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# The Role of Diaphanous in Ring Canal Development in Drosophila melanogaster

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*Butler University*

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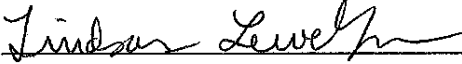
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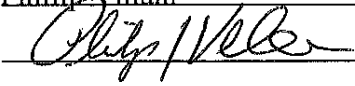
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The Role of Diaphanous in Ring Canal Development in *Drosophila melanogaster*

**Alexandra M. Kindred**
Butler University

**The Role of Diaphanous in Ring Canal Development in *Drosophila melanogaster***

A Thesis

Presented to the Department of Biological Sciences

College of Liberal Arts and Sciences

and

The Honors Program

Of

Butler University

In Partial Fulfillment

of the Requirements for Graduation Honors

Alexandra M. Kindred

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## Abstract

Infertility is a widespread condition that does not always have a known cause, and for which we often do not have a cure. One potential cause of infertility is defects in gametogenesis, or the formation of sperm and egg. During gametogenesis in most organisms, the developing sperm and egg are connected to each other or to supporting cells through intercellular bridges, allowing transfer of materials between cells. Defects in these connections can lead to infertility. The developing fruit fly egg is an excellent model system to study intercellular bridges, or ring canals. Rich in f-actin and actin-binding proteins, ring canals expand ~20 fold during oogenesis, and this expansion is accompanied by a 134-fold increase in the amount of actin in the structure. Ring canal expansion depends on the Arp2/3 complex; mutations in Arp2/3 complex members lead to decreased expansion and ring canal collapse. Interestingly, the Arp2/3 mutant phenotype has been reported to affect later stages of oogenesis (beginning at stage 5). This suggests that other actin nucleators could be involved in promoting ring canal growth prior to this point. I have characterized a role for the formin-family actin nucleator, Diaphanous (Dia), during oogenesis. Depletion of Dia leads to defects in normal ring canal structure and expansion, which are distinct from those observed following depletion of the Arp2/3 complex members. Future work will determine the mechanisms that promote the localization and activation of Arp2/3 and Diaphanous in the context of ring canal formation and expansion.

**Introduction**

*Infertility*

About 6.1 million Americans struggle with infertility, making it a prevalent health issue (12). There are various causes of infertility including cervical abnormalities, ovarian insufficiency, pelvic adhesions, etc. (23) but 25% of infertility cases result from unknown causes (12). Learning more about the basic processes and structures that are important for gamete (sperm, egg) formation could increase our understanding of human infertility and possibly lead to a cure.

*Intercellular Bridges and Gametogenesis*

Intercellular bridges are structures found in the developing gametes of organisms from insects to humans, and defects in intercellular bridges can lead to infertility in many species (6). These structures allow for the sharing of materials (mRNAs, proteins, and even organelles) between neighboring cells (15, 21). During gamete formation, or gametogenesis, intercellular bridges are formed following incomplete cytokinesis; instead of completing the separation between the daughter cells at the end of cell division, cells maintain a cytoplasmic connection, which allows them to communicate with each other, synchronize behaviors, and transfer cytoplasmic materials (5, 15, 20).

The most well-studied intercellular bridges are found within the developing *Drosophila* egg chamber, a multicellular structure which will give rise to the fruit fly egg. Within the ovary of the adult fly, there are 16-18 ovarioles. Each ovariole contains an array of developing egg chambers, with the youngest egg chambers at the anterior, and the oldest egg chambers at the posterior. Each egg chamber is composed of a central

cluster of 16 germ cells (15 nurse cells and one oocyte) that is surrounded by a layer of

epithelial cells called the follicle cells (Fig. 1A, 2B; 20, 21). Formation of the germ cell

cluster begins by division of a germline stem cell, which generates a cystoblast cell; the

cystoblast then undergoes four rounds of incomplete division to form a cluster of 16

interconnected cells (Fig. 1B, 2, 3; 21). In the egg chamber, the intercellular bridges are

called ring canals. Over the course of the 14 stages of oogenesis, the ring canals increase

in size from ~0.5 µm to 10 µm in diameter (21). Because of their large size, the ring

canals in the developing fly egg are easy to visualize using standard fluorescence
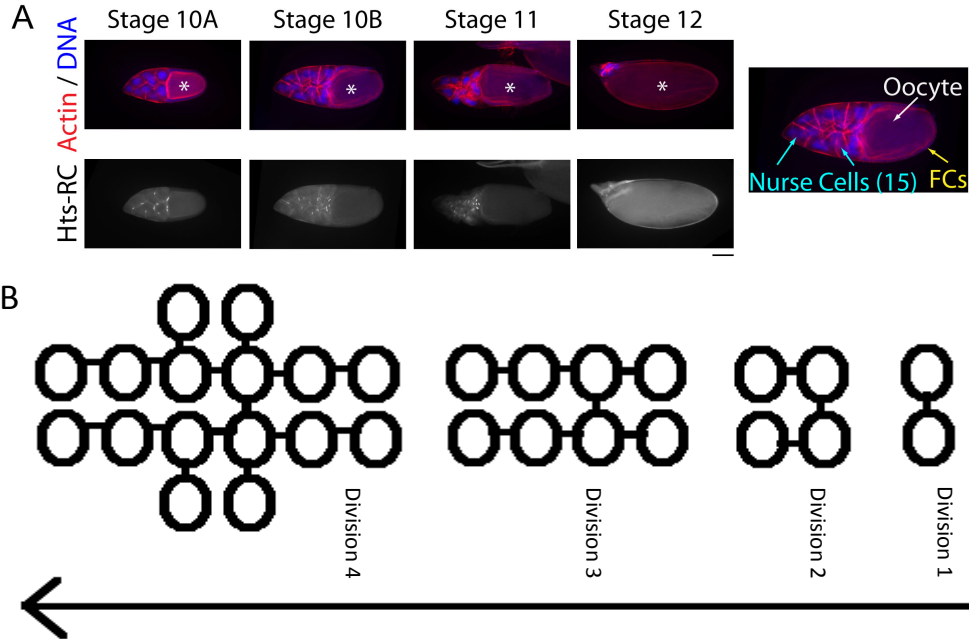


**Figure 1. Egg chambers and ring canal growth.** (A) Late stages of oogenesis showing the process of nurse cell dumping with the nurse cells on the left and the oocyte on the right. Fluorescence images were taken with the same magnification and stained with phalloidin, DAPI and Hts-RC antibody to show the ring canal. From Dr. Lindsay Lewellyn at Butler University (B) Four cell divisions with incomplete cytokinesis resulting in a cluster of 16 cells with intercellular brides connecting them. Arrow represents temporal progression of the divisions. Adapted from Tilney (1996).

microscopy.

　　Ring canal formation and expansion are distinct processes in oogenesis. The beginning stages of egg chamber development occur in the germarium, which is found at the anterior of each ovariole. Formation of the egg chamber within the germarium is complex, involving coordinated division of germline stem cells, somatic stem cells, and then encapsulation of the germline cluster by the somatic follicle cells; careful microscopic analysis of this process revealed that there are 29 stages in the formation of the germline cyst of *D. melanogaster* (11). During the first 20 stages, the germline cells are undergoing mitotic divisions, and the ring canals do not appear to grow in size. It is not until the nona (no nuclear anillin) stages that ring canals begin to expand. This
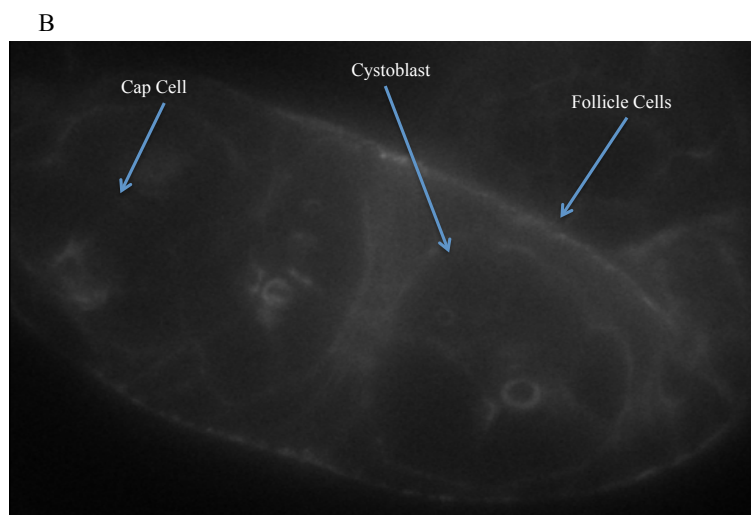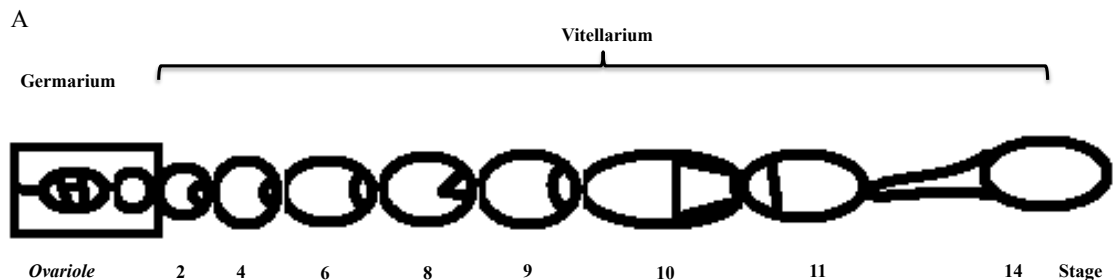


**Figure 2. Germline development in the egg chamber in *Drosophila melanogaster*.** The 14 stages of oogenesis from the germarium (boxed) to a mature egg cell (stage 14). Youngest (left) to oldest (right). (B) Image of maturation from cytoblast to a budding egg chamber surrounded by follicle cells.

suggests that incomplete cytokinesis and ring canal growth are two separate events in the development of the egg chamber (11), which are likely controlled by different molecular mechanisms.

Ring canal growth is essential for normal egg formation. During the early stages of oogenesis, there is a slow transfer of materials from the nurse cells to the oocyte, but at stage 11, the nurse cells squeeze all of their cytoplasmic contents into the oocyte through the ring canals, doubling the oocyte volume in just 30 minutes, a process called "nurse cell dumping" (Fig. 1A; 21). Therefore, it is essential that the ring canals are stable and large enough to accommodate the sudden influx of volume in such a short time (9). Although a number of structural and regulatory proteins have been identified that localize to the ring canals in the egg chamber and are required for their structure and expansion (6, 9, 13), there is still much to be learned about how these conserved intercellular bridges are regulated.

*Actin Nucleators are Necessary for Proper Intercellular Bridge Structure*

Ring canals are made up of filamentous actin (f-actin) and actin-binding proteins. Previous research has shown that the seven-member Arp2/3 actin-nucleation complex is essential for proper ring canal structure (9, 22). The Arp2/3 complex binds to pre-existing actin filaments and initiates the formation of a branch at a 70-degree angle, allowing it to create branched actin networks (17). The Arp2/3 complex must be activated by nucleation-promoting factors (NPFs) (2) as well as post-translational modifications (17). Interaction with members of the WASP/SCAR family (9, 24), as well as phosphorylation of the Arp2 subunit (10) are both important activation steps. Mutations in two of the

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Arp2/3 subunits lead to decreased ring canal size, ring canal deformities, and in some

cases collapse of the entire ring canal, beginning around stage 5 of oogenesis (8, 9, 22).

Preliminary data from our lab has confirmed that depletion of the Arp2/3 complex

member, ArpC2, by RNA interference (RNAi) also led to a severe defect in ring canal

expansion (Fig. 3A,B; 22).
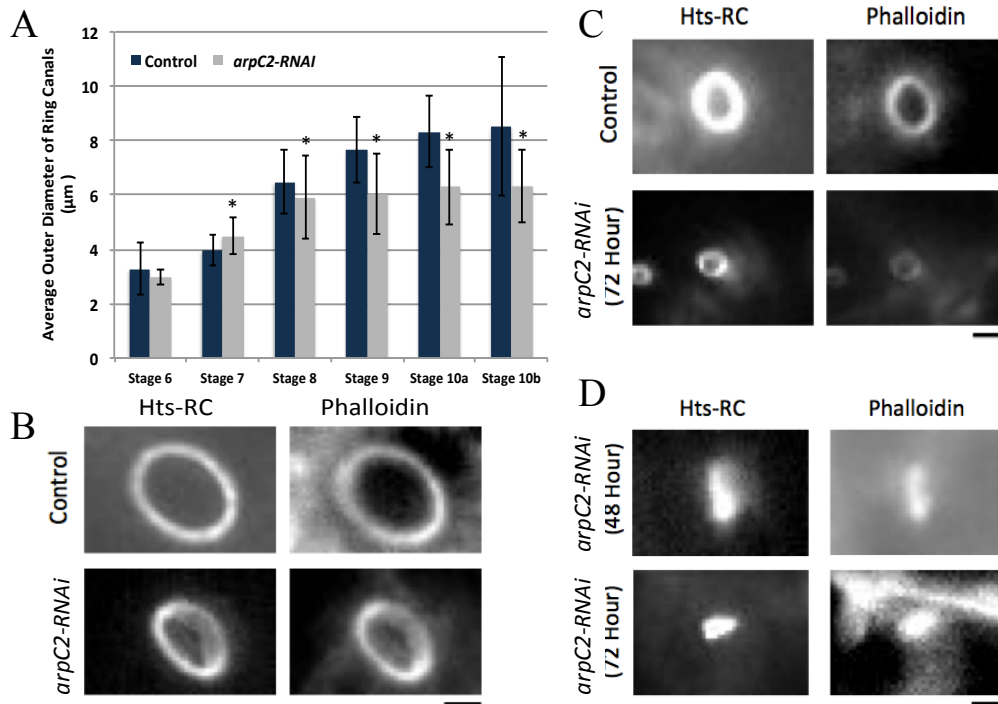


**Figure 3. Ring canals in Arp2/3 depleted egg chambers are smaller than in control egg chambers and show evidence of instability.** (A) Average outer diameter of ring canals during stages 6-10b of oogenesis. The asterisk indicates statistically significant data ($p < 0.05$). $n \geq 33$ at each stage. (B) Stage 10a egg chamber ring canals. Scale bar is 10 μm. (48hrs at 29°C). (C) Ring canals in Arp2/3 depleted egg chambers are smaller than controls. Stage 9 egg chamber ring canals. (72hrs at 29°C) (D) Depletion of the Arp2/3 protein complex can lead to ring canal collapse. Stage 9 egg chamber collapsed ring canals. (48hrs and 72hrs at 29°C) Scale bar is 10 μm. *UAS-ArpC2-RNAi* were crossed with the triple driver (otu-GAL4; nanos-GAL4; nanos-GAL4). Adapted from Tipold (2016).

Electron microscopy studies have characterized two phases of ring canal growth; in the first phase, prior to stage five, actin-containing bundles within ring canal increase in thickness and number. However, in the second phase, from stage 5 until nurse cell dumping at stage 11, the diameter and length of the ring canal increases significantly, without any change in the number of filaments in the ring canal (21). Because Arp2/3 mutants do not show any defects in ring canal structure prior to stage 5 (9), this suggests that the Arp2/3 complex may play a role in the expansion in phase 2 but not in the earlier accumulation of actin filaments; alternatively, Arp2/3 activity during the first phase could lay the groundwork for later expansion.

Diaphanous (Dia) is a member of the formin family of proteins that plays a role in the nucleation and elongation of actin filaments (4). Formins are found in almost all eukaryotes (2). However, rather than functioning in branched actin nucleation like Arp2/3, formins are important in the formation of linear actin filaments (4). Formins have two formin homology (FH) domains, the FH1 and FH2 domains (2). The FH2 domain is involved in actin nucleation (2), whereas the FH1 domain is important in organization and the acceleration of actin elongation, by bringing the protein profilin to the barbed end of the actin filament (3). There are seven different subclasses of formins, and they are classified based on differences in the FH2 sequence. One subclass is Diaphanous, which includes 6 formins in *Drosophila* (19). There are about 15 mammalian formins called DRFs, commonly denoted as mDia (2); the human homolog is Hs Diaphanous (19). Dia has been well-studied in *D. melanogaster*; rather than capping pointed ends of actin like Arp2/3, Dia caps barbed ends, preventing other proteins from forming caps and therefore sustaining elongation (2). Interestingly, in myoblast formation Dia was found to be

necessary for Arp2/3 activation by promoting the localization of SCAR and WASp, two

Arp2/3 activators (4). Although Dia promotes activation of Arp2/3, Dia does not seem to

be dependent on Arp2/3 for activation in this context, suggesting that it may play a role

upstream of SCAR, WASp, and Arp2/3 (4). Furthermore, Dia has been shown to be

important in proper cytokinesis (4); when Diaphanous is depleted, cytokinesis is

disrupted (1). Mutations in Dia lead to multinucleate spermatids and polyploid follicle

cells (1).  Because ring canals are formed through incomplete cytokinesis (21), and

Arp2/3 is required for ring canal expansion beginning at stage 5 (8, 9), I wanted to test

whether Dia could be functioning earlier in oogenesis to promote the formation and early

expansion of ring canals.

**Background on Methods**

*GAL4/UAS System*

To characterize the role of Diaphanous in ring canal development throughout the stages of oogenesis, Dia was depleted using RNAi with a germline-specific nanos-GAL4 driver controlled by a temperature sensitive Gal80 repressor, GAL80[ts]. The UAS-dia-RNAi line was crossed to this germline specific driver line (Gal80[ts]; nanos-GAL4) to specifically reduce the levels of Dia mRNA (and protein) in the germline without affecting its levels in other tissues. The GAL4/UAS system is useful because it allows the tissue-specific expression of a transgene of interest (4). UAS is an upstream promoter that enhances expression of downstream transgenes of interest (18). In this case, the transgene of interest is a non-coding RNA molecule that allows for the RNA interference (RNAi)-based depletion of Dia (18). RNAi is a technique that allows depletion of a target protein through mRNA degradation (18). A short hairpin RNA that is complementary to the target mRNA is designed. This shRNA is cleaved into smaller double stranded siRNAs by the enzyme, Dicer. The antisense strand, as part of the RNA-induced silencing complex (RISC), can bind to the target mRNA within the cell, leading to its degradation (18). Over time, though normal protein turnover, the target protein can be depleted from the tissue being studied.

*Arp2/3 and Formin Inhibitor Treatments*

Preliminary data from our lab suggests that the Arp2/3 complex is continuously required to maintain ring canal expansion (22). Acute treatment with the Arp2/3 inhibitor, CK-666 (7) caused ring canals to shrink significantly compared to treatment with an inactive control molecule, CK-689 (Fig. 4). The Apr2/3 inhibitor works by prohibiting the short pitch conformation between Arp2 and Arp3. Although this drug blocks the formation of filaments, it does not break down preformed actin filaments (7). In addition to characterizing the effect of depleting Dia on ring canal structure and expansion, we determined the effect of acute inhibition of formin activity. The small molecule inhibitor of formin FH2 (SMIFH2) inhibits formin-mediated actin nucleation and elongation (14). This inhibitor works by depolymerizing the barbed end of actin filaments that is the target of formins such as Diaphanous (14).
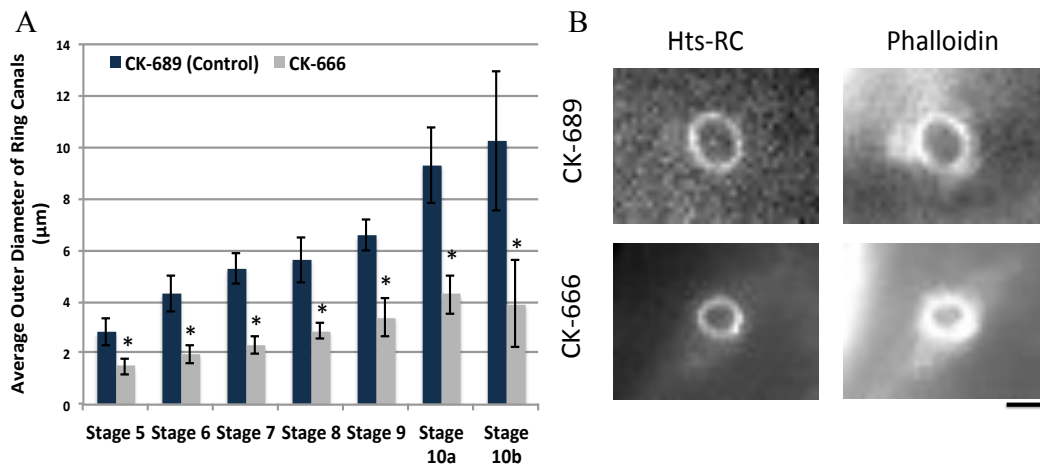


**Figure 4. Inhibition of the Arp2/3 complex using CK-666 leads to egg chambers with smaller ring canals than controls.** (A) Ring canals treated with Arp2/3 inhibitor, CK-666, or treated with control drug, CK-689 for 2 hrs. Average outer diameter of ring canals between nurse cells during stages 5-10b of oogenesis. The asterisk indicates statistically significant data ($p<0.05$). All stages had a $n \geq 29$. (B) Stage 10b egg chamber ring canals. Scale bar is 10 μm. Adapted from Tipold (2016).

*Thesis Research and Hypothesis*

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Research on the formin family member Diaphanous will provide a better understanding of the mechanisms controlling ring canal formation, maintenance, and growth. These findings have the potential to provide insight into gametogenesis in higher organisms. Because intercellular bridges are important for proper gamete formation in many organisms, learning more about the mechanisms controlling their development is important in determining how they impact infertility. The objective of this research was to characterize the role for Diaphanous in ring canal development and compare it to Arp2/3's role in ring canal development. To characterize the role of Diaphanous, depletion and inhibition experiments were performed and the diameters of ring canals were analyzed from the germarium to stage 10b. I hypothesized that Diaphanous is involved in the early stages of ring canal formation and growth. Based on this hypothesis, I predicted that depletion or inhibition of Dia would lead to a significant difference in ring canal number and size.

**Materials and Methods**

      All of the equipment and materials necessary were contained within the Biological Sciences department (fluorescence microscopes) or the Lewellyn lab (anesthesia equipment, constant temperature incubators, and other materials to maintain fly stocks and perform staining, as well as basic molecular biology and biochemistry reagents) at Butler University.

Drosophila melanogaster *Conditions*

      Fly stocks were kept in an incubator at 25°C to maintain a generation time of ~10 days (16). Crosses were set up with 10-12 females and 3-5 males with standard cornmeal-molasses food and live yeast and maintained at 18$^{o}$C. Female offspring from the cross of the appropriate genotype were selected and maintained on yeast for ~1-2 days at either 25$^{o}$C or 29$^{o}$C depending on the condition (16). Different times and temperatures were used to alter the severity of the phenotype, with higher temperatures and longer incubations on yeast resulting in a more severe phenotype. Conditions for each experiment are listed in Table 1.

**Table 1. Genotypes and conditions for *dia-RNAi* experiment.**

| | | | |
|---|---|---|---|
| *UAS-dia-RNAi* x Gal80<sup>ts</sup>; nanos-Gal4 | 18 | 24 | 25 |
| *UAS-dia-RNAi* x Gal80<sup>ts</sup>; nanos-Gal4 | 18 | 24 | 29 |
| *UAS-dia-RNAi* x Gal80<sup>ts</sup>; nanos-Gal4 | 18 | 48 | 25 |
| *UAS-dia-RNAi* x Gal80<sup>ts</sup>; nanos-Gal4 | 18 | 48 | 29 |
| $w^{1118}$ x Gal80<sup>ts</sup>; nanos-Gal4 | 18 | 24 | 25 |
| $w^{1118}$ x Gal80<sup>ts</sup>; nanos-Gal4 | 18 | 24 | 29 |
| $w^{1118}$ x Gal80<sup>ts</sup>; nanos-Gal4 | 18 | 48 | 25 |
| $w^{1118}$ x Gal80<sup>ts</sup>;nanos-Gal4 | 18 | 48 | 29 |

*Dissection and Staining of Tissue*

Flies were anesthetized with $CO_2$ gas and dissected under a stereomicroscope in Schneider's S2 medium using forceps. After dissection, the flies are fixed with a formaldehyde solution (4% formaldehyde in PBS). The ovaries were washed after fixation using PBS + 0.1-0.3% Triton X-100. Fluorescent dyes (DAPI and phalloidin)

were used to visualize DNA and f-actin. Antibodies against the ring canal protein, Hts-RC, were used to stain the tissue.

*Arp2/3 and Formin Inhibitor Treatments (CK-666, CK-689, SMIFH2, DMSO)*

After being maintained on yeast for ~48 hours at 20°C, about five $w^{1118}$ flies were dissected and put in a well with one of four treatments (CK-666, CK-689, SMIFH2, or DMSO). Live imaging medium was made with 1.5 mL FBS and 8.5 mL S2+Pen/Strep media. 5µL CK-666 or CK-689 was added to 2mL live imaging media and 40µL insulin to a final concentration of 250µM. The formin inhibitor SMIFH2 used the same live imaging media. However, only 1µL of SMIFH2 or DMSO was added to 2mL live media and 40µL insulin for a final concentration of 25µM. Egg chambers treated with CK-666 and CK-689 were incubated for either 1 hour or 2 hours prior to fixation. Egg chambers treated with SMIFH2 and DMSO were incubated for 25 minutes. After treatment, the ovaries were fixed and stained as described above. Later stages of oogenesis were imaged and analyzed as described below.

*Imaging and Analysis*

z-stacks were taken of samples using a compound fluorescence microscope (Leica DM5500). Earlier stage egg chambers were imaged using a 63x oil objective (z-step size: 1.00 µm), and later stages were imaged under a 20x objective (z-step size: 2.00 µm). Gain was kept at 4.7 throughout imaging and no binning was used. Images were analyzed using freely available imaging software (*Fiji*).

*Quantification and Statistical Analysis*

        Ring canal outer diameter was measured using the Hts-RC stain in the imaging software, *Fiji*. The output was recorded in microns. For each egg chamber, the stage, number of visible ring canals, outer diameter of ring canals, and number of collapsed ring canals was recorded. The mean size and number of the ring canals at each stage under each condition was determined along with the standard deviation. The target n value for each condition was n≥10 egg chambers at each stage. The germarium stage used was 2a and 2b (11). Statistical significance was determined using a two-tailed t-test assuming unequal variances.

**Results**

*Depletion of Diaphanous Leads to Enlarged Ring Canals*



**Figure 5. Ring canals in Diaphanous-depleted egg chambers are larger than in control egg chambers.** Flies were kept at 25°C for 24 hrs prior to dissection. (A) Average outer diameter of ring canals from the germarium to stage 8 (63x objective). n≥10 egg chambers. (B) Average outer diameter of ring canals from stage 9-10b (20x objective). n≥13 egg chambers. Error bars represent standard deviation. Asterisks indicate statistical significance (p<0.05).

In order to determine the role of Diaphanous in the growth and development of ring canals, RNAi was used to deplete cells in the germline of Dia. Different time points and temperatures were used to vary the severity of the conditions, since in general, longer incubations at higher temperatures increase the expression of UAS transgenes (16). Therefore, we could vary the time and temperature to determine the range of phenotypes caused by depletion of Dia. As was expected, as incubation time and temperature increased, so did the severity of the *diaRNAi* phenotype, evidenced by smaller ovaries in the flies.
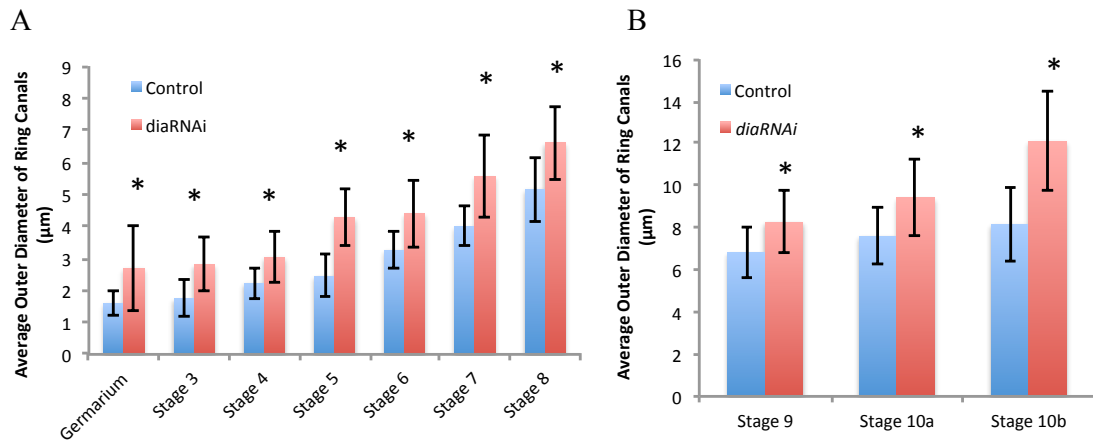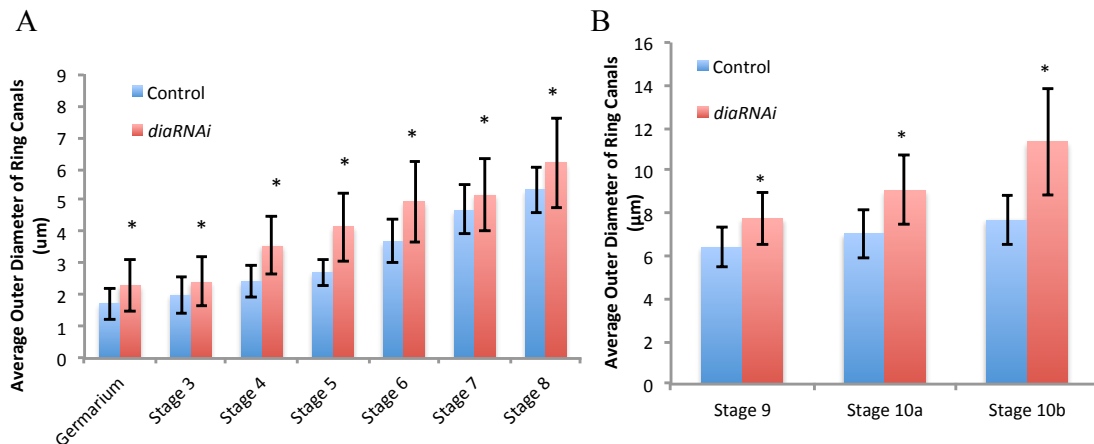
A

B

**Figure 6. Ring canals in Diaphanous-depleted egg chambers are larger than in control egg chambers.** Flies were kept at 29°C for 24 hrs prior to dissection. (A) Average outer diameter of ring canals from the germarium to stage 8 (63x objective). n≥9 egg chambers. (B) Average outer diameter of ring canals from stage 9-10b (20x objective). n≥10 egg chambers. Error bars represent standard deviation. Asterisks indicate statistical significance (p<0.05).

Analysis of ring canals in egg chambers at various stages of oogenesis revealed that there was a significant increase in the average outer diameter of ring canals in the *dia-RNAi* egg chambers compared to controls from the germarium through stage 10b (Fig. 5-8). In the weakest depletion condition, in which flies were maintained on yeast for 24 hours at 25°C, ring canals in control egg chambers had an average diameter of 1.60μm in the germarium stage and 8.18μm at stage 10b. In contrast, egg chambers from the *dia-RNAi* flies had an average ring canal diameter of 2.89 in the germarium stage and 12.09 at stage 10b in this condition (Fig. 5). Interestingly, although the ring canals in stage 10b egg chambers had about the same average outer ring canal diameter (from 11-12 μm) in the four *dia-RNAi* conditions tested, at the germarium stage, the ring canals got

increasingly bigger as the conditions got more severe (from 24 hr at 25°C condition to

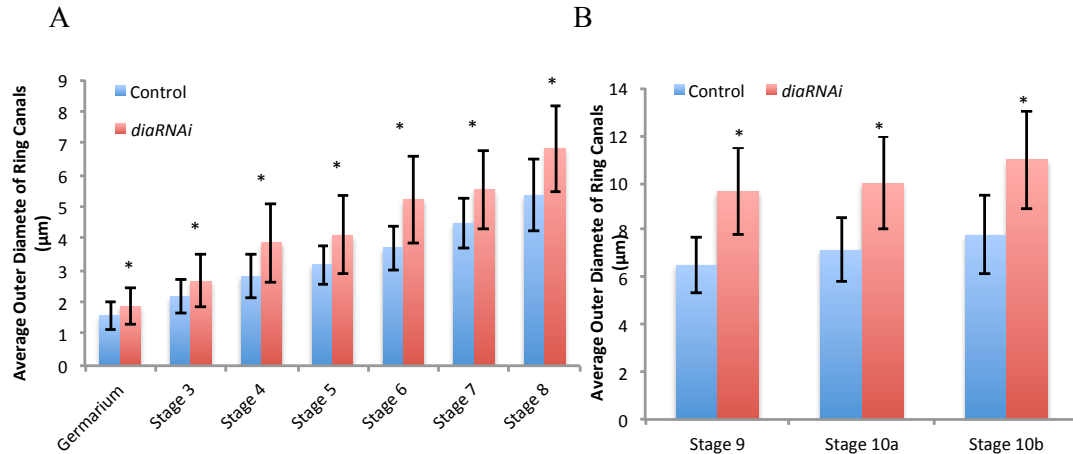the 48 hr at 29°C condition) (Fig. 5-8). Ring canals from germarium stage egg



**Figure 7. Ring canals in Diaphanous-depleted egg chambers are larger than in control egg chambers.** Flies were kept at 25°C for 48 hrs prior to dissection. (A) Average outer diameter of ring canals from the germarium to stage 8 (63x objective). n≥9 egg chambers. (B) Average outer diameter of ring canals from stage 9-10b (20x objective). n≥10 egg chambers. Error bars represent standard deviation. Asterisks indicate statistical significance (p<0.05).

chambers maintained for 48 hours at 29°C ranged from 1μm to over 8μm (compared to

1.6μm in control egg chambers from this condition; Fig. 8A). In addition to the

significant increase in ring canal diameter within the germarium, flies that were kept at

29°C for 48 hours prior to dissection exhibited smaller ovaries with fewer egg chambers,

which could suggest that strong depletion of Diaphanous could ultimately disrupt egg
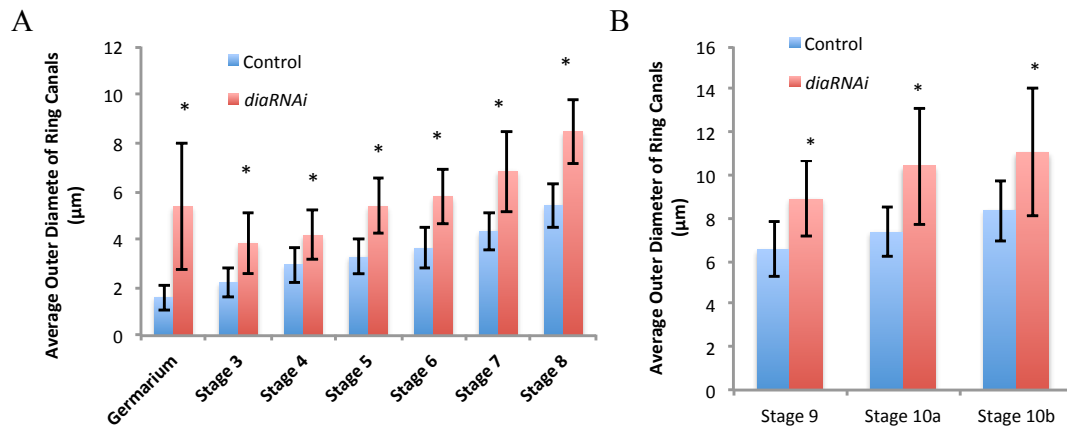
chamber formation.

**Figure 8. Ring canals in Diaphanous-depleted egg chambers are larger than in control egg chambers.** Flies were kept at 29°C for 48 hrs prior to dissection. (A) Average outer diameter of ring canals from the germarium to stage 8 (63x objective). n≥3 egg chambers. (B) Average outer diameter of ring canals from stage 9-10b (20x objective). n≥11 egg chambers. Error bars represent standard deviation. Asterisks indicate statistical significance (p<0.05).

It was observed that many of the younger stage *dia-RNAi* egg chambers (prior to stage 5) had fewer, larger ring canals. Therefore, I wondered whether there was a correlation between the number of ring canals and the average outer diameter of the ring canals. For example, if an egg chamber contains half as many ring canals as a control, would the ring canals be on average twice as large? If an egg chamber has fewer than 15 ring canals, this could indicate either ring canal collapse or defects in incomplete cytokinesis during germline cyst formation. This was especially true for the strongest depletion condition (48 hours at 29°C), where we found some germarium stage egg chambers with 15 ring canals of smaller diameter (~2 μm), while other egg chambers had fewer (2, 3, or 5), larger ring canals (~7-8μm in diameter). Although the largest ring canals were observed in egg chambers with only a few ring canals, there were still many examples of egg chambers with 15 ring canals that were significantly enlarged with an

average outer diameter 2.08µm (Fig. 9). This suggests that the enlargement or expansion

of the ring canals is likely due to a role for Dia in ring canal structure, rather than due to

defects in incomplete cytokinesis.
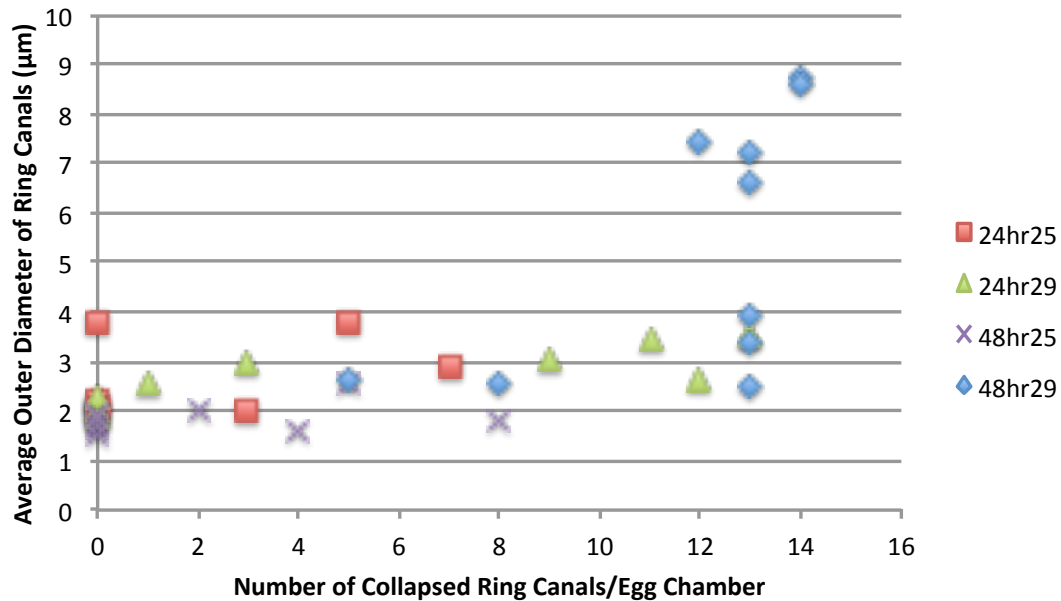


**Figure 9. Average Outer Diameter of Ring Canals Relative to Collapse.** The average outer diameter of the ring canals in each egg chamber was plotted based on the number of collapsed egg chambers. Data was collected from the germarium stage of each condition in *dia-RNAi* flies. n=20.

Qualitative analysis suggests that depletion of Dia alters ring canal structure. In

the expanded ring canals of *dia-RNAi* egg chambers, the actin and Hts-RC stain appeared

thinner than in controls at earlier stages (Fig. 10B), and the recruitment of f-actin

appeared less robust at later stages (Fig. 10A) Additional quantitative analysis of this

observation will be necessary in order to determine whether these observations are

consistent throughout oogenesis.

**Figure 10.**
**Depletion of**
**Diaphanous causes**
**increased ring**
**canal diameter.** (A)
Stage 10b ring
canals acquired with
20x objective. (B)
Stage 4 ring canals
acquired with 63x
objective.
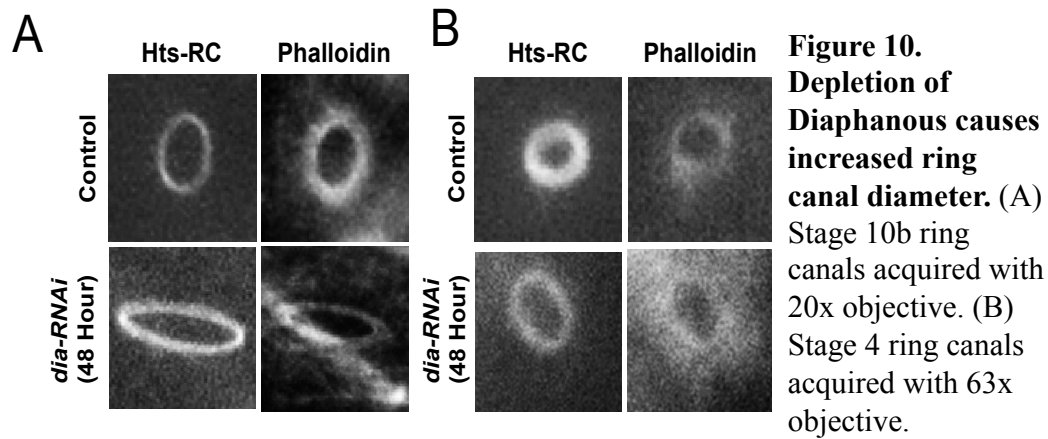
*Apr2/3 and Formin Inhibition Leads to Collapsed Ring Canals*

Previous work in the lab suggests that the Arp2/3 complex activity is continuously

required to maintain ring canal expansion (Fig. 4). Therefore, I wanted to test whether

Diaphanous activity is also continuously required for the maintenance of ring canals.

Upon acute treatment with the formin inhibitor, SMIFH2, there did not appear to be any



**Figure 11. Ring canals after formin inhibition.** (A) Average number of collapsed ring canals from
stages 10a and 10b from drug treatments of CK-666 (1 and 2 hrs), CK-689 (1 and 2 hours) (control),
Formin inhibitor (SM1FH2), and DMSO (control). n≥10 egg chambers. (B) Average outer diameter of
ring canals in control (DMSO) and formin inhibitor (SM1FH2) treated egg chambers. Stages 9-10b.
n≥10 egg chambers. Error bars represent standard deviation. Asterisks denote statistical significance
(p<0.05).

significant changes in the size of the ring canals at stages 9-10b when compared to the controls (Fig. 11). However, there was an increase in the rate of ring canal collapse compared to DMSO treatment (Fig. 11A). However, the rate of collapse was not as high as was observed with the Arp2/3 inhibitor, CK-666.

**Discussion**

*Diaphanous and the Arp2/3 Complex have distinct roles in Ring Canal Development*

I have shown that the formin family member Diaphanous is required for the proper formation, stability, and expansion of ring canals. Beginning as early as in the germarium, ring canals in *dia-RNAi* egg chambers were significantly larger than controls (Fig. 5-8). Further, acute treatment with a formin inhibitor led to low rates of ring canal collapse (Fig. 11), consistent with a role for Dia in maintaining ring canal stability. Interestingly, in the early stages of oogenesis, beginning in the germarium, I observed a large number of egg chambers that contained fewer than 15 ring canals (Fig. 9) and multiple nuclei, which suggests that depletion of Diaphanous could be disrupting the process of incomplete cytokinesis during formation of the germline cyst. Interestingly, in the older *dia-RNAi* egg chambers (stages 9-10b), most egg chambers contain 13-15 ring canals. This could indicate that egg chambers with few ring canals die prior to stage 9-10, or it could be that with our short timepoints (24-48 hours on yeast prior to dissection), I have not captured late stage egg chambers strongly depleted of Dia.

Although there is evidence of defects in formation of ring canals in our *dia-RNAi* egg chambers, the ring canals that do form were significantly larger than in controls (Fig. 5-8). This suggests that Dia-mediated actin nucleation is necessary for proper ring canal formation and later expansion. Dia promotes the formation of straight actin filaments, which is essential for complete cytokinesis in many systems. Actin filaments are a key component of the contractile ring, which, along with active myosin motor proteins, promotes the constriction of a single cell to form two daughter cells (1). Reducing the levels of Diaphanous could therefore decrease the levels of f-actin within the contractile

ring during incomplete cytokinesis. This could lead to a decreased rate and extent of furrow ingression, which would be consistent with the larger ring canal size observed in the germarium. If the ring canals start out larger than in controls, then when the period of ring canal growth begins, this could lead to a further expansion of the ring canals. Qualitative analysis suggests that the ring canals in the *dia-RNAi* egg chambers are thinner than controls (Fig. 10), which would be consistent with decreased recruitment of f-actin to the ring canals during their formation in the germarium. Additional quantitative analysis of the recruitment and organization of f-actin, Hts-RC, and other actin binding and bundling proteins at the ring canal will be necessary in order to determine the effects of Diaphanous depletion on ring canal structure.

The data revealed that Arp2/3 and Diaphanous likely play distinct roles in promoting the formation and expansion of the ring canals during oogenesis. Whereas depletion of Diaphanous leads to expansion of ring canals beginning very early during oogenesis (Fig. 5-8), depletion of the Arp2/3 complex causes a significant defect in expansion beginning in the middle stages of oogenesis (Fig. 3; 15). This temporal relationship between the defects observed upon depletion of Diaphanous or the Arp2/3 protein, ArpC2, is reminiscent of the role for Diaphanous in promoting the activation of the Arp2/3 complex in the context of myoblast fusion. In this system, Dia promotes the localization of the Arp2/3 activator, SCAR (4). Because mutations in SCAR can lead to ring canal collapse (24), this suggests a model in which Diaphanous could be acting during early oogenesis to promote incomplete cytokinesis and localization of SCAR to the nascent ring canals. SCAR activity at the ring canals could then localize the activity of Arp2/3 during later stages of oogenesis to promote ring canal expansion. In the future,

genetic interaction and localization dependency experiments could be performed to test this model.

Additional experiments will need to be performed to determine Dia's role in ring canal development. The decreased numbers of ring canals upon Dia inhibition may suggest that Dia could be continuously required to maintain ring canal stability. Further, analysis of egg chambers over-expressing wild type or constitutively active forms of Diaphanous could provide additional insight into the role of Diaphanous during early oogenesis. It would also be useful to repeat the experiments to increase sample size, since some conditions (48 hours at 29°C flies) were very severe, with the flies exhibiting small ovaries with few egg chambers to analyze. It will also be important to further characterize the formin inhibitor-treated egg chambers. Although we observed some evidence of ring canal instability in this condition, additional analysis of earlier stages will be essential in order to determine whether Dia activity is continuously required at any stage during oogenesis, especially since previous studies found that formin inhibition did result in expanded ring canals similar to what is seen in Dia depleted lines (14).

**Conclusion**

This data provides evidence that Diaphanous is necessary for the proper formation, structure, and growth of ring canals in the developing egg chambers of *Drosophila melanogaster*. This formin appears to be important throughout all stages of oogenesis, particularly the early stages. Depletion of Diaphanous produces enlarged ring canals; which is distinct from the phenotype observed upon Arp2/3 depletion (M. Tipold, Butler University). This suggests that these two actin nucleating proteins play distinct roles in the developing egg. Understanding more about the mechanisms regulating ring canal development may provide future hope of curing infertility.

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