Intracellular compartmentation of CTP synthase in *Drosophila*

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10 Figures and 5 supplementary figures

Abstract

Compartmentation is essential for the localization of biological processes within a eukaryotic cell. ATP synthase localizes to organelles such as mitochondria and chloroplasts. By contrast, little is known about the subcellular distribution of CTP synthase, the critical enzyme in the production of CTP, a high-energy molecule similar to ATP. Here I describe the identification of a novel intracellular structure containing CTP synthase, termed the cytoophidium, in *Drosophila* cells. I find that cytoophidia are present in all major cell types in the ovary and exist in a wide range of tissues such as brain, gut, trachea, testis, accessory gland, salivary gland and lymph gland. In addition, I find CTP synthase-containing cytoophidia in other fruit fly species. The observation of compartmentation of CTP synthase now permits a broad range of questions to be addressed concerning not only the structure and function of cytoophidia but also the organization and regulation of CTP synthesis.

Key words: CTP synthase; cytoophidium; *Drosophila*; organelle; cilium; oogenesis

Introduction

A eukaryotic cell is divided into compartments such as the nucleus, the endoplasmic reticulum, the mitochondrion, Golgi bodies, and the cilium (Alberts et al., 2008). Compartmentation ensures increased structural and functional complexity within a cell's organization. Each compartment is functionally specialized, containing distinct catalytic processes, other specialized molecules, and specific microenvironments. High concentrations of macromolecules localize to these compartments, allowing the cell to perform various metabolic activities efficiently and simultaneously. Specific products are transported between compartments using complex distribution systems (Alberts et al., 2008).

Nucleotides not only act as constituents of nucleic acids, but also serve as energy carriers, participate in cellular signalling, and act as important cofactors of enzymatic reactions (Nelson and Cox, 2000). Synthesis of nucleotides is tightly regulated within the cell. For example, ATP synthase, the enzyme responsible ATP generation, is localized to mitochondria of animal cells and chloroplasts of plants and algae. The compartmentation of ATP synthase into organelles is very important for its function (Alberts et al., 2008).

CTP synthase is a glutamine amidotransferase enzyme that catalyzes the ATP-dependent transfer of the amide nitrogen from glutamine to the C-4 position of UTP to generate CTP (Lieberman, 1956; Long and Pardee, 1967). The CTP synthase reaction product CTP is an essential nucleotide and precursor for the synthesis of RNA, DNA, and sialoglycoproteins. CTP also plays an essential role in the synthesis of all membrane phospholipids in

Saccharomyces cerevisiae and in mammalian cells. CTP is the immediate precursor of the activated, energy-rich phospholipid-pathway intermediates CDP-diacylglycerol, CDP-ethanolamine and CDP-choline . However, in contrast to previous extensive studies on the cellular organization of ATP synthase, little is known about whether and how CTP synthase is compartmentalized within the cell.

Here I describe the identification of a novel intracellular compartment containing CTP synthase in *Drosophila*. I detect CTP synthase using three antibodies specifically targeted to different regions of the CTP synthase molecule, as well as by two GFP-CTP synthase protein trap flies. I consistently detect filamentary structures in *Drosophila* cells using all five CTP synthase markers. I refer to these subcellular snake-like structures as cytoophidia (Greek: *cyto*-, meaning cell, and *ophidia*, meaning serpents). Multiple types of cytoophidia-containing CTP synthase are detected in ovarian cells such as follicle cells, nurse cells and oocytes. In addition, I find that CTP synthase is compartmentalized within cytoophidia in cells from a wide range of *Drosophila melanogaster* tissues, such as brain, gut, testis, accessory gland, salivary gland, trachea, and lymph gland. Finally, I present evidence that cytoophidia containing CTP synthase are present in other species.

MATERIALS AND METHODS

Drosophila stock

Drosophila melanogaster stocks were raised at 21°C on standard cornmeal. The fly strain used in this study was *y w.* Protein traps of GFP-CTP synthase (CA06746 and CA07332) were provided by M. Buszczak and A. Spradling, Carnegie Institution of Washington, Baltimore, Maryland, USA (Buszczak et al., 2007). Fly stock GFP-SAS-4 was provided by J. Raff, University of Oxford, Oxford, UK (Dix and Raff, 2007).

Whole-mount tissue preparation

Tissues were dissected in Grace's medium (Invitrogen Ltd., Paisley, UK) and fixed in 4% paraformaldehyde in PBS at room temperature. Paraformaldehye was washed off with PBS for 30 minutes.

Immunostaining

Whole-mount tissues were stained with antibodies using a previously described protocol (Liu and Gall, 2007). The following antibodies were used in this study. Rabbit anti-CTP synthase, goat anti-CTP synthase, and rabbit anti-phosphorylated CTP synthase (active form) were acquired from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). One batch Rabbit and two batches of rat anti-Cup sera were provided by A. Nakamura, Riken Center for Developmental Biology, Kobe, Hyogo, Japan (Nakamura et al., 2004). One batch from rat-anti Cup serum recognizes sperm tails and was renamed the "anti-Cup* antibody". Rat anti-Cup serum, mouse mAB anti-Lava Lamp (Actin-binding protein 2), and mAB anti-complex II alpha were provided by A.

Spradling, Carnegie Institution, Baltimore, Maryland, USA (Keyes and Spradling, 1997). Mouse mAB anti-acetylated tubulin was T7451 from Sigma-Aldrich Company Ltd., Dorset, UK). A mAB anti-gamma tubulin (T-6559) was also acquired from Sigma. Rabbit anti-SMN was provided by J. Zhou, University of Massachusetts, Amherst, Massachusetts, USA. Alexa 488-, Cy5- or Alexa-633 labelled goat or donkey anti-rabbit, rat or mouse IgG (invitrogen) were used as secondary antibodies. DNA was stained with Hoechst 33342.

Confocal microscopy

Images were acquired under 40X or 63X objectives on a laser-scanning confocal microscope (Zeiss LSM 510 META, Oberkochen, Germany).

Results

Identification of novel filamentary structures, cytoophidia, in *Drosophila*

Both eIF4E and its binding partner Cup are involved in translation regulation (Nakamura et al., 2004). In *Drosophila* ovaries, the Cup protein colocalizes with eIF4E in the same structures, cytoplasmic processing bodies (P bodies) (Sheth and Parker, 2003; Wilhelm et al., 2003; Wilhelm et al., 2005; Liu and Gall, 2007; Lee et al., 2009). In an attempt to find P bodies in male gonads, I stained larval and adult testes using a number of P body markers including multiple antibodies against the Cup protein. As expected, all anti-Cup antibodies showed punctate staining in the cytoplasm of many cells, presumably reflecting P bodies in testes. However, one batch of anti-Cup antibody stained sperm tails in larval and adult testes (Fig. S1). All other anti-Cup antibodies showed no signal on sperm tails, suggesting that the sperm tail staining pattern was likely due to an artefactual cross-reaction. To avoid confusion with the target protein Cup, I refer to that specific batch of antibody with an apparently cross-reacting antibody as the anti-Cup* antibody because of its cross-reactivity. The intensity of the staining on sperm tails was much higher than that seen for P bodies.

The ovary of the fruit fly *Drosophila melanogaster* has been extensively studied and provides an excellent model system for genetics, cellular and developmental biology. A typical ovary in adult flies contains about 16 ovarioles, each being tipped with the germarium which is followed by growing egg chambers (Spradling, 1993). Each egg chamber contains three major cell types: one oocyte, 15 nurse cells, and hundreds of follicle cells. The cuboid-

shaped follicle cells form a monolayer surrounding the germline cells (nurse cells and the oocyte). When staining ovaries in adult flies with the anti-Cup* antibody, I was surprised to observe filamentary structures in follicle cells, nurse cells and oocytes (Fig. 1). These filamentary structures (especially those in germline cells) exhibit a characteristic elongated and serpentine form. For this reason, I shall refer to them as cytoophidia.

The cytoophidium contains CTP synthase

Similar filamentary structures could also be detected by GFP in two protein trap fly stocks, CA06746 and CA07332, from the Carnegie Protein Trap Library (Joseph Gall, personal communication) (Figs. 2, F and S2) (Buszczak et al., 2007). In these two stocks, CTP synthase (CG6854) has been independently engineered to produce protein segments fused to GFP under normal controls. Many protein trap lines have been used to reveal details of protein localization (Morin et al., 2001; Buszczak et al., 2007; Quinones-Coello et al., 2007). Three antibodies against CTP synthase yield an identical pattern in ovarian cells from wild-type flies (Fig. 2, A-D). Moreover, I performed double-labeling experiments and observed that the filamentary structures detected by GFP in both protein trap lines are also labeled by the anti-Cup* antibody (data not shown) and by antibodies against CTP synthase (Fig. 2, E-F). These experiments confirm CTP synthase as a *bona fide* component of the cytoophidium. Cytoophidia in ovarian cells can be detected by an antibody that specifically targets active CTP synthase, suggesting CTP synthase within the cytoophidium is functional.

Growth of cytoophidia in follicle cells

Growing egg chambers in each ovariole are divided into 14 developmental stages with Stage-14 being the most mature stage (Spradling, 1993). Two follicle stem cells in each germarium produce daughters which give rise to all subtypes of follicle cells (Nystul and Spradling, 2007). Follicle cells form a layer of epithelium surrounding 16 germline cells. The germline cells and their associated follicle cells bud off from the germarium to form individual egg chambers. The follicle cells divide and grow during the development of egg chambers.

Follicle cells within the same egg chambers were observed to contain cytoophidia of similar lengths. However, cytoophidia in follicle cells from egg chambers at different stages were found to differ greatly in their lengths (Fig. 3). Short cytoophidia appear in follicle cells within the germarium. Cytoophidia in follicle cells before stage 4 are less than 1 μm in length (Fig. 3, B) but become longer in later stages. Cytoophidia of follicle cells are approximately 4 μm long in stage 8 egg chambers (Fig. 3, E and F) and extend to 5-6 μm in stages 9 and 10 egg chambers (Fig. 3, G and H). Normally only one cytoophidium is observed per follicle cell. From a lateral view, some cytoophidia protrude out from the outside surface of the egg chamber surface (Fig. 1, B).

Cytoophidia in follicle cells are associated with the microtubular network

CTP synthetase 1 has been shown interacted with Tubulin in cultured HEK 293 cells (Higgins et al., 2008). To determine whether cytoophidia in *Drosophila* follicle cells are associated with microtubules, double-labelling experiments were performed with a combination of cytoophidia, and microtubule markers alpha-tubulin and acetylated tubulin. Cytoophidia tend to be located at the boundary of follicle cells with connections made to the microtubular network. Both alpha-tubulin and acetylated tubulin labels exhibit a mesh-like network in follicle cells. Cytoophidia labelled by the anti-Cup* antibody or CTP synthase were always observed to be associated with this network (Fig. 4). Occasionally, the acetylated tubulin signal in the proximity of the cytoophidium was seen to be thinner than the signal derived from the Cup* antibody. Nevertheless, the base of the cytoophidium extends along the rest of the acetylated tubulin network (Fig. 4, M-O).

Cytoophidia in most follicle cells are tilted, which can be clearly visualized through both surface and lateral views, as well as 3-dimensional reconstructions of multiple confocal projections (Figs. 1-4). Cytoophidia in neighbouring follicle cells tilt at various angles, following the paths of neighbouring microtubular structures. From the lateral view of the follicle cells in stage 10 egg chambers, it is seen that the majority of cytoophidia are embedded in the microtubule mesh and point to the posterior direction (Fig. 4, D-I).

Cytoophidia in follicle cells are not associated with centrioles

Given their morphology, position, and number and their association with microtubules, I suspected that the cytoophidia in follicle cells recognized

by anti-Cup* antibody or GFP-CTP synthase might be primary cilia. Each cilium extends from the basal body, a cytoplasmic organelle containing centrioles. In *Drosophila*, the monolayer follicle cells form a polarized epithelium with the apical side facing the germline and the basal side forming the surface of the egg chamber (Spradling, 1993). The centrioles of follicle cells preferentially localize to their apical sides. If the cytoophidium in follicle cells is the primary cilium, it is expected that one end of the cytoophidium should abut the centrioles. To examine this hypothesis, I performed double labelling experiments with a combination of markers for cytoophidia and centrioles. Both gamma-tubulin and SAS-4 are commonly used markers of centrioles and, as expected, they both yielded structures localizing to the apical sides of follicle cells. Although a minority of cytoophidia appear to lie close to centrioles, the majority fail to be associated with centrioles, either in early- or late-stage egg chambers (Fig. 5). Consequently, in follicle cells there appears to be no spatial coincidence of cytoophidia with centrioles, and thus cytoophidia appear to be distinct structures from cilia.

Two types of cytoophidia exist in germline cells: macro- and micro-cytoophidia

Surrounded by the follicle epithelium lie 16 germline cells, of which 15 are nurse cells providing nutrition for the single oocyte present at the posterior end of each egg chamber. Nurse cells undergo multiple endocycles: their DNA is replicated but the nurse cell nucleus does not divide, resulting in the size of nurse cells growing dramatically during oogenesis (Spradling, 1993; Dej and Spradling, 1999).

Two types of cytoophidia were observed in nurse cells and in oocytes. The first (a ‘macro-cytoophidium’) is long and thick, with a bright intensity if labelled by the anti-Cup* antibody or by GFP in the CTP synthase protein trap lines; the second (a ‘micro-cytoophidium’) is short and thin (Fig. S2). Cytoophidium markers show much weaker intensities for micro-cytoophidia than for macro-cytoophidia. When the gain of the confocal microscopy laser is adjusted to low intensity, micro-cytoophidia can become undetectable, yet many macro-cytoophidia remain readily apparent (Figs. S3 and S4).

Macro-cytoophidia exhibit various lengths and shapes

Large nurse cells in late-stage egg chambers appear to have longer macro-cytoophidia than smaller nurse cells at earlier stages (Fig. 6, A). For example, macro-cytoophidia in stage 5 nurse cells are 10-20 μm long, while macro-cytoophidia in stage 10 nurse cells can become 40-50 μm in length, more than 10 times the length of cytoophidia in follicle cells within the same egg chamber. Interestingly, while macro-cytoophidia in early stage nurse cells are straight or just slightly bent, the tips of long macro-cytoophidia in large nurse cells adopt diverse shapes: circular, sharp-bended, twisted, bended and straight (Fig. 6, B-G).

To determine the number of macro-cytoophidia in each nurse cell, I scanned the whole egg chamber from top to bottom under a laser-scanning confocal microscope and reconstructed 3-dimensional images. It appears that each nurse cell contains only one or a few macro-cytoophidia (Figs. 1, C and D; 6, A and S3). Macro-cytoophidia in nurse cells frequently localize to the perinuclear cytoplasm, but they also can lie in the proximity of the cortex.

Macro-cytoophidia are not associated with U bodies or P bodies, two cytoplasmic organelles involved in RNA regulation (Fig. S4).

In the same egg chamber, macro-cytoophidia in the oocyte normally show lengths similar to those in nurse cells (Fig. 6, A). In late-stage egg chambers, the oocyte often contains a greater number of macro-cytoophidia than each nurse cell, which may reflect the large volume of ooplasm.

A macro-cytoophidium is made of multiple filaments

The shape of a macro-cytoophidium is often thick in the middle whilst tapering at each end. Most frequently, a macro-cytoophidium possesses two different ends, one sharp the other blunt. This asymmetry between the two ends suggests that macro-cytoophidia are polarized. In most cases, macro-cytoophidia are tightly contained, within one bundle. Occasionally, a macro-cytoophidium swells, revealing multiple thinner filaments which twist at some points or re-join with what still appears to be a single filament (Fig. 6, H-J).

Discontinuity of CTP synthase along the macro-cytoophidium

For the majority of macro-cytoophidia, GFP-CTP synthase evenly labels the entire length. However, along some macro-cytoophidia GFP-CTP synthase labels appear to be absent within one or a few gaps (Fig. 6, K and L). These gaps are variable in width, but most are < 1 μm. The gaps appear to be randomly distributed along the macro-cytoophidium, and their origins or whether they translocate along the macro-cytoophidium, remain unknown.

Macro-cytoophidia increase in number in apoptotic egg chambers

In healthy egg chambers, there are one or a few macro-cytoophidia in each nurse cell or oocyte hence the overall number of macro-cytoophidia in an egg chamber ranges between 16 and a few dozens. However, the number of macro-cytoophidia increases dramatically when the egg chamber undergoes apoptosis (Fig. 7). Macro-cytoophidia in healthy egg chambers are easily distinguished. By contrast, macro-cytoophidia in apoptotic egg chambers appear to be highly flexible and easily become entangled.

Micro-cytoophidia are associated with Golgi bodies and yolk vesicles

In the cytoplasm of nurse cells and oocytes, there is one or a few macro-cytoophidia. In contrast, thousands of micro-cytoophidia reside in a single germline cell (Fig. 8). In stage 9 or 10 egg chambers, micro-cytoophidia are evenly distributed in the cytoplasm of nurse cells and the oocyte. They are about 1-2 μm long, much shorter than macro-cytoophidia within the same germline cell (Fig. 8, B). Micro-cytoophidia can be straight, a little bent or curved (Fig. 8, C). Double-staining experiments suggest that micro-cytoophidia in nurse cells or oocytes are located close to Golgi bodies as labelled by actin-binding protein 2 (Fig. 8, D-F). For example, two micro-cytoophidia can be linked to one Golgi particle (Fig. 8, D), or one or two Golgi particles can attach to one or both ends of a micro-cytoophidium (Fig. 8, E and F). After stage 8 when yolk vesicles start accumulating in the oocyte, micro-cytoophidia are frequently associated with yolk vesicles (Fig. 8, G).

ATP is generated in the cytosol and in the mitochondrion by ATP synthase. High concentrations of ATP synthase have been found in mitochondria (Alberts et al., 2008). To test whether the CTP synthase-

enriched structures of cytoophidia overlap with mitochondria, I performed a double-staining experiment with a combination of GFP-CTP synthase and an antibody against mitochondrial marker complex II alpha. Even when the germline cytoplasm is full of mitochondria, neither macro-cytoophidia nor micro-cytoophidia were seen to overlap with mitochondria (Fig. 8, H-J). In follicle cells, cytoophidia labelled with CTP synthase are shown to be distinct from mitochondria (Fig S5).

Micro-cytoophidia persist in mature eggs

After stage 10, nurse cells begin dumping their content to the oocyte and to undergo apoptosis. During this period, macro-cytoophidia in nurse cells and the oocyte decrease in number and often disappear in mature eggs. However, micro-cytoophidia persist though oogenesis, and thousands become evenly distributed throughout the entire cytoplasm of a mature egg (Fig. 8, K-M). The length of micro-cytoophidia in mature eggs can reach up to 5-15 μm, much longer than those in egg chambers at earlier stages (Fig. 8, C-F, KM).

Cytoophidia exist in other tissues and other species

Cytoophidia have also been observed in larval tissues such as lymph glands by Joseph Gall (personal communication). To determine the generality of intracellular compartmentation of CTP synthase, I extended my studies to other tissues of the fruit fly *Drosophila melanogaster*. I found cytoophidia containing CTP synthase in all tissues I examined, including lymph gland, salivary gland, testis, accessory gland, brain, trachea, and gut (Fig. 9). Using

multiple antibodies against CTP synthase, I consistently observed cytoophidia in ovaries from other species such as *Drosophila virilis* and *Drosophila pseudoobscura* (Fig. 10).

Discussion

Is the cytoophidium a novel organelle?

In this paper, I have reported that CTP synthase is compartmentalized into a novel and distinctive intracellular structure. Subcellular localization experiments revealed that the cytoophidium might be a new type of organelle. Morphologically, the snake-like structure distinguishes the cytoophidium from many classic organelles such as the mitochondrion, endoplasmic reticulum, and nucleus.

Cytoophidia in the *Drosophila* ovary share several features with cilia. First, the cytoophidium and the cilium are both filamentary structures. Second, only one cytoophidium exists in each follicle cell and one or a few macro-cytoophidium in each nurse cell or oocyte, similar to the numbers of primary cilia in most vertebrate cells. Third, cytoophidia in follicle cells link to microtubule networks, and they sometimes overlap with signals derived from acetylated tubulin, a common marker for cilia in vertebrate cells. Fourth, cilia are highly dynamic structures and the length of a cilium is regulated. Similarly, cytoophidia in *Drosophila* ovarian cells show various lengths at different developmental stages.

Despite these similarities, in other respects cytoophidia differ from cilia. A typical cilium always connects to a basal body, a structure based on a pair of centrioles. In follicle cells, where centrioles are detectable, cytoophidia are not always seen to neighbour the centrioles. In nurse cells, there is no obvious centriole but many cytoophidia are apparent. Also, there is no

indication that macro- or micro-cytoophidia are associated with centrioles in oocytes.

Does the cytoophidium have a role in phospholipid synthesis?

The CTP synthase reaction product CTP is essential for the synthesis of RNA, DNA, and sialoglycoproteins. CTP also plays critical role in the synthesis of membrane phospholipids (Chang and Carman, 2008). Because CTP synthase is enriched in cytoophidia, I speculate that cytoophidia may be critical for the synthesis of CTP and membrane phospholipids. This view is strengthened by the association of micro-cytoophidia and Golgi bodies in germline cells. The Golgi bodies are dynamic structures with considerable membrane flux and protein trafficking (Glick and Nakano, 2009). Recent studies have suggested an interaction between the secretory pathway through Golgi bodies and phosphatidylcholine metabolism – a process regulated by the synthesis of CTP (Kent and Carman, 1999). In late-stage oocytes, yolk vesicles arise from transformed Golgi bodies. Consistent with their association with Golgi bodies, micro-cytoophidia are very close to or touch yolk vesicles in late-stage oocytes (Fig. 8G).

Cytoophidia and mitochondria

ATP is an essential and multifunctional nucleotide, mostly generated by the ATP synthase in the mitochondrion in animal cells (Alberts et al., 2008). Because ATP is the common "energy currency" of cells, the compartmentation of ATP synthase in mitochondria makes mitochondria the power centers of the eukaryotic cell. An analogy could be made for CTP

synthase, which localizes to the cytoophidium. The cytoophidium could thus provide a microenvironment enriched in CTP synthase for the efficient synthesis of CTP and membrane phospholipids. Giving the contrasting compartments for ATP synthase and for CTP synthase, it will be of interest to study the distribution of enzymes for the synthesis of other nucleotides such as GTP and UTP.

Other cytoophidium-like structures

Although cytoophidia considered in this study reside mainly in the cytoplasm, filamentary structures have also found in the oocyte nucleus of *Drosophila* and other insects (Liu et al., 2006). The pathological hallmark of oculopharyngeal muscular dystrophy is the presence of nuclear filaments in skeletal muscle fibers composed of expanded poly(A)-binding protein nuclear 1, ubiquitin, proteasomes and poly(A) RNAs (Tome and Fardeau, 1980; Calado et al., 2000; Abu-Baker and Rouleau, 2007). Nuclear filaments with similar composition have recently been reported in rat supraoptic neurons under physiological and osmotic stress conditions (Villagra et al., 2008). Indeed, nuclear filaments are also found in *Drosophila* nurse cells (unpublished data). It would be interesting to study whether cytoophidia are related to these nuclear filaments. Future studies, including subcellular fractionation, genetics and genomics, should help to understanding the composition, organization and function of the cytoophidium and its related structures.

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Figure legends

Fig. 1. Cytoophidia revealed by an anti-Cup* antibody in *Drosophila* ovarian cells. (A) Surface view of stage-8 follicle cells. Each follicle cell contains a single filamentary structure, the cytoophidium (green). (B) Lateral views of follicle cells in a stage-10 egg chamber. (C) Cytoophidia in a stage-7 egg chamber. Note that there are 15 large cytoophidia in 15 nurse cells. The oocyte is heavily stained as Cup is enriched in the ooplasm. Some cytoophidia in follicle cells are visible under this setting (low gain to avoid oversaturated signal in nurse cells). (D) Surface view of a stage-10 egg chamber. Nurse cells occupy the anterior half of the egg chamber, and the oocyte occupies the posterior half which is covered by follicle cells. Cytoophidia (filamentary structures in green) in three nurse cells stand out from the cytoplasmic background signal. Note that cytoophidia in follicle cells are undetectable under this setting (low gain to avoid oversaturated signal in nurse cells). (E) Stage-10 egg chamber. Note that P bodies (green punctate staining) are enriched in the posterior cortex of the oocyte, and a cytoophidium is located near the ventral cortex of the oocyte. The nucleus of the oocyte is located at the anterior-dorsal corner of the oocyte. Macro-cytoophidia in nurse cells are not captured in this single confocal section because they are out of focus. (F) Zoom-in of E to highlight the cytoophidium in the oocyte. DNA is stained with Hoechst 33342 (magenta in A, B, D and E). Anterior is to the left in C, D and E. Scale bars, 10 μm.

Fig. 2. Cytoophidia contain CTP synthase. (A, B) Surface view (A) and deep view (B) of a stage-8 egg chamber, stained by an antibody against a conserved region at the N-terminal of CTP synthase (green). Cytoophidia are clearly visualized in follicle cells and germline cells. (C, D) Surface view (C) and deep view (D) of a stage-8 egg chamber, stained by an antibody against amino acids 31-118 mapping near the N-terminus of CTP synthase (green). Cytoophidia are clearly visualized in follicle cells and germline cells. (E-H) A deep view of a stage-7 egg chamber from GFP-CTP synthase protein trap fly, stained with an antibody against CTP sysnthase. Note that the CTP synthase antibody (white in G, red in H) provides a pattern identical to the GFP signal (white in F, green in H). DNA is stained with Hoechst 33342 (magenta in A-D, white in E, blue in H). Scale bars, 10 μm.

Fig. 3. Cytoophidia in *Drosophila* follicle cells. (A) A surface view of ovarioles from the protein trap fly CA6746 showing the distribution of GFP-CTP synthase in follicle cells. Note this panel is a montage of two images as the field is too wide for a single shot. (B-H) Zoomed in images of germaria and egg chambers at various stages. (B) Germaria and early-stage egg chambers. (C, D) Stage-7 egg chambers. (E, F) Stage-8 egg chambers. (G) A stage-9 egg chamber. (H) A stage-10 egg chamber. Note the length of cytoophidia in follicle cells increases in late-stage egg chambers. Scale bars, 10 μm.

Fig. 4. Cytoophidia associate with microtubule networks in *Drosophila* follicle cells. (A-C) Surface views of follicle cells. (D-I) Lateral views of follicle cells. (A, D) GFP-CTP synthase. (B, E) Alpha-tubulin. (H) DNA. (C) Merge of

A and B (green, GFP-CTP synthase; red, alpha-tubulin). (F) Merge of D and E (green, GFP-CTP synthase; red, alpha-tubulin). (G) Merge of D and H (green, GFP-CTP synthase; magenta, DNA). (I) Merge of D, E, and H (blue, DNA; green, GFP-CTP synthase; red, alpha-tubulin). (J-L) Surface views of follicle cells. (J) GFP-CTP synthase. (K) Acetylated tubulin. (L) Merge of J and K (green, GFP-CTP synthase; red, acetylated tubulin). (M-O) Zoom-in of a surface view of follicle cells. (M) A cytoophidium stained by the anti-Cup* antibody. (N) Acetylated tubulin. (O) Merge of M and N (green, acetylated tubulin; red, Cup*). Note that the cytoophidium stained by the anti-Cup* antibody (red) overlaps with a thin line of acetylated tubulin (green) from the microtubule network. Scale bars, 10 μm.

Fig. 5. Cytoophidia do not associate with centrioles in *Drosophila* follicle cells. (A-C) a surface view of an early-stage egg chamber. A, GFP-CTP synthase B, gamma-tubulin. C, merge of A and B (green, GFP-CTP synthase; red, gamma-tubulin). (D-F) a surface view of a stage-8 egg chamber. D, cytoophidia stained by an antibody. E, centrioles labelled by sas-4. F, merge of D and E (green, cytoophidia; red, centrioles). Scale bars, 10 μm.

Fig. 6. Macro-cytoophidia in *Drosophila* female germline cells. (A) A deep-view of ovarioles from the protein trap fly CA6746 showing GFP-CTP synthase distribution in the germline cells. Note that these ovarioles are the same as shown in Figure 2A and this panel is also a montage of two images as the field is too wide for a single shot. Inset, a stage-9 egg chamber (green, GFP-CTP synthase; magenta, DNA). (B-G) Macro-cytoophidia with various

shapes. (H, I) Two macro-cytoophidia at tight (H) or loose (I) status. Note that only a part of the macro-cytoophidia is loose in I. (J) A loose macro-cytoophidia showing multiple filaments with many small filamentary structures (micro-cytoophidia) nearby. (K, L) Macro-cytoophidia with gaps not labelled with GFP-CTP synthase. Scale bars, 10 μm.

Fig. 7. Macro-cytoophidia increase upon apoptosis. (A-C) A healthy egg chamber. (D-F) An apoptotic egg chamber. A, D, DNA. B, E, GFP-CTP synthase. C, merge of A and B (green, GFP-CTP synthase; magenta, DNA). F, merge of D and E (green, GFP-CTP synthase; magenta, DNA). Note that there are many more macro-cytoophidia in the apoptotic egg chamber (D-E) than in the healthy egg chamber (A-C). Scale bars, 10 μm.

Fig. 8. Micro-cytoophidia in *Drosophila* germline cells associate with Golgi bodies and yolk granules. (A-F) GFP-CTP synthase (green) and ABP2 (red) in follicle cells (A) and germline cells (B-F). (A, B) Surface (A) and deep (B) views of an egg chamber. (C) A zoom-in of the oocyte in B. (D-F) High magnifications of the oocyte to show the link between micro-cytoophidia and Golgi bodies. (G) Micro-cytoophidia associate with yolk vesicles (dark holes) in the ooplasm. (H-J) Micro-cytoophidia (H) do not overlap with mitochondria (I) in nurse cells. J, merge of H and I (green, GFP-CTP synthase; red, complex II alpha). (K-M) Micro-cytoophidia in a mature egg. K, the anterior half. L, the posterior half. M, a zoom-in view of l. Scale bars (if not indicated), 10 μm.

Fig. 9. Cytoophidia exist in multiple tissues. Tissues were derived from wild-type animals and stained by antibodies against CTP synthase (green). DNA is stained with Hoechst 33342 (magenta). (A) Larval lymph gland. Note there are many needle-like and ring-like structures. Many rings are open including that shown in the inset. (B) Larval testis. (C) Adult testis. (D) Accessory gland. (E) Optical lobe of a larval brain. (F) Trachea. Note there are two needle-like and one ring-like (inset) structures. (G) The root of a salivary gland from a third instar larva. (H, I) Anterior (H) and posterior (I) regions of mid-gut from larvae. (Inset in H) Four-time zoom-in of an island in mid-gut, showing small cells have distinctive cytoophidia. Scale bars, 10 μm.

Fig. 10. Cytoophidia exist in other *Drosophila* species. (A, B) Surface (A) and deep (B) view of an egg chamber from *Drosophila virilis*. (C, D) Surface (C) and deep (D) view of egg chambers from *Drosophila pseudoobscura*. All samples were stained by an antibody against a conserved region of CTP synthase (green). DNA is stained with Hoechst 33342 (magenta). Scale bars, 10 μm.

Supplementary figure legends

Fig. S1. An antibody recognizes sperm tails in *Drosophila melanogaster*. (A-C) Testis from a third-instar larva. Cephalad pole to the left. A, DNA staining only. B, the anti-Cup* antibody stains sperm tails as well as the cytoplasm in most cells of the testis. However, the signal intensity on sperm tails is much higher than that in the cytoplasm. C, merged from A and B (DNA, magenta; Cup*, green). (D-F) part of an adult testis. D, DNA staining only. E, sperm tails are stained brightly with the anti-Cup* antibody while sperm heads (needle-like structures stained with Hoechst 33342 in D and F) are negative for this antibody. F, merged from D and E (DNA, magenta; Cup*, green). DNA is stained with Hoechst 33342. Scale bars, 50 μm.

Fig. S2. Macro- and micro-cytoophidia in an egg chamber. (A) DNA, stained with Hoechst 33342. (B) GFP-CTP synthase. Macro-cytoophidia are the long and thick filaments in the germline cells. Micro-cytoophidia are short and thin filaments in the germline cells. Note that cytoophidia in follicle cells are also visible. (C) Merge of A and B (green, CTP synthase; magenta, DNA). Scale bar, 10 μm.

Fig. S3. Macro-cytoophidia and actin in an egg chamber. (A) DNA, stained with Hoechst 33342. (B) CTP synthase. Note that the gain was adjusted so only macro-cytoophidia in germline cells are detected. (C) Actin labelled with phalloidin. Note that actin networks outline the boundary of nurse cells and the oocyte. There are 15 actin-based ring canals connecting the cytoplasm of

the 15 nurse cells and the oocyte. (D) merge of A, B and C (blue, DNA; green, CTP synthase; red, actin). Note that there are one or two macro-cytoophidia in each germline cell in this egg chamber. Scale bar, 10 μm.

Fig. S4. Macro-cytoophidia are not associated with U bodies or P bodies in the germline cells. (A) Macro-cytoophidia (green, CTP synthase) are not associated with U bodies (red, SMN). (B) Macro-cytoophidia (green, CTP synthase) are not associated with P bodies (red, Cup). Scale bar, 10 μm.

Fig. S5. Cytoophidia and mitochondria show distinct distributions in follicle cells. (A) Cytoophidia labelled wtih GFP-CTP synthase. (B) Mitochondria labelled with complex II alpha. (C) Merge of A and B (green, GFP-CTP synthase; red, complex II alpha). Note that GFP-CTP synthase shows a different pattern to that of complex II alpha in the follicle cells. Scale bar, 10 μm.

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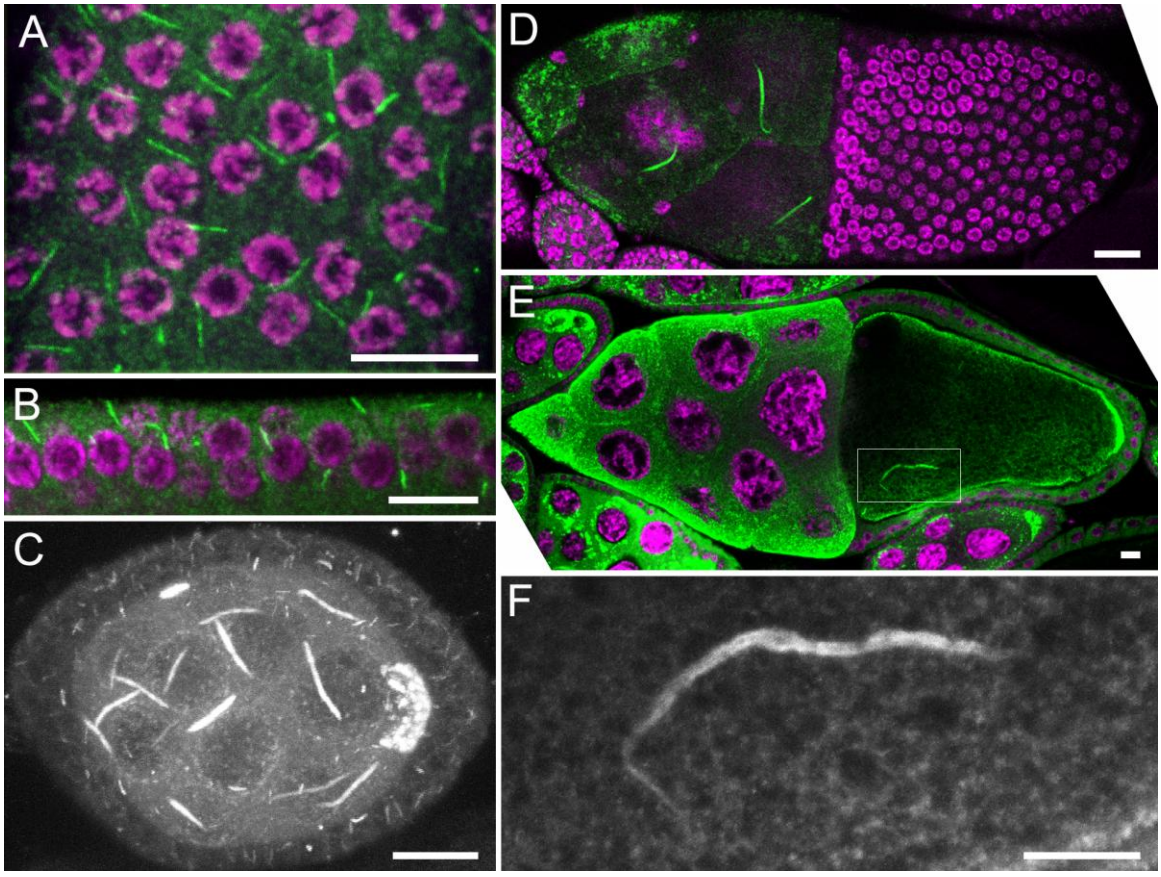
Figures 1-10, S1-S5



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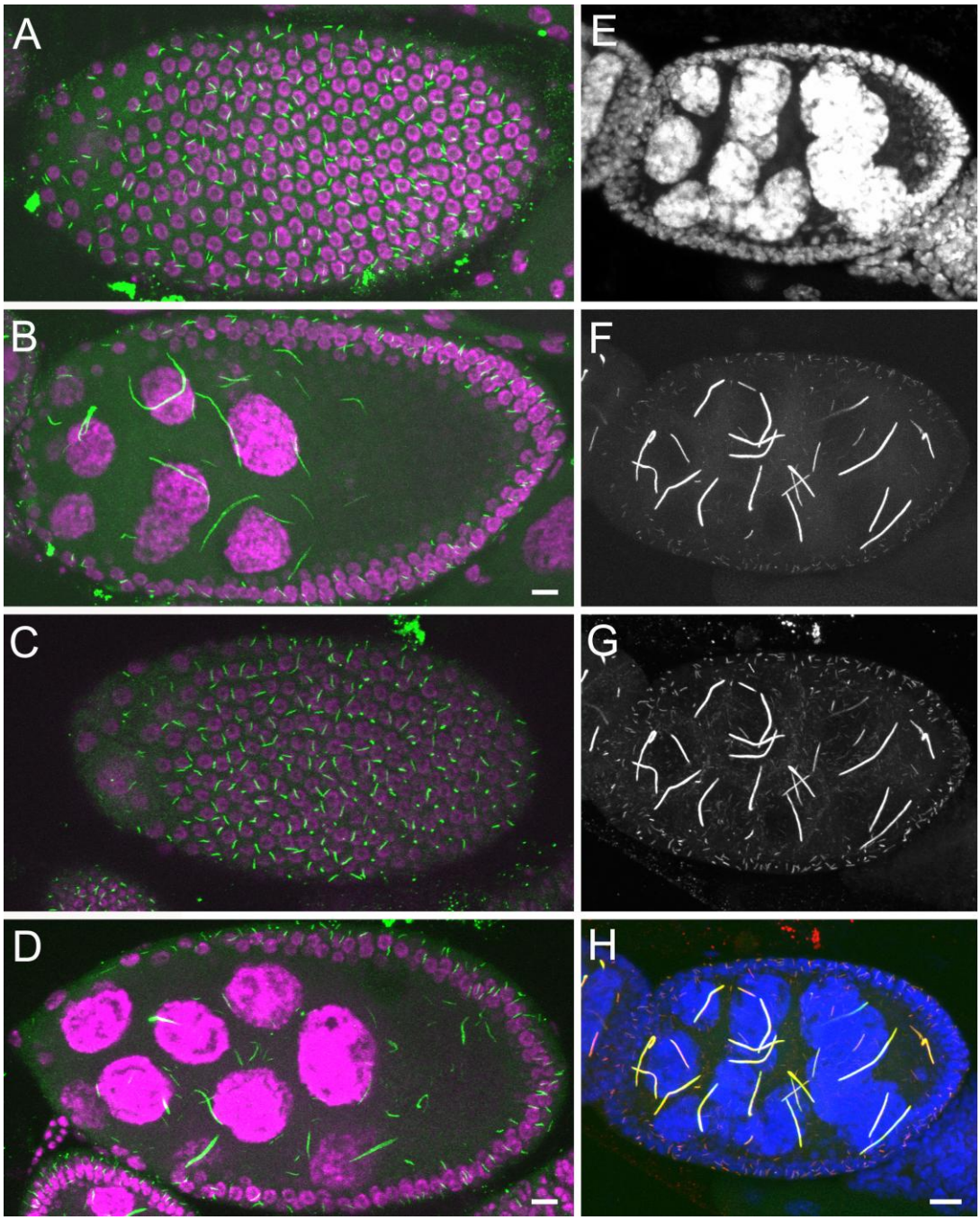

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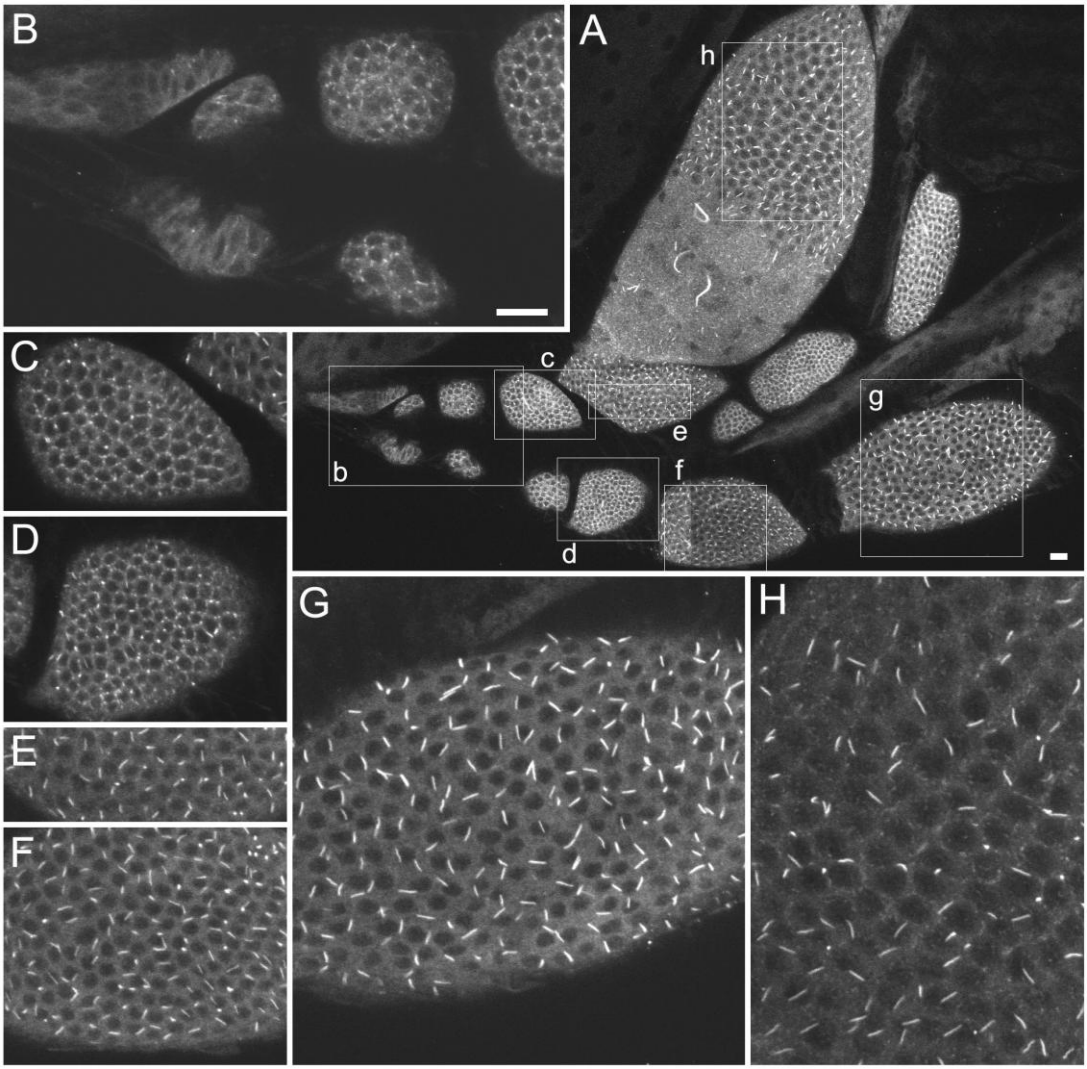

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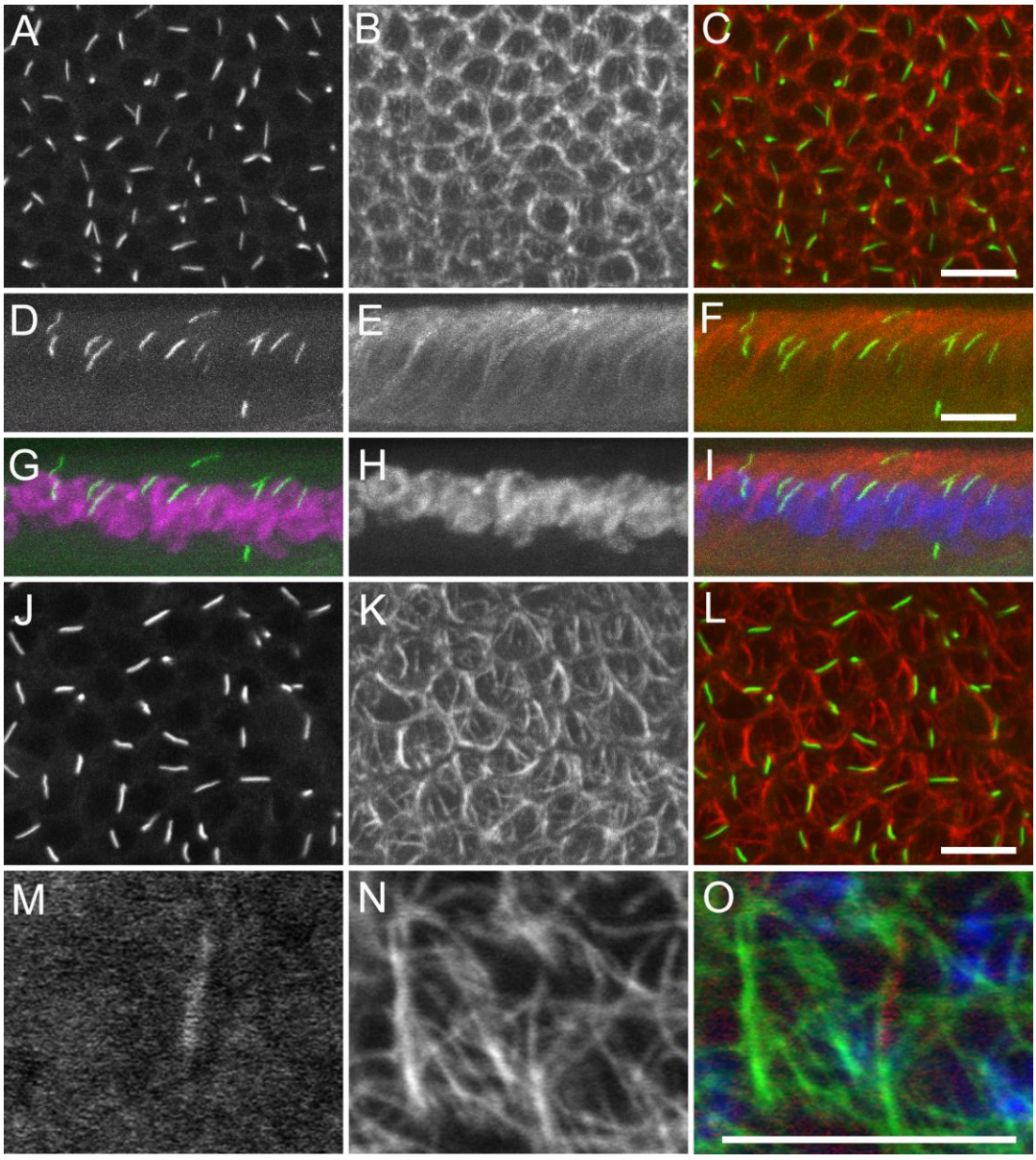

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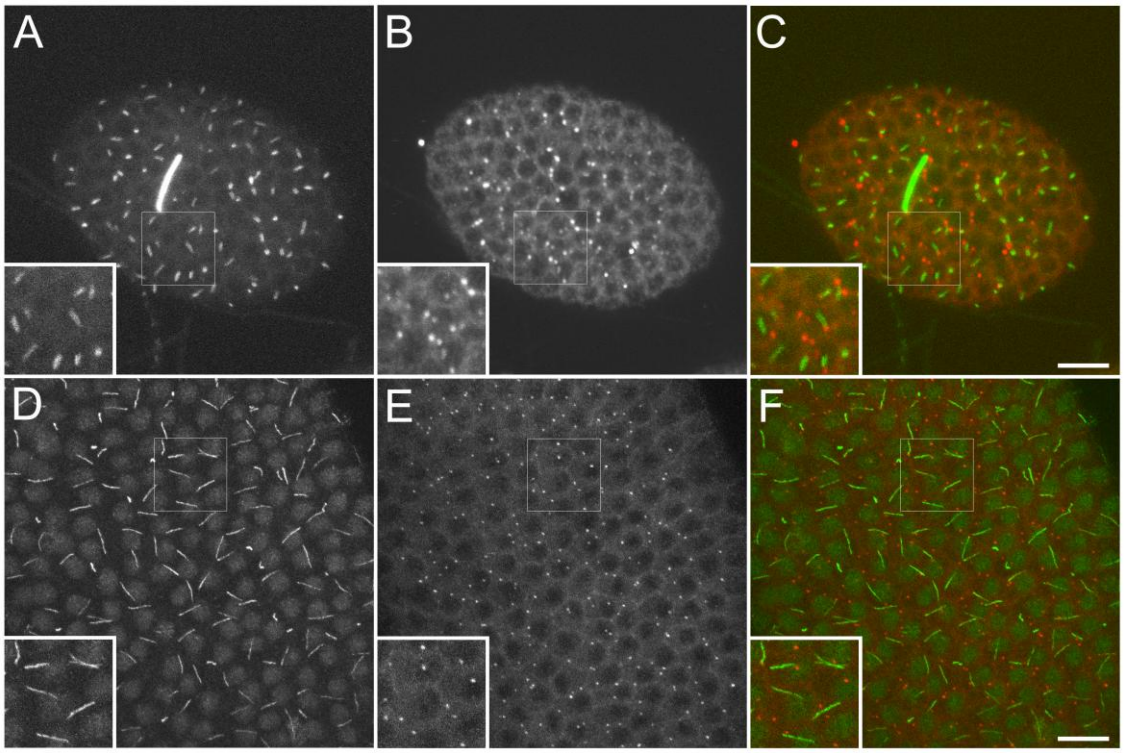

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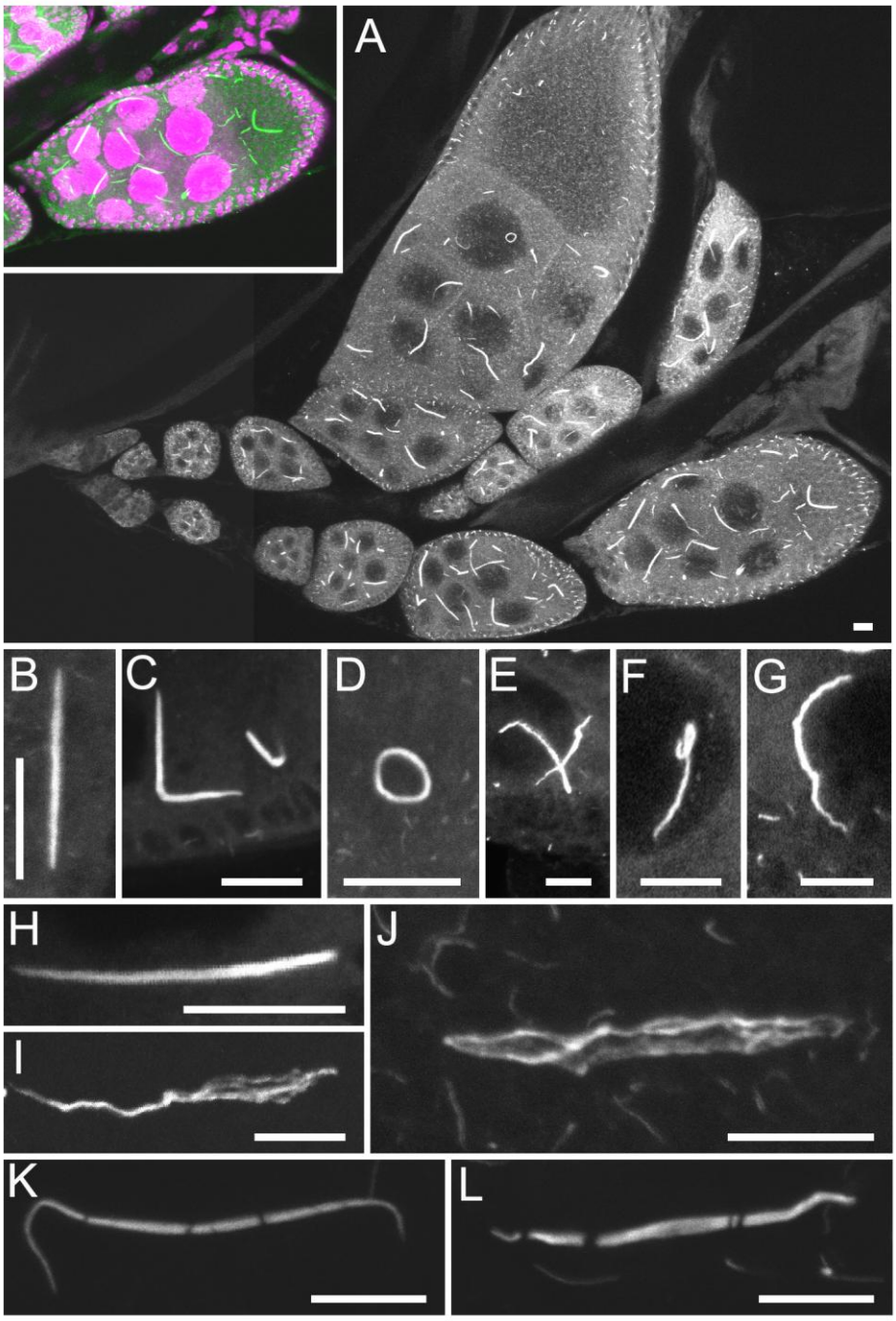

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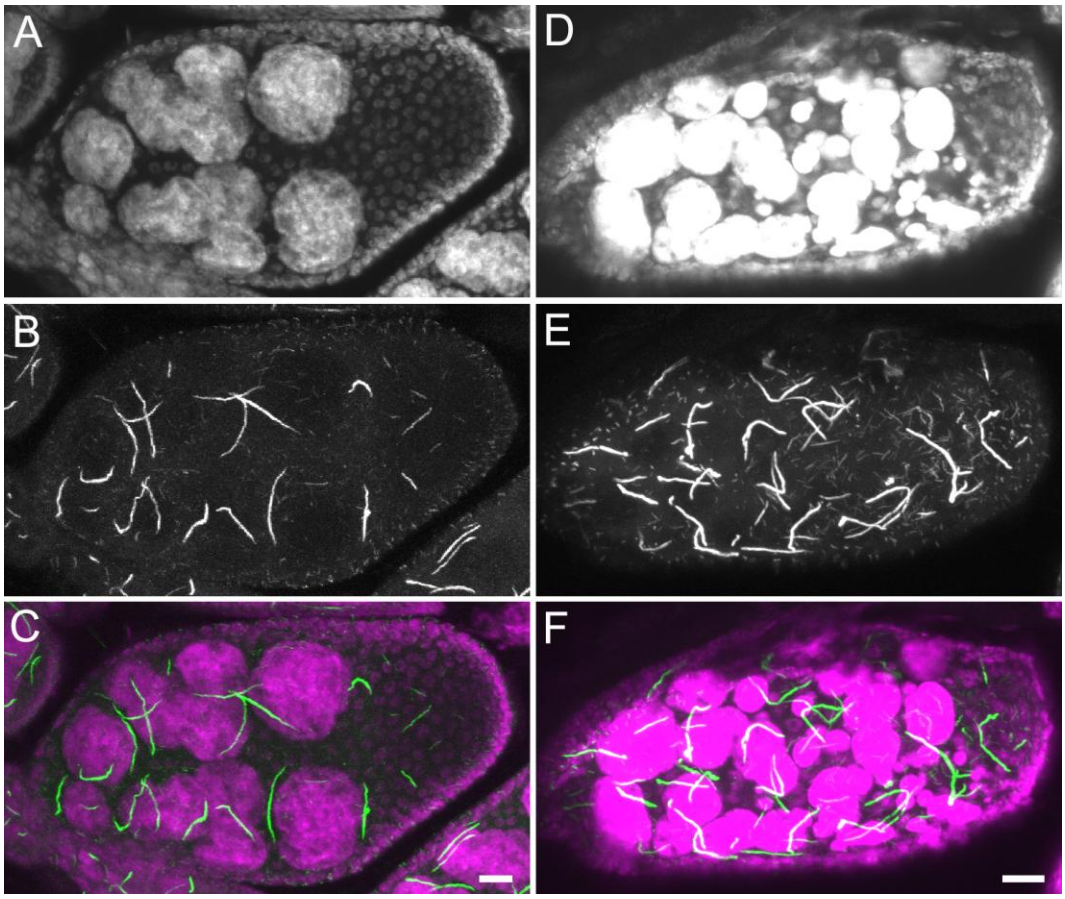

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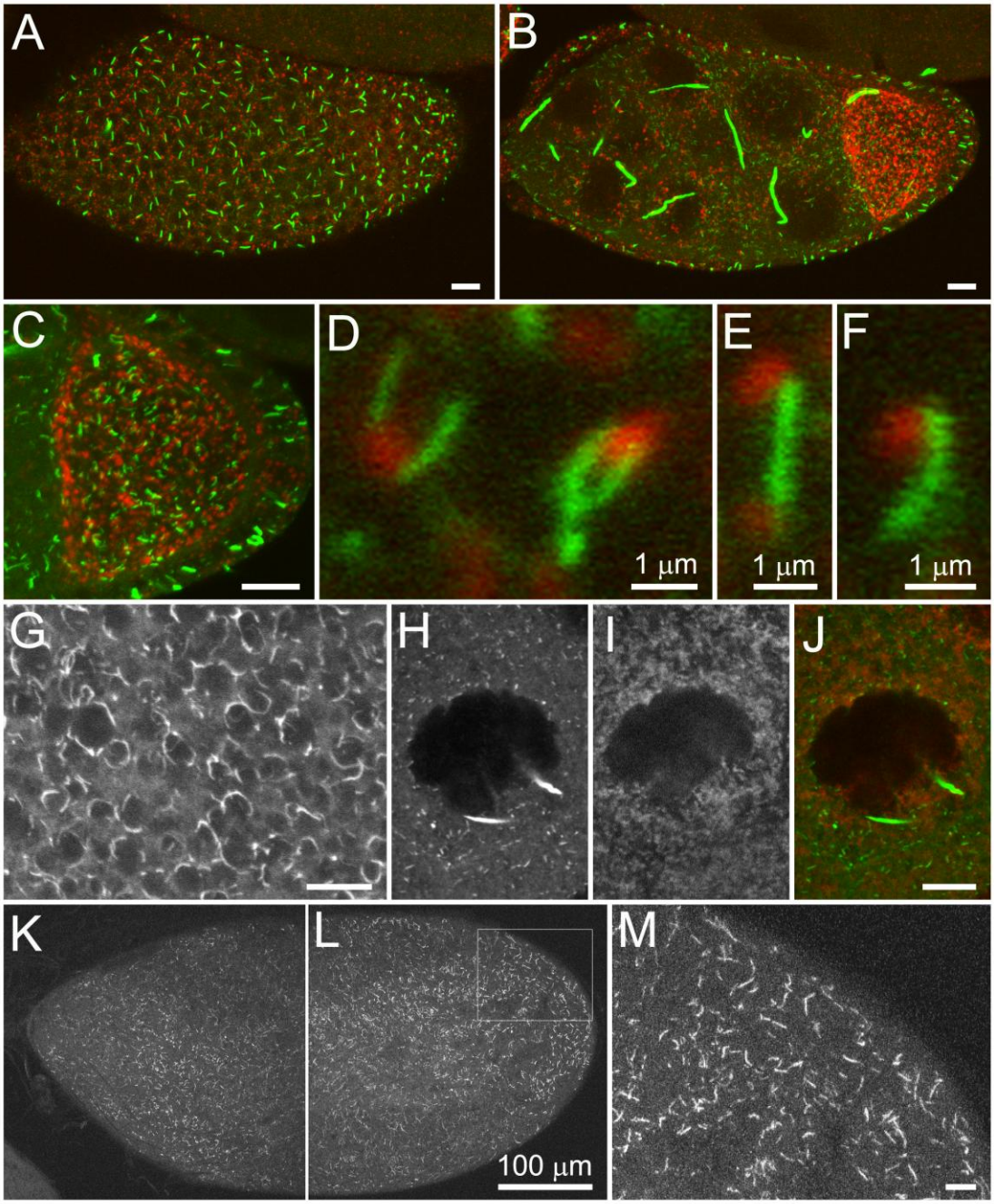

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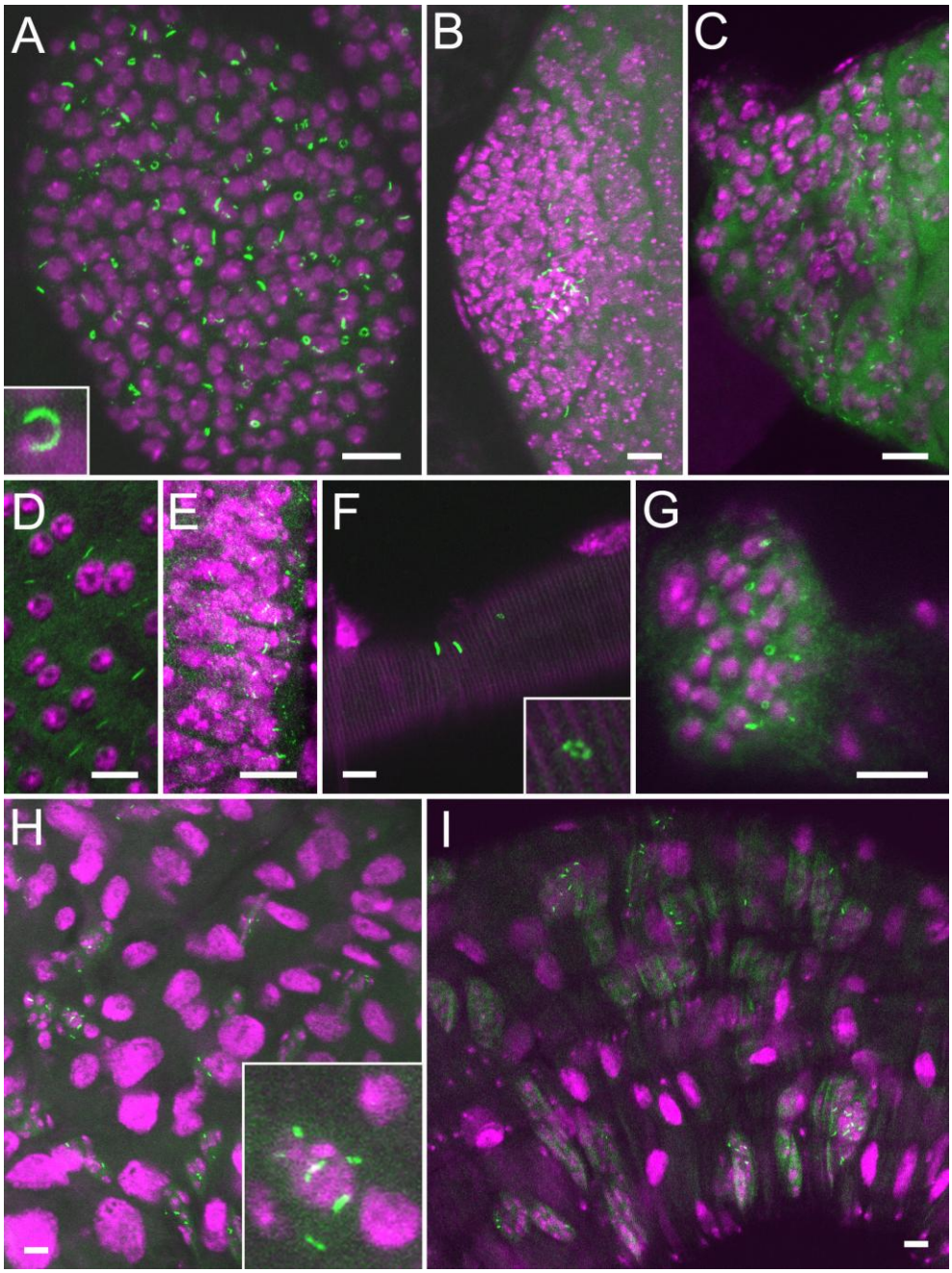

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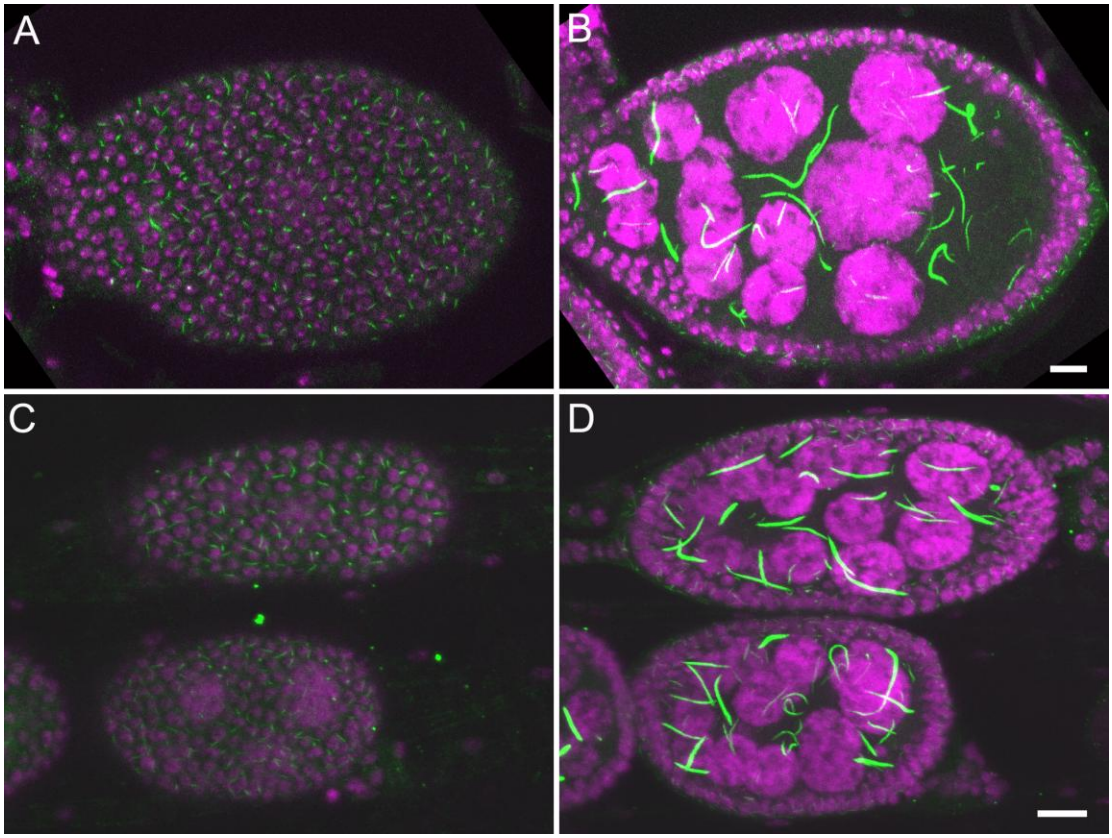

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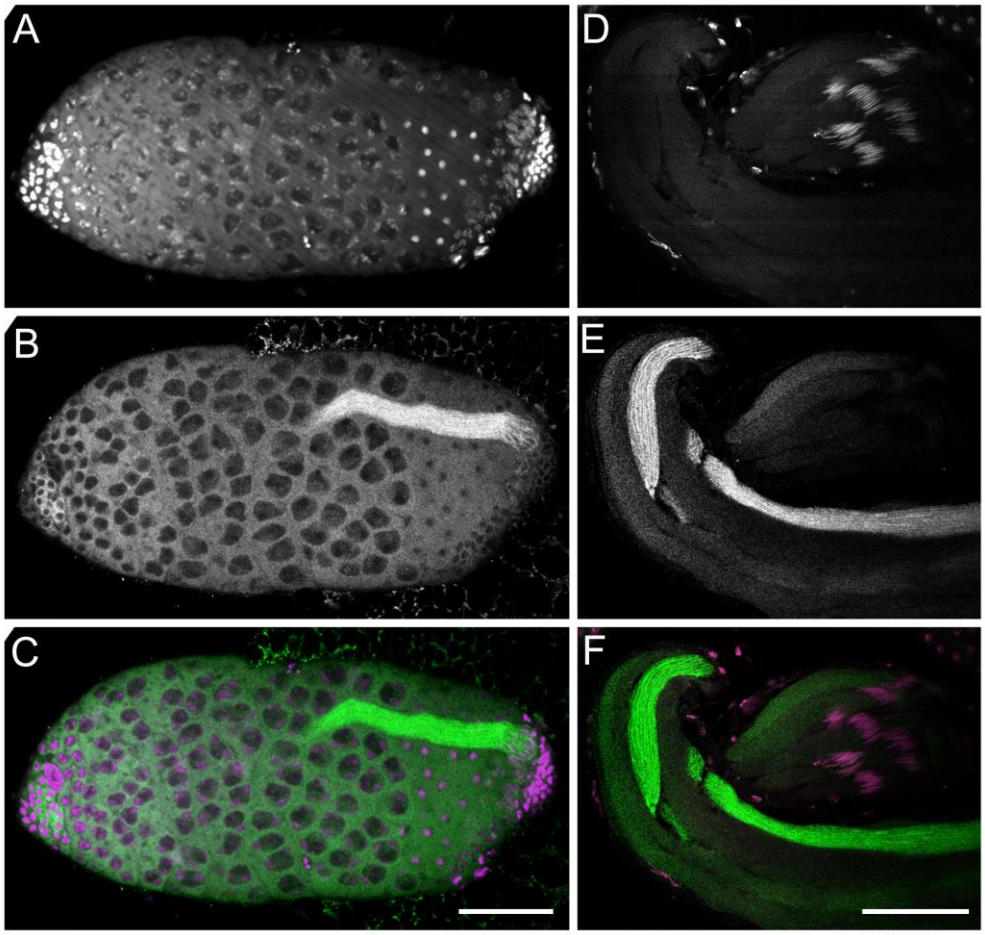

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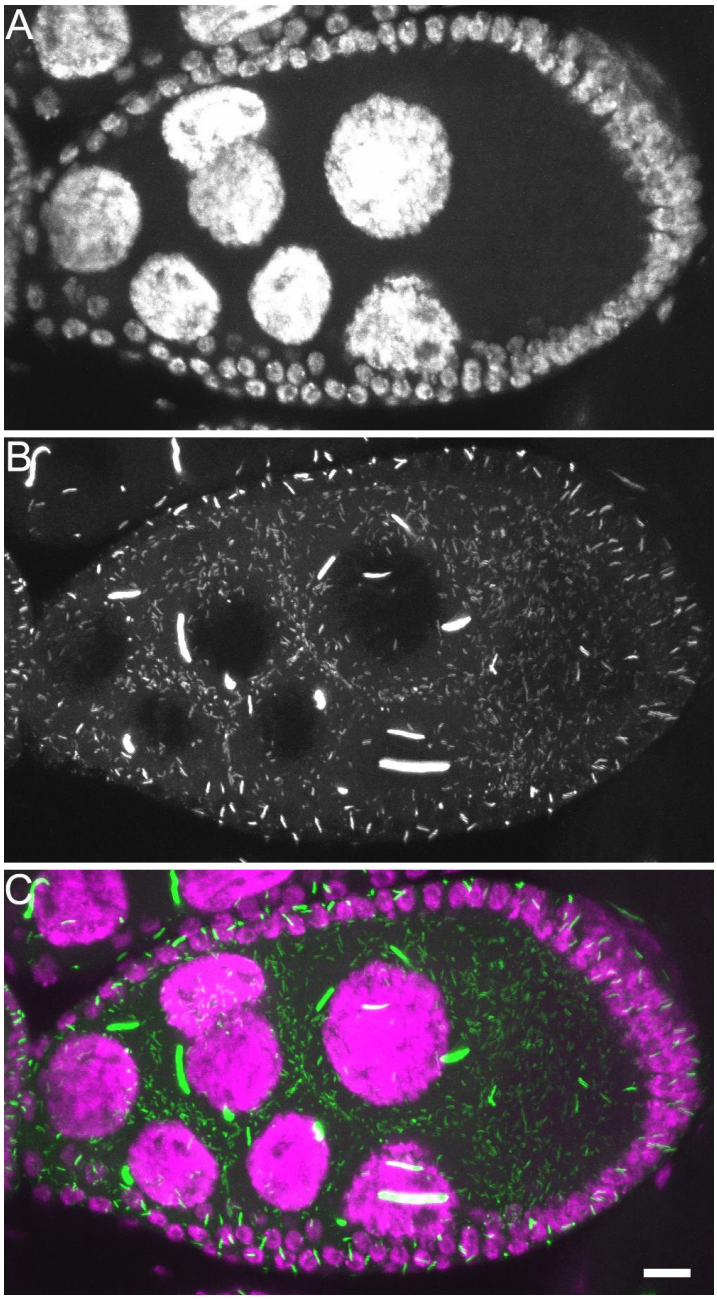

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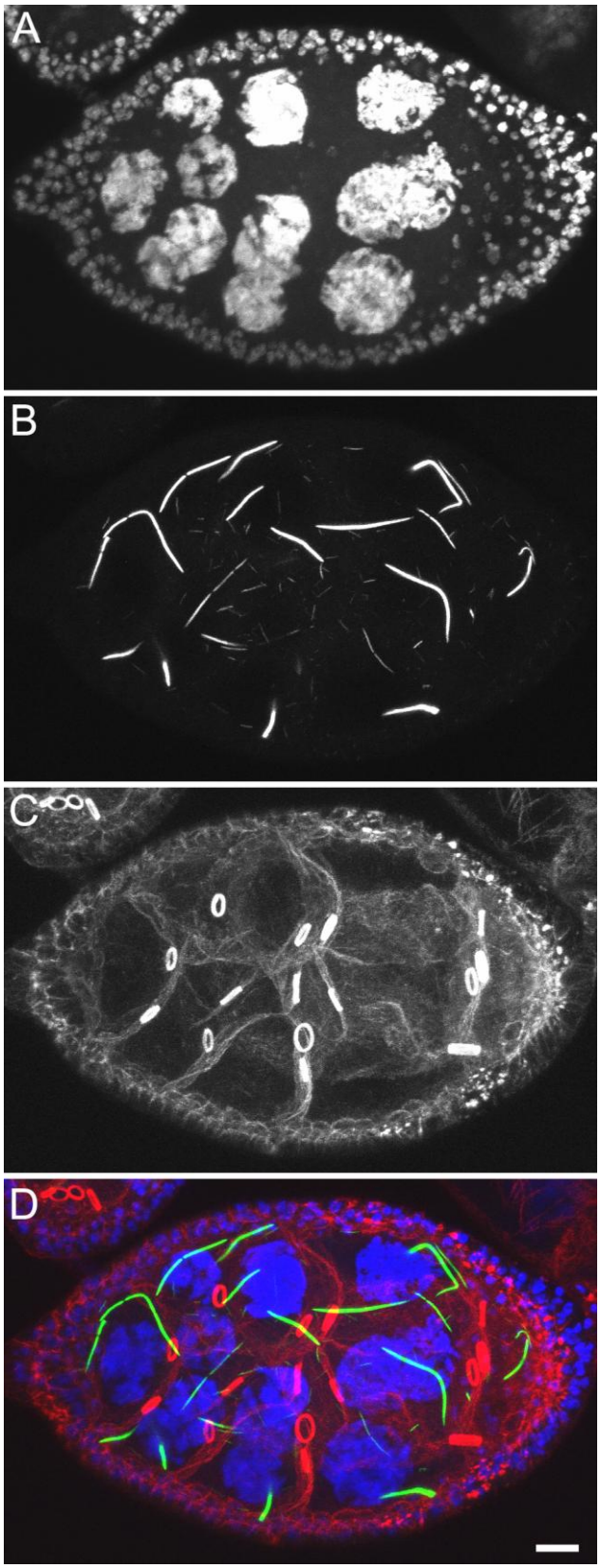

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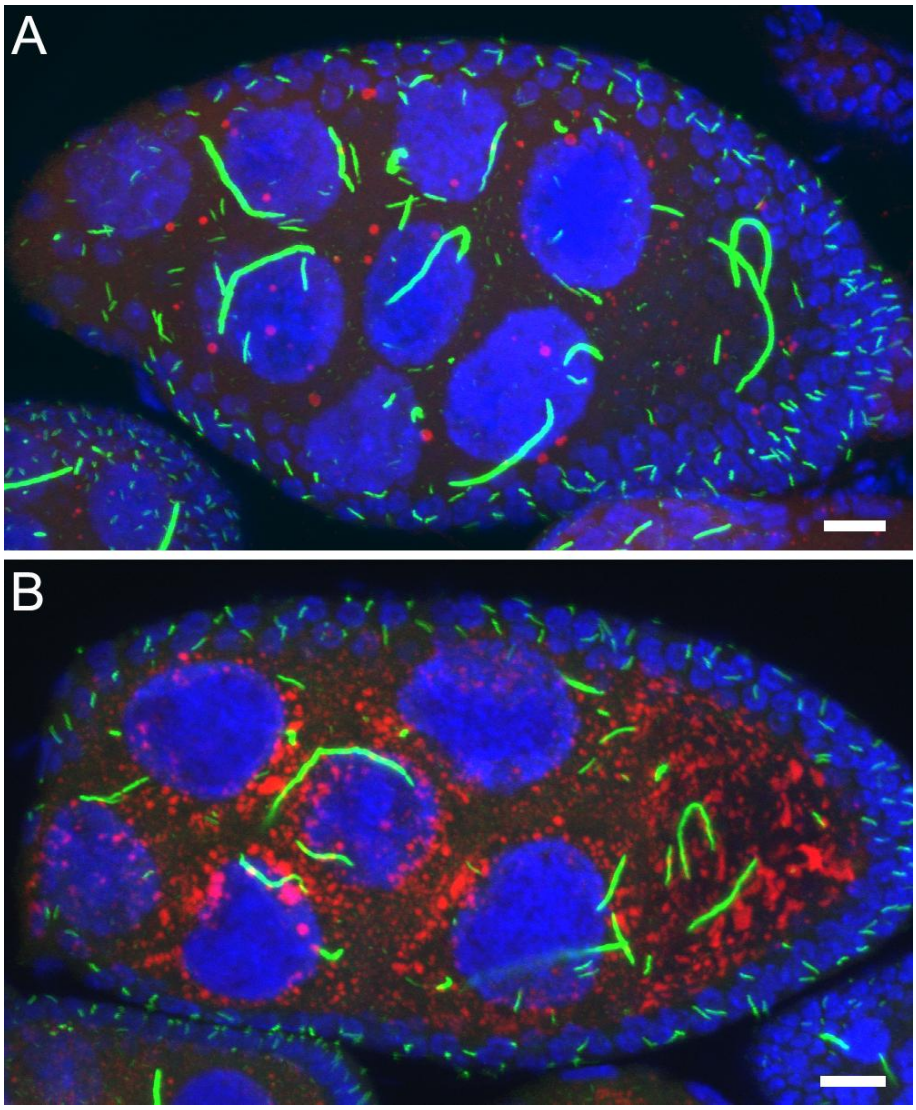

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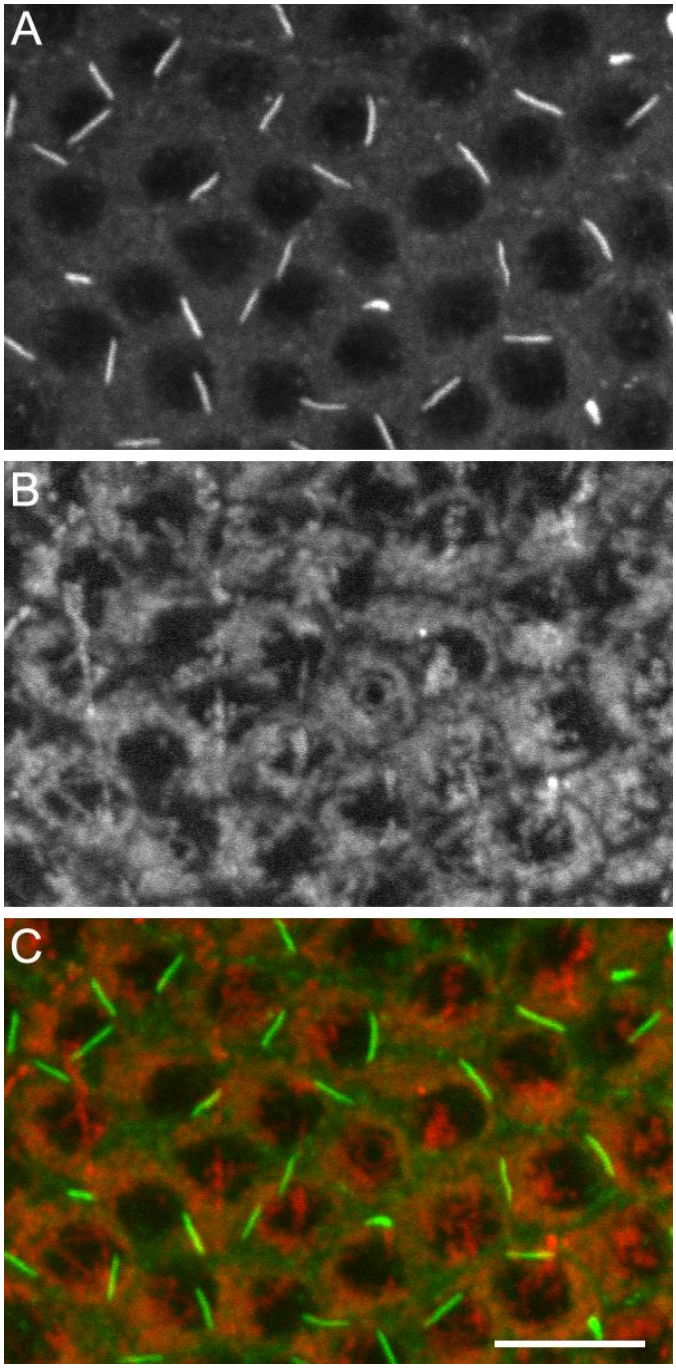

Figure S5