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Identification of genes regulating RNP foci formation in oocytes of *Caenorhabditis elegans*

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August 25th, 2008

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Abstract

Oocytes, or egg cells, of *C. elegans* contain ribonucleoprotein granules, or RNP foci, localized within the cytoplasm. These granules are composed of RNA and RNA binding proteins. Older worms that have delays in oocyte fertilization have RNP foci with increased complexity and size. It is believed that these RNP foci may be similar in function to stress granules in mammalian cells; in mammals, stress granules help to maintain molecular integrity of cells during environmental stresses. The hypothesis is that these RNP foci help to maintain molecular integrity of the oocytes, specifically of the RNA in the oocytes, when there is a delay in fertilization. In other words, if these granules did not form, then the oocytes' cellular integrity would not be maintained and viable embryos could not be generated after fertilization. While RNP foci have been initially characterized, how they are regulated is still not well understood.

In order to better understand the regulation of the assembly of RNP foci, we set out to identify the genes that control their formation. The technique that we used is called RNAi (RNA interference). RNAi is used to reduce the function of a chosen gene and to determine the effect on the worm. *C. elegans* were fed specially prepared *Escherichia coli* strains that each contained gene-specific dsRNA, which knocked-down expression of the gene in the worm. We used a genetic strain of worms that contained a fluorescent marker that caused RNP granules to fluoresce in living worms under a fluorescence microscope. We tested 28 genes and identified 2 that upon RNAi prevented the assembly of large RNP granules. Further characterization of the phenotypes will be calculated in future experiments.

Introduction

The oocytes of vertebrate animals, including humans, are arrested for extended periods of time. Human females are born with the complete and finite number of eggs they will have for their entire life, and an underlying molecular mechanism is believed to contribute to continued oocyte viability. However, female fertility decreases with age and has been attributed to a decrease in the quality of and defects in old-aged eggs (Sherins et al., 1995). Studies of female infertility are checked by both ethical and practical reasons and are thus extremely hard to complete. As a result, model organisms are used for experimentation. Model organisms generally have short generation times, are very small in size, and are easily kept and maintained within a laboratory, making them advantageous organisms to work with in a lab setting.

Caenorhabditis elegans is a model organism that has been used to identify and characterize several developmental processes that are conserved from worms to humans. The species consists of males and hermaphrodites, where hermaphrodites produce both sperm and oocytes and are able to self-fertilize. In hermaphrodites eggs are fertilized every 23 minutes, which is in stark contrast to the decades that human and other vertebrate eggs may remain unfertilized (McCarter et al., 1999). However, in aging *C. elegans* hermaphrodites, sperm become depleted and ovulation of the remaining unfertilized oocytes arrests. Oocytes lay dormant unless the sperm supply is replenished by mating with a male. This arrest of oocytes is very similar to that which occurs in human oocytes, making *C. elegans* an ideal model system to better understand infertility.

When oocytes of *C. elegans* arrest, changes occur in the cytoplasm. Large foci consisting of RNA and RNA binding proteins form and are termed ribonucleoprotein (RNP) granules or RNP foci (Schisa et al., 2001; Anderson & Kedersha, 2006). Specific RNA binding proteins,

such as poly (A) binding protein (PABP) and MEX-3, are found within RNP granules (Schisa et al., 2001; Jud et al., 2008). When worms are undergoing normal fertilization, these proteins are found evenly throughout the cytoplasm of oocytes; however, they aggregate into large foci in the absence of sperm. If sperm are reintroduced, the large foci dissociate and the oocytes return to their original state. Fertilization of the previously arrested oocytes results in viable offspring (Jud et al., 2008).

The RNP foci that form in *C. elegans* cells may have similarities to stress granules that form within humans and other vertebrates. RNP foci form not only in the absence of sperm within an oocyte but also in response to several environmental stresses, which is similar to the formation of stress granules within mammalian cells (Jud et al., 2008). To date, the function of RNP foci in *C. elegans* oocytes is unknown. The current hypothesis is that they may help to protect the molecular integrity of the oocyte, more specifically the molecular integrity of the RNA, when there is a delay in fertilization. Therefore, if old oocytes do not form RNP foci then oocytes may be unable to maintain cellular integrity or produce viable offspring after fertilization. To better understand the importance of RNP foci in aging oocytes, I conducted an RNA interference screen to identify genes that regulate the formation of RNP foci. I identified 2 of 28 tested genes that regulate their formation.

Literature Review

The model organism *C. elegans* is an ideal choice for researching infertility. As *C. elegans* hermaphrodites age, the sperm supply becomes depleted and ovulation arrests. Ribonucleoprotein (RNP) granules, or foci, form in the cytoplasm of the arrested oocytes, which are believed to contribute to the molecular integrity of the oocytes (Schisa et al., 2001).

Caenorhabditis elegans is a species of small, microscopic worms measuring 1 mm in length. The species' genome is completely sequenced, and ~19,000 genes have been identified. The worms consume *E. coli*, and are easily cultured and maintained on simple lab Petri dishes. The adults have only 959 somatic cells while still having a complete digestive system, nervous system, sensory system, and reproductive tract. All cellular processes are easily seen within worms as they are transparent. *C. elegans* is an androdioecious species, consisting of hermaphrodites and males. Hermaphrodites can mate with males, or can self-fertilize. Development from egg to fertile adult takes only 3 days, and a single hermaphrodite worm can produce approximately 300 offspring using only self-fertilization. Fertilization of eggs occurs once every ~ 23 minutes (Riddle et al., 1997; McCarter et al., 1999; Hawley & Walker, 2003).

Germline development in *C. elegans* can be divided into three separate phases: specification, growth, and maintenance. Newly fertilized eggs undergo embryogenesis, then hatch into larvae which molt four times, resulting in the larval stages L1-L4 and the adult stage (Riddle et al., 1997). During embryogenesis and the early L stage1, germline specification occurs. During the late L1 to L3 stages, germline proliferation and differentiation occur. In the late L2 stage, distinct hermaphrodite or male somatic gonad primordium form, and during the L3 stage, sex determination and meiotic prophase begins. In the late L4 and adult stages, gametogenesis occurs. Spermatogenesis occurs during the late L4 stage in males, or during early L4 stage in hermaphrodites, and oogenesis occurs during the adult stage. Oogenesis causes an increase in cytoplasmic volume and produces an oocyte that has been prepared to support embryogenesis (Hubbard & Greenstein, 2005).

In adult hermaphrodites, oogenesis and meiotic maturation occur in the proximal gonad arms (Figure 1). During oogenesis, apoptosis of germ cells occurs near the ventral bend in the

gonad tube, which is believed to help in the rapid loading of maternal factors into the remaining oocytes (Hubbard & Greenstein, 2005). The dead cells are engulfed by the gonadal sheath cells, which cover the gonad arm, regulate meiotic maturation, and contract to help drive ovulation (Greenstein, 2005).

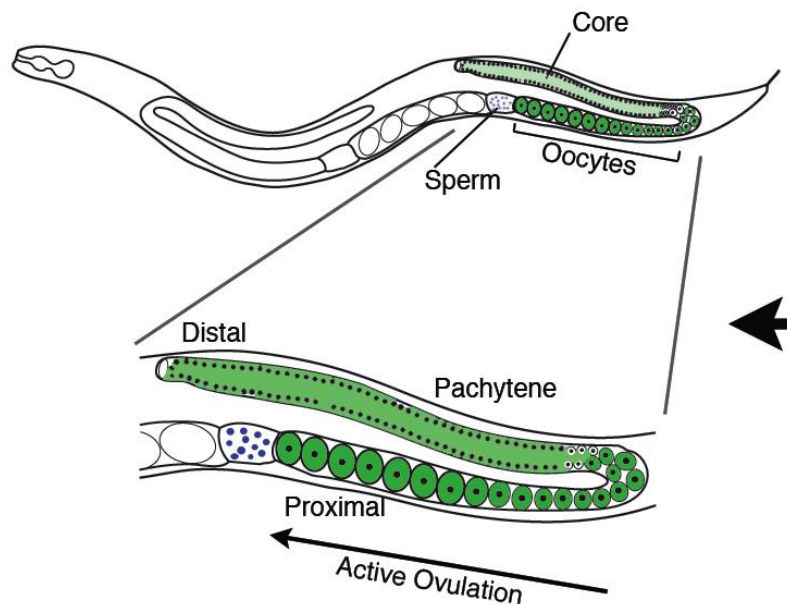


Fig. 1 Adult hermaphrodite gonad arm. Oocytes begin forming in the core, at the distal end of the gonad arm. As the oocytes are pushed around the ventral bend in the gonad arm, apoptosis occurs. As the oocytes reach the proximal end of the gonad arm, meiotic maturation occurs. Oocytes are pushed through the spermatheca, being fertilized in the process, and develop in the uterus until expelled as embryos. (Jud et al., 2008)

In *C. elegans* oocytes, meiotic maturation arrests at prophase I. Oocyte meiotic maturation begins again when stimulated by major sperm protein (MSP), which also activates mitogen-activated protein kinase (MAPK) and sheath cell contractions (Miller et al., 2001). MSP promotes oocyte meiotic maturation by two separate actions. MSP binds the VAB-1 Eph receptor protein-kinase on oocytes, and also antagonizes a key inhibitory cellular pathway (Miller et al., 2003). Initially, meiotic maturation rates are relatively high due to an abundance of sperm; however, these rates drop significantly as sperm is used up for fertilization. This

control mechanism works as a way to stop oocyte production when no sperm is present, which conserves many maternal components (Greenstein, 2005).

Meiotic maturation, ovulation, and fertilization are temporally coupled in *C. elegans* (Hubbard & Greenstein, 2005). Meiotic maturation takes place in an assembly-line fashion, so that the most proximal oocyte matures first. After receiving the MSP signal, the maturing oocyte signals its ovulation by modifying proximal gonadal sheath cells to increase the contraction rate and intensity during ovulation and inducing the spermatheca to dilate during ovulation. Gonadal sheath cells push the oocyte into the spermatheca, where the oocyte is fertilized (Greenstein, 2005).

In oocytes actively undergoing meiotic maturation, ovulation, and fertilization, P granules, which are small ribonucleoprotein (RNP) granules, become cytoplasmically distributed throughout much of development (Pitt et al., 2000). P granules contain maternal mRNAs that are required for germ cell specification (Schisa et al., 2001; Anderson & Kedersha, 2006). When sperm are unavailable, oocyte meiotic maturation arrests at prophase I, and subsequently ovulation and fertilization do not occur. When oocytes arrest, large RNP foci form within the cytoplasm of the arrested cells. RNA binding proteins normally found evenly throughout the cytoplasm of oocytes aggregate into these large RNP foci. If sperm are reintroduced into the system, the RNP foci dissociate and the oocytes return to their previous state. Subsequent fertilization results in viable offspring (Schisa et al., 2001; Jud et al., 2007). Therefore, the RNP foci do not appear to be deleterious to subsequent embryonic development.

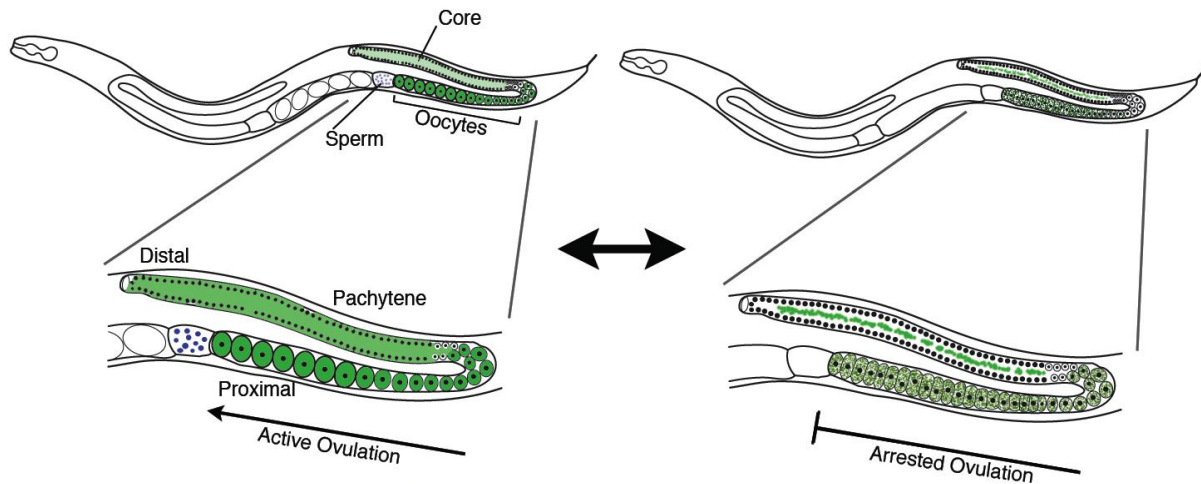


Fig. 2 RNP foci formation in hermaphrodites. On the left is a young-aged hermaphrodite with sperm in the spermatheca going through active ovulation. RNA binding proteins and mRNAs can be found evenly distributed throughout the cytoplasm of the oocytes and throughout the core (green). On the right is an old-aged worm or worm without a sperm supply in arrested ovulation. Note that the RNA binding proteins and mRNAs are now aggregated into large RNP foci throughout the oocytes and the core. This is a reversible process. (Jud et al., 2008)

The current hypothesis for the function of RNP foci formation in arrested oocytes is that the foci help to maintain molecular integrity of the oocytes while there is a delay in fertilization. It is believed that if the foci did not form, the arrested oocytes could not maintain their cellular integrity, and would not form viable offspring upon fertilization (Schisa et al., 2001, Jud et al., 2008).

To better understand the importance of RNP foci in aging oocytes, this project identified genes that appear to regulate the formation of foci. The gene set for this project consisted of genes identified as oogenesis-enriched (Reinke et al., 2003). These genes with high expression during oogenesis probably encode proteins required in oocyte differentiation, as well as maternal factors required for proper development of early embryos. The gene set includes 1,030 genes, of which a subset was evaluated in my project (Reinke et al., 2003; Reinke, 2006). I tested 28 genes and identified two that appear to be required for the assembly of RNP foci in arrested oocytes.

Methods

Of 1,030 oogenesis-enriched genes (Reinke et al., 2003), I examined a subset of 28. Approximately 600 bacterial strains were obtained from an RNAi library at the University of Michigan. The RNAi library contains the ~19,000 genes of the *C. elegans* genome that have been cloned into vectors with the T7 promoter and transformed into the HT115 strain of *E. coli*. The library is stored in 96 well plates.

RNAi by feeding was performed using the bacterial strains corresponding to each of the 28 genes. The *C. elegans* strain GFP::*MEX-3*; *fog-2* was used so that females with arrested ovulation could be assayed and the distribution of MEX-3 was evident by examining GFP expression in living worms.. This transgenic strain contains a translational fusion of green fluorescence protein (GFP) and MEX-3, which allows for RNP foci to fluoresce under a fluorescence scope (Jud et al., 2008). The background of *fog-2* creates “female” worms, hermaphrodites that do not produce sperm (Clifford et al., 2000).

The RNAi process consisted of an 8-day cycle, or RNAi round (Fig 3). The RNAi protocol was slightly modified from a protocol in Kamath & Ahringer (2003). On Day 1, NGM + carbenicillin + IPTG media was prepared and poured into 12-well plates. On Day 3, the *ceh-18* bacterial strain and 11 bacterial strains from the RNAi library were grown in 1 ml of LB + carbenicillin liquid media, overnight at 37°C. On Day 4, the 12-well plate was seeded with 60 µl of the cultured bacteria and incubated overnight at 37°C. On Day 5, the 12-well plate was removed from the incubator and placed into a 20°C incubator. Worms were synchronized by bleaching 5 plates of post-L4 GFP::*MEX-3*, *fog-2* worms (Stiernagle, 2006). Bleached eggs and embryos were collected in M9 buffer, placed in a 60 mm, empty plate and put into a 20°C incubator for at least 24 hours. On Day 6, L1 worms were collected from the bleached hatch-off.

Approximately 10 L1's were orally pipetted onto each well of the 12-well plate. The plate was then incubated at 24°C for 28 hours, in order to produce early-stage L4 worms. On Day 7, the L4's were inspected under a light microscope, and males were removed to ensure no fertilization of hermaphrodites occurred. The plate was then placed into a 24°C incubator for 24-48 hours. On Day 8, adult hermaphrodites were scored under a Leica fluorescence dissecting microscope. Worms were immobilized using levamisole. Changes in oocytes and RNP foci formation were recorded.

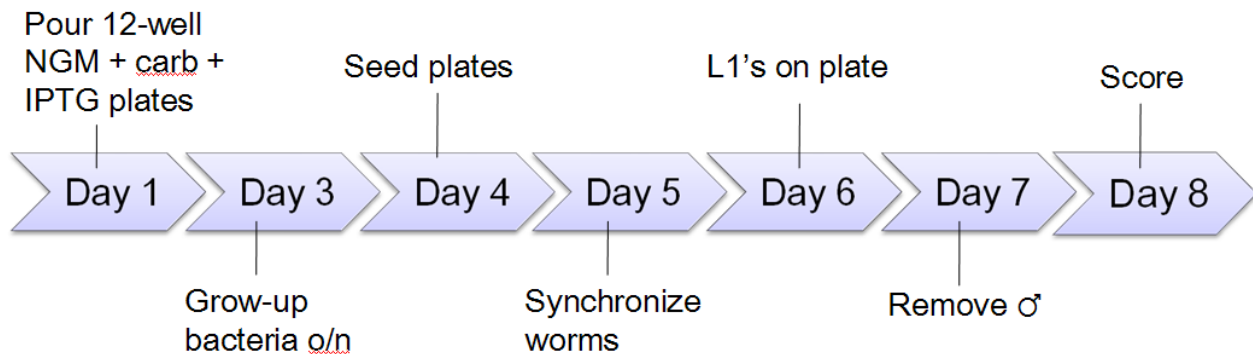


Fig. 3 Flowchart of one round of RNAi. 12-well NGM + carbenicillin + IPTG plates created on Day 1. On Day 3, 11 bacterial strains and control *ceh-18* bacterial strain were grown in 1 ml LB + carbenicillin stocks. On Day 4, the 12-well plate was seeded with 60 μ l of the cultured bacteria and incubated overnight at 37°C. On Day 5, bleaching of adult GFP::*MEX-3*; *fog-2* worms was performed to synchronize worms. On Day 7, male worms were removed from the plates. On Day 8, the adult hermaphrodites were scored.

Results

A subset of 28 “oogenesis-enriched” genes was screened to find genes that regulate RNP foci formation. *ceh-18* was used as a positive control, based on previous studies (Jud et al., 2008). In *ceh-18*, *fog-2* hermaphrodite worms, an even distribution of MEX-3 is observed throughout oocytes. Similarly, after RNAi of *ceh-18* in the GFP::*MEX-3*; *fog-2* hermaphrodite worms, the GFP failed to assemble into large foci (Fig. 4A). This is in contrast to the negative control, where the GFP has localized to large foci in GFP::*MEX-3*; *fog-2* females.

Of 28 genes tested, two appear to be required for MEX-3 foci to assemble. RNAi of 6

genes-K09H9.6, R05D11.7, K07A12.2, W06D4.6, *dcr-1*, and *kgb-1*-resulted in an intermediate phenotype. Penetrance rates for the intermediate phenotype were 75% with three worms, 33% with two worms, 25% with one worm, 60% with three worms, 29% with two worms, and 10% with one worm, respectively. Screens of five genes were unscorable, due to bacterial contamination of plates or the presence of males. RNAi of the remaining 15 genes, resulted in an unchanged distribution of GFP. RNP foci were clearly seen within the oocytes of the GFP::MEX-3; *fog-2* worms (Fig. 4C)(Table 1).

RNAi of the genes *inx-14* and *goa-1* resulted in GFP::MEX-3; *fog-2* worms displaying a “mutant” phenotype or cytoplasmic distribution of GFP in arrested oocytes (Table 1). GFP failed to assemble into large foci in these oocytes (Fig. 4B). RNAi of *goa-1* resulted in a phenotype with penetrance of 36%; after being done in triplicate, and four worms displayed the “mutant” phenotype. RNAi of *inx-14* resulted in a phenotype with a penetrance of 25%; after being done in duplicate, two worms displayed the “mutant” phenotype. When screened, five genes-R119.4, T20F5.6, T01G9.5, K021B2.5, and R05D11.8-were unable to be scored. The remaining 15 genes displayed the “normal” phenotype of RNP foci formation.

Results of RNAi Screen					
Schisa #	Gene	Sequence Name	Cytoplasmic	Penetrance %	<i>n</i> (worms)
1-A3		R119.4	NA		
1-A4		R119.7	N		
1-A6		M01D7.6	N		
1-A7		Y23H5A.3	N		
1-A8		K09H9.2	N		
1-A9		K09H9.6	int	75	3
1-A10		M01D7.6	N		
1-A11		W05F2.3	N		
1-A12		H26D21.2	N		
1-B1		T12F5.2	N		
1-B3		T20F5.6	NA		
1-B4		T21E3.1	N		
1-E10	<i>inx-14</i>	F075A.1	yes	25	2
1-E12	<i>goa-1</i>	C26C6.2	yes	36	4
1-F10		T01G9.5	NA		
1-F11		F16D3.4	N		
1-F12		F02E9.4	N		
1-G1		F01D11.2	N		
1-G2		D1081.7	N		
1-G3		D1081.8	N		
1-G4		K021B2.5	NA		
1-G5		R05D11.7	int	33	2
1-G6		R05D11.8	NA		
1-G7		K07A12.2	int	25	1
1-G8		W06D4.6	int	60	3
2-D5		F14D2.8	N		
4-D8	<i>dcr-1</i>	K12H4.8	int	29	2
4-H10	<i>kgb-1</i>		int	10	1
6-G6	<i>ceh-18</i>		yes	91	21

Table 1. *ceh-18* was used as a positive control and has a 91% penetrance rate. Genes *inx-14* and *goa-1* were found to be possible regulators of RNP foci formation. Genes K09H9.6, R05D11.7, K07A12.2, W06D4.6, *dcr-1*, and *kgb-1* were found to be intermediate in regulating RNP foci formation. Genes R119.4, T20F5.6, T01G9.5, K021B2.5, and R05D11.8 were unscorable, due to bacterial contamination or presence of males. Remaining genes displayed the “normal” phenotype of RNP foci formation.



Fig. 4 Fluorescent images of RNAi screen. *ceh-18* GFP::MEX-3, *fog-2* hermaphrodite, positive control. GFP failed to assemble into large foci, and instead remained cytoplasmically distributed. (A) *inx-14* GFP::MEX-3, *fog-2* hermaphrodite. A “mutant” phenotype-GFP failed to assemble into large RNP foci. Possible regulator of RNP foci formation. (B) Gene K07A12.2 GFP::MEX-3, *fog-2* hermaphrodite. A “normal” phenotype-GFP aggregates into large RNP foci. The arrow points out one of these RNP foci. (C)

Discussion

In order to better understand the regulation of RNP foci formation in arrested oocytes, genes controlling their formation were identified. Very few genes are expected to regulate RNP foci formation. Following this hypothesis, only two genes, *inx-14* and *goa-1*, of 28 tested genes were found to possibly regulate the formation of RNP foci. The gene K09H9.6, an intermediate phenotype gene, may be another possible regulator of RNP foci formation in arrested oocytes. This gene has only been examined once to date, with a penetrance of 75% (Table 1). This gene will be screened in triplicate in order to analyze a larger data set.

Identifying the genes involved in RNP foci formation in *C. elegans* oocytes will allow for further studies to be conducted examining the molecular pathways controlling RNP foci formation. Performing a secondary genetic screen with the positive hits *inx-14* and *goa-1* or characterizing the positive hits are two future steps. Antibody staining against MEX-3, the P granule proteins PGL-1 and GLH-1, and the P body proteins CAR-1, CGH-1, and DCAP-2 will allow us to determine if only the GFP marker for MEX-3 is failing to assemble into RNP foci. Although 26 genes were found to not affect RNP foci formation within my gene set, this project will help the overall project by narrowing down possible genes that affect RNP foci formation within the larger gene set, allowing more resources to be spent on identifying additional new genes. These future studies could lead to a better understanding of how *C. elegans* oocytes maintain their molecular integrity, and similar mechanisms in mammals may then be understood further due to the research on the *C. elegans* model organism.

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