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Dreadlocks (Dock) is necessary to regulate growth of the germline ring canals in the developing *Drosophila melanogaster* egg chamber

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Abstract

Infertility is a prevalent issue in the United States, impacting 1.5 million women (1). A possible cause of infertility is defects in gametogenesis, or the formation of sperm and egg. Therefore, understanding the basic mechanisms that promote normal gamete formation could impact our understanding of infertility. The *Drosophila melanogaster* egg develops from an organ-like structure called an egg chamber. The egg chamber is composed of a central cluster of 16 germ cells that are connected to one another by intercellular bridges, called ring canals. These ring canals are composed of filamentous actin and allow the transfer of materials from supporting nurse cells to the developing oocyte. The ring canals form during early oogenesis and then expand 20-fold. Defects in ring canal formation or expansion can lead to infertility. The purpose of this project was to determine the role of the SH2/SH3 adaptor protein, Dreadlocks (Dock), in the germline ring canals of the developing *Drosophila* egg. Dock is involved in the formation of other actin-rich structures and has been shown to interact with other known ring canal proteins; thus, I examined whether depletion or mutation of Dock affected the process of nurse cell dumping or the size of the ring canals throughout development. Depletion of Dock by RNA interference (RNAi) caused an over-expansion of the outer diameter of the ring canals in egg chambers between the stages of 6 and 10b of oogenesis. Reducing Dock levels also enhanced the phenotype caused by depletion of two other ring canal components, the kinase Misshapen or the Arp2/3 complex. This led me to propose that Dock functions with Misshapen and the Arp2/3 complex to promote normal ring canal expansion and stability. Because of the conserved nature of these intercellular bridges and the proteins being studied, this work could provide significant insight into gametogenesis in higher organisms.

Introduction

Intercellular Bridges in Gametogenesis

Gametogenesis, or the formation of egg and sperm, is essential for proper development. In most sexually reproducing organisms, the developing germ cells go through a stage in which they are connected to other developing germ cells or to supporting cells through intercellular bridges. Intercellular bridges are formed through incomplete cytokinesis. At the end of mitosis, the two daughter cells are connected by a thin cytoplasmic connection, the intercellular bridges. However, this bridge is short-lived in most somatic cells, quickly being cleaved by a syntaxin2 and endobrevin/VAMP8-dependent mechanism. However, in germ cells the intercellular bridges can be maintained in order to provide a method for materials to be moved between developing germ cells or from supporting cells (2).

Intercellular bridges are conserved in a wide variety of organisms, ranging from insects to humans, and are essential for the maturation of both sperm and egg. Intercellular bridges allow for synchronization of mitotic cell divisions in male mice. Intercellular bridges allow developing sperm to remain phenotypically diploid, despite being genetically haploid, by sharing gene products (3). Mutation of testis-expressed gene 14 (TEX14) results in the absence of intercellular bridges and male sterility in male mice (2), which highlights the important role these intercellular connections play in fertility. Intercellular bridges are especially important during the process of oogenesis, where large amounts of proteins, RNAs, and organelles must be loaded in order to support early development. The oocyte itself is unable to carry out many essential cellular functions, such as glycolysis or amino acid transport, due to its low level of

transcriptional activity (4, 5). Therefore, the developing egg is dependent on supporting germ cells to provide nutrients for its proper development (4). In some organisms, like the frog, *Xenopus laevis,* the germ cell nest contains oocytes connected to one another by intercellular bridges (6). In other organisms like the mouse and the fly, *Drosophila melanogaster*, the developing oocyte receives nutrients from supporting germ cells by using intercellular bridges (7, 5). These intercellular bridges are essential for intercellular communication; disruption of intercellular communication halts oocyte development and results in premature ovulation and subsequent degradation of the oocyte (4, 7). Although intercellular bridges are essential for fertility in many organisms, there is still much to be learned about how intercellular bridges are formed, stabilized, and remodeled during gametogenesis.

The developing fruit fly egg provides an excellent model system to study intercellular bridges during oogenesis. Because mammalian oogenesis occurs in the embryo, attempting to study the role of intercellular bridges in higher organisms is challenging. The intercellular bridges found in the developing *D. melanogaster* egg chamber are some of the largest and most well-characterized that have been observed (5). Female flies can lay up to 100 eggs per day and larvae develop into adults within 10 days when kept at 25°C (8). In addition to their rapid development, many powerful genetic tools have been developed in the fly that allow precise manipulations to be easily performed (9).

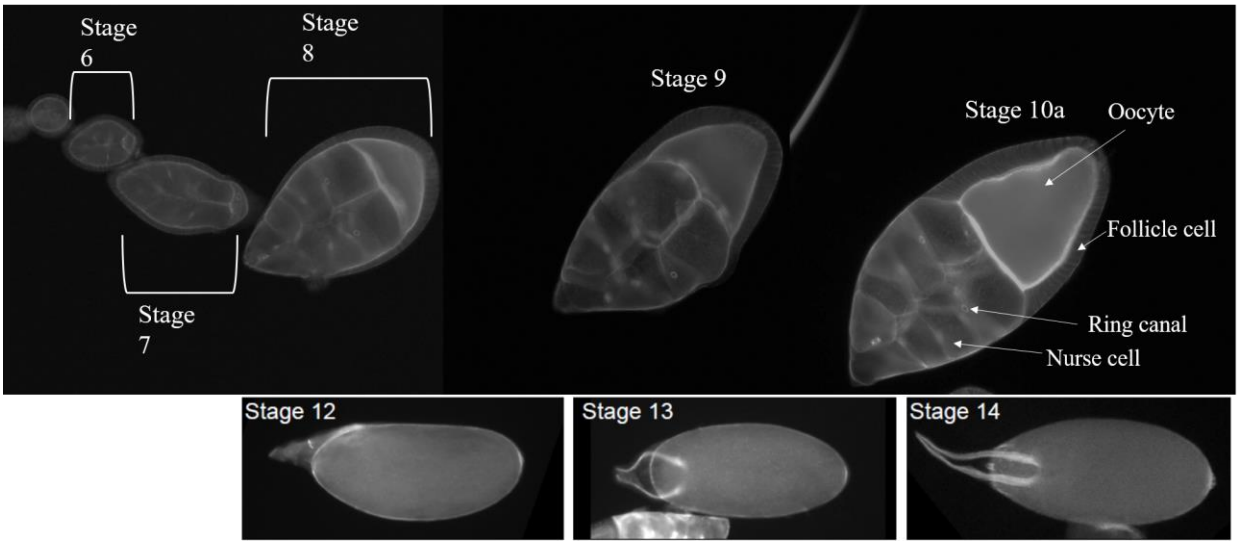

Figure 1: Eight of the fourteen morphological stages of egg chamber development. Images are of control egg chambers. Flies were kept on yeast at 29˚C for 72 hrs prior to dissection.

Egg Chamber Development

Each fruit fly egg develops from a multicellular structure called an egg chamber. The *D. melanogaster* egg chamber is composed of a central cluster of germ cells (1 oocyte and 15 supporting nurse cells) surrounded by a layer of approximately 1000 somatic epithelial cells called follicle cells. Egg chambers will progress through 14 distinct morphological stages prior to becoming a mature egg (Fig. 1). The egg chamber is initially spherical but will begin to elongate starting at stage 5, and it will undergo a thousand-fold increase in volume throughout maturation (10). Egg chamber formation begins in the germarium with the asymmetrical division of a germline stem cell, which gives rise to a cystoblast cell. The cystoblast will then undergo four rounds of mitosis followed by incomplete cytokinesis to produce a germ cell cyst (11). At the end of mitosis, instead of completing cytokinesis and physically separating the two daughter cells, the connection between the two cells is maintained by the recruitment of additional filamentous actin (f-actin) and actin binding proteins, forming a stable intercellular

bridge, or ring canal. After the four rounds of division, the germ cell cluster will contain 16 interconnected cells; two cells will have four ring canals (one of which will become the oocyte), two cells will have three ring canals, four cells will have two ring canals, and the remaining eight cells will each have a single ring canal. The size of the ring canals varies slightly based on their age; the youngest ring canals, which are near the periphery of the egg chamber, are the smallest, whereas the more centrally localized ring canals are older and slightly larger (5). Once they are formed, the ring canals will expand throughout oogenesis.

Transfer of materials through the ring canals occurs throughout oogenesis. During the early stages, there is a slow transfer of materials from the nurse cells to the oocyte, which depends on the action of microtubule-based motors. At stage 11, a bulk cytoplasmic transfer from the nurse cells to the oocyte, known as nurse cell dumping, occurs; this leads to a doubling of the oocyte volume over a period of 30 minutes (12). The growth and structural integrity of the ring canals is essential during this process to prevent the nurse cell nuclei from being dumped along with their cytoplasmic contents.

Throughout oogenesis, the ring canals expand ~20-fold in diameter; this expansion can be divided into two distinct phases. During the first phase, through stage 5, the diameter of the ring canals does not increase substantially (from only 0.65 μm to 1.53 μm); however, they become thicker due to an increase in the number of actin filaments (from 82 filaments at stage 2 to 717 filaments at stage 6). During the second phase, beginning at stage 6, the diameter of the ring canals increases considerably (from 2.3 μm at stage 6 to 10 μm at stage 11), but the number and density of actin filaments remains fairly constant (~700-770 filaments/ring canal with a density of 4000-5000

filaments/μm^2). During the second phase (beginning around stage 6), the actin is reorganized to form a net of actin bundles, which has been compared to a fisherman's net; this reorganization may allow the ring canals to expand without requiring a dramatic increase in actin filament number. Overall, there is a 134-fold increase in the length of the f-actin filaments and an increase in the number of filaments from 80 to 700 per ring canal (5); therefore, understanding how actin filament nucleation and growth are controlled during oogenesis is essential.

Activity of the Arp2/3 Complex and Misshapen are required for proper ring canal growth

A number of different proteins have been identified that localize to the ring canals and promote their formation and/or growth (13, 14, 15, 16), and many of those directly or indirectly regulate actin. One important actin regulator is the Arp2/3 complex, which promotes the formation of branched actin networks. The Arp2/3 complex is composed of seven different subunits: Arp2, Arp3, and ArpC1-ARPC5 (17). Although the Arp2/3 complex is not required for the initial formation of the ring canal, it is essential for ring canal expansion. Mutation of members of the Arp2/3 complex, such as Arpc1 or Arp3, leads to failure of ring canal expansion (reported to begin around stage 5/6), defects in nurse cell dumping, formation of a smaller egg, and female sterility. The ring canals in mutant egg chambers at stage 10a were up to 30% smaller in diameter (18). Although it is known that the Arp2/3 complex is required for ring canal growth, there is still much to be learned about how the activity of this complex is regulated during oogenesis.

Activation of the Arp2/3 complex requires phosphorylation of the Arp2 subunit. The Nck-interacting kinase, or NIK, has been shown to phosphorylate and activate the Arp2/3 complex (19). The *Drosophila* homolog of NIK is the Ste20 family kinase, Misshapen (Msn), which localizes to the ring canals. If Msn is depleted from the germline by RNA interference (RNAi) or if a membrane-tethered form of Msn (Myr-Msn) is expressed, this leads to significant defects in ring canal expansion, signs of collapsed ring canals, and defects in nurse cell dumping (*A. Kline data not shown*), which resembles the phenotypes observed in the Arp2/3 mutant egg chambers (5). Therefore, Misshapen could activate the Arp2/3 complex to promote ring canal expansion.

Full activation of the Arp2/3 complex not only requires phosphorylation but also binding to a nucleation promoting factor, or NPF. There are two different types of NPFs that are classified based on the presence or absence of a complete VCA domain. The VCA domain is made up of three sections: a verprolin-homology, cofilin-homology, and acidic domain. Type 1 NPFs (SCAR, WASP, WASH, JMY in flies) have a complete VCA domain, whereas Type 2 NPFs simply have an acidic domain (17). Analysis of NPF function in the germline revealed that only mutation of SCAR altered ring canal structure, leading to signs of collapsed ring canals (20). Therefore, this suggests that SCAR is the primary activator of the Arp2/3 complex and is necessary to promote ring canal expansion or stability.

The SH2/SH3 adaptor protein, Dreadlocks (Dock), could provide an important link between Misshapen, SCAR, and the Arp2/3 complex. During fly spermatogenesis, sperm differentiation is promoted by the recruitment of Dock to the ring canals in primary spermatocytes (21). Dock physically interacts with Msn (22), and while these

two proteins genetically interact during the final step of gastrulation (dorsal closure) in *Drosophila*, their interaction is not required for photoreceptor development in the eye (23). Dock also physically interacts with SCAR, and genetic analysis has demonstrated that this interaction promotes the fusion of two myoblast types in the formation of the body wall musculature (24). Here, we show that Dreadlocks localizes to the germline ring canals, and depletion of Dock leads to an increase in ring canal diameter. Further, partial reduction of Dock levels significantly enhances depletion of the Arp2/3 complex member, ArpC1, or the kinase, Msn. Therefore, we hypothesize that Dock cooperates with Msn to regulate ring canal growth through interaction with the Arp2/3 activator, SCAR.

Thesis Research and Hypothesis

Intercellular bridges are an evolutionarily conserved structural feature that is essential for fertility in many organisms from insects to mammals (6). Despite the importance of these structures, there is still much to be learned about the proteins required for proper growth and expansion of these actin-rich structures. The developing *Drosophila melanogaster* egg provides an excellent model system to study intercellular bridges due to their large size, wide range of genetic tools available, and their fast generation time (8). We have identified a novel ring canal protein, the SH2/SH3 adaptor protein, Dreadlocks (Dock). Although Dock has been studied in the development of other actin-rich structures, its role in growth and expansion of the ring canal has not yet been explored (24). Dock physically and genetically interacts with the kinase Misshapen in other contexts and has also been shown to physically interact with the Arp2/3 activator

SCAR in the formation of the body wall musculature (22, 23, 24). Both the Arp2/3 complex and Misshapen are essential for normal ring canal expansion and stability. Therefore, I hypothesized that a reduction in the levels of Dock would lead to improper expansion of the ring canals. Interestingly, I found that depletion of Dock leads to significant over-expansion of the ring canals, which is a phenotype that has only been described with one other mutation. Genetic experiments suggest that Dock could cooperate with Misshapen and the Arp2/3 complex to promote normal ring canal expansion and stability.

Materials and Methods

GAL4/UAS System

To characterize the role of specific proteins in germline ring canal formation and/or growth, target proteins were depleted from the germline of the developing *Drosophila* egg chamber using the GAL4/UAS system. GAL4 is a yeast-specific transcription factor that will bind to the enhancer element UAS (upstream activating sequence), effectively turning on expression of genes downstream of that UAS (9). The expression of GAL4 protein can be controlled by tissue-specific promoters, and thousands of different GAL4 "driver" lines are available from the Bloomington *Drosophila* Stock Center (BDSC). Two different germline-specific GAL4 lines were used in this study – the maternal triple driver, or MTD-GAL4, which contains three different GAL4 transgenes (otu-GAL4; nanos-GAL4; nanos-GAL4) or the single nanos-GAL4 driver line. The MTD-GAL4 line expresses GAL4 throughout oogenesis beginning in the germline stem cells, whereas the nanos-GAL4 line initially expresses GAL4 in the germline stem cells, shows a drop in GAL4 expression during early/mid oogenesis, and then expression of GAL4 resumes around stage 5 (25). These GAL4 driver lines were crossed with *Drosophila* UAS-RNAi lines, which drove expression of a short hairpin RNA (shRNA) that was complementary to the target mRNA. The shRNA is processed into 20 nucleotide long fragments by the enzyme, Dicer. The antisense strand of these fragments is incorporated into the RNA-induced silencing complex (RISC) and will act as a degradation template (26). This causes the target mRNA to be degraded and due to protein turnover, the target protein will be depleted specifically within the

germline. Here, the germline drivers were crossed to stocks containing UAS-Dock-RNAi, UAS-Msn-RNAi, and UAS-ArpC2-RNAi transgenes.

Drosophila melanogaster Conditions

All fly stocks were stored at 25°C; at this temperature, flies had a generation time of 10-12 days (9). Flies were maintained on cornmeal molasses food, consisting of cornmeal, molasses, yeast, Tegosept, and propionic acid. Crosses were set up by combining virgin females from one stock and male flies from another stock in a new vial with ground active dry yeast and maintained at 25°C. In preparation for dissection, approximately 10 females and 3-5 males were placed in a vial containing cornmeal molasses fly food and ground yeast to increase egg production. These vials were then incubated at 29°C for either 50 hours (UAS-dock-RNAi x MTD-GAL4) or 72 hours (all other conditions) prior to dissection.

Fly Lines Used

Dock was depleted from the germline using the GAL4/UAS system. UAS-Dock-RNAi/TM3 (Bloomington #43176) was crossed to either the maternal triple driver (otu-Gal4; nanos-Gal4; nanos-Gal4, Bloomington #31777) or nanos-Gal4 (Bloomington #32563). UAS-Msn-RNAi/CyO (Bloomington #42518) or UAS-ArpC2-RNAi/TM3 (Bloomington #43132) was crossed to nanos-Gal4. White-eyed flies, w^{1118}, are commonly used as a background when producing transgenic flies; thus, w^{1118} flies were crossed to either the maternal triple driver or nanos-Gal4 to serve as a negative control. Progeny with the desired genotype were identified by selecting against the balancer chromosomes, which contained dominant mutations for curly wings (CyO) or stubble (TM3). Dock levels were reduced by crossing a line containing a heterozygous *dock*

mutation with nanos-GAL4 (*dock*^{04723}*FRT40/Cyo; nanos-GAL4*) to either control (w^{1118}), UAS-msn-RNAi, or UAS-ArpC2-RNAi.

Dissection Protocol

Ovaries from females of the appropriate genotypes were dissected in Schneider's S2 media using a stereomicroscope and two pairs of forceps. The ovaries were fixed using a 4% formaldehyde solution (in PBS) and washed with PBT (PBS + 0.1-0.3% Triton X-100). They were then stained with TRITC-conjugated phalloidin (ECM Biosciences) to visualize the actin, DAPI (Life Technologies) to visualize the DNA, or an antibody against Hts-RC (1:20, DSHB) or Dock (1:200, (27)) to visualize the ring canals. Tissues were mounted on slides using Slowfade Antifade (Invitrogen). Tissues were mounted on slides using Slowfade Antifade (Invitrogen).

Microscopy and Image Analysis

z-stacks of the egg chambers were acquired using a compound fluorescent microscope (Leica DM5500) and LASX software. Measurements were performed using the line tool in *Fiji*. The number of ring canals was also assessed to determine whether ring canal collapse had occurred. To compare differences in ring canal diameter, egg chambers were staged by monitoring changes in follicle cell morphology and DNA. For each stage, the average outer diameter of the ring canals was determined as well as the standard deviation of the means. To identify significant difference between two conditions, a two-sample t-test was conducted.

Results

Dock localizes to the ring canals and depletion of Dock leads to ring canal overexpansion

Our lab has found a novel role for the Ste20 kinase, Misshapen, in the stability and growth of the germline ring canals. Because Misshapen and Dock physically and genetically interact in other contexts, we wanted to determine whether Dock localizes to the ring canals as well. Egg chambers stained with phalloidin and an α-dock antibody (27) showed that Dock localizes to the ring canals beginning during early oogenesis (Fig. 2A). Dock was then depleted using RNAi under the control of the maternal triple driver (MTD-Gal4), which provides strong GAL4 expression throughout oogenesis (25). Ring canals were significantly overexpanded between stages 6 to 10a of oogenesis; there was a slight difference in the average diameter at stage 10b, but it was not significant (Fig. 2B, C). The largest difference in ring canal size was observed in stage 10a egg chambers. At this stage, ring canals from control egg chambers had an average outer diameter of 6.83 μm, whereas ring canals from the *dock-RNAi* egg chambers showed an average outer diameter of 7.81 μm.

The maternal triple driver provides fairly uniform expression of GAL4 throughout oogenesis Therefore, we wanted to determine whether depletion of Dock using the single nanos-Gal4 driver, which expresses GAL4 in the germline stem cells and then again around stage 5 (25), would show the same phenotype. Although the nanos-GAL4 driver likely provides a weaker depletion, ring canals were still significantly over-expanded in *dock-RNAi* egg chambers from stages 6 through 10b of oogenesis (Fig. 2D). Ring canals in stage 10b *dock-RNAi* egg chambers had an average outer diameter of 7.51 μm,

compared to 7.05 μm of control ring canals. Because the *dock-RNAi* phenotype was reproducible, but not as strong as other ring canal perturbations, we combined Dock depletion with a heterozygous mutation in $dock^{04723}$, to see if we could enhance the phenotype. While ring canals in both $dock^{04723}$/+ and *dock-RNAi*; $dock^{04723}$/+ egg chambers were significantly overexpanded in comparison to controls, enhancement of the heterozygous mutation was not observed when Dock was also depleted by RNAi at most stages (Fig. 2E). Taken together, these data suggest that Dock is required to regulate ring canal size and prevent over-expansion.

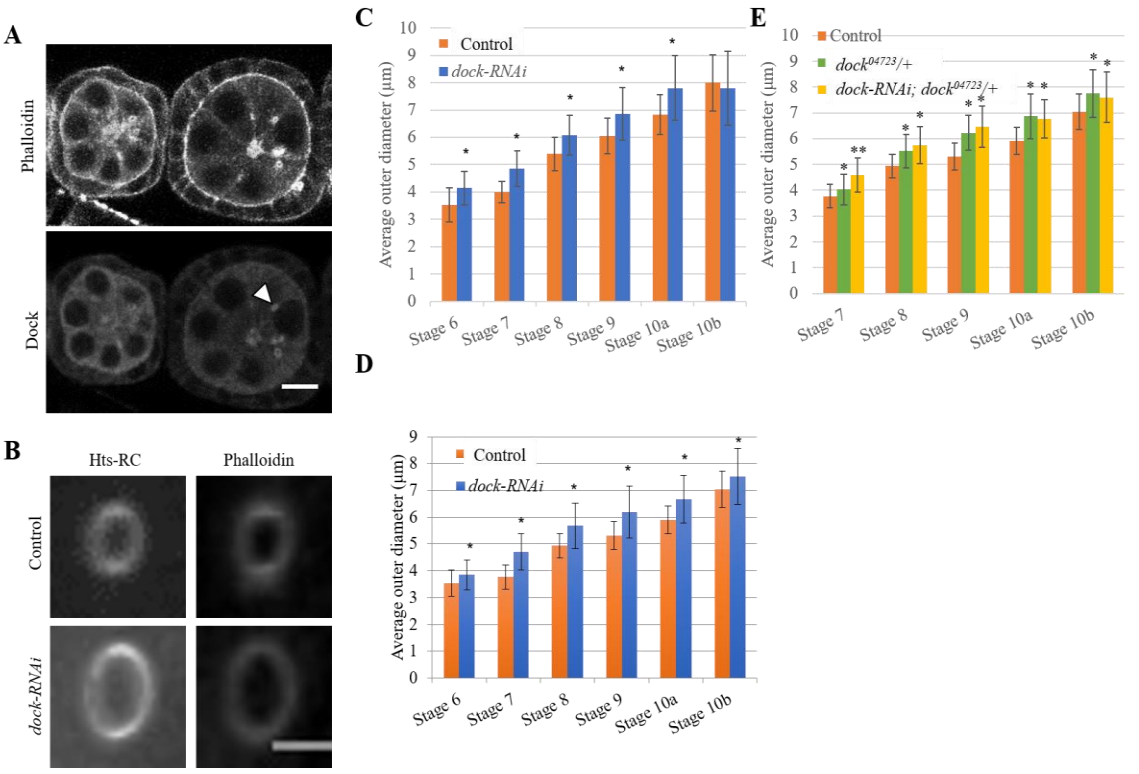

Figure 2: Dock localizes to the ring canals and reduction of Dock levels leads to significantly over-expanded ring canals. (A) Transverse confocal section of egg chambers stained with phalloidin (top panel) and α-dock antibody (bottom panel). Dock localizes to the germline ring canals (arrowhead). Scale bar is 10 μm. (B) Ring canals in stage 9 control and Dock-depleted egg chambers. w^{1118} or UAS-dock-RNAi were crossed to MTD-Gal4. Flies were kept at 29°C for 50 hrs prior to dissection. Scale bar is 5 µm. (C) Average outer diameter of ring canals in Control and Dock-depleted egg chambers from stage 6 through 10b (20x objective). w^{1118} or UAS-dock-RNAi crossed to nanos-Gal4. Flies were kept at 29°C for 72 hrs prior to dissection.. (D) Average outer diameter of ring canals in Control and Dock-depleted egg chambers from stage 6 through stage 10b (40x objective). w^{1118} or *UAS-dock-RNAi* crossed to MTD-Gal4. Flies were kept at 29°C for 50 hrs prior to dissection (E) Average outer diameter of ring canals in Control, $dock^{04723}/+$, and $dock^{04723}/+$ *; dock-RNAi* egg chambers from stage 7 through stage 10b (20x objective). Lines were crossed to nanos-Gal4. Flies were kept at 29°C for 72 hrs prior to dissection. For (C), (D), and (E) n≥33 ring canals and n≥3 egg chambers for most conditions at each time point. Error bars represent standard deviation. Single asterisk indicates difference compared to Control (w^{1118}); double asterisk indicates significant difference from all other conditions at that timepoint (t-test, $p<0.05$).

Reducing Dock levels enhances the *msn-RNAi* phenotype

Based on established connections between Misshapen and Dock and the recently characterized role for the homolog of Msn, NIK, in phosphorylating the Arp2/3 complex, genetic interactions between Msn and Dock were further investigated. Depletion of Msn by RNAi under the control of the MTD-Gal4 driver led to ring canals with significantly larger outer diameters than those in controls from stages 6 through 10b (Fig. 3A) Previous experiments have shown that depletion of Msn using the MTD-GAL4 driver leads to smaller ring canals and signs of collapse. This difference could be due to the small sample size in this experiment. Co-depletion of both Dock and Msn by RNAi under the control of the nanos-Gal4 driver also led to significantly expanded ring canals from stages 6 through 10a (Fig. 3B). While the difference at stage 10b was not significant, ring canals were still larger in *msn-RNAi* & *dock-RNAi* egg chambers than controls. An examination of average outer ring canal diameter at stage 9 for control, *dock-RNAi*, *msn-RNAi*, and *msn-RNAi* & *dock-RNAi* egg chambers showed that ring canals were significantly larger in the co-depleted egg chambers in comparison to all other conditions (Fig. 3C). Ring canals in egg chambers depleted of both Msn and Dock had an average outer diameter of 7.42 µm, while those in egg chambers depleted of solely Msn or Dock had diameters of 6.88 or 6.72 µm, respectively.

The relationship between Msn and Dock was further examined by reducing Dock levels with a heterozygous mutation in *msn-RNAi* egg chambers (*msn-RNAi*, $dock^{04723}$). Consistent with the RNAi data, ring canals in *msn-RNAi*, $dock^{04723}$ egg chambers had a significantly larger outer diameter than those in *msn-RNAi*, $dock^{04723}$/+, or control egg chambers (Fig. 3). However, there were no signs of ring canal collapse. This data

suggests that Msn and Dock could function within the same pathway to promote normal ring canal expansion.

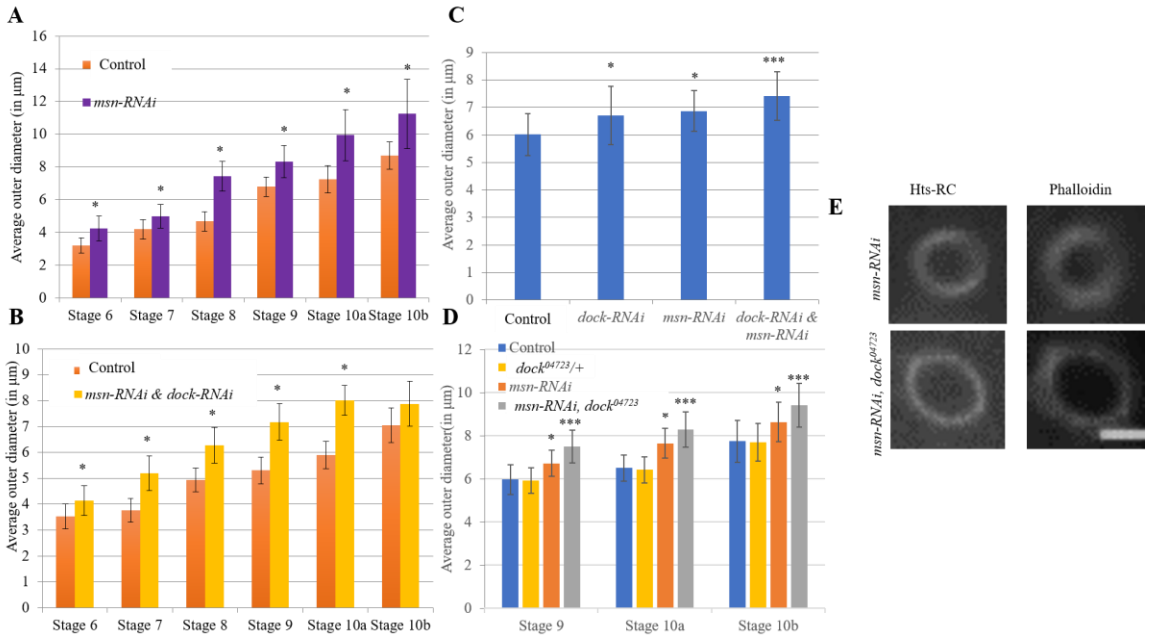

Figure 3: Reducing Dock levels enhances the *msn-RNAi* phenotype.(A) Average outer diameter of ring canals in Control and Msn-depleted egg chambers from stage 6 through 10b (20x objective). w^{1118} and UAS-msn-RNAi were crossed to nanos-Gal4. n≥22 ring canals and n≥2 egg chambers for most time points. (B) Average outer diameter of ring canals in Control egg chambers and those depleted of both Msn and Dock from stage 6 through 10b. All lines were crossed to nanos-Gal4. n≥22 ring canals and n≥2 egg chambers for most time points. (C) Average outer diameter in stage 9 Control, Dock-depleted, Msn-depleted, or Msn- and Dock-depleted egg chambers. All lines were crossed to nanos-Gal4. n≥55 ring canals and n≥5 egg chambers for each time point. (D) Average outer diameter of ring canals in Control, $dock^{04723}$/+, *msn-RNAi*, and *msn-RNAi,* $dock^{04723}$ egg chambers from stage 6 through 10b. All lines crossed to nanos-Gal4. n≥88 ring canals and n≥8 egg chambers for most time points. (E) Ring canals in stage 10b *msn-RNAi* and *msn-RNAi,* $dock^{04723}$ egg chambers. Lines were crossed to nanos-Gal4. Scale bar is 5 µm. For all figures, flies were kept at 29°C for 72 hrs prior to dissection. Error bars represent standard deviation. Single asterisk indicates significant difference compared to Control (A, B, C) or $dock^{04723}$/+ (D). Triple asterisk indicates significant difference from all other conditions at that timepoint (t-test, $p<0.05$).

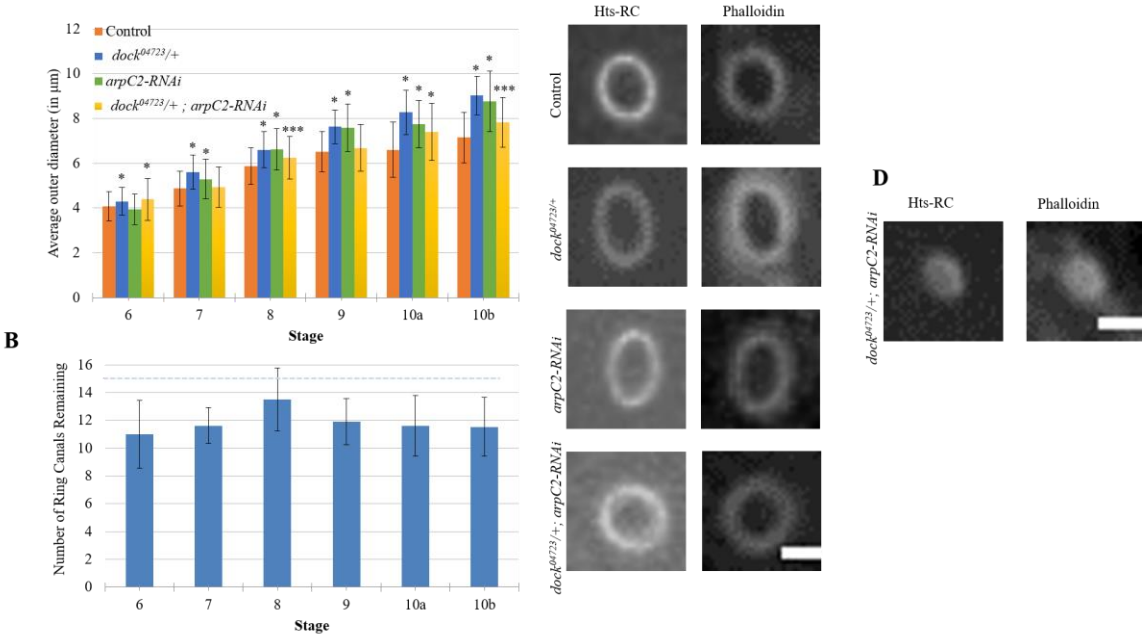

Figure 4: Reducing Dock levels enhances the *arpC2-RNAi* phenotype. (A) Average outer diameter of ring canals connecting nurse cells at various stages of development in Control, *arpC2-RNAi, dock*^{04723}*/+, and dock*^{04723}*/+; arpC2-RNAi* egg chambers. n≥44 ring canals and n≥4 egg chambers for most time points. (B) Number of remaining ring canals in *dock*^{04723}*/+; arpC2-RNAi* egg chambers at various stages of development. All other conditions did not display any change in the average number of ring canals at each developmental stage. n> 5 egg chambers at each time point. (C) Ring canals in stage 9 Control, *arpC2-RNAi, dock*^{04723}*/+, and dock*^{04723}*/+; arpC2-RNAi* egg chambers. Scale bar is 5 µm. (D) Collapsed ring canals in *dock*^{04723}*/+; arpC2-RNAi* egg chambers. Scale bar is 5 µm. For all figures flies were kept at 29°C for 72 hrs prior to dissection and lines were crossed to nanos-Gal4. Error bars represent standard deviation. Single asterisk indicates significant difference compared to Control (*w*^{1118}); triple asterisk indicates significant difference from all other conditions at that timepoint (t-test, p<0.05).

Reducing Dock levels also enhances the *arpC2-RNAi* phenotype

Another possible mechanism through which Dock could be controlling ring canal size is through regulation of the Arp2/3 complex. Dock was shown to interact with the Arp2/3 activator, SCAR, in other contexts (24), therefore, we hypothesized that Dock

could promote Arp2/3 activation in the context of ring canal expansion as well. Depletion of the Arp2/3 complex member, ArpC2, using the nanos-GAL4 driver led to an expansion of the outer diameter of the ring at all stages measured expect stage 6. By stage 10b, ring canals had expanded in *arpC2-RNAi* egg chambers to an average outer diameter of 8.76 µm, compared to 7.14 µm in controls (Fig. 4A,C). Depletion of ArpC2 throughout oogenesis using the maternal triple driver led to a significant decrease in the average outer diameter and a significant amount of ring canal collapse (28); therefore, this weaker phenotype provides a sensitized background to look for enhancement or suppression by a Dock mutation. As was shown previously (Fig. 2E), reducing Dock levels with a heterozygous mutation ($dock^{04723}$) led to expansion of the ring canals in egg chambers between stages 6 through 10b of oogenesis. However, when Dock levels were reduced in the *arpC2-RNAi* egg chambers ($dock^{04723}$*/+; arpC2-RNAi*), there was a dramatic effect on ring canal stability. Egg chambers in most stages analyzed had, on average, 3 collapsed or missing ring canals (Fig. 4B, D), a phenotype that was not observed in any of the other conditions, but which is strikingly similar to that observed with a stronger depletion of ArpC2 using the MTD-GAL4 (28). Interestingly, the remaining ring canals in the $dock^{04723}$/+; *arpC2-RNAi* egg chambers were partially rescued in their size. Although additional experiments will be necessary to understand how this condition alters ring canal size, this data suggests that Dock and the Arp2/3 complex could function within the same pathway to promote ring canal stability.

Discussion

In *Drosophila melanogaster*, egg chamber development is dependent on the transfer of materials from supporting nurse cells to the oocyte through intercellular bridges (2). The growth and expansion of these intercellular bridges is tightly regulated by many different proteins (13, 14, 15, 16). Here we have characterized a novel role for the SH2/SH3 adaptor protein, Dreadlocks, in regulation of the germline ring canals. Dock localizes to the ring canals, and a reduction in Dock levels leads to a significant over-expansion of ring canal outer diameter (Fig. 2, 4).

Depletion of Dock leads to a modest, yet consistent expansion of the germline ring canals

RNAi-based depletion of Dock from the germline using either the maternal triple driver, which expresses GAL4 throughout oogenesis, or the single nanos-GAL4 driver, which expresses GAL4 in the germarium and then again after stage 5, led to a similar, significant expansion in ring canal outer diameter at most stages. This suggests either that there is perdurance of the RNAi effect during early oogenesis in the nanos-GAL4 experiment, or that the *dock-RNAi* over-expansion phenotype results from depletion of Dock in the germline stem cells or after stage 5, but that Dock protein is not essential during the middle stages of oogenesis (from ~stage 2-5). To distinguish between these two possibilities, additional experiments using other GAL4 driver lines will be required, for example using the matα4-GAL4, which is not expressed until stage 2 of oogenesis, after the egg chamber has budded from the germarium (29).

Our data demonstrates that Dock appears is necessary to prevent ring canal over-expansion; yet the question remains how over-expansion impacts fertility. Thus far, the only other protein known to negatively regulate ring canal growth is Parcas (Pcs). Homozygous *pcs* mutants display over-expanded ring canals, and mutation of Parcas was able to rescue a ring canal expansion defect observed in *Btk29* mutants. Btk29, a known ring canal protein, was recently shown to promote ring canal expansion through negative regulation of cadherin-based cell-cell adhesion. The model put forth was that Pcs negatively regulates Btk29A to promote normal ring canal expansion (30). The presence of negative regulators of ring canal expansion suggests that over-expansion can have detrimental effects on proper oocyte development, perhaps by causing ring canal instability. Additional experiments will be necessary to directly test whether Dock is required for fertility. Further, additional experiments could explore whether Dock could be either interacting with or recruiting Parcas to the ring canals as a way of negatively regulating Btk29A. Parcas contains an SH3-binding domain (31), which could mediate binding to one of Dock's SH3 domains. Btk29 and Msn have been shown to physically interact (22); therefore, it is possible that Dock, Msn, Btk29, and Parcas form a network to promote activation of Arp2/3 and to down-regulate cadherin-based adhesions during ring canal expansion (Fig. 5).

Dock, Misshapen, and the Arp2/3 complex likely cooperate to promote ring canal expansion and stability

Based on connections between Dreadlocks and Misshapen in other developmental contexts (22, 23), we wanted to test whether the two could be functioning together in the

germline. Misshapen also localizes to the ring canals, and depletion of Misshapen protein throughout oogenesis using the maternal triple driver led to a significant changes in ring canal diameter and evidence of ring canal collapse (*A. Kline data not shown*). Interestingly, depletion of Msn in the germline stem cells and then after stage 5 using the nanos-GAL4 driver led to a significant increase in ring canal outer diameter (Fig. 3D) without evidence of ring canal collapse, which resembled depletion of Dock. To determine whether Dock and Misshapen could function within the same pathway to promote proper ring canal expansion, Dock levels were reduced in *msn-RNAi* egg chambers (*msn-RNAi*, $dock^{04723}$). Reducing Dock levels significantly enhanced the *msn-RNAi* phenotype, leading to an even greater over-expansion (Fig. 3D); however, it did not lead to ring canal instability and collapse. This suggests that Misshapen and Dock could function within the same pathway (Fig. 5), but that Misshapen is likely involved in Dock-independent pathways as well.

One additional connection that was explored was to the Arp2/3 complex, which is essential for ring canal expansion and stability (18). The homolog of Msn, NIK, has been shown to phosphorylate and activate the Arp2/3 complex (19), and the Arp2/3 activator, SCAR physically and genetically interacts with Dock (24). Therefore, Dock and Msn could be cooperating to activate the Arp2/3 complex at the ring canal to promote expansion. Depletion of the Arp2/3 complex member, ArpC2, using the nanos-GAL4 driver led to a similar ring canal expansion (Fig. 4A). Interestingly, when Dock levels were reduced in *arpC2-RNAi* egg chambers ($dock^{04723}$*/+;arpC2-RNAi*), there was a synergistic defect in ring canal stability, with egg chambers at all stages analyzed displaying collapsed ring canals (Fig. 4B). This phenotype resembles a stronger depletion of ArpC2 throughout oogenesis using the maternal triple driver. Overall, these data suggest that Dock, Msn, and

the Arp2/3 complex play a minor role in the first phase of normal ring canal morphogenesis, prior to stage 5. Reducing the levels of any one of them individually from the germline stem cells leads to a modest expansion of the ring canals. Misshapen and the Arp2/3 complex additionally play an essential role during later stages, as depletion throughout oogenesis leads to failure of ring canal expansion and collapse.

Greater insight into these differences could be gained by assessing the effects of Dock depletion or over-expression on the localization of the Arp2/3 activator, SCAR. If one important role for Dock is to promote SCAR localization to the ring canals (Fig. 5), then depletion of Dock should lead to lower levels of SCAR and thereby a decrease in Arp2/3 activity. However, because depletion of Dock alone does not resemble a strong depletion of the Arp2/3 complex member, ArpC2, using the MTD-GAL4 line, it suggests that there might be alternative mechanisms to activate Arp2/3 at the ring canals. However, the observation that reducing Dock levels in egg chambers "weakly" depleted of ArpC2 resembles a stronger depletion of ArpC2 using the MTD-GAL4 line suggests that the alternative Arp2/3 activation mechanism is not robust enough to compensate when levels of the Arp2/3 complex itself are reduced.

Additional experiments will be necessary to determine how Dock, Msn, and the Arp2/3 complex cooperate to promote ring canal expansion. In order to assess the hierarchy of Dock, Msn, and the Arp2/3 complex, it will be important to determine how over-expression of Dock affects ring canal structure. I have created two different fly lines that overexpress an affinity-tagged, wild-type form of Dock (UAS-HA-Dock) or a membrane-tethered, affinity-tagged form (UAS-Myr-HA-Dock). HA is a region of the influenza hemagglutinin protein that allows us to monitor the localization of these over-expressed

proteins. Preliminary qualitative analysis indicates that overexpression of HA-Dock, especially under the control of the maternal triple driver, leads to a very severe phenotype, much stronger than was observed in *dock-RNAi* egg chambers. Quantitative analysis needs to be performed to determine the impact on ring canal size when Dock is over-expressed or tethered to the membrane. Future experiments can utilize these tools to determine genetic and localization dependencies between Dock, Msn, and the Arp2/3 complex

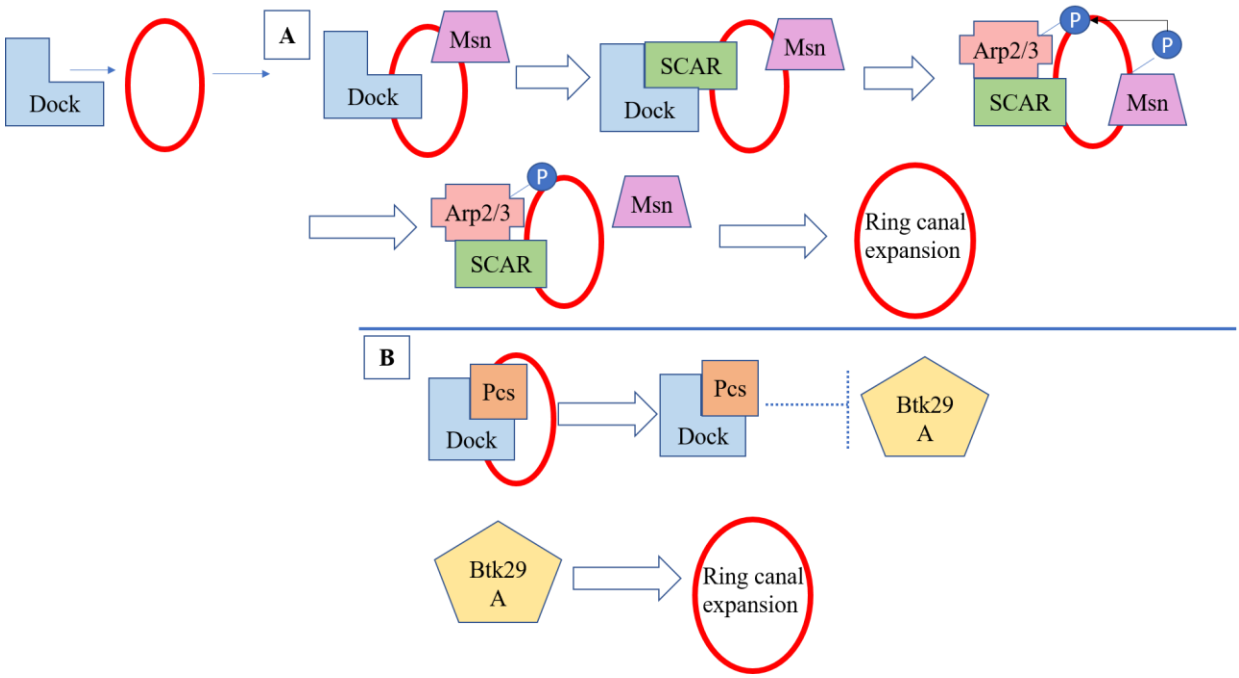

Figure 5: Two possible roles of Dock at the ring canals. (A) Dock localizes to the ring canal and recruits Msn. SCAR then binds to Dock and recruits the Arp2/3 complex. Msn phosphorylates the Arp2 subunit, thus fully activating the Arp2/3 complex. (B) Dock localizes to the ring canal and then binds to Pcs using its SH3 binding domain. Pcs then inhibits Btk29A, limiting ring canal expansion.

Here we described the role of Dock at the ring canals and proposed that it may be interacting with two previously known ring canal proteins, Msn and the Arp2/3 complex (Fig. 5). Intercellular bridges are largely conserved and their proper growth and expansion is required for fertility in *D. melanogaster* and male mice (2). Gaining a greater understanding of the protein pathways required for intercellular bridge development, and thus gametogenesis, provides important insight about the causes of infertility and identifies potential targets for infertility treatments.

Conclusion

This study characterized a novel role of the SH3/SH2 adaptor protein, Dreadlocks, in the growth and expansion of intercellular bridges by using *D. melanogaster* as a model system. Depletion of Dock led to a modest, but consistent, over-expansion of the outer diameter of the ring canals. In addition, reduction of Dock levels in egg chambers depleted of Msn led to a significant enhancement of the single perturbation. When Dock was reduced in egg chambers already depleted of the Arp2/3 complex, there was a synergistic defect in ring canal stability. Future experiments will need to be carried out to determine how Dock, Msn, and the Arp2/3 complex are interacting to regulate ring canal growth and expansion. Gaining more information about the protein pathway required for proper ring canal development could help to identify potential targets for infertility treatments in the future.

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