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Nuclear Migration and Spermatid Elongation During Spermiogenesis in Drosophila Pseudoobscura

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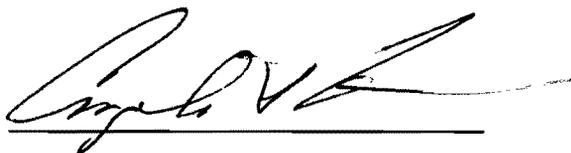# NUCLEAR MIGRATION AND SPERMATID ELONGATION DURING SPERMIOGENESIS IN *DROSOPHILA PSEUDOOBSCURA*

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Submitted in partial fulfillment of the requirements for the Degree of Masters of Science in Biology from Department of Biological Sciences at Seton Hall University

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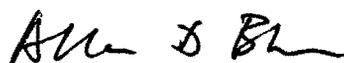
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Abstract

Drosophila is a model genus that can be used to improve our understanding of mammalian spermatogenesis as the cellular and molecular processes in mammalian and fruit fly spermatogenesis are very similar. In Drosophila pseudoobscura spermatogenesis, 128 round spermatids arise via five mitotic and two meiotic divisions within an encapsulating cyst and then transform into mature, elongate sperm during a post-meiotic stage termed spermiogenesis. Prior to spermiogenesis, the 128 spermatid nuclei relocate to one side of the cyst, however the mechanism by which this relocation occurs is unknown. Cytoskeletal and motor proteins have been reported to be responsible for different nuclear migration events across biological systems. Because actin structures are involved in later stages of sperm maturation, we hypothesized that an actin-based structure may be responsible for the nuclear migration event. Also, actin has been investigated in nuclear migration events in different models including Drosophila, and results prove it is an important player. Our results indicate that the nuclei in the 128-cell stage relocate to one side of the cyst during spermatogenesis in D. pseudoobscura. Also, we report the presence of a distinct, spherical actin-based structure associated with the sperm nuclei in pre-migratory 128-cell cysts. The nuclei appear to congregate around this structure prior to migration. Furthermore, actin is detected as a punctate cytoplasmic signal prior to this stage. Cell culture techniques were also used to investigate D. pseudoobscura sperm cell viability after 24 hours. These cysts were stained with Hoechst 33342 at the beginning of the culture, and tested for viability by calcein-AM staining. Interestingly, we detected multiple cytoplasmic 'elongation bulges' in early post-migration cysts using this protocol. Multiple bulges were detected on each developing sperm precursor before the nuclei had undergone nuclear migration. Also, cytoplasmic 'voids' were detected within the head cyst cells associated with this stage; the function of these structures is unknown.

Introduction

I. Overview

Drosophila has been used as a model genus for complex genetic, evolutionary and cell biological studies for the past century. Moreover, *Drosophila* can be considered a powerful model for spermatogenesis studies. Spermatogenesis is a highly regulated and complex cellular process in which diploid germ line stem cells divide mitotically and meiotically to produce haploid round spermatids. These haploid cells will eventually pass paternal genetic material to the egg during formation of the diploid zygote in fertilization. Before fertilization can occur properly, these spermatids must undergo a highly complex internal restructuring event called spermiogenesis. Spermiogenesis includes nuclear and chromatin remodeling, the assembly of the acrosome, and elongation of the sperm tail (Fuller 1998). Like many biological processes, these internal restructuring events are highly regulated and are required for production of mature spermatozoa (Cheng and Mruk 2011).

II. *Drosophila* as a powerful model for spermatogenesis

Drosophila melanogaster has been the species of choice for spermatogenesis analyses since the 1970s (Cross and Shellenbarger 1979; Cenci *et al.* 1994; Fuller 1998; Gregory *et al.* 2008; Ueishi *et al.* 2009). *Drosophila* flies are cheap and easy to handle in the lab and have a very convenient generation time. *Drosophila* is a genus in the *Drosophilidae* family that contains the well-understood *melanogaster* subgroup to which *D. melanogaster* belongs. That, along with the fact that twelve *Drosophila* species genomes have been sequenced (Markow

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2007), makes *D. melanogaster* an extremely powerful model for many analyses in which human or other mammalian test subjects would be difficult (White-Cooper 2009).

Spermatogenesis in *Drosophila* begins in a characteristic locale in the apical end of the testis called the "stem-cell niche" (White-Cooper 2009). Pigmented and smooth muscle cells make up the outer tissue of *Drosophila* testes. The lumen of the testis is packed with the stem cells and somatic cells necessary for the spermatogenesis process to occur properly. In the *D. melanogaster* stem-cell niche, one diploid germ line stem cell (GSC) undergoes mitotic and meiotic divisions to ultimately form 64 spermatids within an intact syncytium (Fuller 1998). The mitotic divisions are incomplete, leaving the sperm precursors connected via cytoplasmic bridges. These bridges may be important in cell cycle regulation and coordinated development of the sperm precursors in *Drosophila* (Fuller 1998). Beginning in the 32-cell stage, mitosis ceases, all cells undergo a pre-meiotic S-phase, and then meiotic divisions eventually form the round spermatids. During each meiotic interphase, *Drosophila* cysts undergo a prolonged G2 phase in which the cells grow substantially, but remain associated via cytoplasmic connections (Fuller 1998).

A single mitotic division of the GSC into a gonialblast in the stem cell niche commits a cyst to spermatogenesis. The niche also contains specialized cyst progenitor cells (CPCs), flanked by the GSCs, which give rise to the two cyst cells that will encapsulate the developing sperm cells as they undergoes spermatogenesis. The CPCs divide as the GSCs divide to give rise to the encapsulated gonialblast, primed for spermatogenesis. The GSCs and CPCs are arranged in a "rosette" fashion within the niche (Figure 1). When mitosis of the GSCs and CPCs occurs,

one of the daughter cells remains a stem cell (Fuller 1998). The somatic cells within the niche that are responsible for maintaining the GSCs and CPCs in their stem cell state are called hub cells. The hub cells also provide anchors to keep the germline cells in the vicinity of the stem cell niche (Yamashita *et al.* 2005). Illustrations of spermatogenesis in *Drosophila pseudoobscura* are shown in Figures 1 and 2.

Cell culture techniques for *D. melanogaster* cysts have been developed (Cross and Shellenbarger 1979). However, because of the difficulties associated with dissection of *D. melanogaster* testes, and with obtaining a substantial amount of cysts from the testes, *D. pseudoobscura* became the species of the choice in our lab for cyst culture. *D. pseudoobscura* testes in the abdomen of male flies are easily visible by the naked eye due to their vibrant red color (Njogu *et al.* 2010). Also, a protocol for culturing *D. pseudoobscura* cysts has been developed in our lab that allows for higher cyst viability than *D. melanogaster* (Njogu *et al.* 2010; Ricketts *et al.* 2011). *D. melanogaster* testes are pale yellow coiled tubular structures; whereas, *D. pseudoobscura* testes are ellipsoidal, and are thus much easier to handle and allow for obtaining many more cysts. The ellipsoidal morphology, bright red color, and ease of cyst acquisition from *D. pseudoobscura* testes make this species an attractive model for spermatogenesis studies.

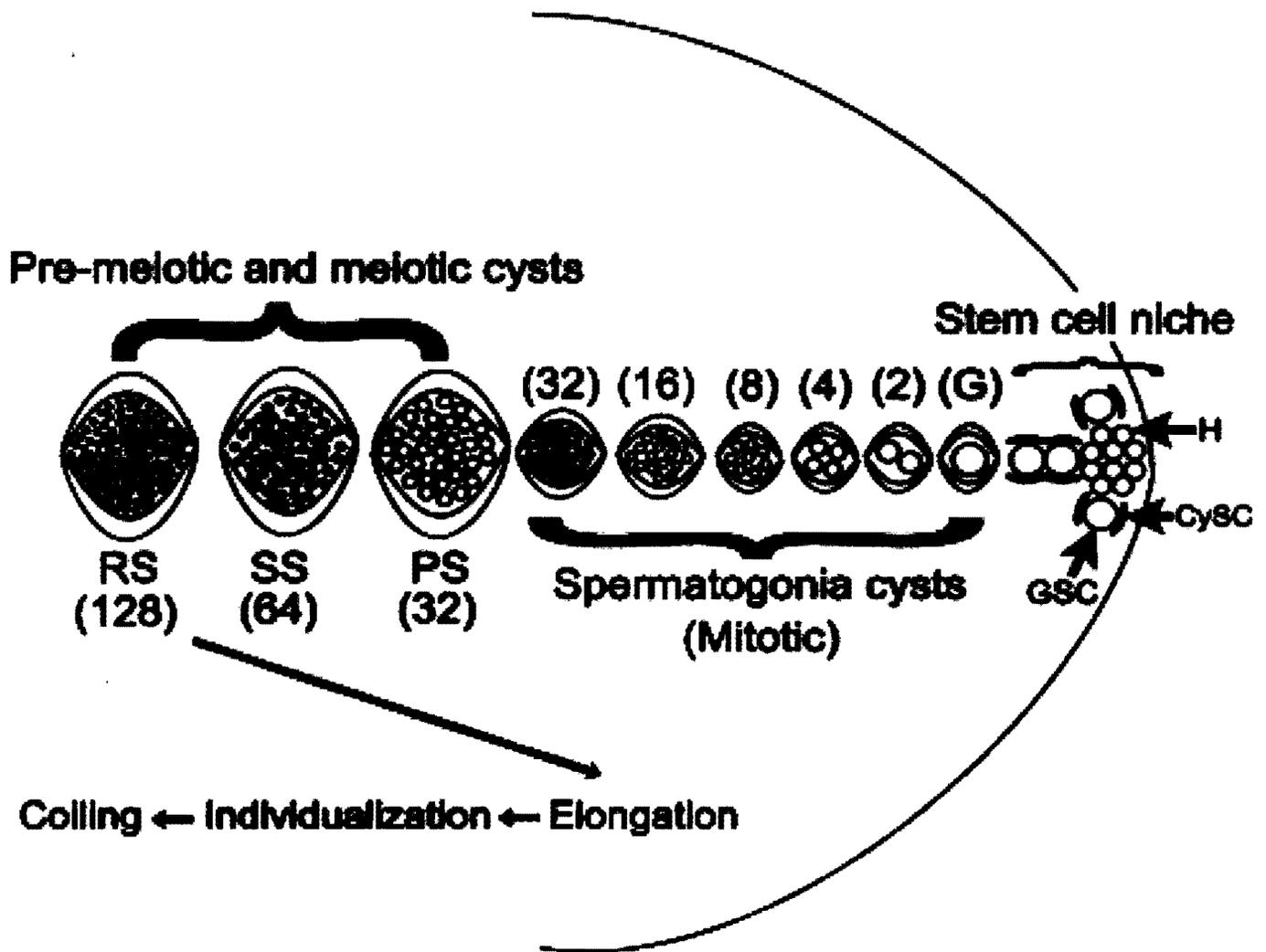**Figure 1:** Illustration of the progression of spermatogenesis in *Drosophila pseudoobscura* testes. A gonialblast (G) forms via a complete mitotic division of a GSC. Then five mitotic divisions produce a 32-cell stage. Next, two meiotic divisions produce the round spermatids containing 128 nuclei (RS). The post-meiotic stage called spermiogenesis produces mature spermatozoa. From Ricketts *et al.* 2011.

Like *D. melanogaster*, spermatogenesis in *D. pseudoobscura* commences in the stem cell niche. In *D. pseudoobscura,* a GSC undergoes a mitotic division to form a single gonialblast in the stem cell niche, which is not unlike *D. melanogaster*. In *D. pseudoobscura* testes, however, a gonialblast undergoes five mitotic divisions and two meiotic divisions to form a cyst of round spermatids containing 128 individual nuclei instead of the 64 formed in *D. melanogaster* testes.

Spermatogenesis in *Drosophila* can be compared to mammalian spermatogenesis in many ways, but there are some extremely important differences. In the mammalian testis, Sertoli cells line the blood-testis barrier and provide nutrients for the cells undergoing the many stages of spermatogenesis. These cells are required for testis formation and development as well as spermatogenesis in mammals. They are also involved in the development of germ line cells into functioning spermatozoa (Griswold 1998). These highly important cells in mammalian spermatogenesis are not present in *Drosophila* testes, but the cysts cells that encapsulate each individual cyst may be considered functional equivalents (Fuller 1998).

Spermatogenesis in *Drosophila* involves large-scale internal restructuring before the sperm are functional. As mentioned earlier, in *D. pseudoobscura,* 128 haploid round spermatids develop within an encapsulating cyst, formed from one single diploid gonial precursor through mitotic and meiotic divisions in the stem cell niche. There are three main steps in spermatogenesis in fruit flies: spermatid formation through mitotic and meiotic divisions, the post-meiotic transformation event called spermiogenesis, and coiling. Upon

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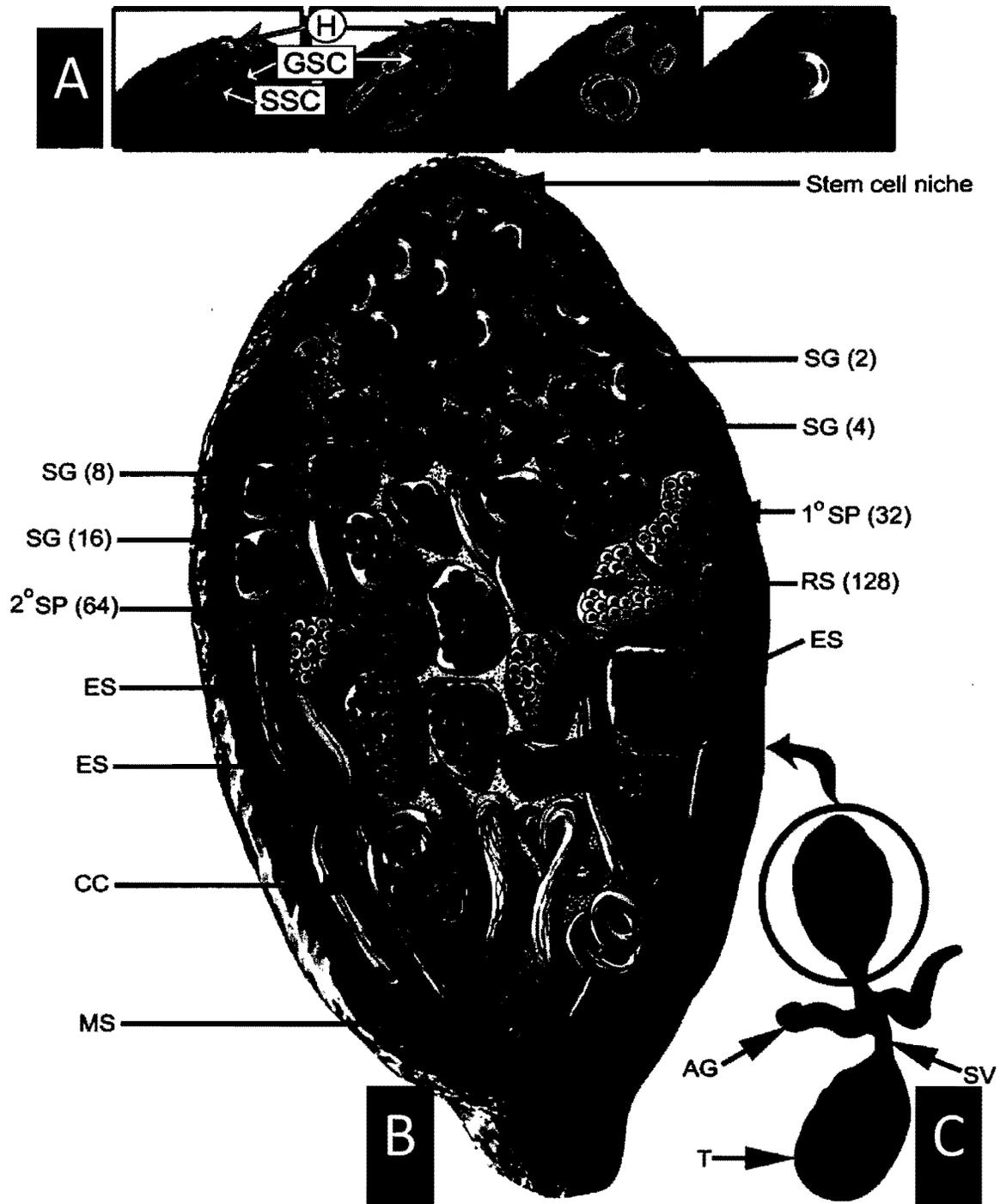
Figure 2: Illustration of spermatogenesis in *Drosophila pseudoobscura*. A representation of the stem cell niche in the apical end of the testis is shown in A. B shows spermatogenesis as it occurs in *Drosophila pseudoobscura* from a gonialblast to mature sperm. The number of nuclei in each cyst stage is indicated in parentheses. An intact pair of testes is shown in C. AG=Accessory Gland, T=Testis, SV=Seminal Vesicle. From Njogu *et al.* 2010.

spermatid formation via meiotic divisions, these cells undergo highly specialized reconstruction events to form motile spermatozoa capable of fertilization in a process called spermiogenesis. In *Drosophila*, spermiogenesis can produce functional spermatozoa that can reach 2 mm in length (Barreau *et al.* 2008).

Effective production of motile sperm includes nuclear transformation, axoneme construction, spermatid individualization, and large-scale mitochondrial reorganization (Riparbelli and Callaini 2007; Noguchi and Miller 2003). Nuclear volume is reduced 200-fold in *Drosophila* because, in *Drosophila* chromatin, histones are first replaced by transition proteins then eventually by proteins called protamines during nuclear transformation (Fuller 1993). This chromatin condensation event protects the DNA from breakage during the sperm transmission to the maternal egg (Rathke *et al.* 2009). The reduction in nuclear volume occurs in mammalian spermatids, but to a much lesser extent (Braun 2001, Rathke *et al.* 2010). Specific cell stages during spermatogenesis can be identified based on histone modification and replacement by protamines (Rathke *et al.* 2009). Transformation also comprises morphological changes as nuclei change from round to "needle-shaped." Spermatids also acquire a microtubule-based flagellum containing an axoneme that is involved with sperm motility. Immotile sperm mutants are available and are incapable of fertilizing a maternal egg (Gao *et al.*, 2003). Also, two mitochondria aggregate to form a huge structure known as a *nebenkern* during spermiogenesis in *Drosophila*, and this structure eventually forms the mitochondrial derivatives present in elongate spermatids (Clark *et al.* 2006).

In late stages of spermatogenesis in *Drosophila*, elongate spermatids proceed through the individualization process. In individualization in *Drosophila*, new plasma membranes are deposited on every single spermatid as one characteristic 'individualization complex' per spermatid navigates its way down the sperm tails (Timakov and Zhang 2001, Noguchi and Miller 2003). In *Drosophila melanogaster,* individual "investment complexes," comprised of 64 "actin cones," deposit plasma membranes on each individual spermatid (Noguchi and Miller 2003). As the 'individualization complex' traverses the tail, a distinctive 'cystic bulge' forms ahead of the complex and excess cytoplasm, organelles, and cyst cell membranes are extruded from the tails as a waste bag when the bulge detaches from the tail (Noguchi and Miller 2003, Huh *et al.* 2004). Interestingly, cytoplasm removal may be a way for the male gamete to prevent transmission of parasites to the egg (Randerson and Hurst 2001). The removal of the cytoplasmic connections between spermatids and caspases usually involved with apoptosis are essential for individualization to successfully transform spermatids into functioning sperm even though individualization is non-apoptotic (Huh *et al.* 2004). Inhibition of certain effector caspases at specific stages inhibits the non-apoptotic process of spermatid individualization in *Drosophila* (Arama *et al.* 2003).

Many proteins are implicated and interact with each other during the elaborate individualization process of *Drosophila* spermatogenesis, but F-actin seems to play a major role. Noguchi and Miller (2003) specifically characterized the importance of F-actin in individualization *in vitro* and its polymerization by the Arp2/3 complex is highly important in this process. In a dose-dependent fashion, 'cystic bulge' and 'individualization complex'

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movement stopped when cultures were treated with F-actin inhibitors (Noguchi and Miller 2003). Certain F-actin stabilizing drugs have been shown to effect movement slightly. As the 'individualization complexes' began movement, microtubules were not visible, and microtubule inhibitors did not prevent complex or bulge movement down the sperm tail (Noguchi and Miller 2003). Moreover, kinesin and myosin inhibitors showed similar results to the microtubule inhibitors where bulge and complex movement were concerned (Noguchi and Miller 2003). These results implicated F-actin polymerization as the main driving force in the movement of the bulge and complex, an actin-based structure itself, down the tail during individualization in *Drosophila*.

III.   Nuclear Migration

Moving nuclei bundles to specific locations is an extremely complex process that occurs across all kingdoms (Van Bruaene *et al.* 2004, Kosodo 2012, and Schillaci *et al.*2012). Until recently, very little had been known or reported regarding nuclear migration. For example, the microtubule motors dynein and kinesin in *C. elegans* have been described as important players in the nuclear migration event that occurs during embryogenesis (Starr 2011). Explicitly, Starr (2011) showed via live-cell imaging analysis that movement of nuclear bundles was dependent on *UNC-83*, a kinesin-1 and dynein recruiter, as *unc-83* mutations inhibited migration. In *Drosophila* oocytes, cell asymmetry exists as in *Drosophila* spermatogenic cysts, and microtubules have been shown to coordinately push nuclei to the anterior (Zhao *et al.* 2012). Interestingly, inhibition of a nuclear migration event has been investigated as a possible way to target breast cancer tumors in Chilean women because it has been shown that a migration

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event permits augmented tumor growth (Beguelin *et al.* 2010, Schillaci *et al.* 2012). A completely separate model for a nuclear migration event in asymmetrical fibroblasts has been shown to be actin-dependent. Luxton *et al.* (2010) showed that the *SUN2* and *nesprin2G* nuclear envelope proteins associated with linear 'cables' of actin during migration, and that actin cable assembly provided the force required for nuclear movement (Luxton *et al.* 2010). In fact, *SUN2, nespirin2G,* and these actin cables were all required for proper nuclear migration to progress as their inhibition prevented migration (Luxton *et al.* 2010). Another example of actin-dependent nuclear migration has been reported in mouse neurons (Solecki *et al.* 2009).

In *Drosophila pseudoobscura* spermatogenesis, a nuclear migration event appears to be occurring. Prior to spermiogenesis and elongation of each individual spermatid, all 128 nuclei of the round spermatid stage relocate to one side of the encapsulated cyst. At this stage, the cyst cell associated with the nuclei is termed the "head cyst cell" and the other cyst cell is termed the "tail cyst cell." The mechanism for this relocation is unknown. We sought to investigate the manner by which the round spermatid nuclei relocate to the head cyst cell. F-actin is prominent in the 'investment cone' that endows a plasma membrane on each individual spermatid and displaces superfluous cytoplasm as 'waste bags' during individualization (Noguchi and Miller, 2003). Actin projections from the head cyst cell are also important in sperm maturation in *Drosophila* (Noguchi and Miller, 2003). These projections adhere to the maturing spermatids within the cyst and have been reported to prevent premature release into the seminal vesicle (Desai *et al.*, 2009). Also, as actin is an extremely prominent protein in the sperm acrosome, we hypothesized that actin could be involved in nuclear migration.

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IV. Spermatid Tail Elongation

Sperm motility is extremely important for fertilization to occur properly. Sperm must be able to navigate its way through the female reproductive tract to pass on its genetic material and form a zygote. Microtubules are present in elongating *Drosophila* sperm cells before individualization even has occurred, but disappear during actin cone movement, and eventually reappear again (Noguchi and Miller 2003). The sperm tail of *Drosophila* has the 9 + 2 organization of microtubules (Doyle *et al.*, 2008).

As mentioned earlier, all sperm nuclei remain connected as they develop within an encapsulated cyst in *Drosophila,* and this allows for spermatogenesis to be coordinated. Another obligatory event in spermatogenesis is sperm tail elongation after (or possibly prior to) the 128 nuclei in the round spermatid stage have relocated to the head cyst cell. A *Drosophila-* specific *Dynein Light Chain 1* has been shown to be vital for this to occur as mutants show abnormal spermatid elongation (Ghosh-Roy *et al.* 2005). They also found that axoneme assembly is completely separate from sperm elongation based on ultrastructural analysis findings (Ghosh-Roy *et al.* 2005). In a separate study, the *nebenkern*, or giant mitochondrial complex formed in *Drosophila* spermatogenesis, provides a platform for microtubules to attach, and this is required for the tail elongation process (Noguchi *et al.* 2011).

V.   Current Approach

The purpose of the current work was to (1) establish a protocol for actin staining of *Drosophila pseudoobscura* spermatogenic cysts in culture, (2) investigate the mechanism by

which the 128 nuclei of the round spermatid stage in *Drosophila pseudoobscura* relocate to the head cyst cell in culture through high resolution imaging analysis, and (3) begin to understand the dynamics by which spermatids elongate after nuclear migration but prior to spermatid individualization.

MATERIALS AND METHODS

I. Fly Rearing

Drosophila pseudoobscura flies were obtained from the *Drosophila* Species Stock Center at the University of California-San Diego and grown on Jazz Mix (Fisher Scientific) at room temperature.

II. *Drosophila pseudoobscura* Testis Dissection

All dissection work was performed using a Nikon C-DSLS stereomicroscope on a sterilized lab bench. *Drosophila pseudoobscura* flies were anesthetized manually using carbon dioxide or by placing in the freezer for 60 seconds. Once immobilized, flies were placed in an autoclaved vial containing no food, and the males were separated from the females using sterilized fine forceps. Males were easily distinguishable from their female counterparts due to the intense red pigmentation of their testes located in the fly abdomen. Separated male flies were placed in a few drops of 1X PBS on a sterilized shallow well slide. Testes were removed from males using a sterile injection needle and fine probe. Individual flies were first decapitated, and then the probe was used to remove the pair of testes from the abdomen of the fly. Once removed,

the intact pair of *Drosophila pseudoobscura* testes was placed in a few fresh drops of 1X PBS on a sterilized deep well slide until enough flies were dissected for the specific experiment.

Pupae were also used for dissection purposes in order to maximize the amount of pre-migratory cysts. Pupae testes were dissected as described above; the only difference was that they were soaked in 70% ethanol for 10 minutes prior to dissection in 1X PBS. Healthy pupae were selected based on the presence of bright, red testes visible through the abdomen and pupal case. Upon dissection, remaining fat bodies as well as the seminal vesicle and accessory glands were carefully removed with a sterile injection needle and fine forceps.

III. Fluorescence Staining of non-cultured *Drosophila pseudoobscura* Cysts

Prior to bursting, pairs of *Drosophila pseudoobscura* testes were fixed in 200 μL of 4% paraformaldehyde in 1X PBST for eight minutes in a deep well glass slide. Then, the testes were washed three times in fresh 1X PBST for three minutes each. Whole testes were then placed in a single plastic well with a coverslip and cysts were burst from each testis using fine forceps and probe in 300 μL of stain cocktail. By holding the basal end of each testis with a sterile fine forceps, a sterile injection needle was used to slice open the apical end and tease out as many cysts as possible.

Cysts were burst in a shallow well formed by a silicon form attached to a coverslip containing a staining cocktail of 150 μL of Hoechst 33342 (Sigma-Aldrich) (final concentration, 5 μg/mL) and 150 μL of phalloidin-AlexaFluor 568 (Invitrogen) to a final concentration of 6

Units/mL. Cysts were incubated in this cocktail for 20 min. After incubation, samples were taken to the Olympus FVI000 confocal laser scanning microscope (CLSM) for high resolution imaging.

In some cases, acridine orange, another nuclear stain, was used instead of Hoechst. All steps described above were used for the acridine orange procedure. The final acridine orange concentration in the well for confocal imaging was 2 µg/mL. Cysts were incubated for 1 hour before imaging with the CLSM.

IV. In vitro Culture of *Drosophila pseudoobscura* Cysts

The culture medium used was as described by Njoju et al. (2010). 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin was added to supplement M3 insect culture medium (Sigma Aldrich). After soaking in 70% ethanol for 15 minutes, pupae were decapitated with a sterilized probe, and bodies were removed from the pupal case. Then, after testes were removed from the abdomen, cysts were teased out of the testes in 20 µL of fresh culture medium on a sterilized shallow well slide on a sterile lab bench. Three pairs of testes were burst for each culture well. After three pairs of testes were burst in 20 µL of media, 30 µL of media was added around the cysts to centralize them in the well. The glass coverslip containing the cysts was then taken to a sterilized laminar flow hood. A 200 µL pipet was used to transfer cysts in 30 µL increments very carefully into one well of a 24-well culture plate with 100 µL of sterile culture media already in the well. Formation of bubbles was avoided when transferring cysts. Each well was fused with a glass coverslip so as to eventually visualize cultures on the Olympus

FVI000 confocal laser scanning microscope. Cysts were transferred in 30 µL increments 10 times for a final volume of around 400 µL of culture media with cysts.

V.   Fluorescent Staining of 24-hour *Drosophila pseudoobscura* cultures

After successful transfer of cysts to a culture well, Hoechst 33342 was added for a final concentration of 0.02 µg/mL and the cultures were left in the hood overnight (Pristell and Klaus 2012). 24-well plates with coverslip bottoms were used. The next day, the cultures were visualized using the Olympus CLSM. Cysts were stained for cell viability after the 24-hour period with 0.1 µg/mL calcein-AM (Sigma-Aldrich) and visualized under the CLSM.

After 24 hours of culture in the hood, cysts remained adherent to the glass of the culture wells. Culture media was carefully removed from the culture wells to avoid removing the cysts from the well. Cysts were fixed with 4% paraformaldehyde for 8 minutes followed by three 1X PBST washes. Finally, phalloidin-AlexaFluor 568 (Invitrogen) was added to each well to a final concentration of 6 Units/ml, and cultures were visualized on the Olympus CLSM.

VI. Confocal Imaging and 3D reconstruction

An Olympus FV1000 CLSM was used for all high resolution imaging analysis of *Drosophila pseudoobscura* spermatogenic cysts. Samples were imaged using either a 40x or 60x oil immersion lens. For Hoechst 33342, a 405 nm laser was used for excitation. For Alexa Fluor 568 excitation, a 543 Helium/Neon laser was used. Optical sections were collected by

setting the top and bottom of the volume to be imaged, then optimizing the slice thickness using the "optimize" feature in the Olympus software. Datasets were collected at a sampling speed of 8 microseconds per pixel. Image resolution was set to either 512 x 512 or 1024 x 1024.

The computer program Imaris® (Bitplane AG) was used to convert high resolution stacks acquired from the Olympus CLSM into 3-D volume reconstructions. Videos files were created using Imaris® to more easily see the actin structure present in late-stage pre-migratory *Drosophila* cysts. Then, using Windows Movie Maker, screenshots were taken of the video files for use in figures.

Results

I. Wide-field fluorescence microscopy of *Drosophila pseudoobscura* spermatogenic cysts from testes

We first sought to characterize the pre- and post- migratory *Drosophila pseudoobscura* cysts in the testes using Hoechst 33342 to stain the individual nuclei. Figure 3 shows examples of *Drosophila pseudoobscura* cyst stages. In Figure 3A, an 8 or 16-cell spermatogonia cyst is clearly visible.   Figures 3B and 3C show cysts that are further along in the *Drosophila* spermatogenesis process as compared to the cyst in Figure 3A, but they have not yet undergone the nuclear migration event. Note that the individual nuclei in Figure 3A are much larger than those shown in Figures 3B and 3C. Figures 3D-3F and Figures 3G-3I show cysts where the nuclei have undergone the nuclear migration event to align at one side of the cyst. In Figure 3J, the cyst shown is further along in the spermatogenesis process than all the other cysts shown in Figure 3. The nuclei bundled in the cyst in Figure 3J have begun the elongation process (See Figure 1).

II. Confocal analysis of *Drosophila pseudoobscura* non-cultured spermatogenic cysts

The next step in our analysis was to investigate the possible role that actin may play in nuclear migration. We first had to develop a protocol to visualize the cysts using confocal microscopy. As mentioned above, we hypothesized that actin may be playing a role in the migration event because of its importance later on in spermatogenesis. Figure 4 shows confocal micrographs of *Drosophila pseudoobscura* cysts stained with Alexa Fluor 568 conjugated to phalloidin, and Hoechst 33342. Figure 4A shows an intact pre-migratory

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spermatogonia cyst very early in the spermatogenic process. Note the intense and punctate

actin signals in various parts of the cyst of Figure 4A. In other parts of that cyst, the actin signal

is much more diffuse and less intense than the signals marked with the arrow. In the burst cyst

shown in Figure 4B, there seems to be an actin signal surrounding individual nuclei that have

yet to undergo the migration event. Additionally, extended filamentous-like actin signals were

detected in this cyst (heavy arrows). 'Voids' were also observed which may represent the

mitochondrial derivative, or *nebenkern* (light arrows). The pre-migratory round spermatid cyst

in Figure 4B has more nuclei than the cyst in 4A. Figure 4C also shows a cyst in the early gonial

stages very similar to the cyst in Figure 4A. As evident in Figure 4A, the intact pre-migratory cyst

in Figure 4C also exhibits intense and punctate actin signals scattered throughout the cyst. In

Figure 4C, the punctuate signals seem to be more prevalent around the nuclei. The cyst in

Figure 4C is also clearly one that remained intact during the staining process. In Figure 4D, a

cyst containing nuclei that have already undergone the nuclear relocation event to one side is

shown. Note here the intense actin signal nearest the nuclei. This actin signal is in close

proximity to where the nuclei relocate to. This intensely orange signal was not present on the

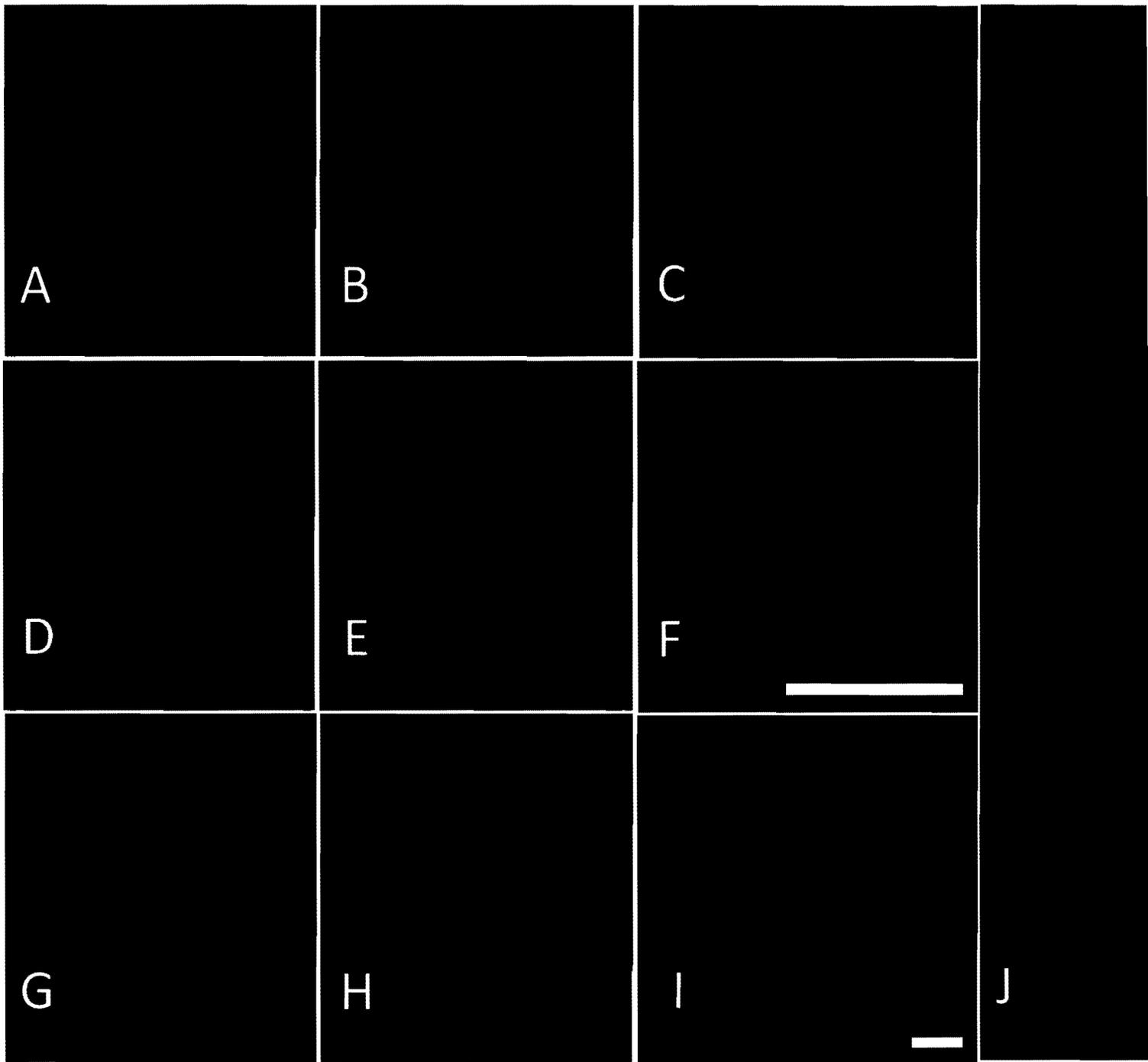other side of the cyst opposite where the nuclei align.

Figure 3: Fluorescent microscopy of *Drosophila pseudoobscura* cysts stained with Hoechst 33342 only. Various stages of spermatogenic cysts taken from *D. pseudoobscura* pupa stained with Hoechst 33342. **A-F** and **J** were taken at 400X magnification, and **G-I** were taken at 100X. In **A-C**, cysts in which the nuclei have yet to migrate are visible. In **D-I**, cysts that have undergone the nuclear migration event are visible, and **J** shows a stage in which nuclei have begun to elongate. Scale bar for all panels = 30 μm (**A-F** and **J** correspond to bar in **F**, and **G-I** correspond to bar in **I**)

In panel E, a developing cyst later on the spermatogenic process is shown. This cyst is similar to the cyst shown in Figure 3J. The nuclei have clearly migrated and elongated. The sperm tails also are evident in Figure 4E. Here, the actin signal is quite strong at the tips of the individual nuclei. These signals represent what will eventually become the acrosome. Figure 4E served as a control for the Alexa Fluor 568 confocal analysis.

Figure 5 also shows confocal micrographs of *Drosophila pseudoobscura* spermatogenic cysts taken from pupae. The nuclei in these four cysts have undergone migration and the tails have started to elongate. As shown in the cyst in Figure 4D, all these cysts show an intense actin signal along the plane of the cyst where the nuclei align. Also, the orange actin signal in the cysts of Figures 5A-5D is unlike the punctate actin signals present in the pre-migratory cysts shown in Figures 3A-3C. Figure 5B shows a whole cyst with the actin signal nearest the nuclei. Note in Figure 5B that the actin signal is not nearly as evident on the opposite side of the cyst away from where the nuclear relocate. This indicates that the strong actin signal nearest the nuclei in round spermatid cysts that have undergone nuclear migration may be separate from the actin signal present in the head cyst cell reported by (Desai *et al.* 2009). F-actin cones in *Drosophila* that form around individual nuclei and deposit individual plasma membranes while traversing the axonemes have been reported the conical structures visible in Figure 5C may represent these F-actin-based conical structures (Desai *et al.*, 2009; Noguchi and Miller 2003). Also, in Figure 5C there is some background staining present along the sperm tails, but the signal is most strong where the nuclei arise.
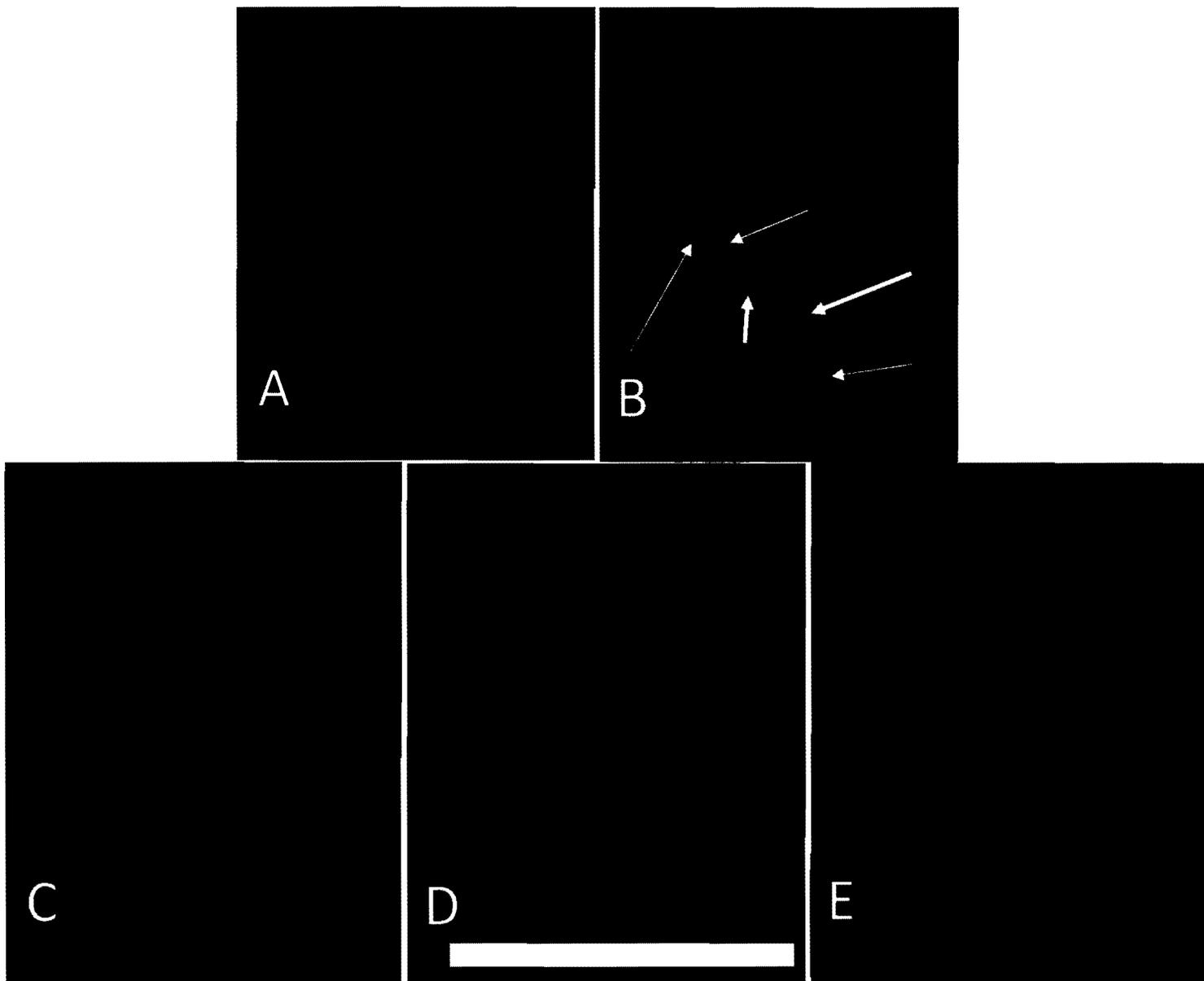
Figure 4: High resolution images of various stages of *Drosophila pseudoobscura* spermatogenic cysts. All cysts are stained with 6 u/mL Alexa Fluor 568 (orange) and 5 µg/mL Hoechst 33342 (blue) *in vitro*. **A** and **C** show intact pre-migratory cysts, **B** shows a burst pre-migratory cyst, **D** consists of a cyst that has undergone the nuclear migration event, and **E** shows a cyst that is further along in spermatogenesis that has begun formation of the sperm tail. Also, note in panel **B** multiple 'voids' that may represent the mitochondrial derivative, or *nebenkern* (light arrows) and the filamentous actin structures present within this cyst (heavy arrows). Scale bar = 50 µm for all panels.
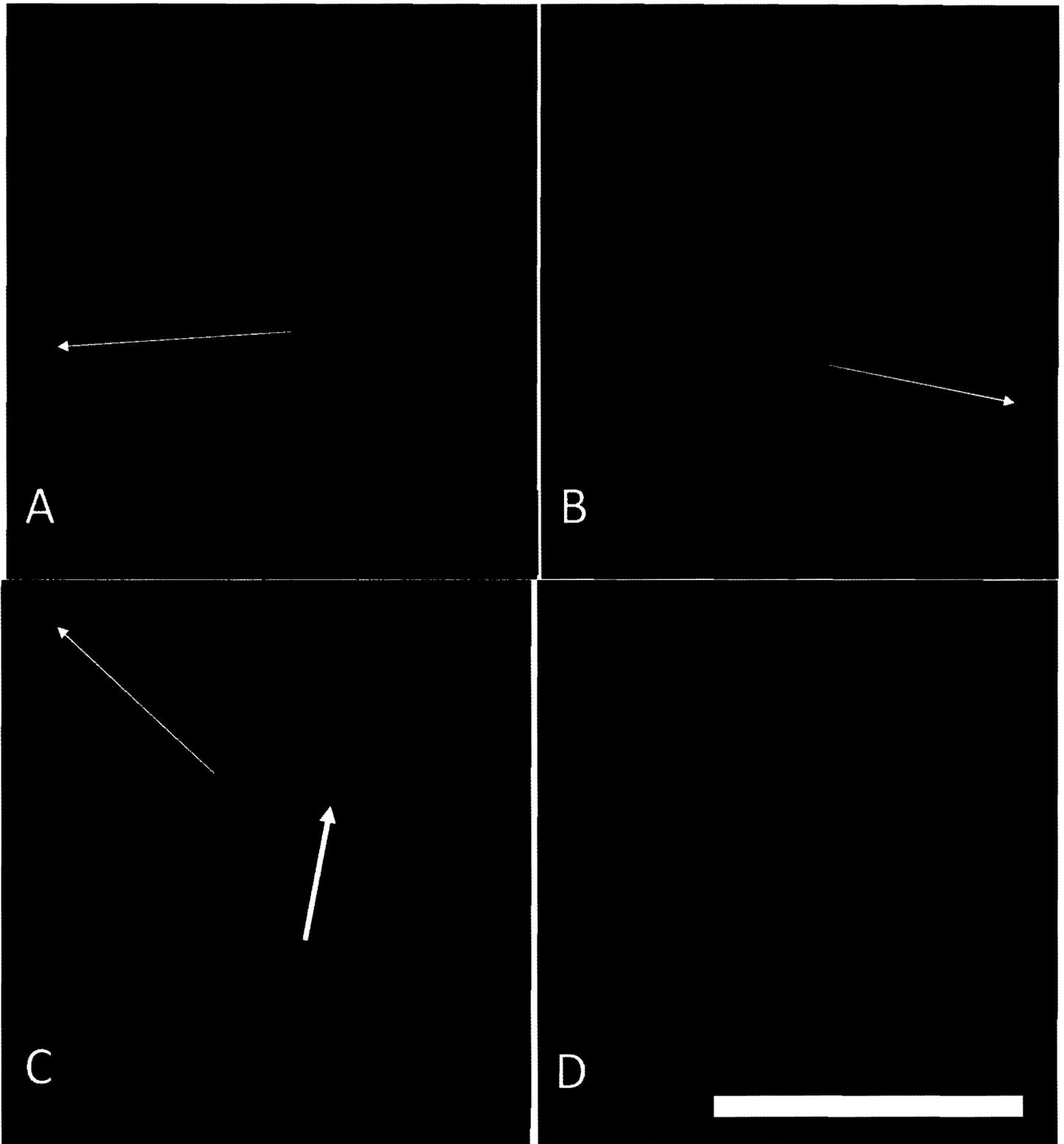
Figure 5: High resolution images of *Drosophila* pseudoobscura spermatogenic cysts that have undergone migration. Each panel shows cysts stained with 5 µg/mL Hoechst 33342 (blue) and 6 Units/mL Alexa Fluor 568 (orange). Panels **A, B, and D** show individual cysts in which the nuclei have migrated and begun to elongate. Panel **C** shows a cyst in which the nuclei have migrated, but have not yet begun to elongate. Also, note in panel **C** the pointed actin structures visible in the center portion of the cyst (heavy arrow). Scale bar= 50 µm for all panels

III. Analysis of *Drosophila pseudoobscura* cultured pupal spermatogenic cysts

The next phase of the investigation included cell culture analysis and confocal visualization on *Drosophila pseudoobscura* pupal cysts. In order to visualize filamentous actin structures, the cysts first needed to be fixed and rinsed before staining, however, the cysts are non-adherent to plastic culture plates. Additionally, higher resolution imaging must be performed through a coverslip. Culturing cysts for 24 hours in coverslip bottom culture plates facilitated the adherence of cysts to the plates. This allowed us to fix the cysts with formaldehyde and rinse without a massive loss of material.

Figure 6 shows phase contrast micrographs of individual cysts overlaid with the Hoechst 33342 nuclear stain (blue). The cysts were stained (at hour 0 of culture) with 0.02 μg/mL Hoechst 33342, and placed in culture medium for 24 hours. Then phase contrast microscopy was used to visualize the 24-hour old cysts. In Figure 6A, a cyst that has undergone nuclear migration is visible. Figures 6B-6C and Figure 6F show further examples of cysts whose nuclei have migrated. Figure 6D shows another cyst that is further along in the spermatogenesis process. Its nuclei have undertaken migration, begun forming the individual sperm tails, and begun to elongate. Figure 6E is a cyst in the spermatogonia stage that has yet to undergo nuclear migration.

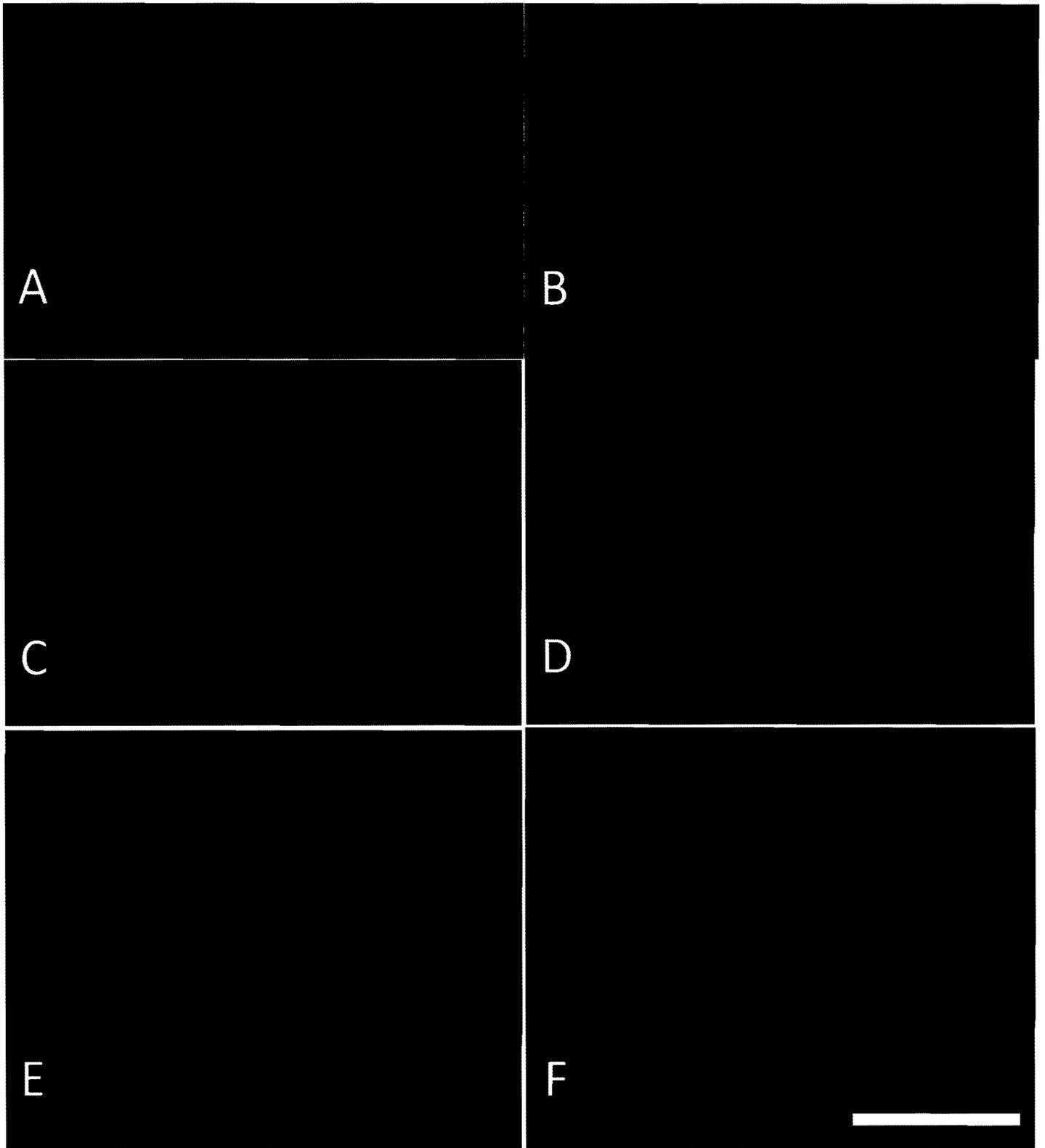**Figure 6:** Phase contrast micrographs of cultured *Drosophila pseudoobscura* spermatogenic cysts after 24 hours. All cysts were stained with 0.02 µg/mL Hoechst 33342 at 0 hours. **A-D** and **F** show cysts that have undergone nuclear migration, and **E** is a cyst earlier in the spermatogenic process that has yet to undergo migration. Scale bar for all panels = 25 µm

Figure 7 again shows cultured spermatogenic cysts taken from *Drosophila pseudoobscura* pupa, but these cysts were stained with 0.1 μg/mL of calcein-AM after being cultured for 24 hours. As before, these cysts were stained with 0.02 μg/mL Hoechst 33342 at the start of the culture. Calcein-AM stains for cell viability, and clearly all these cysts remained alive after 24 hours as evidenced by the green fluorescence of all cysts shown in Figure 7. Most of the cysts stained with 0.02 μg/mL Hoechst 33342 from all the culture trials remained viable after 24 hours.

Figure 7A shows a cyst that has undergone nuclear migration. What is fascinating about all the cysts in Figure 7 is the presence of the multiple "elongation bulges" on all developing sperm tails (light arrows). Note the presence of cytoplasmic 'voids" in the head cyst cell in Figures 7A and 7D where the nuclei align (heavy arrows). Figure 7B is an example of cysts where these voids were not detected. Figure 7C is a cyst where the tail cyst cell has burst but the head cyst cell seems to be intact. The nuclei have migrated in this cyst, and the "elongation bulges" are clearly visible on each individual developing sperm tail. Figure 7D shows a cyst similar to the cyst in Figure 7A. Figure 7E is another example of a cyst similar to the one in 7A. Finally, Figures 7F and 7G show cysts that have much more developed sperm tails than the other cysts in Figure 7.

Figure 8 shows examples of 24-hour cultured cysts stained with Alexa Fluor 568-phalloidin and Hoechst 33342. Figure 8A and Figure 8D show slices of two different pre-migratory *Drosophila pseudoobscura* spermatogenic cysts stained for F-actin with Alexa Fluor 568 and DNA with Hoechst 33342. Similar to the cysts displayed previously (Figures 3, 4, and 6),

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these cultured cysts also appear to have characteristic actin structures surrounding the nuclei that have yet to migrate. The confocal image stacks were reconstructed into three dimensional volumes using Bitplane Imaris (Figures 8B, 8C, 8E, and 8F).

After using Imaris® to create the files, Windows Movie Maker® was used to take screenshots of the clips at different angles. These 3-D reconstructions are displayed in Figures 8B, 8C, 8E, and 8F. Figures 8B and 8C are snapshots at different angles of the same video created from the cyst in Figure 8A. Figures 8E and 8F are snapshots at different angles of the video of the cyst in Figure 8D created by Imaris®. In all of these panels (8B, 8C, 8E, and 8F) large spherical actin structures are present (light arrows).Visualizing these spherical structures is difficult without reconstructing the image stacks into three dimensional volumes. Single two dimensional confocal image slices do not allow for visualization the spherical actin structures present in late stage pre-migratory cysts. Separate from the large spherical actin structures present in Figures 8B, 8C, 8E, and 8F, note the presence of the actin signals surrounding individual nuclei in Figure 8A. These actin structures were not present in early pre-migratory *Drosophila pseudoobscura* spermatogenic cysts (Figure 4A-4C).
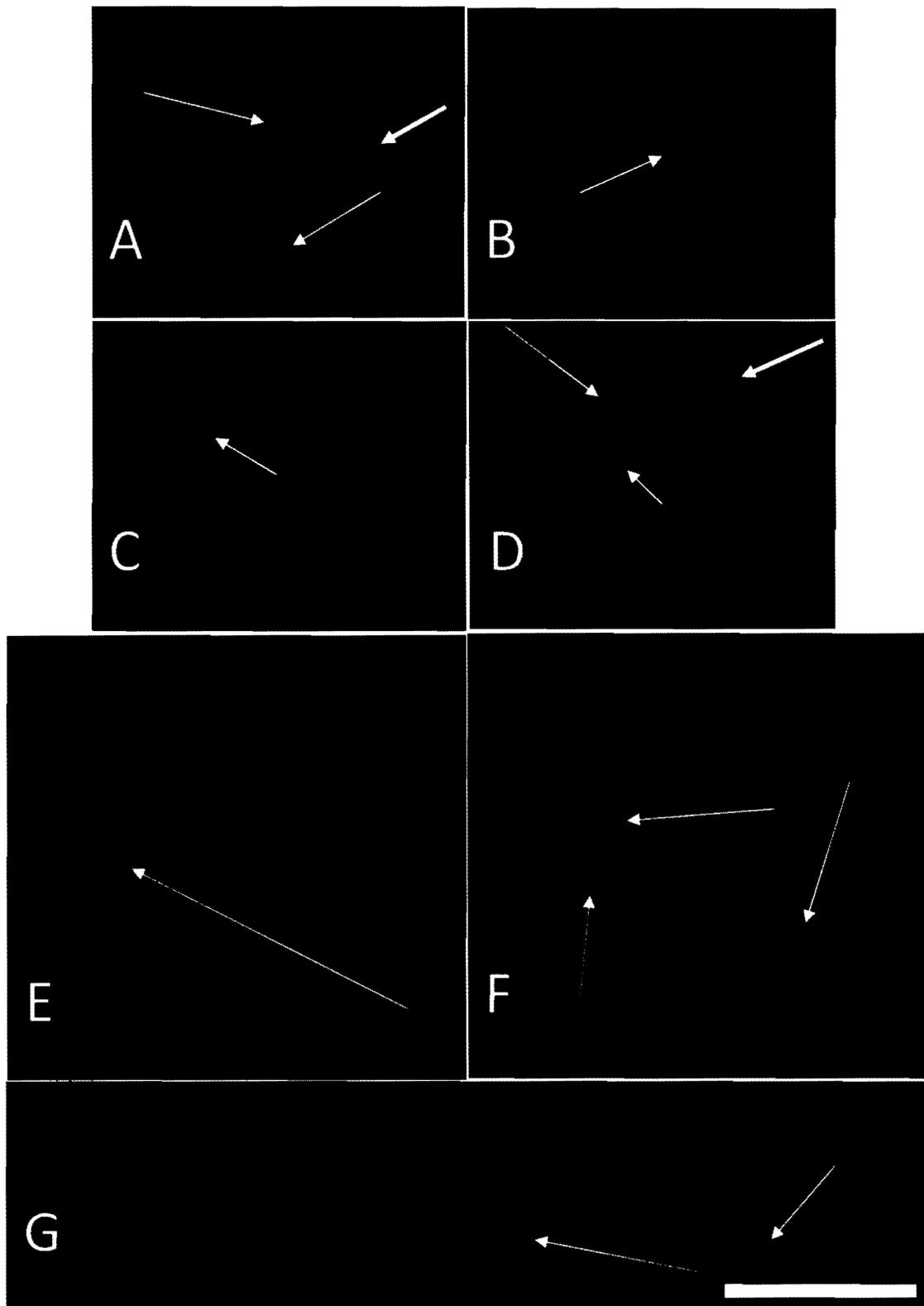
Figure 7: High resolution images of cultured *Drosophila pseudoobscura* cysts after 24 hours stained with 0.02 µg/mL Hoechst 33342 (blue) and calcein-AM (green). Calcein-AM allows for visual verification of cell viability. Note the presence of many "elongation bulges" in all panels and multiple bulges on each individual sperm precursor (light arrows). Panels **A-E** show cysts that have just undergone the nuclear migration event; whereas, **F** and **G** show cysts later on in spermatogenesis in which nuclear elongation has begun. The calcein-AM signal has faded after prolonged imaging in Panels A, C, and F. Panels A and **D** also show characteristic voids in the head cyst cell (heavy arrows). Scale bar= 50 µm in all panels
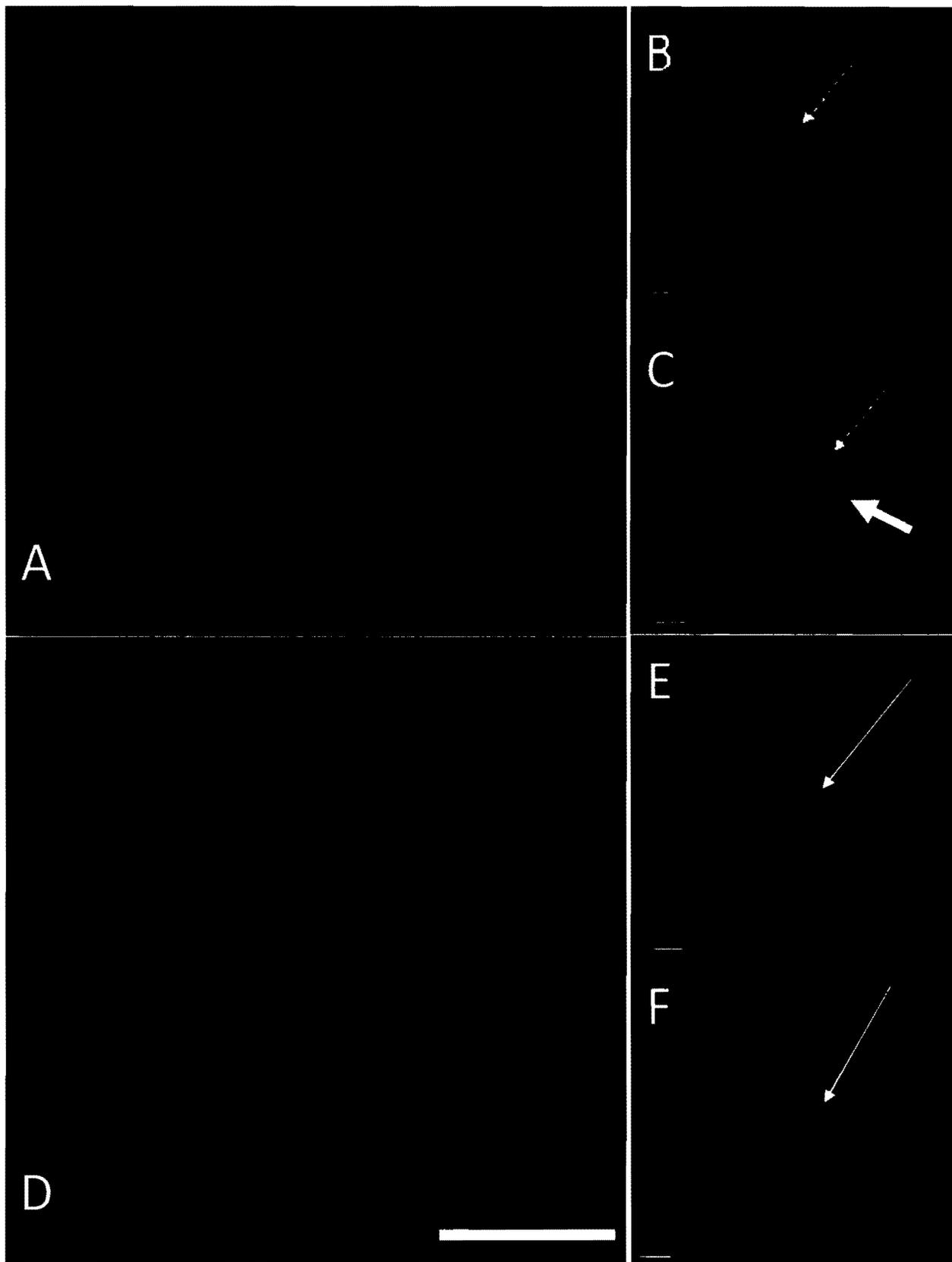
Figure 8: High resolution images of 24 hour-old *Drosophila pseudoobscura* cultured cysts and screenshots of 3D reconstructions. All cysts are stained with 6 u/mL Alexa Fluor 568 (orange) and 0.02 µg/mL Hoechst 33342. **A** and **D** are slices taken with a confocal microscope. Panels **B** and **C** are 3D representations of the cyst in panel **A** shown at two different angles, and panels **E** and **F** correspond to the cyst in **D**. The large, globular actin structure present in panels **B, C, E, and F** that may be playing a mechanical role in the nuclear migration are indicated (light arrows). Scale bar for panels **A** and **D**= 25 µm. Scale bar for panels **B, C, E,** and **F**= 10 µm

DISCUSSION

I. Pre-Migratory Round Spermatid Cysts Have an Actin Structure not Present in Earlier Stages

Spermatogenesis is a highly complicated and regulated process thzat involves whole-scale cellular changes. *Drosophila* has been a model genus for studying this highly complex progression of a single gonialblast to mature sperm for more than thirty years (Cross and Shellenbarger 1979). The purpose of this work was to show that in spermatogenesis in *Drosophila pseudoobscura*, the nuclei that develop within an encapsulating cyst and eventually become mature sperm must relocate to one side of the cyst before the process can continue. We also sought to investigate the importance F-actin may play in this event due to findings that identified it as an important player in other nuclear migration events. Moreover, a characteristic "individualization cone" made primarily of actin is present in developing *Drosophila* sperm that aids in the individualization process of spermatogenesis by depositing individual cell membranes on each sperm precursor (Noguchi *et al.*, 2003). Actin is also known as a major component in acrosome biogenesis. In *Drosophila*, F-actin based projections from the head cyst cell have been found to aid in the prevention of premature spermatid release (Desai *et al.*, 2009). Finally, we hoped to investigate a separate event in spermatogenesis in *Drosophila pseudoobscura* in which the sperm tails form and elongate.

In vitro analysis using Alexa Fluor 568 conjugated with phalloidin, a known F-actin probe with very high affinity, and Hoechst 33342, a nuclear dye, show F-actin may be a player in nuclear migration in *Drosophila pseudoobscura* spermatogenesis. Through phase-contrast and confocal microscopy, we show here that the nuclei definitely relocate to one side of the cyst during spermatogenesis (Figures 3-5). After developing the methodology required to stain cysts with the Alexa Fluor 568 and Hoechst 33342, we used this procedure in cultured *Drosophila pseudoobscura* cysts. The culture method was developed previously in our lab (Njogu *et al.*, 2010, Ricketts *et al.*, 2012). 24-hour-old cultured cysts show a large actin structure in round spermatid spermatogenic stages prior to the nuclear migration event (Figure 8). These large actin structures may be playing some sort of role in the nuclear migration process in *Drosophila* spermatogenesis. The nuclei seem to congregate around this spherical actin structure before migration (Figure 8B, 8C). In addition, there seems to be a higher volume of nuclei surrounding the spherical actin structure in 8C (heavy arrow), but this is not as evident in 8E or 8F. Separately, it is important to note the presence of the distinct actin signals in Figures 8A that seem to surround each individual nucleus. Perhaps these structures are involved in the nuclear migration event because these structures are also not as prominent in earlier cyst stages.

Actin has been associated with nuclear migration and positioning in many systems (Starr and Han 2003). In fact, actin has been shown to provide the physical force required to displace nuclei in *Drosophila* and in *Caenorhabditis elegans* (von Dassow and Schubiger 1994, Starr and Han 2003). In *C. elegans* embryos, actin disruption has been shown to lead to anomalous relocation of mitochondria, and they argue that actin may be important in nuclear migration

(Starr and Han 2003). Previous time-lapse experiments have shown that incomplete disassembly of a filamentous actin structure in close proximity to nuclei is required for a nuclear migration event in the *Drosophila* embryo (von Dassow and Schubiger 1994). Figure 5 shows cysts that have undergone nuclear migration in which the nuclei seem to aggregate near an actin signal. As mentioned earlier, this signal most likely is not an extraneous signal from the head cyst cell because the tail cyst cell does not show a similar signal. Most if not all of the cysts that have undergone migration show this sort of signal where the nuclei seem to relocate. Perhaps, then, actin is not only the main protein present in the 'individualization cone' and acrosome, but it may also be serving as the structural element that enables the nuclei to migrate to the head cyst cell prior to individualization and maturation of the spermatids into functioning spermatozoa. Maybe disassembly of this large structure we see in pre-migratory *Drosophila pseudoobscura* spermatogenic cysts facilitates the nuclear relocation, and the signal we see in Figure 5 actually is the dissembled form of the spherical structure that we see in the 3-D reconstructions in Figure 8.

In *Saccharomyces cerevisiae*, an actin-related gene, *ACT5*, was discovered to be required for a nuclear migration event. Null mutants of *ACT5* and overexpression of a similar gene, *Act5p*, resulted in irregular nuclear migration (Muhua *et al.*, 1994). In the *Neurospora* fungus, unusual hyphae aggregates arise due to defects in a different gene, *RO-4*, with sequence homology to vertebrate actin. These aggregates arise due to the result of a disrupted nuclear migration event (Robb *et al.*, 1995). The large actin structure present in the 24-hour pre-

migratory cultured cysts provides more evidence that actin may be an important player in

nuclear migration events in spermatogenesis.

II. Many "Elongation Bulges" Form on Elongating Sperm Tails in

Drosophila pseudoobscura

Using a methodology previously developed in our lab (Njogu *et al.*, 2010, Ricketts et al.,

2012) we were able to culture *Drosophila pseudoobscura* spermatogenic cysts and test their

viability after 24 hours. Previous testing in our lab investigated the optimal concentration of

Hoechst 33342 to permit culture viability (Pristell and Klaus 2012). A final concentration of 0.02

µg/mL Hoechst 33342 was added at zero hours to the cultures. After 24 hours calcein-AM

staining and confocal microscopy showed that the cysts remained viable and revealed many

"elongation bulges" on cysts where the nuclei had already migrated (Figure 7). To the best of

our knowledge, these structures have not been reported for elongating spermatids in

Drosophila. Their role is currently unknown, but they could possibly be related to the synthesis

of the sperm tail. Fertilization is the production of a diploid zygote from haploid paternal and

maternal gametes. One extremely important component of a sperm cell is the sperm tail that

permits utility of the cell through the female reproductive tract to facilitate fertilization. In fact,

Drosophila sperm can vary in length from a few microns to centimeters in length (Noguchi *et al.*

2011). The sperm tail is so vital to fertilization that a previous study that investigated the

correlation between sperm tail lengths and fertilization rate found that *Drosophila* evolved to

have long sperm because females' reproductive tracts select for males with longer sperm

(Miller and Pitnick 2002). The sperm tail, like any other flagellum, is made primarily of microtubules; so, understanding sperm tail elongation dynamics and how microtubules function in this process in *Drosophila* could be useful. Sperm tail elongation is quite an extensive component of spermatogenesis. It should come as no surprise then that microtubule-mitochondria associations are extremely important to the production of elongate sperm. A previous study in *Drosophila* found a microtubule and mitochondria-associated protein, *Milton*, and other microtubule cross-linking proteins whose disruption caused major defects in sperm lengths (Noguchi *et al.*, 2011). Visualizing the mitochondria along with microtubules in *D. pseudoobscura* cultured cysts is a goal for future work.

Here, we found rather distinct cytoplasmic voids are present in the head cyst cell in elongating cysts (Figure 7A, 7D, 7G). Further analysis is required to verify the nature of these voids. Performing fluorescence microscopy with tubulin antibodies may provide convincing evidence may give insight into the function of these voids. These voids may indicate the cells are apoptotic, the voids do not seem to resemble the blebs characteristic of apoptosis. Although these voids do not seem to be apoptotic, further analysis is required to verify this assumption.

III. Future work

Developing a live-cell imaging protocol would allow us to visualize the nuclear migration event as it is occurring in *Drosophila pseudoobscura* cultured cysts. This would provide much more insight into how nuclear migration occurs and enlighten us on the amount of time the

event actually takes. A potentially major drawback to live-cell analysis on cysts would be that because fixation with formaldehyde is required for high resolution imaging, we would not be able to use our Alexa Fluor 568 conjugated with phalloidin. Attempting to incubate the burst cysts with the Alexa Fluor 568 tagged with phalloidin without fixing the samples before proved unsuccessful. Actin inhibitors would also prove indispensible in further understanding of the role actin plays in nuclear migration. Because it appears that staining of the nuclei with very low concentrations of Hoechst 33342 is non-toxic to cultured cysts (Pristell and Klaus, 2012), the effects of actin inhibitors could be assayed by imaging the movement of the nuclei.

Also, due to the fact that other proteins have been implicated in nuclear migration events in different models, we tried performing a similar procedure for incubating *Drosophila pseudoobscura* cultured cysts with α-tubulin antibodies, but time constraints prevented us from perfecting that protocol. Being able to visualize α-tubulin in our cultured cysts would provide much needed insight into the nuclear migration event as well as tail elongation. In fact, evidence shows that some nuclear migration events are not exclusive, but rather dependent on both actin and microtubules together (Spear and Erickson 2012); so, possibly performing a triple stain with Hoechst 33342, Alexa Fluor 568 conjugated with phalloidin, and a tubulin antibody could help to characterize the nuclear migration event in *Drosophila* spermatogenesis.

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