

Formation, architecture and polarity of female germline cyst in *Xenopus*

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Abstract

Little is known about the formation of germline cyst and the differentiation of oocyte within the cyst in vertebrates. In the majority of invertebrates in the initial stages of gametogenesis, male and female germ cells develop in full synchrony as a syncytia of interconnected cells called germline cysts (clusters, nests). Using electron microscopy, immunostaining and three-dimensional reconstruction, we were able to elucidate the process of cyst formation in the developing ovary of the vertebrate *Xenopus laevis*. We found that the germline cyst in *Xenopus* contains 16 cells that are similar in general architecture and molecular composition to the cyst in *Drosophila*. Nest cells are connected by cytoplasmic bridges that contain ring canal-like structures. The nest cells contain a structure similar to the *Drosophila* fusome that is probably involved in anchoring of the centrioles and organization of the primary mitochondrial cloud (PMC) around the centriole. We also find that in contrast to other organisms, in *Xenopus*, apoptosis is a rare event within the developing ovary. Our studies indicate that the processes responsible for the formation of female germline cysts and the establishment of germ cell polarity are highly conserved between invertebrates and vertebrates. The dissimilarities between *Drosophila* and *Xenopus* and the uniqueness of each system probably evolved through modifications of the same fundamental design of the germline cyst.

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Introduction

In many phylogenetically diverse organisms, the development and differentiation of germ cells occur within a cyst (also known as a cluster or nest) of clonal cells interconnected by stable intercellular bridges called ring canals. Although the importance of cyst arrangement is not fully understood, it probably facilitates the exchange of developmentally important molecules among germ cells (Büning, 1994; Fawcett et al., 1959; Gondos and Zamboni, 1969; Mahowald, 1971; Telfer, 1975; reviewed in: de Cuevas et al., 1997; Deng and Lin, 2001; King et al., 1982; Pepling et al., 1999). Since all cells of single

cyst are mitotic descendants of a single precursor cell, the number of cells in the cyst, as a rule, is a power of two (2^n), and exceptions from the 2^n principle observed in certain insects are usually caused either by the death or the fusion of some of the participating cells (Büning, 1994).

The formation and development of the germ cell cluster has been most extensively studied in various insects and especially in *Drosophila*. The germline cyst in *Drosophila* contains 16 (2^4) cells called cystocytes that are the product of four synchronous divisions of a single cystoblast. The cystocytes are connected by ring canals whose rims contain, among other proteins, the F-actin, actin cross-linking protein kelch and adducin like hu-li tai shao (hts) protein (Kelso et al., 2002; Koch and King, 1969; Yue and Spradling, 1992; reviewed in de Cuevas et al., 1997). In *Drosophila*, following each round of cystocyte division, ring canals migrate along the surface of the cells and gather in the center of the

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cyst creating a pattern called “the rosette”. Within the rosette the cystocytes radiate out from the center of the cyst like the petals of a flower. It is believed that the rosette conformation facilitates contact and exchange of molecules or organelles among the cystocytes (Storto and King, 1989; reviewed in: de Cuevas et al., 1997; Telfer, 1975). The germline cyst in *Drosophila* has an intricate branching pattern with two cells containing four ring canals each, two cells containing three ring canals, four cells containing two ring canals and eight cells containing one ring canal (reviewed in: Büning, 1994; King et al., 1982). Such geometry of the germline cyst depends on the nonrandom segregation of the preexisting ring canals and the proper orientation of the planes of consecutive mitotic divisions (reviewed in Telfer, 1975). The polarization of mitoses results from the anchoring of the centrioles and the poles of the mitotic spindles by a germline specific organelle, the fusome (Deng and Lin, 1997).

The fusome, first described in the 19th century in male germline cysts of various insects, contains vesicles and membranous tubules similar in appearance to smooth endoplasmic reticulum. This unique structure is rich in α and β spectrin and the hts protein (de Cuevas et al., 1996; Lin et al., 1994; Matuszewski et al., 1985, reviewed in: de Cuevas et al., 1997; Telfer, 1975). The fusome forms from the spherical spectrin-rich organelle called the spectrosome, which is present in the precursor cell of the cyst (Deng and Lin, 1997). During consecutive mitoses, the fusome grows directionally and branches through the ring canals of the cyst. The mutations that disrupt normal formation or composition of the fusome or ring canals have a profound impact on cyst formation and oocyte differentiation and fertility (de Cuevas et al., 1996; Yue and Spradling, 1992; reviewed in de Cuevas et al., 1997; Deng and Lin, 2001). After the completion of mitoses in most insects, the cystocytes lose synchrony and rosette conformation, the cysts become polar and the process of oocyte differentiation begins. In *Drosophila*, and in many other insect species, the oocyte invariably forms from one of two cells containing the highest number of ring canals (reviewed in de Cuevas et al., 1997; Deng and Lin, 2001; Telfer, 1975). The cyst enters the meiotic prophase, but only two cells with four ring canals develop typical synaptonemal complexes (Carpenter, 1979, 1981; Schmekel et al., 1993). Eventually, only 1 of the 16 cystocytes, the pro-oocyte, maintains the meiotic state and differentiates into the oocyte, the other 15 siblings become the nurse cells.

Although several morphological and ultrastructural studies identified the presence of germline cell clusters in developing vertebrate gonads, including *Xenopus* ovaries, the origin of these clusters remained either hypothetical or, especially in mammals, controversial (Coggin, 1973; Fawcett et al., 1959; Gomperts et al., 1994; Gondos, 1973; Pepling and Spradling, 1998, 2001; reviewed in Pepling et al., 1999). Also, it was not known if in vertebrates all cells of the cyst differentiate into oocytes or if some of them form

nurse cells that eventually degenerate. Pepling and Spradling (1998) showed the presence of clonal germline cysts with ring canals in the ovaries of 11.5–17.5 days postcoitum mouse embryos. These investigators also showed that only 33% of the oocytes survive to form primordial follicles in the ovary of the mouse (Pepling and Spradling, 2001). However, there is no detailed information available on the development, architecture and polarity of the vertebrate female germline cyst.

The ovary of the adult *Xenopus laevis* is composed of asynchronously developing oocytes of various sizes (stage I to stage VI) in the diplotene of first meiotic prophase. The ovary of a postmetamorphic froglet contains nests of synchronously developing oocytes in pachytene of the first meiotic prophase (prestage I oocytes, Hausen and Riebesell, 1991). Prestage I and stage I oocytes exhibit visible signs of morphological asymmetry as well as an underlying molecular polarity. The position of the future vegetal pole of the oocyte is clearly marked by the presence of the mitochondrial cloud (MC) or Balbiani body. The center of the mitochondrial cloud is occupied by the centriole, and the presumptive vegetal apex of the cloud contains various localized RNAs, germinal granules or mitochondrial cement that is believed to be a precursor of germinal granules (Heasman et al., 1984; reviewed in Kloc et al., 2001a, 2002). In the early stages of assembly in developing *Xenopus* gonads, the cloud is often called the primary mitochondrial cloud (PMC) (Tourte et al., 1981) or premitochondrial cloud (Kloc et al., 1998, 2001a).

Oocyte polarity and the establishment of the animal or vegetal axis in oogenesis are the essential foundation for the axial patterning of *Xenopus* embryo. There are indications that oocyte asymmetry is foreshadowed by the polarity of the oogonia and the positioning of the forming mitochondrial cloud. Therefore, a description of how the geometry and polarity of developing cysts is established will be important in our understanding of the mechanisms involved in the patterning of the embryo during development.

There have been only a few reports describing the ultrastructure of the developing gonad in *X. laevis*. The majority of these reports depict sexually undifferentiated gonads in *Xenopus* tadpoles (Ijiri and Egami, 1975; Kalt and Gall, 1974; Wakahara, 1982; Wylie and Heasman, 1976). Several studies followed the ultrastructure of the developing ovary, but they concentrated either on primary oogonia or on the cysts (nests) of meiotic prophase oocytes; however, none of them described the events leading to the formation of such cyst (Al-Mukhtar and Webb, 1971; Coggin, 1973; Tourte et al., 1981). Coggin (1973) assumed a priori, from incidental observations of synchronous divisions and the presence of cytoplasmic bridges connecting oocytes, that the nest contained 16 oocytes and that each nest was the result of four consecutive divisions of a single precursor cell. In the present analysis, we determined the sequence of events culminating in the formation of the female germline cyst, and described the development of cyst polarity in the

Xenopus ovary. Our studies indicate that while there are some differences, the geometry of female germline cyst and the molecules involved in the formation of the cyst and germ cell polarity are highly conserved between invertebrates and vertebrates.

Material and methods

Electron microscopy

The developing ovaries were removed from anaesthetized tailed and tailless froglets (stages 62–66) of wild-type *X. laevis*. For ultrastructural analysis and three-dimensional reconstruction, ovaries were fixed in TEM fixative (2% formaldehyde, 3% glutaraldehyde, EM grade, Ted Pella, Redding, CA, in 0.1 M sodium cacodylate buffer pH 7.3, Polysciences, Warrington, PA) in some instances containing 10 μ M taxol (Cytoskeleton, Denver, CO) to stabilize the microtubules. To enhance the visualization of centrioles and cytoskeleton, the material was stained in 0.5% uranyl acetate and the osmium tetroxide treatment was omitted. This resulted in very light staining of all membranous structures; however, it allowed the visualization of highly contrasted centrioles and microtubules. Embedding and sectioning were done as described by Kloc et al. (2001b). For ultrastructural analysis, the material was sectioned at 70 nm and for the three-dimensional reconstruction at approximately 180 nm. In addition, the analysis of the divisions in the nests was also performed at light microscopy level on semithin 1- μ M sections stained with 1% methylene blue in 1% borax.

Three-dimensional reconstruction

A set of serial EM images was collected using Adobe Photoshop. Images were aligned for three-dimensional reconstruction using Image Vision Library, which is a tool kit for the analysis and processing of two-dimensional images (SGI, <http://www.sgi.com/software/imagervision>). Corresponding registration points were selected within each image and the images aligned according to these markers. Cellular structures such as individual cell membranes, nuclei, mitochondria, centriole and ring canals were identified and marked using the channel feature (Photoshop) as a means to segment the structures. Annotated TIFF images were further processed using Iris Explorer visualization software (NAG) which uses the empirical data and visualizes it in three dimensions (Toll, Numerical Algorithms Group, <http://www.nag.com>). Individual stacks of images were combined into a single three-dimensional dataset that contains the bioinformatics needed to define a biological structure. This integrated dataset, containing segmentation information, was used to extract individual cellular components using various image processing and interpolation modules, some of which were developed by National

Center for Macromolecular Imaging—Baylor College of Medicine (NCMI—BCM <http://ncmi.bcm.tmc.edu>). Extracted cellular components were further processed using a marching cube isosurface module to create suitable three-dimensional Open Inventor geometries (Silicon Graphics, <http://www.oss.sgi.com/projects/inventor>). Color schemes were later applied to the geometries to highlight cellular features and relative placements. The Motion Picture Experts Group (MPEG) animation was generated to create an array of camera placements.

Antibodies

Primary antibodies: hts RC monoclonal antibody and kel 1B monoclonal antibody (both from DSHB, University of Iowa) were used at 1:1 dilution (according to DSHB recommendation) for whole mount and 1:50 dilution for sections, centrin 20H5 monoclonal antibody (gift from Dr. Jeffrey L. Salisbury, Mayo Clinic Foundation, Rochester, MN, USA) was used at 1:1000 dilution and α and β spectrin monoclonal antibody S9645 (Sigma, St. Louis, MO) was used at 1:50 dilution. Anti- α tubulin monoclonal antibody (T 9026, Sigma) was used at 1:500 dilution. Secondary antibodies: anti-mouse FITC (Roche, Indianapolis, IN) and anti-mouse-AP (Calbiochem, San Diego, CA) were both used at 1:200 dilution.

Immunostaining and histology

For immunostaining, ovaries were fixed either in 4% formaldehyde in PBS with 0.1% Triton X-100 or in Bouin's fixative (Sigma). After fixation in Bouin's, ovaries were washed overnight with a few changes of 70% ethanol. For whole mount method, the ovaries were washed a few times in PBS–0.05% Tween 20 and then blocked in casein blocking solution (BioRad) for 1–3 h at room temperature. Subsequently, ovaries were incubated overnight at 4°C in primary antibody in casein blocking solution, washed 4 \times 15 min in PBS–Tween and incubated in secondary antibody conjugated with alkaline phosphatase or with FITC in casein blocking solution, overnight, at 4°C. After washing 4 \times 15 min in PBS–Tween, ovaries were either stained in NBT/BCIP tablet solution (Roche) or in case of FITC they were directly observed with fluorescent microscope. Some of the fixed ovaries were embedded in paraplast, sectioned at 10 μ M and immunostaining or hematoxylin and eosin staining on sections were performed as described by Kloc et al. (1998).

Actin staining

The actin staining method was adapted from the protocol of Mazurkiewicz and Kubrakiewicz (2001). For whole mount staining, ovaries were fixed in 4% formaldehyde (EM grade, Ted Pella) in PBS with 0.1% Triton X-100 for 30 min at room temperature. After two 15-min

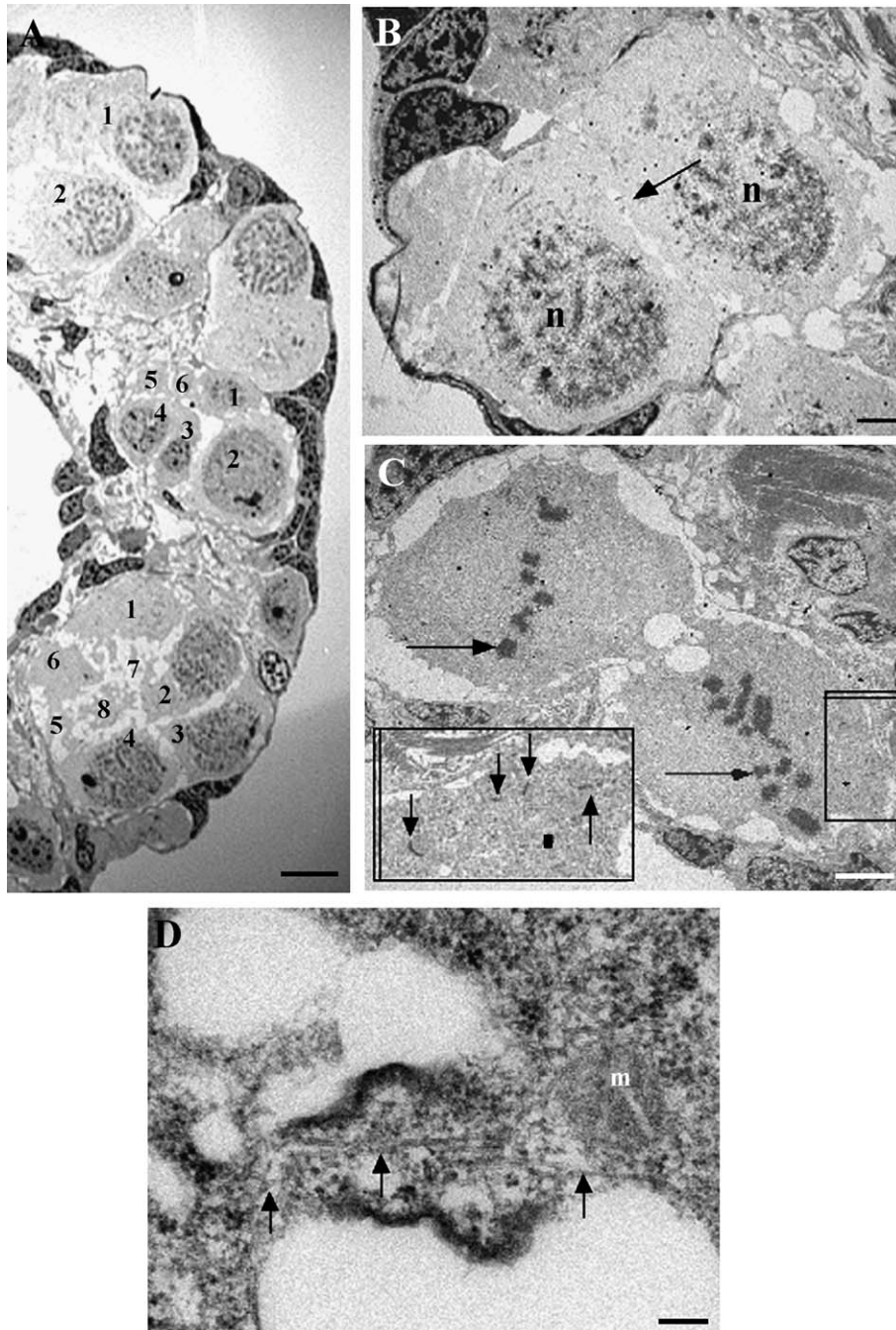


Fig. 1. Ring canals in the *Xenopus* ovary. Electron microscopy image of a fragment of froglet ovary showing several cysts of synchronously developing cystocytes. Numbers indicate individual cells visible in a given cyst (A). Two cystocytes connected by a ring canal (arrow), nuclei (n) (B). Two cystocytes in metaphase of second cystocyte division. Arrows point to the chromosomes in metaphase. Inset shows the region of cell pole with undispersed mitochondrial cement (arrows) (C). High magnification of a ring canal with vesicular cytoplasm and microtubules. Arrows point to the microtubules traversing the canal, mitochondrion (m) (D). Scale bar is equal to 150 μ m in A, 4 μ m in B and C, and 150 nm in D.

washes in PBS–Tween 20, ovaries were blocked for 30 min in 1% BSA in PBS and then stained overnight, in dark, at 4°C, in rhodamine-phalloidin (Molecular Probes, Eugene, OR; 5 μ l of methanolic stock solution of 200 U/ml per 200 μ l of PBS–1% BSA). Ovaries were washed twice, 15 min each in PBS–Tween in dark, mounted in Antifade with 10 μ g/ml of Hoechst (both from Molecular

Probes) and observed with regular or confocal fluorescent microscope.

Whole mount TUNEL staining

Whole mount TUNEL staining was adapted from protocol of [Veenstra et al. \(1998\)](#). Ovaries were fixed in MEMFA

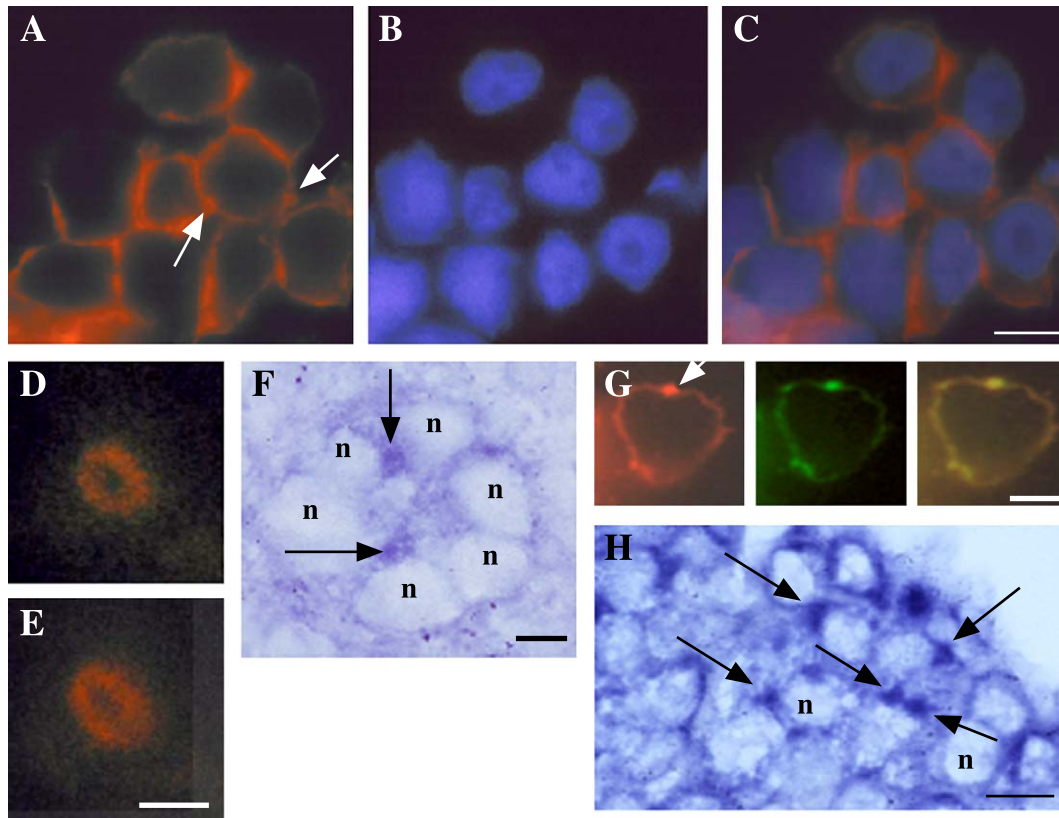


Fig. 2. Ring canals contain actin, kelch and hts. Fragment of a 16-cell cyst, stained for actin (red) with rhodamine-phalloidin. Arrow points to the ring canal (A). Nuclei of the same cyst counter-stained with Hoechst 33258 (B). Merged view of A and B (C). Different views of ring canals stained for actin with rhodamine-phalloidin, single ring canals (D and E). Section of the 8-cell cyst (with only six cells visible) stained with anti-kelch antibody. Arrow points to the localization of kelch protein, nuclei (n) (F). Colocalization of actin (red) and kelch (green) in whole mount staining of a single cystocyte. Merged view (yellow). Arrow points to the ring canal (G). Section of a fragment of ovary stained with anti-hts antibody. Hts is localized in a pattern similar to the location of ring canals (long arrows), nucleus (n) (H). Light microscopy whole mount (A–E and G) and sections (F and H). Scale bar is equal to 10 μm in A–C, 500 nm in D–E, 7 μm in F, 25 μm in G and 10 μm in H.

(100 mM MOPS buffer pH 7.4); 2 mM EGTA; 1 mM MgSO_4 ; 4% formalin for 1–2 h, at room temperature and then transferred to 100% Methanol, at -20°C . The next day, ovaries were washed twice, 15 min each, in PBS–0.05% Tween 20 and equilibrated for 30 min in $1\times$ terminal deoxynucleotidyl transferase (TdT) buffer, at room temperature. Subsequently, they were labeled in $1\times$ TdT buffer containing 0.5 μM digoxigenin-dUTP (Roche) and 150 U/ml of recombinant terminal deoxynucleotidyl transferase (rTdT), overnight, at 37°C . The TdT buffer and rTdT were both from Invitrogen (Carlsbad, CA). Labeled ovaries were washed twice, 1 h for each wash, with PBS containing 1 mM EDTA, at 65°C , and then four times, 1 h for each wash, in PBS, at room temperature. After washing, the digoxigenin label was detected as follows: ovaries were blocked for 1 h in 2% blocking solution in G1 buffer (both from Roche) and incubated overnight with anti-digoxigenin-AP antibody (1:5000 dilution) in 2% blocking solution at 4°C . The next day ovaries were washed in several changes of G1 buffer (for 2 h total) and color reaction was performed using NBT/BCIP tablet solution (Roche). Color reaction was stopped in MEMFA and, to counter-stain the nuclei, ovaries were

mounted in Antifade containing Hoechst 33258 (Molecular probes).

Results

Female germ cells in Xenopus develop synchronously within the cyst and are connected by ring canals

The developing gonads of stage 62–66 *X. laevis* post-metamorphic froglets are already sexually differentiated and the ovaries can be unmistakably distinguished from the testes by their undulated appearance. Ovaries are paired organs approximately 2 mm long and 0.75 mm in width attached to the mesentery at the ventral surface of the kidney. A single ovary contains numerous germ cell cysts surrounded by somatic cells with younger cysts being located at the ovary periphery (cortex) and progressively older cysts located towards its lumen (Fig. 1). Analysis at the light level of serial sections of ovaries showed the presence of 2-, 4-, 8- and 16-cell cysts. By comparing the stage of individual cells within approximately 200 cysts we

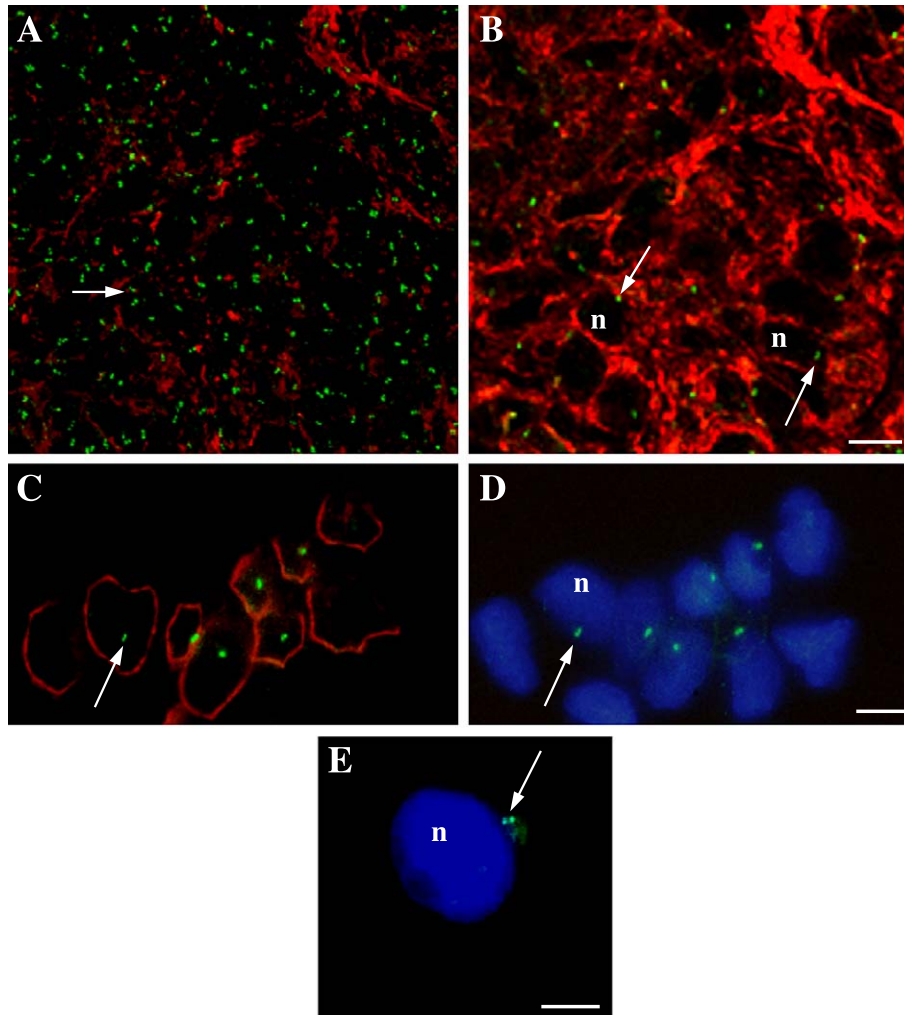


Fig. 3. Distribution of centrioles in the froglet ovary. Confocal image of a whole mount of an ovary from a froglet stained with anti-centrin antibody (green) and costained for actin with rhodamine-phalloidin (red). Numerous centriole doublets (arrow) of somatic cells are visible on the surface optical section of the ovary (A). Deeper optical sections of the same region of ovary showing germline cysts with the centrioles (arrows) at the base of the nuclei (n) (B). Fragment of 16-cell cyst showing centriole doublets (arrow), actin-stained (red) cell boundaries (C) and Hoechst-stained nuclei (n) (D). Single cystocyte showing centriole doublet (arrow) within the PMC, Hoechst-stained nucleus (n) (E). Scale bar is equal to 10 μm in A and B, 8 μm in C and D, and 5 μm in E.

concluded that all the cells within an individual cyst always divide and progress through the cell cycle in synchrony (Figs. 1A–C). Ultrastructural analysis showed that the cystocytes within individual cysts were connected by intercellular bridges that contained an electron-dense rim adjacent to the plasma membrane. However, in contrast to *Drosophila*, the bridges were devoid of an inner rim. The bridges in *Xenopus* were much smaller than ring canals in *Drosophila* (Robinson et al., 1994) and had a diameter ranging from 0.5 to 1 μm compared to 0.5–8 μm in *Drosophila*. The size range depended on the phase of the cell cycle and cystocyte age. *Xenopus* ring canals contained an electron dense rim, and were traversed by numerous microtubules (Figs. 1D and 4D; Tilney et al., 1996; Warn et al., 1985; Woodruff and Tilney, 1998). Since the ring canals in 16 cell cysts in *Drosophila* contain F-actin, the actin cross-linking protein kelch and hts protein (Robinson et al., 1994), we determined if the same

molecules were found in the bridges of *Xenopus* (Kelso et al., 2002; Koch and King, 1969; Robinson and Cooley, 1997; Warn et al., 1985; Xue and Cooley, 1993; Yue and Spradling, 1992). Actin staining with phalloidin-rhodamine and immunostaining with an antibody directed against the first 330 amino acids of the *Drosophila* kelch ORF (kel 1B; Xue and Cooley, 1993) showed that, indeed, the ring canals in *Xenopus* 8- and 16-cell cysts contain actin immunoreactive proteins and kelch (Figs. 2A–G). We also performed co-immunolocalization for actin and kelch to show the colocalization of these two proteins within the ring canals (Fig. 2G). Fig. 2H shows the pattern of immunostaining with an antibody that was raised against the carboxy terminal domain of *Drosophila* hts protein (hts RC; Ding et al., 1993; Robinson et al., 1994). In *Xenopus*, the hts protein was localized in very distinct spots resembling the location of ring canals among the cells of the cysts (Fig. 2H).

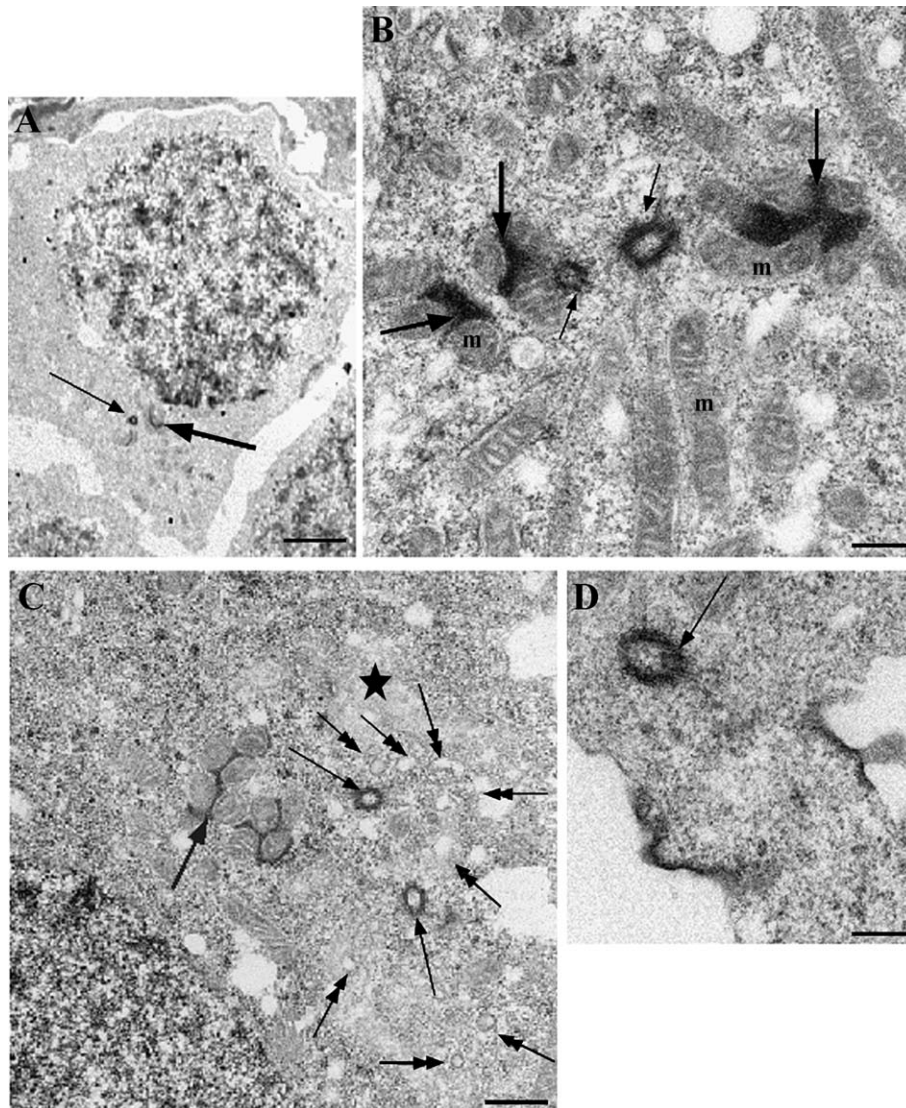


Fig. 4. Localization of centrioles in the cystocytes. Electron microscopy image of cystocyte showing round nucleus and the centriole (thin arrow) within the PMC. The PMC is in the basal part of the cell, thick arrow points to the cement that is visible between the mitochondria (A). High magnification of the central part of PMC showing pair of centrioles (thin arrows) surrounded by mitochondria (m) with mitochondrial cement (thick arrows) (B). Fragment of the cystocyte showing vesicles (double arrows) and electron light cytoplasm (star) of the fusome. Slightly parted centrioles (thin arrows) are located within the fusome. Mitochondria with mitochondrial cement (thick arrow) are visible on the fusome periphery. Fragment of the nucleus is visible in left lower corner (C). High magnification of the centriole (arrow) near the ring canal. Vesicular cytoplasm within the ring canal and numerous microtubules surrounding the centriole are also visible (D). Scale bar is equal to 2 μm in A, 300 nm in B, 600 nm in C and 300 nm in D.

The position of centrioles within the cyst and their spatial relationship to the primary mitochondrial cloud and ring canals

Previous studies showed that in *Xenopus*, the oogonia and prestage I oocytes contain a primary or premitochondrial cloud that is a precursor of the mitochondrial cloud (MC) of stage I oocytes (Al-Mukhtar and Webb, 1971; Coggins, 1973; Kloc et al., 1998, 2001a; Tourte et al., 1981). Many different terms were used for the description of the precursor of the mitochondrial cloud of stage I oocytes such as premitochondrial cloud, precloud or mi-

tochondrial aggregate (Kloc et al., 1996; Kloc et al., 1998). To unify the terminology we will use the following terms: mitochondrial aggregate—the precursor of the mitochondrial cloud present in germ stem cells and primary oogonium; primary mitochondrial cloud (PMC)—the precursor of the mitochondrial cloud present in cystocytes. The PMC contains numerous mitochondria, mitochondrial cement that is believed to be a precursor of germinal granules, and centrally positioned centrioles (Al-Mukhtar and Webb, 1971; Coggins, 1973; Heasman et al., 1984; Kloc et al., 1998, 2001a; Tourte et al., 1981). In the germline cyst of *Drosophila*, the positioning of the cen-

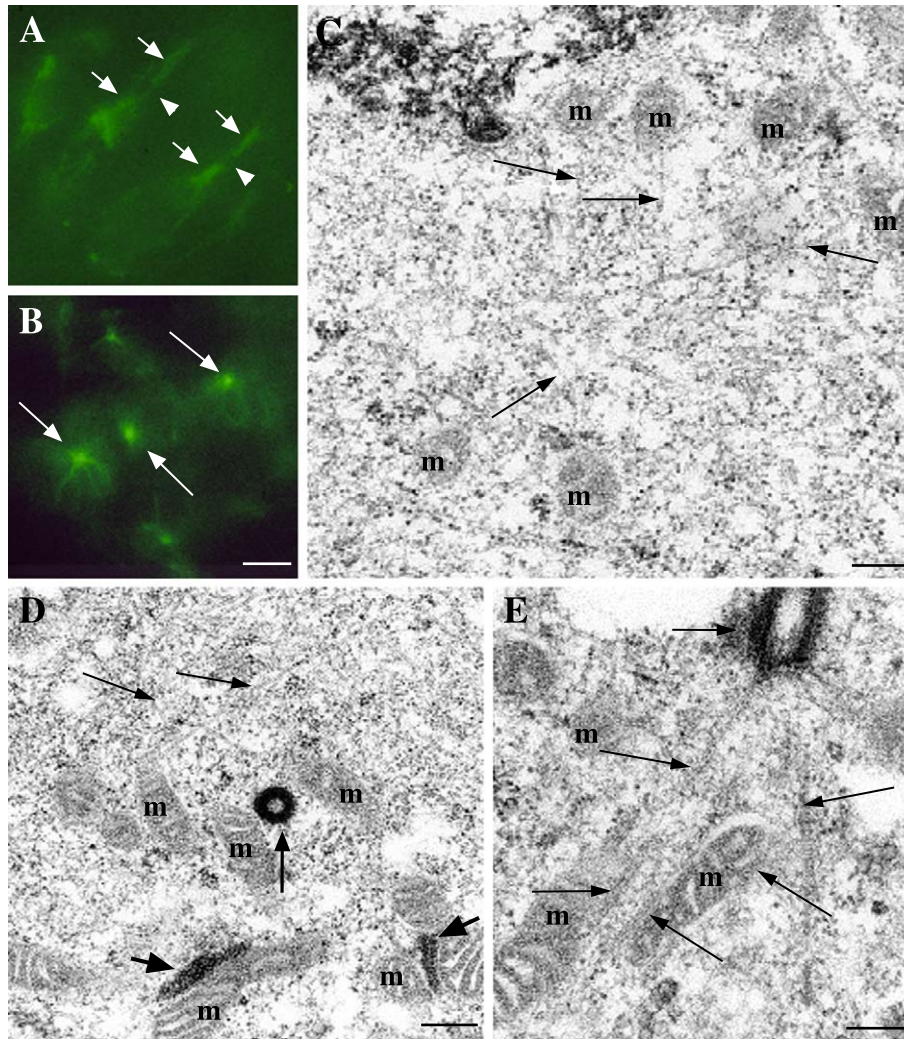


Fig. 5. MTOC activity of centrioles. Whole mount of froglet ovary stained with anti-tubulin antibody (A and B). Microtubules (green, arrows) are visible in the basal parts of cystocytes and traversing the bridges between cystocytes (arrowheads) (A). Asters and spindles (arrows) in mitotic cystocytes (B). Electron microscopy image of microtubules in cystocytes (C–E). Microtubules (arrows) branching toward the mitochondria (m) in the PMC region of the cystocyte. Fragment of the nucleus is visible in the upper left corner (C). Numerous microtubules (arrows) are visible in the PMC region. The centriole (thick arrow) is at the center of the PMC between mitochondria (m) and mitochondrial cement (thick short arrows) (D). Fragment of PMC with the microtubules (arrows) radiating from the centriole (short arrow) toward the mitochondria (m) (E). Scale bar is equal to 14 μm in A and B, 300 nm in C and D, and 200 nm in E.

trioles during cystocyte divisions plays a crucial role in the establishment of cyst polarity. Therefore, we were interested in determining the behavior and spatial arrangement of centrioles in *Xenopus* cysts. Whole mount immunostaining of *Xenopus* ovaries with antibody against centrin allowed us to visualize the spatial distribution of the centrioles within the whole ovary and within individual cysts. Centrin is a low molecular weight (M_r approximately 20000) protein that belongs to the EF-hand superfamily of calcium-binding proteins and is a ubiquitous component of centrioles, centrosomes and mitotic spindle poles (reviewed in Salisbury, 1995; Salisbury et al., 2002). Confocal analysis of immunostained ovaries showed that during the mitotic interphase of all cystocytes the pairs of bodies (presumed to be centrioles based on staining) were

located within the PMC adjacent to the nucleus (Fig. 3). Ultrastructural analysis showed that, indeed, these centrin staining bodies represent a pair of centrioles that are in the center of the PMC, in close contact with mitochondria and the mitochondrial cement (Figs. 4A and B). Interestingly, we found that the mitochondrial cement had not dispersed during cell division but remained attached to mitochondria, and together segregated toward the poles of the spindle (Fig. 1C, inset). In early prophase cystocytes, we detected a pair of slightly parted centrioles that were located more toward the periphery of the cloud and were surrounded by a unique vesicle-rich cytoplasm that we believe represents the fusome ((Fig. 4C; see below). In late telophase cystocytes, we detected a solitary centriole near the ring canal (Fig. 4D).

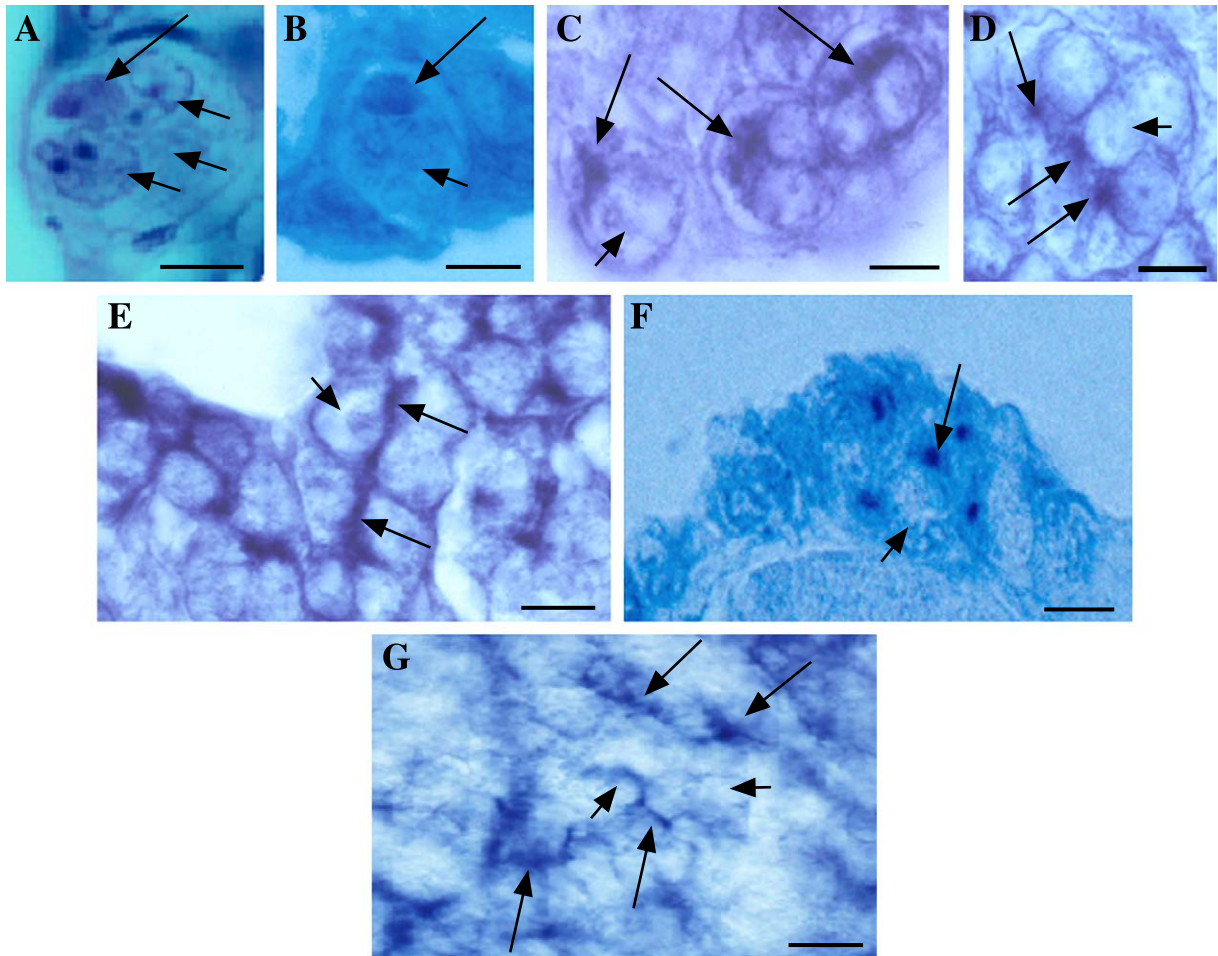


Fig. 6. The fusome contains spectrin and hts. Immunostaining with anti-spectrin and anti-hts antibody on sections (B–F) and whole mount (G) of froglet ovary. Cystoblast showing the mitochondrial aggregate (long arrow) and several lobes of the nucleus (short arrows), hematoxylin and eosin staining (A). Spectrin is present in the mitochondrial aggregate (long arrow) of the cystoblast, short arrow points to the unstained nucleus (B). 2- and 4-cell (C), 8-cell (D) and 16-cell (E) cysts showing the presence of spectrin (long arrows) in the cytoplasm where PMCs and fusome are located. Short arrows point to the nuclei. Cyst of prophase oocytes with spectrin visible as the spots (long arrow) in the middle of the PMCs. Short arrow points to unstained nucleus (F). Several 16-cell germline cysts with branching fusomes stained for the presence of hts protein (arrows). Short arrows point to the nuclei (G). Scale bar is equal to 27 μm in A and B, 10 μm in C–F and 20 μm in G.

Microtubule organizing center (MTOC) activity of cyst centrioles

In *Drosophila*, the centrioles of the cyst display interphase MTOC activity at the early stages of cystocyte formation and also later at the beginning of oocyte differentiation (Grieder et al., 2000; reviewed in Megraw and Kaufman, 2000). Therefore, we wanted to determine the MTOC activity of the centrioles in the developing cyst of *Xenopus*. Using whole mount immunostaining with anti-tubulin antibody and ultrastructural analysis we were able to follow the presence and distribution of microtubules in different stage cysts (Fig. 5). We found that centrioles were active in the nucleation of microtubules not only during cyst divisions, when they generated mitotic spindles (Fig. 5B), but also in cysts in the interphase of consecutive mitoses (Figs. 5A, C–E). Immunostaining and electron microscopy showed the presence of microtubules concentrated at the

basal parts of cystocytes and also traversing the bridges between adjacent cystocytes (Figs. 1D and 5A). In addition, we observed the microtubules emanating from the centrioles that were positioned in the center of the PMC (Figs. 5C and E). Interestingly, these microtubules formed a network that penetrated the interior of the PMC. Microtubules were also visible branching out toward the surface and appeared to dock with the mitochondria (Figs. 5C–E).

*The presence of fusome in the *Xenopus* female germline cyst*

Our previous study on the organization of the germ cell lineage in *Xenopus* showed that the PMCs and MCs of different staged oocytes contained spectrin (Kloc et al., 1998). In *Drosophila*, the fusome is not only rich in spectrin but also in hts protein that is an ortholog of mammalian cytoskeletal protein adducin (Ding et al., 1993; Yue and Spradling, 1992). Therefore, we performed immu-

nostaining with these two antibodies on different stage cysts in *Xenopus*. We found the presence of spectrin in 2, 4, 8 and 16-cell cysts in the areas of cytoplasm also occupied by PMC (Figs. 6C–E). In 8- and 16-cell cysts, the spectrin distribution assumed a characteristic branching pattern in the center of the cyst (Figs. 6D and E). Hts protein was localized near the ring canals (see above and Fig. 2) and also exhibited a branching pattern in the center of the cysts (Fig. 6G). Interestingly, in the nests of meiotic oocytes, as we described previously (Kloc et al., 1998), the spectrin protein was limited to the very center of PMC (Fig. 6F). We were also able to visualize the presence of spectrin in cells that we believe were either germ cell stem cells or the cystoblasts (primary oogonia, the precursor cells of the cyst). These cells were readily recognizable by their large size and lobulated nucleus. They contained a kidney-shaped mitochondrial aggregate that is a precursor of PMC in cystocytes (Fig. 6A; Coggins, 1973; Tourte et al., 1981). We found that the mitochondrial aggregate in these cells contained spectrin (Fig. 6B). Our ultrastructural analysis of female germline cysts in *Xenopus* clearly showed the presence of a structure with similar features as the *Drosophila* fusome. The fusome in *Xenopus*, like the fusome in insects, had a very characteristic appearance, containing numerous vesicles within the electron-light cytoplasm, and very often it was visible surrounding the centrioles (Fig. 4C) and in vicinity of nuclear envelope and inside the ring canals (Figs. 1D and 4D). In *Xenopus*, like in insects, the mitochondria were in close contact with the periphery of the fusome but were always excluded from its interior.

Reconstruction of the architecture and geometry of the cyst

The information that can be extracted from the examination of single sections of the cyst, either at the light or electron microscopy level, is not sufficient for the reconstruction of the architecture of a cyst as a whole. The positioning of the ring canals in the cysts makes it impossible to visualize simultaneously more than two ring canals on the single section. Therefore, to visualize the spatial relationships between the ring canals and other structures within a developing cyst, we performed a computer-aided three-dimensional reconstruction of serial semithin and thin sections from interphase and dividing cysts. This allowed us to count the number of bridges connecting the cystocytes and to recapitulate the branching pattern and geometry of the female germline cyst in *Xenopus*. Fig. 7 shows several examples of such reconstructions from thirty-eight 180 nm thin sections. We were able to determine the position and the number of the bridges present in a given cyst as well as the relationship among the centrioles, PMC and ring canals. These observations are summarized in Fig. 8. Analysis of 40 germline cysts on thin and semithin sections showed that the 16-cell cyst in *Xenopus* contains two cells with four ring canals

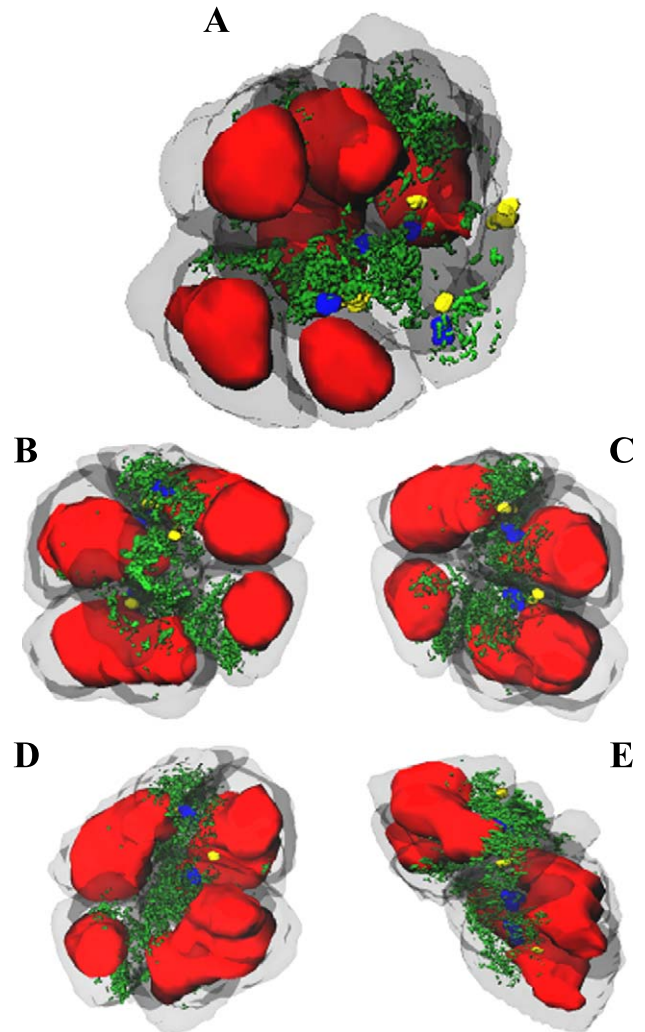


Fig. 7. Three-dimensional reconstruction of interphase cysts. Two different 8-cell cysts with six nuclei visible. Cyst 1 (A) and four different views of cyst 2 (B–E). Cytoplasm is gray, nuclei are red, mitochondria of PMC are green, centrioles blue and ring canals are yellow. In cyst 1, five ring canals and four centrioles near the PMC and ring canals are visible. Spatial relationships between mitochondria, centrioles and ring canals are visible in all reconstructions. Also note the constant distance (2 μm) between the centrioles and ring canals in all cystocytes (see text). PMC, ring canals and centrioles face each other and are located centripetally in “the rosette” conformation (see text). These reconstructions were from 38 serial ultrathin sections similar to the section shown in Fig. 1B.

each, two cells with three ring canals, four cells with two ring canals and eight cells with one ring canal each. Observation of semithin sections of 60 dividing cysts between stages 2 and 8 cells showed that the first two cystocyte divisions in *Xenopus* resulted in the formation of U-shaped cluster of four cells, which is also typical for *Drosophila* (Fig. 8). However, the planes of the next two divisions had different orientations than *Drosophila*, resulting in the formation of a cyst with spatial arrangements of the cystocytes different than *Drosophila* (Fig. 8). In addition, the analysis of serial sections and the rotation of the whole cysts enabled us to examine the spatial

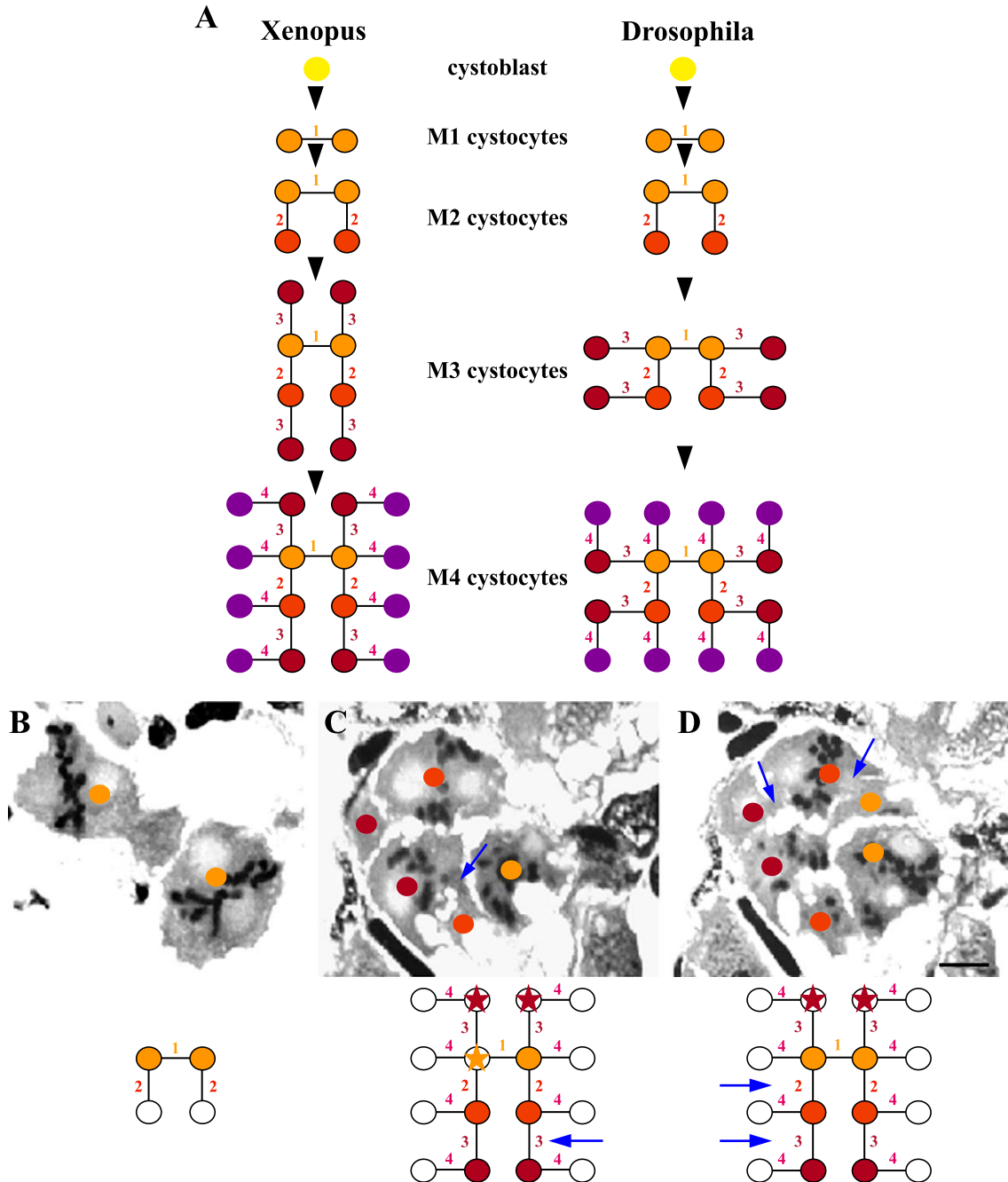


Fig. 8. Geometry of the cyst. Comparison among the pattern of divisions, ring canal distribution and ultimate geometry of female germline cyst in *Xenopus* and *Drosophila*. Numbers from 1 to 4 indicate successive generations of ring canals and cystocytes. Different colors indicate cystocytes of the same generation (A). Actual light microscopy image of two M1 cystocytes (yellow circles) in metaphase of the second division. Drawing underneath shows a 4-cell cyst that will result from this division (B). Two different sections of 8-cell cyst in metaphase of fourth division (C, D). Drawings underneath show a 16-cell cyst that will result from this division. Color circles represent successive generation of cystocytes visible in the section, arrows indicate ring canals visible in the section, stars indicate cells that are present in this cysts but are not visible in the section, white circles represent cystocytes that will form as the result of this particular division. Scale bar is equal to 20 μ m.

relationship among the PMC, centrioles and ring canals. We found that in all cells of an individual cyst all of the clouds faced the center of the cyst (Figs. 7 and 9). We also observed that in different cystocytes the distance between the centrioles and ring canals was always constant (ap-

proximately 2 μ m) (Fig. 7). This observation, together with the fact that centrioles were often situated within the fusome, strongly implied that the position of centrioles relative to the ring canal was fixed by the fusome (summarized in Fig. 10).

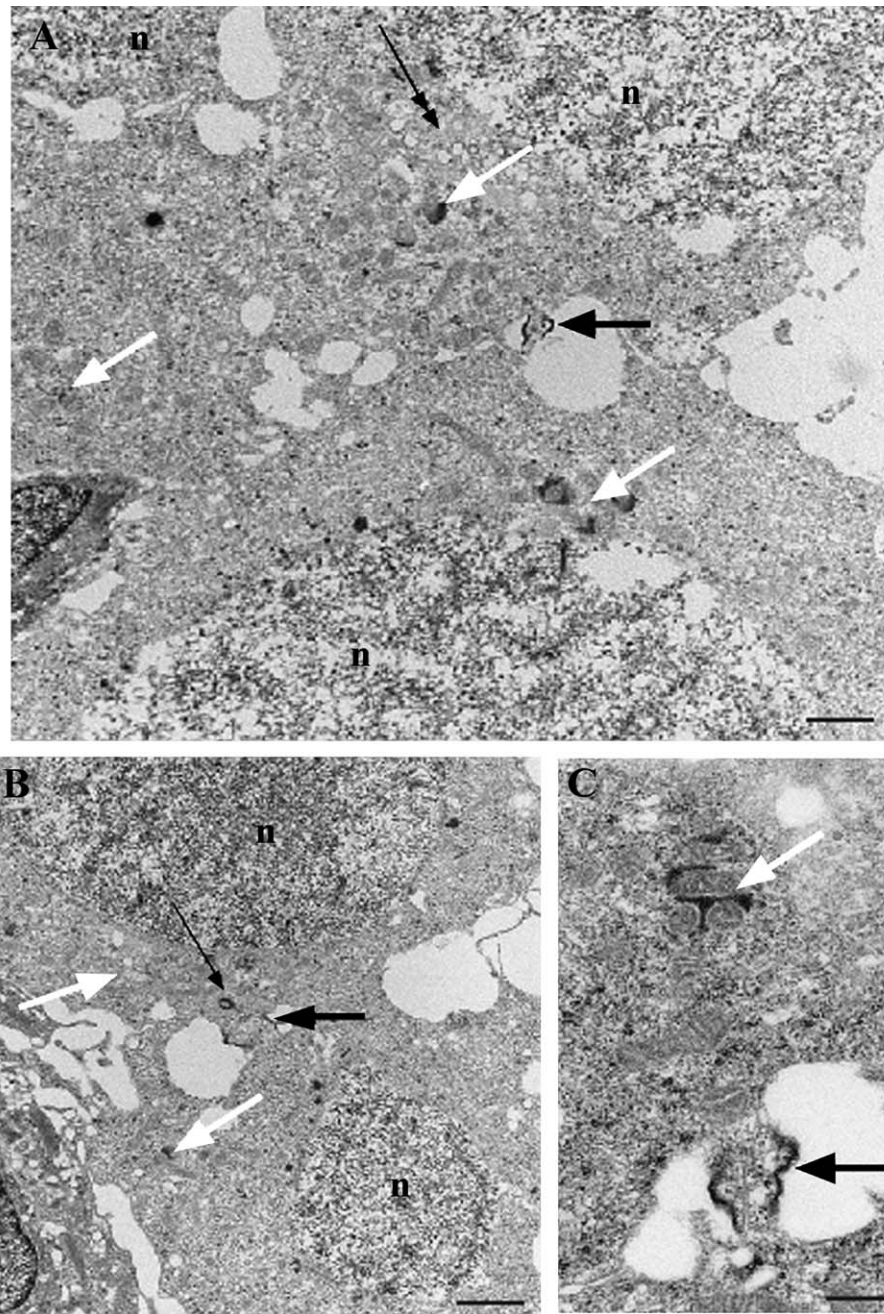


Fig. 9. Spatial relationship among PMCs, centrioles and ring canals. Electron microscopy image of three neighboring cystocytes. In all three cells, the PMCs with mitochondrial cement (white arrows) face the center of the cyst. Ring canal (arrow) is visible between two cells. Double arrow points to the fusome adjacent to the nucleus (A). Two cystocytes connected by the ring canal, with centriole (arrow) at the vicinity of the ring canal. PMC with mitochondrial cement (white arrow) is facing the center of the cyst (B). PMC with mitochondrial cement (white arrow) facing the ring canal (black arrow) (C), nucleus (n). Scale bar is equal to 2.5 μm in A, 1.4 μm in B and 500 nm in C.

Ultimate polarity of the meiotic oocyte cyst

In *Xenopus*, after the final cystocyte division, the 16-cell cyst enters prophase of the first meiotic division and all cystocytes develop into oocytes. It has been well documented that, unlike *Drosophila* where there is a break in synchrony of cyst division immediately after the last cystocyte division, in *Xenopus*, the oocytes progress through

leptotene, zygotene and early pachytene phases of first meiotic prophase in complete synchrony. In late pachytene, oocytes become separated by invading prefollicular cells that break the bridges, and from this moment they develop asynchronously (Al-Mukhtar and Webb, 1971; Coggins, 1973; Hausen and Riebesell, 1991; Tourte et al., 1981). We analyzed the polarity of early pachytene oocytes in 16-cell cysts. Pachytene oocytes are characterized ultrastruc-

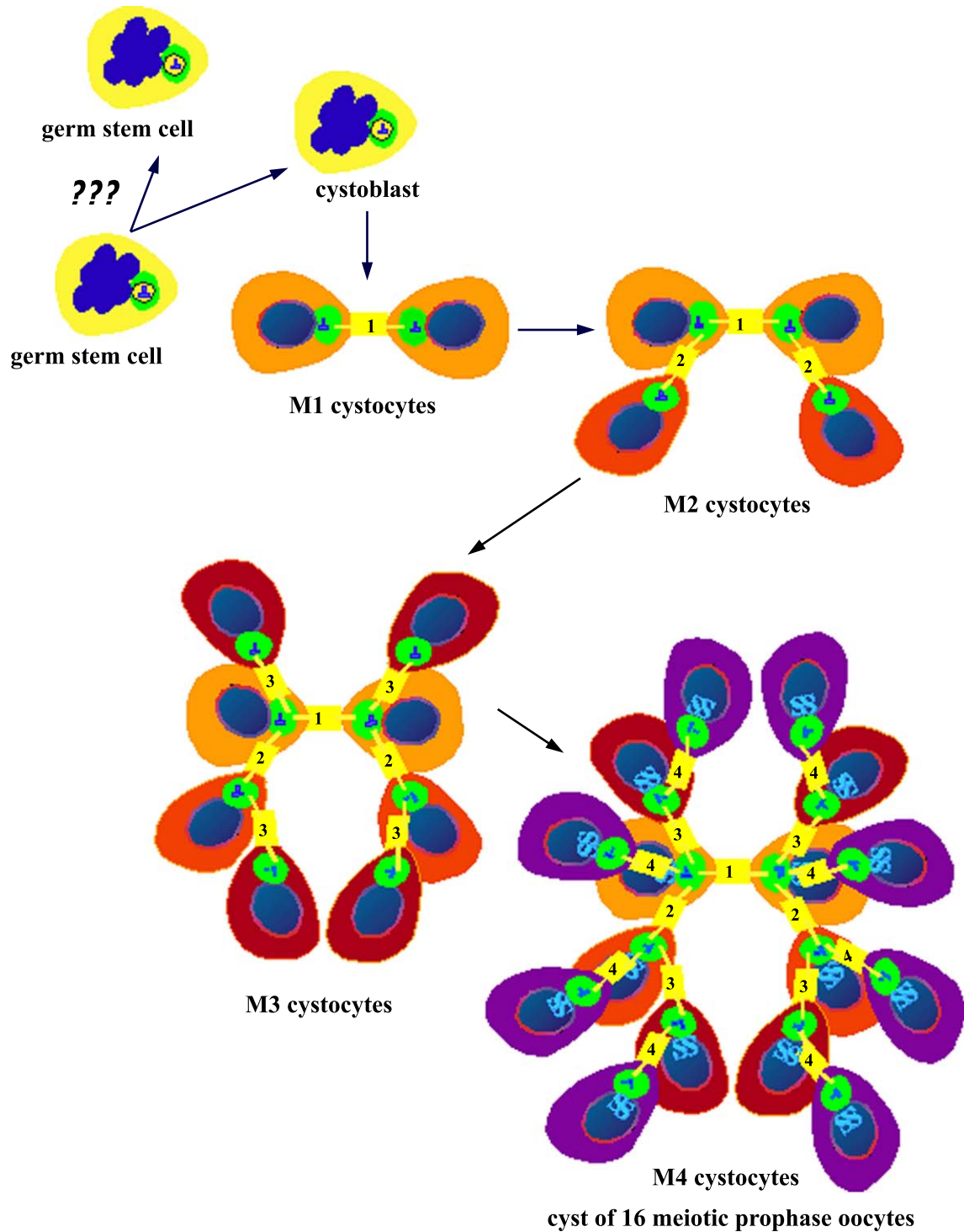


Fig. 10. Summary of the architecture of female germline cyst in *Xenopus*. Germ stem cell and cystoblast that is a precursor cell of the cyst are light yellow. They have a lobulated nucleus and a mitochondrial aggregate that contains spectrin and might be an equivalent of *Drosophila* spectrosome. There is no information available on the pattern of division of the germ stem cell. It is not clear if the division of the germ stem cell results in another stem cell and cystoblast (this is more plausible) or just two cystoblasts that will both form the cysts. Cystocytes of the same generation (M1–M4) are labeled with the same color, ring canals and fusomes are yellow, PMC with centrosomes in the center are green, nuclei are dark blue. Synaptonemal complexes (SS) are blue. Numbers indicate successive generations of ring canals. All ring canals, PMCs, centrosomes and synaptonemal complexes are located centripetally and face the center of the cyst (see text).

turally by the presence of synaptonemal complexes in the nucleus. Synaptonemal complexes contact the nuclear envelope, and they are arranged on the side of the nucleus that

is facing the PMC and the centrosome (Figs. 11A–C). This arrangement has been described in the literature as a bouquet stage. We found that the PMC contacted the nuclear

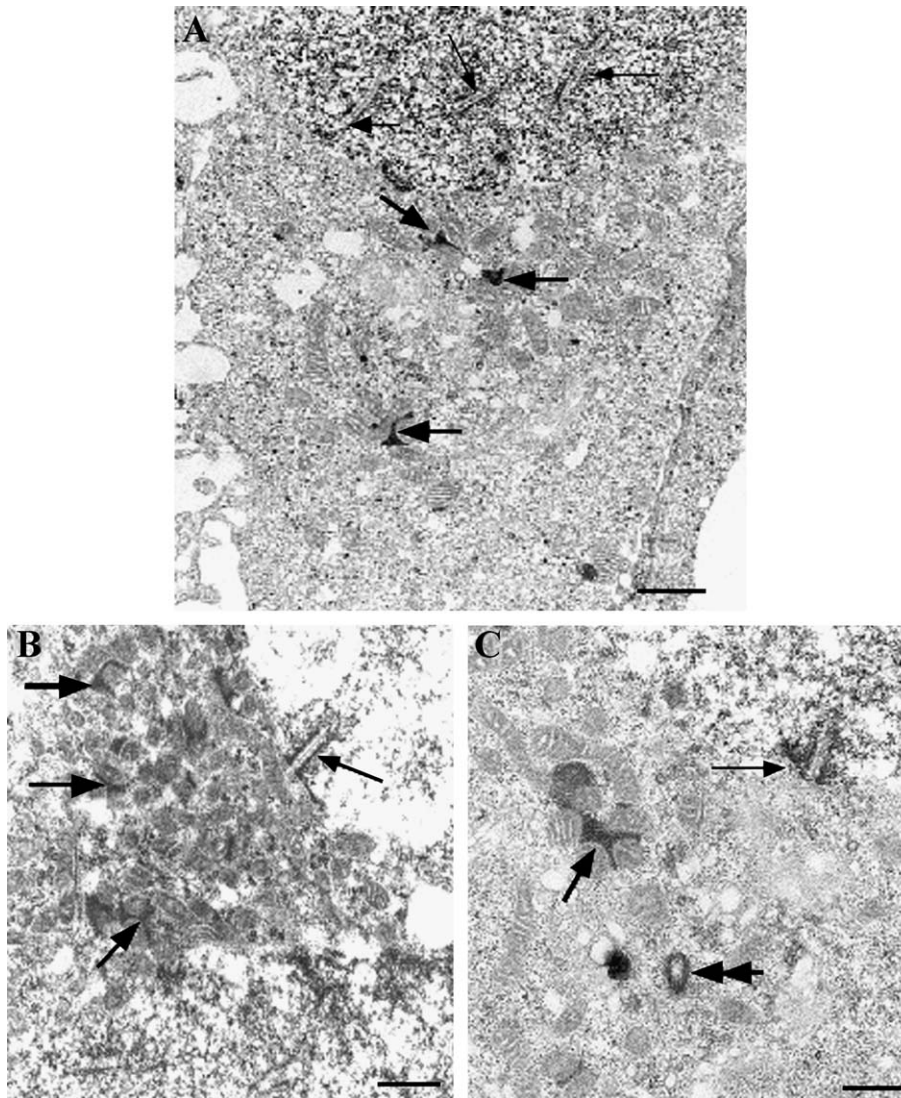


Fig. 11. Axial polarity of prophase oocytes. Fragment of pachytene oocyte with the synaptonemal complexes (thin arrows) facing the PMC. Thick arrows point to the mitochondrial cement within PMC (A). Fragment of pachytene oocyte. Synaptonemal complex (thin arrow) and PMC adjacent to the nuclear envelope. Thick arrow points to the mitochondrial cement of PMC, double arrow points to the centriole (B, C). Scale bar is equal to 900 nm in A and B, and 700 nm in C.

envelope in the region of the attachment of the synaptonemal complexes (Fig. 11B). This association created a very conspicuous axial polarity of the oocyte (Figs. 10 and 11). This polar conformation with all PMCs gathered at the center of the cyst was observed in all oocytes examined within an individual cyst. Thus, the whole cyst assumed the conformation that we believe is an equivalent of “the rosette”—a conformation typical of the cyst in *Drosophila* and other insects (reviewed in Telfer, 1975).

Do all cystocytes in the individual cyst survive and differentiate into oocytes?

We attempted to address the question of whether all cells in individual cysts survive and differentiate into oocytes in *Xenopus*. Recently, Pepling and Spradling (2001) showed that the perinatal reduction in germ cell number in mouse is

the result of programmed breakdown of germline cyst between 20.5 and 22.5 dpc and that only 33% of the oocytes survive to form primordial follicles. To address the issue of loss of oocytes within a cyst we performed whole mount TUNEL staining on differentiating *Xenopus* ovaries (Fig. 12). TUNEL staining allows labeling of the free ends of broken DNA molecules that are customarily considered as indicators of cell death and apoptosis (Veenstra et al., 1998). We found that there are a few apoptotic cells especially in the cortical region of the ovary where mostly younger cysts are located (Fig. 12A). However, the distribution and frequency of apoptotic cells did not follow any periodic pattern and apoptotic cells were usually located outside of and between the individual cysts. This suggested that the apoptotic cells were probably apoptotic prefollicular cells (Figs. 12C and D). This observation as well as the observation that the part of the ovary containing older cysts

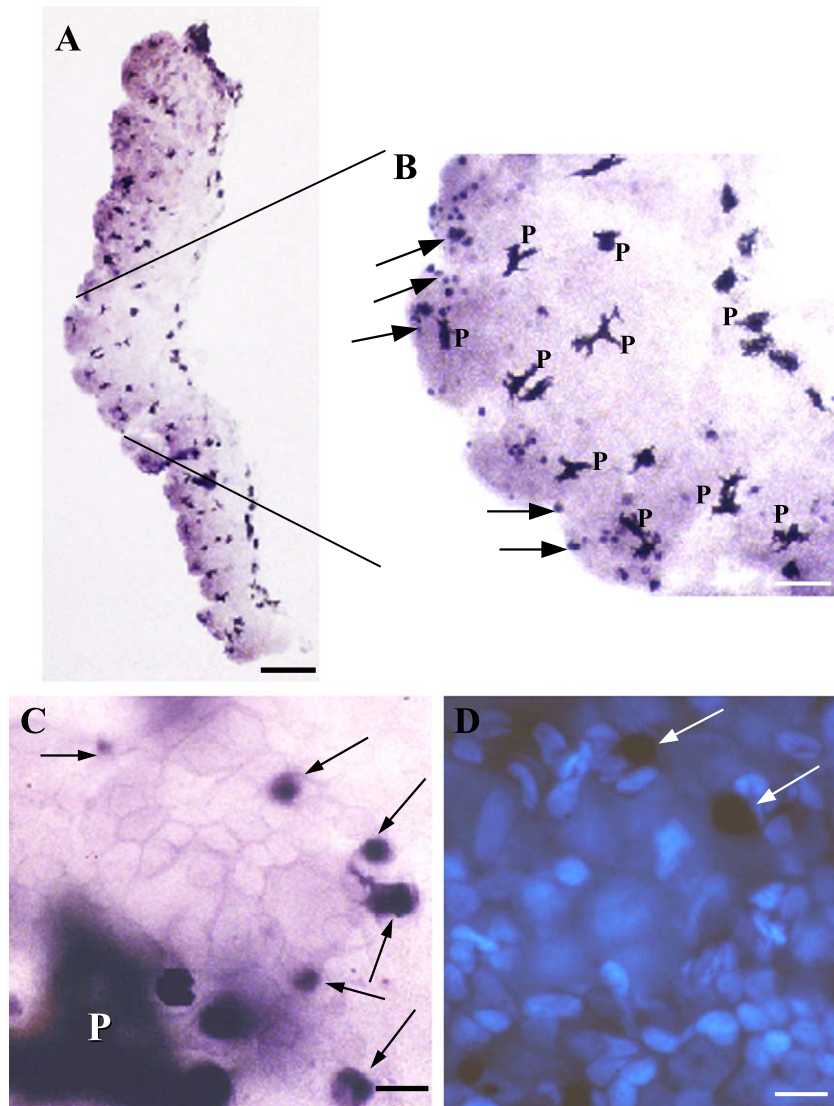


Fig. 12. Apoptosis in froglet ovary. Whole mount TUNEL staining. Whole ovary (A) and its fragments showing randomly distributed apoptotic cells (arrows) in the cortex of the ovary. P—pigment in the malanocytes (B). Higher magnification of the ovary showing unstained cyst in the center and apoptotic cells (arrows) located at the periphery and between the cysts, TUNEL staining (C) and Hoechst and TUNEL double staining (D). Scale bar is equal to 350 μm in A, 200 μm in B and 80 μm in C and D.

showed no or very little apoptosis suggested that in *Xenopus*, all cells of the cyst survive and differentiate into oocytes (Fig. 12).

Discussion

Although the formation and architecture of the female germline cyst has been comprehensively studied in *Drosophila* and several other insect species, very little is known about these processes in vertebrates. We found that the female germline cyst in *Xenopus* forms according to the 2ⁿ rule, by four consecutive mitotic divisions with incomplete cytokineses. Also, similar to female cystocytes in *Drosophila* and mouse, female cystocytes in *Xenopus* are connected by intercellular bridges called the ring canals

(Koch and King, 1969; Mahowald, 1971; Pepling and Spradling, 1998, reviewed in de Cuevas et al., 1997). The fact that some of the elements of ultrastructure and composition (presence of actin, kelch and, probably, htsRC protein) of ring canals in *Xenopus* are similar to those in *Drosophila* also points to the similarities in these structures between vertebrates and invertebrates. We found that the geometry of the first two cystocyte divisions in *Xenopus* is identical to that in *Drosophila*, resulting in the formation of U-shaped clusters of four cystocytes. However, the two subsequent divisions have different planes in *Xenopus* than in *Drosophila*. As a result, the final spatial geometry of the cyst of *Drosophila* and *Xenopus* is slightly different. The cyst in *Xenopus*, like the cyst in *Drosophila*, is highly branched and has the same number and pattern of ring canals: two oldest cystocytes with four ring canals, two with

three ring canals, four with two and eight cystocytes with one ring canal.

In insects, a species-specific geometry and branching pattern of the germline cyst result from the anchoring of centrioles and the poles of mitotic spindles to the fusome. The fusome is a specialized germline-specific structure consisting of membranous vesicles and tubules that contain membrane cytoskeletal proteins such as spectrin and adducin-like hts protein, motor proteins and cyclins (reviewed in de Cuevas et al., 1997). The fusome not only dictates the planes of divisions but is also responsible, probably through the activity of cyclin A, for counting the total number of cystocyte divisions. Also, it is involved in the transport of molecules within the differentiating cyst (Lilly et al., 2000).

In *Drosophila*, the fusome originates in the spherical, spectrin-rich organelle called the spectrosome that is present in the cystoblast (Deng and Lin, 1997). Pepling and Spradling, (1998) observed a cytoplasmic structure that may correspond to the spectrosome in early female germ cells of the mouse; however, they were not able to find any ultrastructural or biochemical evidence of the fusome in the mouse ovary. This led them to conclude that the requirement for a fusome in the mouse germline cyst has been altered or eliminated. Our studies demonstrate that in *Xenopus*, a precursor cell of the cystocytes (cystoblast or germ stem cell) contains a single, spectrin-rich mitochondrial aggregate that is a precursor of the PMC. Therefore, the PMC may be the equivalent of the spectrosome of *Drosophila*.

We also found that the female *Xenopus* germline cyst contains a fusome which under the light microscope is less distinct than that of *Drosophila*. However, the ultrastructural features of the *Xenopus* fusome are similar to those of *Drosophila* and other insects in that it contains numerous vesicles similar in appearance to the smooth endoplasmic reticulum located within an electron-light cytoplasm (reviewed in Telfer, 1975). In *Xenopus*, this structure is found within the PMC in contact with centrioles as well as being near the nuclear envelope. We found that the region of the cytoplasm that contains the PMC, fusome and ring canals, also contains α and β spectrin and hts protein. This suggests that not only the ultrastructure, but also the biochemical composition of the fusome is similar in insects and *Xenopus*. In 8- and 16-cell *Xenopus* cysts, the spectrin and hts staining show a continuous and branching pattern in the center of the cyst. In addition, in the EM, vesicle containing cytoplasm typical for the fusome is visible within *Xenopus* ring canals. This may indicate that the *Xenopus* fusome, like its counterpart in the insect, spans the ring canals between the cystocytes and forms an interconnected and ramified polyfusome (Storto and King, 1989). We showed that in early prophase cystocytes of *Xenopus* the centrioles are often found within the fusome. The inference of this finding is that like insects, the fusome in *Xenopus* plays a role in the anchoring of centrioles and thus guiding the planes of division and spatial geometry of the cyst.

In interphase *Xenopus* cysts, the centrioles are located at the center of the PMC and are surrounded by mitochondria and mitochondrial cement. Also, we observed numerous microtubules radiating from the centrioles spreading throughout the PMC and in close contact with mitochondria and mitochondrial cement. This suggests that, similar to *Drosophila*, *Xenopus* centrioles display an interphase MTOC activity before the final (fourth) cystocyte division. After the final division of the female germline cysts of *Drosophila*, 1 of the 16 cystocytes becomes an oocyte, the rest of the 15 cells become the nurse cells (reviewed in de Cuevas et al., 1997; King et al., 1982). In *Drosophila*, the MTOC activity of the pro-oocyte centrioles is responsible for the creation of cyst polarity and the directional transport of organelles and molecules into the future oocyte (Mahowald and Strassheim, 1970; Theurkauf et al., 1993; reviewed in Cooley and Theurkauf, 1994). In *Xenopus*, there are no nurse cells and all 16 cystocytes differentiate into oocytes. This indicates that there is probably no need for directional transport in the *Xenopus* cyst, although there is probably a need for the transport of synchronizing factors among the early cystocytes. We suggest that the MTOC activity of centrioles in the *Xenopus* cyst is responsible for the organization of the PMC around the centrioles. This would include microtubule-directed transport of mitochondria with attached mitochondrial cement toward the centrioles after each division. The close contact between the branching network of microtubules and mitochondria and the positioning of the centriole in the very center of the cloud that we found in *Xenopus* cystocytes strongly support this hypothesis. The organization of nuage and germinal granules around the centrioles was also found in *C. elegans* and *Drosophila* (Counce, 1963; Mahowald, 1962, 1968; Pitt et al., 2000).

The roles of MTOC and microtubules in the organization and the movement of mitochondria have been reported for many different animal and plant species and various cell types (Fuchs et al., 2002; Knabe and Kuhn, 1996; Ligon and Steward, 2000; McDaniel and Roberson, 2000; Morris and Hollenbeck, 1995). It is also believed that the “docking” of mitochondria to microtubules stabilizes their pertinent distribution within the cell (Hollenbeck, 1996). We believe that in *Xenopus*, like in *Drosophila*, the positioning of the centrioles within the cystocytes is determined by anchoring at the fusome. We observed an intrinsic spatial relationship among the fusome, centrioles, microtubules and PMC in *Xenopus*. We postulate that the fusome is responsible, via positioning of the centriole, for the positioning and the organization of the PMC. In *Drosophila*, it appears that the fusome is able to aggregate the mitochondria on its surface supporting this possibility (Storto and King, 1989). An alternative is that in *Xenopus*, the fusome may be involved in the positioning and the organization of the PMC through the direct organization of microtubules. Recently, it has been shown that in *Drosophila*, the fusome has direct contact with the microtubule network in developing cysts and it was suggested that the fusome may be directly

involved in the polarization or even nucleation of the microtubules (Grieder et al., 2000).

In *Xenopus*, the fusome is often visible near the nuclear envelope of cystocytes and oocytes on the side where the synaptonemal complexes of the bouquet stage oocyte are attached, that is, on the side of the nucleus facing the PMC. This suggests the possibility that in *Xenopus* the fusome is not only involved in the polarization of the divisions in the cyst, but is also responsible for the polarity of the internal organization of the chromosomes within the oocyte nucleus and ultimately for the establishment of axial polarity of the whole oocyte.

A fascinating question is what are the mechanisms and factors responsible for the differentiation of cystocytes into pro-oocytes and ultimately into oocytes. For a long time, it was believed that the number of ring canals was an indicator of the cystocyte fate. This was supported by the fact that in insects, out of 16 cystocytes, only two cystocytes with the highest number of ring canals enter the pro-oocyte pathway of differentiation. The most recent theory is that the determination of the cystocyte fate occurs even before the formation of the cyst during the very first division of the cystoblast (reviewed in: de Cuevas et al., 1997; Deng and Lin, 2001). It has been postulated that during this first division, the spectrosome of the cystoblast becomes asymmetrically divided between two daughter cells, and the cystocyte that receives the bulk of spectrosome becomes the future oocyte (Lin and Spradling, 1995). Our analysis of cyst formation and oocyte determination in *Xenopus* adds some valuable information to these interpretations. In *Xenopus*, all cystocytes of the cyst, independent of the number of ring canals, enter synchronously into the prophase of meiosis and progress through the oocyte differentiation pathway. This suggests that there is no relationship between the number of ring canals and the cell fate. It will be extremely interesting to see how the first division of the cystoblast looks in *Xenopus*. If it is true that the segregation of the spectrosome dictates the fate of daughter cells, then we postulate that, in contrast to *Drosophila*, the spectrosome (i.e., the mitochondrial aggregate) in *Xenopus* should be symmetrically divided between two daughter cystocysts.

Another important question is which aspects of the *Xenopus* model of cyst formation and oocyte differentiation are unique to *Xenopus* and which are universal for all vertebrates. It was commonly believed that the presence of the mitochondrial cloud in oocytes of *Xenopus* is an oddity shared by a very limited number of invertebrate and vertebrate species (Kloc et al., in press). It was also believed that in the majority of vertebrates including all mammals, the oocytes are symmetrical and that their differentiation proceeds through a pathway unrelated to that in *Xenopus* (reviewed in de Smedt et al., 2000). However, in many vertebrates, including all marsupials and majority of mammals, with the noticeable exception of mouse, oocytes are clearly asymmetrical and contain a prominent mitochondrial

cloud (Falconnier and Kress, 1992; Toyooka et al., 2000; Young et al., 1999; reviewed in: de Smedt et al., 2000; Motta et al., 2000). Recently, Pepling and Spradling (2001) showed that in mouse germline cyst (just before its programmed breakdown) mitochondria and ER aggregate perinuclearly and can be seen within the ring canals joining adjacent germ cells. This suggests that the mechanisms and factors operating in the formation of female germline cyst and of early polarity of the oocytes in *Xenopus* are probably more universal than previously thought. Our studies indicate that the processes responsible for the formation of female germline cysts and the establishment of germ cell polarity are highly conserved between invertebrates and vertebrates. It seems that the dissimilarities that we found between *Drosophila* and *Xenopus* and the uniqueness of each system evolved through the modifications of the same fundamental design of the germline cyst.

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