conserved phase of germ cell development? Trends Cell Biol 1999; 9:257–262.
22. Pepling ME, Spradling AC. Female mouse germ cells form synchronously dividing cysts. Development 1998; 125:3323–3328.
23. Pepling ME, Spradling AC. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. Dev Biol 2001; 234: 339–351.
24. Borum K. Oogenesis in the mouse: a study of the meiotic prophase. Exp Cell Res 1961; 24:495–507.
25. McGuinness MP, Orth JM. Reinitiation of gonocyte mitosis and movement of gonocytes to the basement membrane in testes of newborn rats in vivo and in vitro. Anat Rec 1992; 233:527–537.
26. Nebel BR, Amarose AP, Hacket EM. Calendar of gametogenic development in the prepuberal male mouse. Science 1961; 134:832–833.
27. Huckins C. The spermatogonial stem cell population in adult rats, I: their morphology, proliferation and maturation. Anat Rec 1971; 169:533–557.
28. De Pol A, Vaccina F, Forabosco A, Cavazzuti E, Marzona L. Apoptosis of germ cells during human prenatal oogenesis. Hum Reprod 1997; 12:2235–2241.
29. Orwig KE, Ryu BY, Avarbock MR, Brinster RL. Male germ-line stem cell potential is predicted by morphology of cells in neonatal rat testes. Proc Natl Acad Sci U S A 2002; 99:11706–11711.
30. Carmena M, Riparbelli MG, Minestrini G, Tavares AM, Adams R, Callaini G, Glover DM. *Drosophila* polo kinase is required for cytokinesis. J Cell Biol 1998; 143:659–671.
31. Minestrini G, Mathe E, Glover DM. Domains of the Pavarotti kinesin-like protein that direct its subcellular distribution: effects of mislocalisation on the tubulin and actin cytoskeleton during *Drosophila* oogenesis. J Cell Sci 2002; 115:725–736.
32. Tilly JL. Ovarian follicle counts: not as simple as 1, 2, 3. Reprod Biol Endocrinol 2003; 1:e11.
33. Enders GC, May JJ II. Developmentally regulated expression of a mouse germ cell nuclear antigen examined from embryonic day 11 to adult in male and female mice. Dev Biol 1994; 163:331–340.
34. Sellitto C, Kuriyama R. Distribution of a matrix component of the midbody during the cell cycle in Chinese hamster ovary cells. J Cell Biol 1988; 106:431–439.

35. Rajkovic A, Pangas SA, Ballow D, Suzumori N, Matzuk MM. NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. Science 2004; 305:1157–1159.
36. Xue F, Cooley L. kelch encodes a component of intercellular bridges in *Drosophila* egg chambers. Cell 1993; 72:681–693.
37. Yue L, Spradling AC. hu-li tai shao, a gene required for ring canal formation during *Drosophila* oogenesis, encodes a homolog of adducin. Genes Dev 1992; 6:2443–2454.
38. Roulier EM, Panzer S, Beckendorf SK. The Tec29 tyrosine kinase is required during *Drosophila* embryogenesis and interacts with Src64 in ring canal development. Mol Cell 1998; 1:819–829.
39. Dodson GS, Guarnieri DJ, Simon MA. Src64 is required for ovarian ring canal morphogenesis during *Drosophila* oogenesis. Development 1998; 125:2883–2892.
40. Kuriyama R, Gustus C, Terada Y, Uetake Y, Matuliene J. CHO1, a mammalian kinesin-like protein, interacts with F-actin and is involved in the terminal phase of cytokinesis. J Cell Biol 2002; 156:783–790.
41. Matuliene J, Kuriyama R. Kinesin-like protein CHO1 is required for the formation of midbody matrix and the completion of cytokinesis in mammalian cells. Mol Biol Cell 2002; 13:1832–1845.
42. Matuliene J, Kuriyama R. Role of the midbody matrix in cytokinesis: RNAi and genetic rescue analysis of the mammalian motor protein CHO1. Mol Biol Cell 2004; 15:3083–3094.
43. Lu N, Guarnieri DJ, Simon MA. Localization of Tec29 to ring canals is mediated by Src64 and PtdIns(3,4,5)P3-dependent mechanisms. EMBO J 2004; 23:1089–1100.
44. Hedges SB. The origin and evolution of model organisms. Nat Rev Genet 2002; 3:838–849.
45. Hawkins NC, Thorpe J, Schupbach T. Encore, a gene required for the regulation of germ line mitosis and oocyte differentiation during *Drosophila* oogenesis. Development 1996; 122:281–290.
46. Pauli D, Mahowald AP. Germ-line sex determination in *Drosophila melanogaster*. Trends Genet 1990; 6:259–264.