The role of gap junctions in *Caenorhabditis elegans* oocyte maturation and fertilization

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Received for publication 16 May 2006; revised 3 August 2006; accepted 17 August 2006

Available online 22 August 2006

Abstract

We have investigated the role of gap junctions in *Caenorhabditis elegans* oocyte maturation and fertilization. Gap junctions are observed between oocytes and the surrounding ovarian sheath cells in wild-type gonads. The sheath transcription factor CEH-18 is required to negatively regulate oocyte maturation, mitogen-activated protein kinase (MAPK) activation, and ovulation. Transmission electron microscopy (TEM) indicates that sheath/oocyte gap junctions are rare or absent in *ceh-18(mg57)* null mutant gonads. To test the hypothesis that gap junctions negatively regulate oocyte maturation, we performed an RNAi screen of innexin genes, which encode channel-forming proteins. Here we show that INX-14 and INX-22 are required in the female germ line to inhibit oocyte maturation, MAPK activation, and ovulation. Genetic analysis and TEM are consistent with INX-14 and INX-22 being components of sheath/oocyte gap junctions. Our results support the hypothesis that gap junctions maintain oocytes in meiotic prophase I when sperm are absent. We also implicate these channels in regulating sheath cell contractile activity and sperm recruitment to the spermatheca, the site of sperm storage and fertilization. Together with previous studies, our results help establish the *C. elegans* gonad as a model system for investigating the molecular mechanism(s) by which gap junctions regulate meiosis and fertilization.

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Keywords: Gap junction; Innexin; Meiosis; Oocyte maturation; Ovarian contraction; Mitogen-activated protein kinase; Fertilization; Sperm motility

Introduction

An evolutionarily conserved feature of metazoan reproduction is that oocytes arrest in meiotic prophase (reviewed in Masui, 1985; Masui and Clarke, 1979). Mammalian oocytes arrest in the diplotene stage of prophase I during prenatal development, then resume meiosis in adults by a process called oocyte maturation, which prepares the oocyte for fertilization and embryogenesis. A surge of luteinizing hormone (LH), released from the anterior pituitary gland, stimulates oocyte maturation and the coordinated release of the oocyte from the mature Graafian follicle, called ovulation. LH is a peptide that binds to a G-protein-coupled receptor expressed in mural granulosa cells, but not in oocytes or the cumulus cells surrounding them (reviewed in Mattioli and Barboni, 2000). Therefore, LH action on oocyte maturation is indirect (reviewed in Mehlmann, 2005). In mural granulosa cells, LH induces the expression of epidermal growth factor-like ligands, which function to promote oocyte maturation (Park et al., 2004). There is also evidence for an inhibitory signal that maintains oocytes in prophase I, as precocious oocyte maturation results when oocytes are separated from antral follicles (Edwards, 1965; Pincus and Enzmann, 1935). These studies suggest that follicle cells are the source of multiple signals that control the timing of oocyte maturation.

Gap junctions are intercellular channels that form at sites of close cell–cell apposition and allow passage of small molecules (Nicholson, 2003; Unger et al., 1999). In the mammalian Graafian follicle, gap junctions are observed between oocytes and cumulus cells (Anderson and Albertini, 1976; Gilula et al., 1978). It has been proposed that these gap junctions transmit a...
signal from cumulus cells to oocytes that negatively regulates oocyte maturation (Dekel, 2005; Dekel and Beers, 1980). An alternative possibility is that gap junctions transmit a maturation-promoting signal (Eppig, 2001). Testing these models in vivo has been difficult owing to pleiotropy and redundancy of connexin genes, which encode channel-forming proteins in chordates. At least five connexins are expressed in the mouse follicle (Kidder and Mhawi, 2002). Oocytes fail to reach meiotic competence and mature Graafian follicles fail to develop in mice with a targeted deletion of Cx37, a connexin expressed at the interface between oocytes and developing granulosa cells (Simon et al., 1997). Therefore, the in vivo role of Cx37 and gap junctions in oocyte maturation is not completely understood.

We have been using the nematode Caenorhabditis elegans as a simple model to investigate the molecular mechanisms that regulate oocyte maturation. The transparent epidermis enables direct visualization of oocyte maturation and ovulation in wild-type and mutant animals (Fig. 1A). Oocytes in diakinesis of meiotic prophase are located in the proximal gonad arm adjacent to the spermatheca, the site of sperm storage and fertilization (Fig. 1A) (Hirsh et al., 1976; McCarter et al., 1999; Ward and Carrel, 1979). Ovarian sheath cells surround the developing oocytes (Fig. 1A) and form gap junctions with them (Hall et al., 1999). Sperm release a paracrine hormone called the major sperm protein (MSP) to stimulate oocyte maturation, MPK-1 mitogen-activated protein kinase (MAPK) activation, and sheath cell contraction (Kosinski et al., 2005; Miller et al., 2001). During maturation, the oocyte signals for dilation of the spermathecal valve, resulting in ovulation and fertilization (Iwasaki et al., 1996). Oocytes can arrest in meiotic prophase for days in the absence of sperm (McCarter et al., 1999). The hermaphrodite gonad undergoes spermatogenesis prior to oogenesis (Hirsh et al., 1976; Ward and Carrel, 1979), so maturation and ovulation occur constitutively in adults until sperm are depleted. In mutant hermaphrodites that do not produce sperm (i.e., females), and closely related nematode species with separate male and female sexes, oocytes arrest in meiotic prophase until insemination occurs and sperm migrate to the spermatheca (Hill and L’Hernault, 2001; McCarter et al., 1999).

MSP binds to multiple receptors, including the VAB-1 Eph receptor protein-tyrosine kinase on oocyte and sheath cell plasma membranes (Corrigan et al., 2005; Miller et al., 2003). Binding antagonizes two inhibitory signaling pathways, one mediated by canonical VAB-1 ligands called ephrins and the other dependent on the CEH-18 POU-class transcription factor (Miller et al., 2003). These pathways act in parallel to negatively regulate oocyte maturation and MAPK activation in the absence of sperm. CEH-18 is expressed in sheath cells, but not in oocytes (Greenstein et al., 1994; Rose et al., 1997), suggesting that it is required to transduce a signal from sheath cells to oocytes that maintains oocytes in prophase I. The role of CEH-18 in this process could be indirect, as sheath differentiation and structure are disrupted in ceh-18 null mutants. Ultrastructural studies indicate that CEH-18 is required for close apposition of sheath and oocyte plasma membranes and formation or maintenance of sheath/oocyte gap junctions (Rose et al., 1997). These data raise the possibility that gap junctions negatively regulate oocyte maturation.

Gap junctions in invertebrates are formed by innexins, which are analogous to vertebrate connexins (reviewed in Phelan, 2005; Starich et al., 2001). Innexins and connexins have similar structures consisting of four membrane-spanning domains, two extracellular loops, and cytoplasmically located N- and C-termini (Fig. 1B). Innexins localize to gap junctions in vivo and are sufficient to form intercellular channels in Xenopus oocytes (Landesman et al., 1999; Phelan et al., 1996, 1998b; Starich et al., 2003; Starich et al., 1996). Here, we conducted an RNA-mediated interference (RNAi) screen of the 25 innexin loci in the C. elegans genome and identified INX-14 and INX-22 as negative regulators of oocyte maturation, MPK-1 MAPK activation, and ovulation. Genetic studies, site of action analyses, and transmission electron microscopy support the hypothesis that INX-14 and INX-22 are components of sheath/oocyte gap junctions. In addition to oocyte maturation, we implicate these structures in regulating sheath cell contractile activity and sperm recruitment to the spermatheca.

**Materials and methods**

**C. elegans culture and genetics**

C. elegans variety Bristol, strain N2 is the wild-type strain. Worm cultures were maintained on NGM plates at 20 °C or 25 °C (Brenner, 1974). Males were generated from N2 crosses or by using the fog-2(q71) strain, which segregates “female” and normal male progeny (Schedl and Kimble, 1988). fog-2(q71) and fog-3(q443) null feminizing mutations that eliminate sperm production in hermaphrodites were used for all studies (Ellis and Kimble, 1995; Schedl and Kimble, 1985). The following mutant strains were used: SS104 [glp-4(bn2)], CB4108 [fos-2(q71)], DG1743 [fas-3(q443)/hT2(qd48)], PD8488 [ref-1(pk1417)], DG1744 [ref-1(pk1417)/hT2(qd48)], GR1034 [ceh-18(mg57)], GD1604 [ceh-18(mg57); fog-2(q71)], inx-22(tm1661); fog-2(q71), and inx-22(tm1661). The hT2(qd48)I balancer chromosome is dominantly marked with pharyngeal GFP.

The inx-22(tm1661) strain was generated by the National Bioresource Project (Tokyo). Homozygous animals were backcrossed to the wild type eight times prior to constructing inx-22(tm1661); fog-2(q71) mutants. Genotypes were determined from single worms by nested PCR using the primer sequences ACGCGTATCGTGAATCCCGA, GCAGGCGATAGAATCTGCT, TTCCGATTCGCGTCCGCT, and TGGGAGAGATCGACGCGAT.

**Phenotypic analysis and rate determination**

Oocyte maturation is characterized by distal migration and subsequent breakdown of the nuclear envelope, cortical cytoskeletal rearrangement, and meiotic spindle assembly. During maturation, sheath cell contractile activity increases and the spermathecal valve dilates. In unmat ed females that fail to inhibit oocyte maturation and ovulation, unfertilized oocytes fill the uterus and are found in large numbers on the culture plates (Miller et al., 2003, 2004). This phenotype was scored using a stereomicroscope in the RNAi screen. Detailed analysis of oocyte maturation and ovulation was performed on live anesthetized animals (0.1% tricaine and 0.01% tetramisole in M9 buffer) using DIC high-resolution optics (McCarter et al., 1999). Oocyte meiotic progression was evaluated by DAPI staining of dissected gonads. For each strain, oocyte maturation and sheath contraction were monitored by direct observation (or time-lapse recording). Basal sheath contraction rates (contractions per minute) are measured between ovulations by monitoring distal migration of the oocyte nuclear envelope (Corrigan et al., 2005; McCarter et al., 1999; Miller et al., 2004). Oocyte maturation and sheath contraction rates were measured in adult worms 24–36 h past the L4 stage. Contractions whose total sheath displacement is greater than 1.5 μm were scored. glp-4(bn2) was grown at the restrictive temperature (25 °C). Examination of their gonads revealed no oocytes. Total sheath displacement during contractions in glp-4(bn2) animals is greater than...
Fig. 1. The *C. elegans* hermaphrodite gonad and innexin structure. (A) Diagram of the anterior reproductive tract. The transparent epidermis makes the events of oocyte maturation and fertilization directly observable in the intact animal (reviewed in Yamamoto et al., 2005). There are two U-shaped gonad arms connected to a common uterus. In the proximal gonad (the anterior arm is shown in the schematic representation), oocytes (Ooc) in diakinesis of meiotic prophase are surrounded by ovarian sheath cells, which form gap junctions with them (Hall et al., 1999). Within the spermatheca, sperm (red) release extracellular MSP to induce oocyte maturation and spermathecal valve dilation, resulting in ovulation and fertilization. Embryos (Emb) develop in the uterus before being laid through the vulva into the external environment. During mating, males inject sperm through the vulva into the uterus. Amoeboid sperm crawl around fertilized eggs to the spermatheca. (B) Innexin protein structure. Innexin genes encode proteins containing four membrane-spanning domains (blue) and two extracellular cysteine-containing loops. Both the N- and C-terminus are predicted to be intracellular (Phelan et al., 1998a; Starich et al., 2001). (C) *inx-14* and *inx-22* gene structure. The *inx-14* gene contains seven predicted exons (purple blocks), whereas *inx-22* contains six predicted exons. The locations of membrane-spanning regions, determined by the TMHMM algorithm (Krogh et al., 2001), are shown below each gene structure. The *tm1661* mutation (red bar) deletes two membrane-spanning domains and is predicted to be a strong loss-of-function or null allele.
the wild type. Brood sizes were measured from RNAi animals at 25 °C or mutant and wild-type animals at 20 °C. The unc-4 RNAi feeding clone was used as a negative control, as unc-4 is not expressed in the adult gonad and unc-4 mutants do not exhibit reproductive defects. Tables show average oocyte maturation or sheath contraction rates ± standard deviation. A two-sample t-test was used to test for significance.

RNA-mediated interference (RNAi)

RNAi feeding clones were cultured overnight at 37 °C in 2 ml of LB supplemented with tetracycline (10 μg/ml) and ampicillin (100 μg/ml). One milliliter of overnight culture was transferred to 25 ml of LB/Amp/Tet and grown for 1 h. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After 4 h, the bacteria were centrifuged and resuspended in 1 ml M9 buffer (3.0 g KH2PO4, 6.0 g Na2HPO4, and 5.0 g NaCl in 1 L ddH2O) containing ampicillin, tetracycline, and 400 mM IPTG. 250 μl bacteria were dropped onto NGM plates (plus Amp/Tet/IPTG) and allowed to dry. L4 animals were cultured on the RNAi plates for 24–36 h at 25 °C. HT115 bacterial feeding strains were obtained from the genome-wide library (Kamath et al., 2003) and checked for accuracy by sequencing or PCR using primers internal to the cloned region.

Fluorescence microscopy

MAPK activation was evaluated using monoclonal MAPK-YT antibodies (Sigma) as previously described (Miller et al., 2001). MAPK-YT recognizes the mpk-1 gene product. Briefly, gonads were dissected from adult animals 24–36 h past the L4 stage and rapidly fixed in 2% neutral-buffered paraformaldehyde. After an overnight incubation at 4 °C, the gonads were washed 4 times in PBT (phosphate-buffered saline + 0.05% Tween-20) and incubated in PBT containing 1 mg/ml BSA for 1 h. MAPK-YT antibodies (1:2000) were incubated with gonads for 4 h at room temperature, then washed 4 times with PBT. Rabbit anti-mouse FITC-conjugated antibodies (1:500) were used for detection. An Axiovert 200M Zeiss Photomicroscope equipped with epi-fluorescence, an Axioscan digital camera, and a PC computer was used for imaging. Alexa Fluor 660 phalloidin (Molecular Probes) and DAPI were used to visualize F-actin and DNA, respectively.

Sperm migration analysis

MitoTracker Red CMXROS (Molecular Probes) was used to label male sperm by modification of a method described in previous studies (Kosinski et al., 2005). Briefly, MitoTracker (MT) was diluted in DMSO to 1 mM. Fifty males were placed in 297 μl M9 buffer in a watch glass. 3 μl MT was added to a final concentration of 10 μM. Males were incubated in the dark for 2 h, then transferred to fresh plates and allowed to recover overnight. About 25 males were placed with 6–8 anesthetized hermaphrodites (0.1% tricaine and 0.01% tetramisole in M9 buffer) and incubated on NGM plates containing a 1 cm in diameter drop of bacteria. After mating for 20–30 min, anesthetized animals were removed from males and incubated for 1 h in the dark. The MT mated hermaphrodites were mounted for microscopy on 2% agarose pads. To visualize sperm migration in the uterus, MT labeled hermaphrodites were mounted immediately after mating. Sperm migration traces were generated from time-lapse videos taken at 30-s intervals. Axiosvision software was used to measure distances. Anesthesia does not affect sperm motility. MT sperm are indistinguishable from non-labeled sperm in appearance and motility. Additional details on sperm migration intervals. Axiovision software was used to measure distances. Anesthesia does not affect sperm motility. MT sperm are indistinguishable from non-labeled

Transmission electron microscopy (TEM)

TEM was performed as described previously with a few modifications (Hall et al., 1999). L4 stage animals were cultured for about 24 h at 25 °C. Fifty to sixty whole or dissected worms were fixed in 2.5% glutaraldehyde/1% paraformaldehyde/0.1 M sucrose/0.05 M cacodylate overnight at 4 °C. Animals were post-fixed in 0.5% osmium tetroxide/0.5% potassium ferrous cyanate and stained with 1% uranyl acetate/0.1 M sodium acetate. Worms were embedded in 3% agarose blocks and then dehydrated using a series of ethanol dilutions followed by 100% propylene oxide. The agarose blocks were embedded in epoxy resin and baked for 48 h at 70 °C. Gonad-containing thin sections were counterstained with uranyl acetate and/or lead citrate and examined using a Hitachi 7000 (Hitachi High Technology America, Pleasanton CA) electron microscope. Sections from three to five gonads were analyzed for each genotype. Dissection does not alter the integrity or structure of the gonad. Similar results are observed in extruded and non-extruded gonads. All female strains analyzed are in the fog-2(q71) background.

Results

RNAi screen of the 25 innexins in the C. elegans genome

Gap junctions have been observed by transmission and freeze fracture electron microscopy between oocytes and the surrounding sheath cells in the proximal gonad (Hall et al., 1999). The sheath transcription factor CEH-18 is required to negatively regulate oocyte maturation, MPK-1 MAPK activation, and ovulation in the absence of sperm (Miller et al., 2003). Sheath cell differentiation and function are disrupted in ceh-18 (mg57) null mutants. Transmission electron microscopy (TEM) indicates that sheath/oocyte gap junctions are rare in ceh-18 (mg57) mutant gonads (Rose et al., 1997). We hypothesized that the oocyte maturation defect in ceh-18 mutants results from loss of gap junctions. To test this hypothesis, we conducted an RNAi screen of the 25 innexin loci in the C. elegans genome (Table 1; Starich et al., 2001). The screen was performed in the fog-2 (q71) background, which generates XX females and XO males (Schedl and Kimble, 1988). L4 stage females were fed bacteria expressing dsRNA for each innexin and scored 24 to 48 h later. In uncontrolled female cultures, females accumulate in the proximal gonad, while few oocytes that have completed oocyte maturation and ovulation are found in the uterus and on the culture plates (Fig. 2A and data not shown). By contrast, fewer oocytes accumulate in the proximal gonads of unmated ceh-18 (mg57) females (Fig. 2B). The oocyte maturation and ovulation rate is high, causing unfertilized oocytes to fill the uterus and often the plates (Miller et al., 2003). We screened 25 innexin RNAi clones for those that phenocopied ceh-18 (mg57) females and identified two genes, inx-14 and inx-22 (Table 1; Figs. 2A–D). Innexins with redundant functions or those that are refractory to RNAi could not be identified using this strategy.

inx-14 and inx-22 encode proteins with features typical of innexin family members, including four putative membrane-spanning regions and two extracellular loops (Figs. 1B and C). Innexins that mediate gap junction assembly in oocytes should have mRNAs expressed in the hermaphrodite germ line during oogenesis. DNA microarray and in situ hybridization analyses are consistent with inx-14 and inx-22 having this mRNA expression pattern, along with four other innexins predicted to function in the germ line or embryo (Table 1) (Kohara, 2001; Reineke et al., 2004). To further validate the RNAi screen, we generated inx-22(tm1661); fog-2(q71) double mutants. tm1661 deletes 765 bp, including the second and third membrane-spanning domains, and is predicted to be a strong loss-of-function or null allele (Fig. 1C). The gonads of unmutated inx-22 (tm1661) females are nearly identical to those of unmutated inx-
INX-14 RNAi, inx-22 RNAi, and ceh-18(mg57) females (Figs. 2B–E). We conclude that our RNAi screen has identified two candidates for mediating sheath/oocyte gap junction assembly.

INX-14 and INX-22 negatively regulate oocyte maturation and MAPK activation

Analysis of anesthetized unmated females by DIC microscopy indicates that loss of INX-14 or INX-22 causes precocious nuclear envelope breakdown, cortical cytoskeletal rearrangement, and spermathecal valve dilation, culminating in ovulation. This phenotype is identical to that of unmated ceh-18(mg57) females, except CEH-18 is required for normal dilation of the spermathecal valve (Rose et al., 1997). DAPI staining demonstrates that innexin RNAi and mutant oocytes progress normally through meiotic prophase before entering metaphase during oocyte maturation. The proximal gonads contain fewer oocytes than those from unmated controls, resembling wild-type gonads containing sperm and unmated ceh-18(mg57) female gonads (Fig. 2). Quantitative analysis indicates that the oocyte maturation rates of unmated inx-14 and inx-22 RNAi and mutant females (hereafter referred to as loss-of-function females) are similar to unmated ceh-18(mg57) females and significantly higher than unmated controls (Table 2, compare lines 1–5; P<0.001). We conclude that INX-14 and INX-22 negatively regulate oocyte maturation and ovulation in the absence of sperm.

The sperm signal MSP stimulates MPK-1 MAPK activation in the most proximal oocytes of wild-type hermaphrodites, as evidenced using MAPK-YT antibodies, which recognize the diphosphorylated, active form of MPK-1 (Miller et al., 2001). CEH-18 acts in parallel to ephrin/VAB-1 signaling to negatively regulate MAPK activation in the absence of sperm (Miller et al., 2003). Oocyte MAPK-YT staining is often faint in unmated ceh-18(mg57) females, but slightly more intense on average than unmated controls (Figs. 3A, B). The oocytes of unmated inx-14 RNAi, inx-22 RNAi, and inx-22(tm1661) female gonads contain activated MAPK, resembling the oocytes of wild-type hermaphrodite and mated female gonads (Figs. 3A–E). These results indicate that INX-14 and INX-22 negatively regulate oocyte MAPK activation in the absence of sperm.

INX-14 and INX-22 are required in the female germ line

DNA microarray studies support the hypothesis that inx-14 and inx-22 mRNAs are enriched in the germ line during oogenesis (Reinke et al., 2004). In situ hybridization analysis is also consistent with germ line expression (Kohara, 2001). However, it is difficult to determine whether these innexins are also expressed in sheath cells, due to their close proximity to oocytes and the thinness of the sheath. A given innexin could be expressed in oocytes and sheath cells, but its function could be required in the sheath only. To test the hypothesis that INX-14 and INX-22 are required in oocytes, we used rrf-1(pk1417) mutants, which are sensitive to RNAi in the female germ line, but resistant to RNAi in the soma (Sijen et al., 2001). By comparing the RNAi phenotype of a given gene in control females to rrf-1(pk1417) females, we can determine whether gene function is required in the female germ line or somatic sheath cells (Corrigan et al., 2005; Miller et al., 2003). The oocyte maturation rate of unmated inx-14 RNAi rrf-1(pk1417) females is similar to unmated inx-14 RNAi females and significantly higher than unmated controls (Table 2, compare line 9 to lines 6–8; P<0.001). Furthermore, oocytes accumulate to the same extent in unmated inx-14 RNAi rrf-1(pk1417) female and unmated inx-14 RNAi female gonads, much less than unmated female controls (Fig. 2 and data not shown). Data for inx-22 fit the same site of action profile as inx-14 (Table 2, compare line 11 to lines 6, 7, and 10; P<0.001). These results indicate that INX-14 and INX-22 are required in the female germ line to negatively regulate oocyte maturation. The possibility that either innexin is also required in sheath cells cannot be eliminated.

INX-14, INX-22, and CEH-18 act in a common genetic pathway

CEH-18 is required in sheath cells for the assembly or maintenance of a subset or possibly all sheath/oocyte gap junctions. If INX-14 is a component of CEH-18-dependent gap junctions, then the oocyte maturation rate of unmated ceh-18(mg57) females should be similar to unmated inx-14 RNAi ceh-18(mg57) females. Alternatively, if the maturation rate of unmated inx-14 RNAi ceh-18(mg57) females is significantly higher than unmated ceh-18(mg57) controls, then ceh-18 and inx-14 likely act in parallel pathways. Comparison of maturation rates in single and double loss-of-function backgrounds supports the hypothesis that inx-14 and ceh-18 act in the same genetic pathway (Table 2, compare line 12 to lines 2 and 3). The same relationship is observed for inx-22 and ceh-18 (Table 2, compare line 13 to lines 2 and 4). We conclude that INX-14 and INX-22 function is dependent upon CEH-18, a result consistent with the idea that these innexins are components of sheath/oocyte gap junctions.

Gap junctions can form from homomeric or heteromeric assemblies of innexin proteins (see Discussion). To test whether inx-14 and inx-22 act in the same pathway to regulate oocyte maturation, we examined maturation rates in single and double loss-of-function backgrounds. The rate of unmated inx-14 RNAi inx-22(tm1661) females is not higher than unmated inx-22(tm1661) controls (i.e., non-additive rates), suggesting that inx-14 and inx-22 function in the same pathway (Table 2, compare line 14 to lines 3 and 5). Taken together, these data are consistent with INX-14 and INX-22 forming heteromeric channels between oocytes and sheath cells.

INX-14 functions in the sperm-sensing mechanism

The sperm signal MSP antagonizes an inhibitory pathway dependent upon CEH-18 function. If a given innexin acts in the sperm-sensing mechanism, then it should be required to inhibit oocyte maturation in the absence of sperm, but not in the presence of sperm. To test this prediction, we compared
oocyte maturation rates of innexin RNAi and mutant gonads in the presence and absence of sperm. The maturation rate of inx-14 RNAi hermaphrodites is significantly higher than unmated inx-14 RNAi females (Table 2, compare line 16 to 3; \( P < 0.001 \)), but is not significantly different from the rate of wild-type hermaphrodites (Table 2, compare line 16 to 15 and data not shown; \( P > 0.10 \)). Therefore, INX-14 likely functions in a sperm-dependent pathway. By contrast, INX-22 function is at least in part independent of sperm presence, as the maturation rates of mated inx-22 RNAi females and inx-22 (tm1661) hermaphrodites are higher than the rate of wild-type hermaphrodites (Table 2, compare lines 17 and 18 to 15; \( P < 0.005 \)). Identical results are observed in mated females.

The number of oocytes containing activated MAPK can also be used to test for sperm-dependent function. The MAPK activation pattern in inx-14 RNAi hermaphrodites is similar to wild-type hermaphrodites (data not shown), while consistently more oocytes contain activated MAPK in inx-22(tm1661) hermaphrodites compared to the wild type (Figs. 3E, F). These results suggest that INX-14 functions in the MSP sperm-sensing mechanism.

INX-14 and INX-22 negatively regulate sheath cell contraction

MSP binds to receptors on the sheath cell plasma membrane to promote an increase in the sheath contraction rate (Corrigan et al., 2005; McCarter et al., 1999; Miller et al., 2003). In unmated female gonads, the sheath contraction rate is low (Table 3, line 1). We noticed that loss of INX-14

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**Table 1**

Results summary from the innexin RNAi screen

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<td>24. unc-7</td>
<td>R07D5.1</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>25. unc-9</td>
<td>R12H7.1</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

Note. The 25 innexin genes in *C. elegans* genome were screened (Starich et al. 2001). Germ line expression data are from DNA microarray (Reinke et al., 2004) and in situ hybridization analyses (Kohara, 2001). Only strong inhibition phenotypes were considered in the screen. This screen was not designed to identify innexins with redundant functions. Abbreviations: N, not detected; AH, adult hermaphrodite germ line.

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**Fig. 2.** DIC micrographs of unmated females. (A) In unmated fog-2(q71) female controls, the oocyte maturation rate is low and prophase-arrested oocytes accumulate in the proximal gonad distal to the spermatheca. Few oocytes that have completed oocyte maturation and ovulation are observed in the uterus. The spermatheca is outlined in black in all images. (B) In unmated ceh-18(mg57) females, the oocyte maturation rate is high. Fewer prophase oocytes accumulate in the proximal gonad, while numerous unfertilized oocytes having recently completed maturation and ovulation are observed in the uterus (arrowheads). (C–E) The oocyte maturation and ovulation rate is also high in unmated inx-14 RNAi females (C), unmated inx-22 RNAi females (D), and unmated inx-22(tm1661) females (E). (F) The proximal gonads of unmated innexin loss-of-function females (C, D) resemble those of mated fog-2(q71) females (F) and wild-type hermaphrodites (not shown). Fertilized eggs are observed throughout the uterus in panel F. Gonads are oriented as in Fig. 1A. Scale bar corresponds to 20 μm.
causes higher sheath contraction rates in unmated females, suggesting that gap junctions negatively regulate sheath contraction (Table 3, compare line 2 to 1; \(P < 0.001\)).

Consistent with this hypothesis, the contraction rate in unmated \(ceh-18(mg57)\) females is similar to unmated \(inx-14\) RNAi females (Table 3, compare line 3 to 2). The sheath contraction rate in unmated \(inx-14\) RNAi \(ceh-18(mg57)\) females is not significantly different than unmated \(inx-14\) RNAi females and unmated \(ceh-18(mg57)\) females, consistent with \(inx-14\) and \(ceh-18\) functioning in a common genetic pathway (Table 3, compare line 4 to lines 2 and 3; \(P > 0.10\)).

Despite the high oocyte maturation rate of unmated \(inx-22\) mutant and RNAi females, the sheath contraction rate in these females is not significantly different than unmated controls (Table 3, compare lines 5 and 6 to line 1; \(P > 0.10\)). Therefore, high oocyte maturation rates do not necessarily result in high basal sheath contraction rates. These data indicate that \(INX-14\) and \(CEH-18\) are required to negatively regulate sheath cell contractile activity in the absence of sperm.

To test whether \(INX-14\) and \(INX-22\) negatively regulate sheath contraction in the presence of sperm, we compared contraction rates in mutant and RNAi hermaphrodites to their respective controls. The sheath contraction rate in \(inx-14\) RNAi hermaphrodites is significantly higher than wild-type hermaphrodites (Table 3, compare line 8 to 7; \(P < 0.001\)). Similar results are observed in \(inx-22\) RNAi and \(inx-22(tm1661)\) hermaphrodites compared to the wild type (Table 3, compare line 9 to 7 and data not shown; \(P < 0.001\)). The actin cytoskeleton is disorganized in \(ceh-18(mg57)\) sheath cells, resulting in decreased contractile activity (Rose et al., 1997). Actin disorganization is not observed in \(inx-14\) RNAi and \(inx-22(tm1661)\) hermaphrodites, as evidenced by phalloidin staining. We conclude that \(INX-14\) and \(INX-22\) are required to inhibit sheath cell contraction in the presence of sperm and \(INX-14\) is required in the absence of sperm.

### Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sperm (Y/N)</th>
<th>Maturations per hour</th>
<th>(N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (fog-2(q71))</td>
<td>N</td>
<td>0.10±0.08</td>
<td>29</td>
</tr>
<tr>
<td>2. (ceh-18(mg57); fog-2(q71))</td>
<td>N</td>
<td>1.20±0.60</td>
<td>17</td>
</tr>
<tr>
<td>3. (inx-14) RNAi (fog-2(q71))</td>
<td>N</td>
<td>0.77±0.27</td>
<td>21</td>
</tr>
<tr>
<td>4. (inx-22) RNAi (fog-2(q71))</td>
<td>N</td>
<td>0.87±0.24</td>
<td>10</td>
</tr>
<tr>
<td>5. (inx-22(tm1661); fog-2(q71))</td>
<td>N</td>
<td>1.57±0.68</td>
<td>20</td>
</tr>
<tr>
<td>6. (fog-3(q443))</td>
<td>N</td>
<td>0.12±0.11</td>
<td>14</td>
</tr>
<tr>
<td>7. (rprf-1(pk1417)) (fog-3(q443))</td>
<td>N</td>
<td>0.14±0.12</td>
<td>13</td>
</tr>
<tr>
<td>8. (inx-14) RNAi (fog-3(q443))</td>
<td>N</td>
<td>0.86±0.28</td>
<td>7</td>
</tr>
<tr>
<td>9. (inx-14) RNAi (rprf-1(pk1417)) (fog-3(q443))</td>
<td>N</td>
<td>0.94±0.19</td>
<td>10</td>
</tr>
<tr>
<td>10. (inx-22) RNAi (fog-3(q443))</td>
<td>N</td>
<td>0.98±0.32</td>
<td>9</td>
</tr>
<tr>
<td>11. (inx-22) RNAi (rprf-1(pk1417); fog-3(q443))</td>
<td>N</td>
<td>1.01±0.42</td>
<td>10</td>
</tr>
<tr>
<td>12. (inx-14) RNAi (ceh-18(mg57); fog-2(q71))</td>
<td>N</td>
<td>0.71±0.25</td>
<td>19</td>
</tr>
<tr>
<td>13. (inx-22) RNAi (ceh-18(mg57); fog-2(q71))</td>
<td>N</td>
<td>0.95±0.32</td>
<td>13</td>
</tr>
<tr>
<td>14. (inx-14) RNAi (inx-22(tm1661); fog-2(q71))</td>
<td>N</td>
<td>1.11±0.36</td>
<td>9</td>
</tr>
<tr>
<td>15. Wild type</td>
<td>Y</td>
<td>2.45±0.45</td>
<td>19</td>
</tr>
<tr>
<td>16. (inx-14) RNAi</td>
<td>Y</td>
<td>2.59±0.52</td>
<td>10</td>
</tr>
<tr>
<td>17. (inx-22) RNAi</td>
<td>Y</td>
<td>4.33±0.79</td>
<td>17</td>
</tr>
<tr>
<td>18. (inx-22(tm1661))</td>
<td>Y</td>
<td>3.37±0.75</td>
<td>24</td>
</tr>
</tbody>
</table>

Note. See text for details. The average number of oocyte maturations and ovulations per hour ± standard deviation is shown. For comparison, the maturation rate of mated \(ceh-18(mg57); fog-2(q71)\) females is 1.89±0.31 mat/h (Miller et al., 2003).

Fig. 3. MPK-1 MAPK activation in proximal gonads. Fluorescent images of proximal gonads stained with antibodies against the activated or diphosphorylated form of MPK-1 MAPK (Miller et al., 2001). (A) In unmated \(fog-2(q71)\) females, oocytes contain undetectable levels of activated MAPK. (B) The oocytes of unmated \(ceh-18(mg57)\) females contain low, but sometimes detectable levels of activated MAPK. (C, D) MAPK activation is clearly detected in the most proximal oocytes of unmated \(inx-14\) RNAi females. (E) Oocyte MAPK-YT staining in wild-type gonads containing sperm (E) has a distribution and intensity similar to unmated innexin loss-of-function female gonads (C, D). (F) In \(inx-22(tm1661)\) hermaphrodite gonads, more oocytes have activated MAPK on average than the wild type (E). One intensely and two weakly stained oocytes are visible in panel E, while five stained oocytes are visible in panel F. Gonads are oriented as in Fig. 1A. Scale bar corresponds to 20 \(\mu m\).
INX-14 is required in the female germ line to regulate sheath contraction

We reasoned that sheath/oocyte gap junctions negatively regulate sheath contraction. To begin testing this prediction, we examined sheath contraction rates in glp-4(bn2) mutant gonads, which contain sheath cells, but neither oocytes nor sperm (Beanan and Strome, 1992). The sheath contraction rate in glp-4(bn2) gonads is significantly higher than unmated female control gonads (Table 3, compare line 10 to 1; P<0.001) and is similar to the rate in unmated ceh-18(mg57) and inx-14 RNAi females (Table 3, compare line 10 to lines 2 and 3). These data are consistent with a model in which an oocyte to sheath communication mechanism inhibits sheath contraction. If this mechanism involves sheath/oocyte gap junctions, then loss of INX-14 in oocytes, but not sheath cells is predicted to cause increased sheath contraction rates. We tested this prediction by using rrf-1(pk1417) females, which are refractory to RNAi in the somatic sheath cells, but sensitive in the female germ line (Corrigan et al., 2005; Sijen et al., 2001). The sheath contraction rate of unmated inx-14 RNAi rrf-1(pk1417) females is significantly higher than unmated controls (Table 3, compare line 13 to lines 11 and 12; P<0.001) and similar to inx-14 RNAi females (Table 3, compare line 13 to 2). We conclude that INX-14 is required in the female germ line. Taken together, our results support the hypothesis that sheath/oocyte gap junctions negatively regulate sheath contraction.

INX-14 and CEH-18 are required for sperm recruitment to the spermatheca

The brood size of inx-14 RNAi hermaphrodites is much lower than control RNAi hermaphrodites (Fig. 4A). Brood sizes in inx-22 RNAi and inx-22(tm1661) hermaphrodites are significantly lower than their respective controls (P<0.005), but are higher than inx-14 RNAi hermaphrodites (Fig. 4A). inx-14 RNAi does not appear to affect spermatogenesis, as young adults before the first ovulation contain an abundance of spermatids that undergo activation into motile spermatozoa capable of fertilization. When young adult hermaphrodites, which have already completed spermatogenesis, are exposed to inx-14 double-stranded RNA for 24 h, unfertilized eggs begin to accumulate in their uteri. Analysis of staged hermaphrodites at increasing time intervals following exposure indicates that spermatozoa are lost from the spermatheca and uterus over time. Sperm loss is rare in wild-type hermaphrodites because sperm that are swept into the uterus by fertilized eggs crawl back to the spermatheca (Kubagawa et al., in press; Ward and Carrel, 1979). These observations raise the possibility that loss of INX-14 causes sperm migratory defects.

We hypothesized that sheath/oocyte gap junctions are necessary for the production or release of sperm guidance signals. To test this hypothesis, we used a sperm tracking assay to examine sperm migration patterns in vivo (Kubagawa et al., in press). MitoTracker (MT), a fluorescent dye that specifically stains mitochondria, is incubated with wild-type males to fluorescently label their sperm. When labeled wild-type males are mated to non-labeled wild-type hermaphrodites, fluorescent sperm accumulate in the spermatheca over time (Fig. 4B). Sperm migration paths in the uterus were generated from time-lapse videos of freshly mated hermaphrodites. Wild-type sperm in control hermaphrodites move around fertilized eggs toward the spermatheca (Fig. 4C). To test whether INX-14 is required for sperm recruitment, we mated MT wild-type males to inx-14 RNAi hermaphrodites. Sperm rarely reach the spermatheca in these animals, with most sperm located throughout the uterus (Fig. 4B). Time-lapse image analysis indicates that sperm are motile, but fail to migrate consistently toward the spermatheca (Fig. 4C). Similar motility defects are observed in inx-14 RNAi rrf-1(pk1417) hermaphrodites, suggesting that INX-14 is required in oocytes to control sperm targeting (data not shown). Weak sperm migration defects are observed in inx-22 (tm1661) hermaphrodites, while ceh-18(mg57) hermaphrodites exhibit an intermediate phenotype (Fig. 4B). We conclude that sheath/oocyte gap junctions are required for sperm recruitment to the spermatheca.

### Table 3: Basal sheath contraction rates

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sperm (Y/N)</th>
<th>Contractions per minute</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. fog-2(q71)</td>
<td>N</td>
<td>1.8±0.7</td>
<td>23</td>
</tr>
<tr>
<td>2. inx-14 RNAi; fog-2(q71)</td>
<td>N</td>
<td>6.4±2.0</td>
<td>14</td>
</tr>
<tr>
<td>3. ceh-18(mg57); fog-2(q71)</td>
<td>N</td>
<td>7.0±2.2</td>
<td>11</td>
</tr>
<tr>
<td>4. inx-14 RNAi ceh-18(mg57); fog-2(q71)</td>
<td>N</td>
<td>8.0±1.4</td>
<td>11</td>
</tr>
<tr>
<td>5. inx-22 RNAi; fog-2(q71)</td>
<td>N</td>
<td>2.6±1.9</td>
<td>7</td>
</tr>
<tr>
<td>6. inx-22(tm1661); fog-2(q71)</td>
<td>N</td>
<td>2.0±1.1</td>
<td>12</td>
</tr>
<tr>
<td>7. Wild type</td>
<td>Y</td>
<td>9.6±1.5</td>
<td>30</td>
</tr>
<tr>
<td>8. inx-14 RNAi</td>
<td>Y</td>
<td>14.7±1.5</td>
<td>7</td>
</tr>
<tr>
<td>9. inx-22(tm1661)</td>
<td>Y</td>
<td>16.7±2.5</td>
<td>11</td>
</tr>
<tr>
<td>10. glp-4(bn2)</td>
<td>Y</td>
<td>8.5±2.5</td>
<td>10</td>
</tr>
<tr>
<td>11. fog-2(q71)</td>
<td>N</td>
<td>2.1±0.8</td>
<td>14</td>
</tr>
<tr>
<td>12. rrf-1(pk1417) fog-3(q443)</td>
<td>N</td>
<td>1.9±1.0</td>
<td>16</td>
</tr>
<tr>
<td>13. inx-14 RNAi rrf-1(pk1417) fog-3(q443)</td>
<td>N</td>
<td>6.1±1.7</td>
<td>10</td>
</tr>
</tbody>
</table>

Note. See text for details. The average number of sheath contractions per minute±standard deviation is shown. The contraction rate of mated ceh-18 (mg57); fog-2(q71) females is similar to unmated ceh-18(mg57); fog-2(q71) females. This result is difficult to interpret because cytoskeletal defects in ceh-18(mg57) sheath cells limit contractile activity (Rose et al., 1997).

Ultrastuctral studies

We used transmission electron microscopy to examine the ultrastructure of the sheath cell and oocyte interface. Consistent with previous reports, we observed close apposition of sheath and oocyte plasma membranes in wild-type gonads (Figs. 5A, B). This close association is also observed in unmated female gonads (Figs. 5C, D). Sheath/Oocyte gap junctions occur at regions where the sheath cell and oocyte membranes are in contact (a putative gap junction is shown in Fig. 5D), including regions where finger-like sheath projections extend between neighboring oocytes (Hall et al., 1999). We were able to detect clear evidence of sheath/sheath gap junctions (Fig. 5E), but equally compelling evidence of sheath/oocyte gap junctions requires tilting of the specimen or freeze fracture.
Previous TEM studies of ceh-18 (mg57) gonads demonstrated that sheath cell and oocyte plasma membranes are not closely apposed, preventing gap junction formation or maintenance (Rose et al., 1997). We see a similar architecture in unmated ceh-18(mg57) female gonads, where large stretches of the sheath and oocyte interface have separated and sheath projections extending between neighboring oocytes are rare (Figs. 6A, B). The sheath and oocyte interface of inx-14 RNAi gonads resembles ceh-18(mg57) gonads (Figs. 6C, D), contrasting significantly with controls (Fig. 5). Similar results are found in inx-22(tm1661) gonads (Figs. 6E, F). Loss of CEH-18, INX-14, or INX-22 function does not result in loss of sheath and oocyte membrane apposition at all interface sites, raising the possibility that gap junctions are not completely absent (Fig. 6). Taken together, these results are consistent with a model in which sheath/oocyte gap junctions, assembled from INX-14 and/or INX-22 complexes, are required for close membrane apposition.

We noticed that inx-14 RNAi gonads contain numerous thin (typically less than 200 nm) cytoplasmic extensions filling the extracellular spaces between oocyte and sheath membranes (Figs. 6C, D). Most appear to originate from oocytes (Fig. 6D, inset), although the possibility that a fraction originates from sheath cells cannot be excluded. Similar extensions are also
observed in ceh-18(mg57) and inx-22(tm1661) gonads, but they are much less abundant. We rarely see such structures between sheath cells and oocytes in wild-type and unmated female gonads. However, cytoplasmic extensions are observed between neighboring oocytes in regions lacking sheath contact (Figs. 5F, G). Although the role of these structures is not understood, our results suggest that their formation at the sheath and oocyte interface is regulated at least in part by INX-14.

Discussion

Two types of gap junctions have been identified in the hermaphrodite proximal gonad, sheath cell to oocyte and sheath cell to sheath cell (Hall et al., 1999). The CEH-18 POU-class transcription factor is required for formation of sheath/oocyte gap junctions (Rose et al., 1997). Here we provide genetic and ultrastructural evidence that the innexins INX-14 and INX-22

Fig. 5. Transmission electron micrographs from wild-type and unmated female proximal gonads. (A, B) Oocyte (Oo) and sheath cell (Sh) membranes are closely apposed in wild-type proximal gonads. A close-up image of the boxed region in panel A is shown in panel B. Sheath/oocyte gap junctions can be found at regions of close membrane apposition, including the interface of finger-like sheath processes extending between neighboring oocytes and the oocytes themselves (Hall et al., 1999). (C–G) In unmated females, oocyte and sheath membranes are closely apposed around the entire circumference of the proximal gonad (C), as seen in the wild type. A close-up image of the boxed region in panel C shows a putative sheath/oocyte gap junction (D, white arrow). While convincing evidence of sheath/oocyte gap junctions requires tilting of the specimen or freeze fracture techniques, sheath/sheath gap junctions are more electron dense and easier to detect (E, white arrow). The gap junction in panel E is between two overlapping sheath cell membranes adjacent to the oocyte plasma membrane. (F, G) Thin cytoplasmic extensions often less than 0.2 μm wide are observed in the extracellular spaces between oocytes. In some images, the extensions form a zipper-like structure (G). These same extensions are rarely observed in the extracellular spaces between oocytes and sheath cells. Gonad dissection does not affect the architecture of the sheath and oocyte interface [not shown and (Hall et al., 1999)]. Scale bar corresponds to 2 μm in panel A, 5 μm in panel C, and 0.5 μm in panels B, D, E, and F.
are components of CEH-18-dependent sheath/oocyte gap junctions. Phenotypic studies demonstrate that these junctions function to negatively regulate oocyte maturation and sheath cell contraction, as well as to promote sperm recruitment to the spermatheca, the site of fertilization.

**Genetic composition of sheath/oocyte gap junctions**

Innexins are structural components of invertebrate gap junctions that are sufficient to form intercellular channels in *Xenopus* oocyte pairs and localize to gap junctions in vivo (Phelan et al., 1996, 1998b; Starich et al., 2003, 1996). We screened by RNAi the 25 innexin loci in the *C. elegans* genome to identify those with a phenotype resembling unmated *ceh-18*(*mg57*) females. CEH-18 is expressed in the sheath cells, but not in the oocytes (Greenstein et al., 1994). We show that loss of INX-14 or INX-22 in the female germ line causes oocyte maturation defects nearly identical to loss of CEH-18. Double knockdown experiments place *inx-14* and *inx-22* in the same genetic pathway as *ceh-18* for oocyte maturation. These results are consistent with gap junction-mediated signaling from sheath cells to oocytes. We show that oocytes are required to inhibit sheath contraction. Loss of INX-14 in the female germ line causes sheath contraction defects nearly identical to *ceh-18*(*mg57*). Double knockdown experiments place *inx-14* and *ceh-18* in a common genetic pathway for sheath contraction. These results are consistent with gap junction-mediated signaling from oocytes to sheath cells. Taken together, loss-of-function and site of action studies demonstrate that INX-14 and INX-22 are required for bi-directional communication from sheath to oocyte and oocyte to sheath.

Fig. 6. Transmission electron micrographs of proximal gonads from unmated mutant and RNAi females. (A, B) In unmated *ceh-18*(*mg57*) females, large regions exist where oocyte (Oo) and sheath cell (Sh) plasma membranes are not closely apposed (A) and finger-like sheath projections extending between neighboring oocytes are absent (B). This architecture was previously reported in *ceh-18*(*mg57*) hermaphrodites (Rose et al., 1997). (C, D) In unmated *inx-14* RNAi females, sheath and oocyte plasma membranes are not closely apposed and sheath projections extending between neighboring oocytes are rare (C), similar to unmated *ceh-18*(*mg57*) females (A, B). Numerous thin cytoplasmic extensions often less than 0.2 μm wide are observed in the extracellular spaces between sheath cells and oocytes. These extensions appear to originate from oocytes and resemble thin extensions observed between neighboring oocytes (Figs. 5F, G). The inset in panel D is a close-up of the region to the left of the asterisk. (E, F) The architecture of unmated *inx-22*(*tm1661*) female gonads is similar to unmated *ceh-18*(*mg57*) female and *inx-14* RNAi female gonads. A close-up image of the boxed region in panel E is shown in panel F. Thin cytoplasmic extensions between oocyte and sheath membranes are less abundant in *ceh-18*(*mg57*) gonads and *inx-22*(*tm1661*) gonads compared to *inx-14* RNAi gonads. Scale bar corresponds to 2 μm.
Innexins can form homomeric channels composed of a single isoform, heteromeric channels composed of multiple isoforms in the same cell, and heterotypic channels composed of different isoforms in neighboring cells (Phelan and Starich, 2001; Stebbings et al., 2000). The Drosophila innexin ZPG is thought to form heterotypic channels with an unknown innexin between germ cells and adjacent somatic cells (Tazuke et al., 2002). Double knockdown experiments suggest that inx-14 and inx-22 act in a common pathway, a result consistent with heteromeric channel assembly. mRNA expression and site of action studies indicate that inx-14 and inx-22 function in oocytes, but it is not clear whether they also function in sheath cells. INX-14 and INX-22 in oocytes could couple to INX-14 and INX-22 in sheath cells or they could couple to other innexins not identified in the RNAi screen. Putative promoter sequences for inx-8 and inx-9 are sufficient to drive expression in sheath cells (Starich et al., 2001), but loss of either gene product alone does not phenocopy loss of INX-14 or INX-22 (Table 1 and M.A. Miller, unpublished data). INX-8 and INX-9 could function redundantly to form heterotypic sheath/oocyte gap junctions or these innexins could assemble sheath/sheath gap junctions. Our RNAi screen was not designed to identify genes with redundant functions. Sheath innexins could be direct transcriptional targets of CEH-18 or alternatively, CEH-18 could regulate transcription of other genes indirectly required for sheath/oocyte gap junction assembly. Comprehensive genetic and subcellular protein localization studies for all innexins will be necessary to determine the complete innexin composition of sheath/oocyte gap junctions.

Gap junctions mediate electrical coupling and direct exchange of small molecules between neighboring cells (reviewed in Phelan, 2005; Phelan and Starich, 2001). Transmission electron micrographs of inx-14 RNAi and inx-22(tm1661) gonads reveal separation of oocyte and sheath cell plasma membranes, an architecture observed in ceh-18(mg57) gonads, but not in controls (Figs. 5 and 6). These data indicate that sheath/oocyte gap junctions are required for close apposition of sheath and oocyte membranes. At least two models of the signal transduction mechanism are plausible. INX-14 and INX-22 channels could mediate the exchange of electrical or chemical signals between oocytes and sheath cells that directly regulate intracellular effectors. Alternatively, these channels could indirectly affect a signaling mechanism(s) requiring close proximity of sheath and oocyte membranes. We could not determine with certainty whether gap junctions are completely eliminated in innexin mutant and RNAi gonads, as the relative proportion of interface regions containing gaps to those not containing gaps varies from section to section. Loss of INX-14, INX-22, or CEH-18 function results in a common set of reproductive defects, but sperm dependence (see below) and phenotypic severity depend on the gene eliminated. These differences could be due to altered signaling properties of remaining channels or to channel-independent functions of innexins. In any event, the results presented here strongly support the hypothesis that INX-14 and INX-22 are structural components of sheath/oocyte gap junctions. The molecular mechanism by which these channels transduce signals is an important subject for future work.

Role of gap junctions in oocyte maturation and ovulation

During evolution, the major sperm protein (MSP) has acquired intracellular cytoskeletal and extracellular signaling functions for reproduction (Bottino et al., 2002; Miller et al., 2001). MSP exported from spermatozoa in membrane-bound vesicles acts as a paracrine hormone that induces oocyte maturation and sheath contraction in the proximal gonad (Fig. 1A), resulting in ovulation (Kosinski et al., 2005). MSP binds to multiple receptors, including the VAB-1 Eph receptor, on oocyte and sheath cell surfaces. Binding triggers UNC-43 Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activation at the oocyte cortex and MPK-1 MAPK activation in the oocyte cytosol (Corrigan et al., 2005; Miller et al., 2003). Ultimately, these events antagonize two inhibitory signaling pathways, one requiring canonical VAB-1 ligands called ephrins and the other requiring the sheath transcription factor CEH-18.

We provide evidence that INX-14 and INX-22 form heteromeric sheath/oocyte gap junctions dependent on CEH-18 function; these junctions negatively regulate oocyte maturation and MAPK activation in the absence of sperm. Sheath/Oocyte gap junctions are observed in wild-type gonads containing sperm and MSP (Hall et al., 1999; Kosinski et al., 2005). Therefore, MSP is not likely to promote physical disruption of gap junctions prior to oocyte maturation and ovulation, when oocytes and sheath cells separate. We speculate that MSP regulates either the signals flowing through gap junctions or effectors downstream of these signals in oocytes. Testing whether innexin function is dependent on sperm is providing insight into the signaling mechanism. INX-14 is required to inhibit oocyte maturation in the absence of sperm, but not in the presence of sperm. By contrast, INX-22 is required in the presence and absence of sperm. These data suggest the hypothesis that sperm signals antagonize a signaling pathway dependent on INX-14 function. If INX-14 and INX-22 form heteromeric gap junctions, as suggested by double knockdown experiments, then these channels may be able to transmit multiple signals. The results of this study, together with those of previous studies, support a model in which MSP binding to its receptors antagonizes an inhibitory signaling pathway mediated by sheath/oocyte gap junctions. Two important questions that need to be addressed are how do gap junctions inhibit oocyte maturation and how does MSP disrupt this mechanism.

MSP binding to its receptors on sheath cells triggers activation of the ITR-1 inositol triphosphate receptor, resulting in increased basal sheath contractile activity (Corrigan et al., 2005; Yin et al., 2004). During oocyte maturation and ovulation, signals from the oocyte transiently stimulate an additional increase in activity, called ovulatory contractions (Iwasaki et al., 1996; McCarter et al., 1999). We provide evidence that gap junctions transmit a signal(s) from oocytes to sheath cells that negatively regulates sheath contraction. Genetic data suggest that sperm signals (i.e., MSP) do not antagonize INX-14 and INX-22 function in the sheath contraction mechanism, contrasting with the oocyte maturation mechanism. For sheath contraction, INX-22 function may be sperm-dependent, but INX-14 function is not. The opposite is
true for oocyte maturation, as INX-14 function is sperm-dependent. Sperm-independent contractile activity could regulate ovulatory contractions, which are dependent on oocytes, not sperm. Physical disruption of gap junctions during oocyte maturation may cause a transient increase in the contraction rate, perhaps contributing to ovulatory contractions. These data suggest that gap junctions are capable of activating pathways in sheath cells and oocytes that can be regulated differently.

**Role of gap junctions in sperm recruitment**

*C. elegans* sperm extend a pseudopod of MSP filaments to crawl across unfertilized oocytes, fertilized eggs, and the walls of the uterus and spermatheca. Both hermaphrodite- and male-derived sperm are actively recruited to the spermatheca, which is separated from immature oocytes by a small constriction (Fig. 1A) (Hill and L’Hernault, 2001; Ward and Carrel, 1979). Results from a recent study show that polyunsaturated fatty acids (PUFAs), the precursors of prostaglandins and other eicosanoid signaling molecules, function in oocytes to control directional sperm motility within the uterus (Kubagawa et al., in press). The data support a model in which oocytes synthesize signals derived from PUFAs that recruit sperm to the spermatheca. It is not understood how oocytes in the proximal gonad release these guidance cues or terminate their release after ovulation. Continued production and release in the uterus is predicted to cause aberrant sperm targeting to the spermatheca.

Here we show that innexin channels are necessary for sperm recruitment. Sperm migration paths from *inx-14* RNAi hermaphrodites (Fig. 4C) are similar to those from mutant hermaphrodites deficient in PUFA metabolism (Kubagawa et al., in press). The biochemical function of INX-14 in sperm recruitment is not currently understood. One possibility is that innexin hemichannels in oocytes mediate the release of sperm-recruiting signals. Connexin-43 hemichannels mediate prostanoid release in cultured mammalian oocytes (Cherian et al., 2005), although the *in vivo* mechanisms by which cells release eicosanoids are not well understood. An alternative possibility is that innexins transport signaling precursors, ATP, or enzyme cofactors to oocytes. The sperm targeting defects in *ceh-18*(*mg57*) mutants suggest that recruitment is regulated at least in part by formation of sheath and oocyte gap junctions. Disruption of these gap junctions during oocyte maturation and ovulation may block signal synthesis or release and therefore, prevent aberrant sperm targeting. The identification of the active guidance factor(s) will facilitate tests of the biochemical mechanism.

**Comparison of the mammalian oocyte maturation mechanism to *C. elegans***

The signal that promotes oocyte maturation in mammals is LH, which travels to the ovary through the bloodstream (reviewed in Mattioli and Barboni, 2000). Within the ovary, mature antral follicles contain a single oocyte surrounded by cumulus cells that are themselves surrounded by the fluid-filled antrum and mural granulosa cells. LH binds to a G-protein-coupled receptor expressed in mural granulosa cells, but not in the oocyte or cumulus cells. Binding induces the expression of epidermal growth factor-like ligands, which act on cumulus cells to promote oocyte maturation (Park et al., 2004). The pathway(s) that transmits the maturation signal from cumulus cells to oocytes is controversial. The G-protein-coupled receptor GPR3 functions in oocytes to maintain meiotic arrest, possibly by stimulating cAMP production (Mehlmann et al., 2002). Physical separation of oocytes from antral follicles promotes oocyte maturation *in vitro* (Edwards, 1965; Pincus and Enzmann, 1935), an observation that supports the existence of a signal derived from follicle cells that inhibits oocyte maturation.

Gap junctions are found between oocytes and cumulus cell processes that traverse the zona pellucida (Anderson and Albertini, 1976; Gilula et al., 1978). It has been proposed that cAMP flowing through these gap junctions from cumulus cells to the oocyte inhibits oocyte maturation (Dekel, 2005). Another model postulates that a positively acting factor transmitted through gap junctions triggers oocyte maturation in response to LH (Eppig, 2001). In either case, the relationship between gap junctions and GPR3 is not understood. The *in vivo* role of gap junctions in oocyte maturation has been difficult to determine because mouse connexin knockouts have folliculogenesis defects that prevent analysis of oocyte maturation and specific inhibitors of oocyte gap junctions are not available (Mehlmann, 2005).

We show in *C. elegans* gonads that gap junctions between sheath cells and oocytes negatively regulate oocyte maturation. These gap junctions are required to transmit a signal from sheath cells to oocytes that maintains oocytes in meiotic prophase I, as proposed for mammalian follicles. *C. elegans* and mammalian oocyte maturation mechanisms share other features in common. Recent evidence supports a role for G protein signaling and cAMP in *C. elegans* oocyte maturation, although there appear to be both similarities and differences in regulation compared to mammals (Govindan et al., 2006). Two kinases implicated in oocyte maturation in *C. elegans* and mammals are CaMKII and MAPK. In *C. elegans*, UNC-43 CaMKII functions in oocytes to promote oocyte maturation and MPK-1 MAPK activation, possibly by blocking gap junctional signaling (Corrigan et al., 2005). The role of MPK-1 is not clear as null mutations in *mpk-1* cause germ cells to arrest in the pachytene stage of meiosis (Church et al., 1995; Hsu et al., 2002). Pharmacological studies in cultured mouse follicles suggest that CaMKII and MAPK function as positive regulators of oocyte maturation (Su et al., 2002, 2003). However, MAPK activity is required in the cumulus cells, not in the oocytes. The oocyte MAPK pathway may act in parallel to another pathway to promote oocyte maturation, but its role is not well understood (Su et al., 2002; Verlhac et al., 2000). The picture emerging from these studies is one in which *C. elegans* and mammalian oocyte maturation mechanisms share some signaling pathways in common, perhaps because they were present in a common ancestral mechanism. The roles of these pathways, their modes of regulation, and their connectivity may have changed extensively during evolution. The *C. elegans* oocyte maturation model will be important for discerning molecular features that
are evolutionarily conserved in vertebrates from those that are derived. Furthermore, it has great promise for addressing fundamental questions of meiotic regulation and gap junctional communication.

Acknowledgments

We thank Leigh Millican for the assistance with transmission electron microscopy, Elizabeth Turnipseed and Rob Steele for critically reading the manuscript, and David Greenstein for the helpful suggestions. Some strains were provided by the Caenorhabditis Genetics Center, which is funded by the NIH. The inx-22 (m1661) strain was created by the Japanese National Bioresource Project, which is supported by the Ministry of Education, Culture, Science, Sports and Technology. Financial support for this study came from the UAB Department of Cell Biology, including HHMI funds delegated by UAB, the Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, a UAB Comprehensive Cancer Center Junior Faculty Development Grant, and an American Cancer Society Basic Research Scholars Grant (RSG-06-151-01-DDC).

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