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The EGR family gene *egrh-1* functions non-autonomously in the control of oocyte meiotic maturation and ovulation in *C. elegans*

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SUMMARY

Oocyte production, maturation and ovulation must be coordinated with sperm availability for successful fertilization. In *C. elegans* this coordination involves signals from the sperm to the oocyte and somatic gonad, which stimulate maturation and ovulation. We have found that the *C. elegans* early growth response factor family member EGRH-1 inhibits oocyte maturation and ovulation until sperm are available. In the absence of sperm, *egrh-1* mutants exhibit derepressed oocyte maturation marked by MAPK activation and ovulation. *egrh-1* mutants exhibit ectopic oocyte differentiation in the distal gonadal arm and accumulate abnormal and degraded oocytes proximally. These defects result in reduced brood size and partially penetrant embryonic lethality. We have found that endogenous EGRH-1 protein and an *egrh-1::gfp* reporter gene are expressed in the sheath and distal tip cells of the somatic gonad, the gut and other non-gonadal tissues, as well as in sperm, but expression is not observed in oocytes. Results of tissue-specific *egrh-1(RNAi)* experiments and genetic mosaic analyses revealed that EGRH-1 function is necessary in the soma and, surprisingly, this function is required in both the gut and the somatic gonad. Based on transformation rescue experiments we hypothesize that EGRH-1 in the somatic gonad inhibits oocyte maturation and ovulation.

KEY WORDS: *C. elegans*, EGR family, Gut, Oogenesis, Somatic gonad

INTRODUCTION

Early growth response (EGR) family genes are immediate early genes induced by mitogenic stimulation that were first identified in mammals (for a review, see O'Donovan et al., 1999). The human and mouse genomes each contain four EGR genes, which encode transcription factors sharing three highly conserved C_2H_2 zinc fingers that bind a GC-rich consensus site (O'Donovan et al., 1999; Swirnoff and Milbrandt, 1995). EGR-family factors regulate a variety of signaling pathways involved in cellular growth and differentiation, as well as the transition from short-term to long-term memory (Alberini, 2009; O'Donovan et al., 1999). Recent evidence suggests that EGR genes function as tumor suppressors (Joslin et al., 2007; Unoki and Nakamura, 2001), and mutations in these genes have been associated with human neuropathies (for a review, see Houlden and Reilly, 2006). EGR gene mutant mice exhibit a variety of defects, including infertility (Lee et al., 1996; Tourtellotte et al., 1999), defective nerve myelination and abnormal hindbrain development (Schneider-Maunoury et al., 1993; Warner et al., 1998), as well as sensory ataxia and an absence of muscle spindles (Tourtellotte and Milbrandt, 1998).

egrh-1 (early growth response factor homolog) is one of four *C. elegans* genes (*egrh-1*, *egrh-2*, *egrh-3* and ZK337.2) closely related to mammalian EGR genes. Like mammalian EGR-family factors, the EGRH-1 protein contains three C_2H_2 zinc fingers near the C-terminus. In situ hybridization studies and SAGE analyses suggest that *egrh-1* mRNA is broadly expressed from embryo through adult stages (Kohara, 2001; McKay et al., 2003). However, neither the function nor expression pattern of *egrh-1* has been previously described. Here, we characterize EGRH-1 function and expression in adult hermaphrodites.

The *C. elegans* hermaphrodite gonad comprises two U-shaped gonad arms containing both somatic cells and germline cells and it provides an excellent model for the regulation of germ cell development (Greenstein, 2005; Hansen and Schedl, 2006; Kimble and Crittenden, 2005). Each arm of the gonad includes the somatic distal tip cell (DTC), located near a population of proliferating germ cells at the distal end of the gonad, and five pairs of somatic sheath cells, located more proximally and surrounding differentiating germ cells. During the L4 stage, the first germ cells produced differentiate as sperm and are stored in the somatic spermatheca after ovulation has begun. In adults, germ cells continue to proliferate and differentiate as oocytes that are ovulated approximately every 20 minutes and fertilized in the spermatheca. As embryos enter the uterus, sperm are swept from the spermatheca and migrate back in response to chemoattractive cues from the oocytes. Germ cells are continuously produced in adults, allowing all stages of proliferation and differentiation to be observed.

Germ cell development and ovulation depend on interactions with the somatic gonad (for a review, see Greenstein, 2005). At the distal-most point of each arm, the DTC maintains a mitotic germ cell population (Kimble and White, 1981). As these cells progress away from the DTC, they exit the mitotic cell cycle and enter meiosis in the transition zone and eventually differentiate as oocytes arrested in meiotic prophase in the proximal gonad arm (Hirsh et al., 1976). In the proximal arm, the sheath cells inhibit oocyte maturation (McCarter et al., 1997; McCarter et al., 1999). Sperm-derived signals overcome this inhibition to promote oocyte maturation and ovulation (McCarter et al., 1999; Miller et al., 2001). In the absence of sperm (either in hermaphrodites that have

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depleted their sperm supplies or in mutants that fail to make sperm), ovulation is inhibited and a mitogen-activated protein kinase (MAPK) associated with maturation is not activated.

Here, we describe a role for EGRH-1 in regulating oocyte development. *egrh-1* loss results in ectopic oocyte differentiation and accumulation of abnormal and degraded oocytes. In the absence of sperm, *egrh-1* mutants exhibit MAPK activation and derepressed ovulation. *egrh-1* is expressed in the DTC and sheath cells of the somatic gonad, as well as in the gut, and we show that *egrh-1* function is required in the somatic gonad and gut to regulate oocyte development. Based on our data, we hypothesize that EGRH-1 functions in the somatic gonad to inhibit oocyte maturation and ovulation.

MATERIALS AND METHODS

Nematode handling and strains

C. elegans were grown under standard conditions (Lewis and Fleming, 1995) and the following strains were used: N2 (wild-type); JK574 [*fog-2(q71)*] (Schedl and Kimble, 1988); JK560 [*fog-1(q253)*] (Barton and Kimble, 1990); NL2099 [*rrf-3(pk1426)*] (Simmer et al., 2002); NL2098 [*rrf-1(pk1417)*] (Sijen et al., 2001); OK0600 [*fog-1(q253); egrh-1(tm1736)*]; OK0639 [*fog-2(q71); egrh-1(tm1736)*]. OK0559 [*egrh-1(tm1736)*] was outcrossed from a strain obtained from S. Mitani (National BioResource Project). Animals were genotyped for *egrh-1(tm1736)* by single worm duplex PCR using primers PO761 (GGCAC-CATCATCGTCATACAAGG), PO762 (GGCAGCAGATAAGCC-TGAAAATTC) and PO762 (GCTGTAGTCATCCATTGGCTCGG) under conditions similar to those previously described (Beaster-Jones and Okkema, 2004).

Oocyte maturation rates for *fog-2(q71)* and *fog-2(q71); egrh-1(tm1736)* females were determined by counting total ovulations over a 4-hour timepoint at 25°C (Miller et al., 2003). Newly molted adults were used to avoid oocyte degradation found in older *fog-2(q71); egrh-1(tm1736)* females.

To test the mating efficiency of *egrh-1(tm1736)* sperm, *fog-1(q253)* females were mated with either N2 or *egrh-1(tm1736)* males. Single crosses were set up at 25°C overnight, males were then removed from plates and mated females were transferred once every 24 hours for 3 days. The total number of embryos, as well as hatched larvae, were counted.

General methods for nucleic acid manipulations

Standard methods were used to manipulate plasmid DNAs and oligonucleotides (Ausubel, 1990), and all plasmid sequences are available from the authors. The *egrh-1*-containing cosmid C27C12 was kindly provided by Alan Coulson (Sanger Institute). Plasmid pOK236.01 contains an *egrh-1(+)* genomic DNA fragment (bp 10647-19888 of C27C12; Accession No. Z69883). Plasmid pOK240.01 contains the same genomic fragment as pOK236.01 with the *gfp* open reading frame (ORF) from pPD103.87 (kindly provided by A. Fire, Stanford University) digested with *Xba*I inserted in-frame into an *Nhe*I site at bp 17454 in *egrh-1* exon 5. Plasmid pOK262.03 contains the *egrh-1* cDNA from pOK165.12 digested with *Xma*1 and PspOMI ligated to *Xma*I- and *Not*I-digested pJM16 (kindly provided by J. McGhee, University of Calgary), which contains the *ges-1* promoter fragment.

RNAi analyses

RNAi analyses were performed essentially as previously described (Fire et al., 1998). dsRNAs produced by in vitro transcription (Ambion) from the *egrh-1* cDNA yk484c12-pOK165.12 were injected at approximately 200-500 ng/μl into the germline of young adult hermaphrodites, which were allowed to recover and were transferred each day to freshly seeded plates. Phenotypes were scored in F1 progeny as young adults 20 and 40 hours post-L4 stage. RNAi experiments in wild-type and *rrf-1* mutant backgrounds were performed at 20°C, whereas those in *rrf-3(pk1426)* were performed at 16°C.

Transgene analyses

All transgenic lines were generated by microinjection using pRF4 (100 ng/μl) carrying a dominant *rol-6* allele as a transformation marker (Mello and Fire, 1995). OK0601 *egrh-1(tm1736); cuEx503[C27C12(+)]* contains cosmid C27C12 (2 ng/μl). OK0653 *egrh-1(tm1736); cuEx542[egrh-1(+)]* contains plasmid pOK236.01 (2 ng/μl). OK0658 *egrh-1(tm1736); cuEx547[egrh-1::gfp]* contains plasmid pOK240.01 (2 ng/μl). For mosaic analyses, OK0636 *egrh-1(tm1736); cuEx531[C27C12 + sur-5::gfp]* contains cosmid C27C12 (2 ng/μl) and *sur-5::gfp* (100 ng/μl).

Antibodies and microscopy

The *egrh-1* ORF encoding amino acids 1-120 was amplified from yk484c12-pOK165.12 and inserted into pMal2c (NEB). Antibodies were raised in rabbits D1919 and D1920 (Open Biosystems) against the MBP::EGRH-1 fusion purified from *E. coli* BL21 (Ausubel, 1990). EGRH-1 antibodies were purified by sequentially depleting against *E. coli* extracts expressing maltose-binding protein (MBP) and enriching against purified MBP::EGRH-1 bound to nitrocellulose filters (Duerr, 2006). D1919 reacted strongly with MBP::EGRH-1 on western blots and was used in these studies.

For EGRH-1 antibody staining, adult hermaphrodites were dissected, fixed for 10 minutes in 1.25% freshly prepared formaldehyde (Grant and Hirsh, 1999) and stained with EGRH-1 antibodies diluted to 1:1000 overnight at 4°C. EGRH-1 was localized by indirect immunofluorescence using goat anti-rabbit secondary antibodies labeled with Alexa 488 or Alexa 546 diluted to 1:500 for 2 hours at room temperature. MAPK activation in dissected hermaphrodites was detected using monoclonal MAPK-YT antibodies (1:2000 dilution; Sigma) (Miller et al., 2001) and Alexa 488-labeled goat anti-mouse secondary antibody (1:500 dilution). Worms were visualized using a Zeiss Axioskop microscope equipped for differential interference contrast (DIC) and fluorescence microscopy. Images were captured using an Axiocam camera and AxioVision software.

Mosaic analysis

Mosaic analyses were carried out using *sur-5::gfp* expression to identify lineages lacking an *egrh-1(+)* transgene (Yochem and Herman, 2003). L4 and young adult animals from the strain OK0636 *egrh-1(tm1736); cuEx531[C27C12 + sur-5::gfp]* were screened using a fluorescence dissecting microscope for candidate mosaic animals that had lost the extrachromosomal transgene in the MS, E and EMS lineages. Candidates were maintained for 20 hours and 707 animals were examined using a compound microscope to identify the lineage that had lost the *gfp(+)* transgene and to score the Egrh-1 phenotype. Transgene loss in MS, E and EMS lineages was verified by loss of GFP in spermatheca and the posterior pharynx for MS, intestinal cells for E and all of these cells for EMS. Animals were then scored in DIC for the presence of abnormal and degraded oocytes in the proximal gonad and an accumulation of oocytes in the distal gonad. In addition, 1119 hermaphrodites were cloned and examined for loss of the array in the germline and presence of the mutant phenotype but we were unable to identify an animal with a germline-specific loss.

RESULTS

C. elegans EGRH-1 is similar to EGR-family zinc-finger proteins

The *C. elegans* gene *C27C12.2* encodes a predicted 461 amino acid protein containing three C_2H_2 zinc fingers near the C-terminus and is most closely related in reciprocal BLAST searches to mammalian early growth response (EGR) family proteins and *Drosophila* Stripe (Fig. 1A,B) (Lee et al., 1995; O'Donovan et al., 1999). We have therefore named *C27C12.2 egrh-1* for early growth response factor homology. EGRH-1 protein shares high sequence identity with EGR-family factors in the zinc-finger region, including residues that contact DNA (Wolfe et al., 2000), but it has no obvious similarity elsewhere. This high identity within the zinc-finger DNA-binding domain suggests that EGRH-1 probably binds the same GC-rich sequence motif (GCGKGGGCG) recognized by

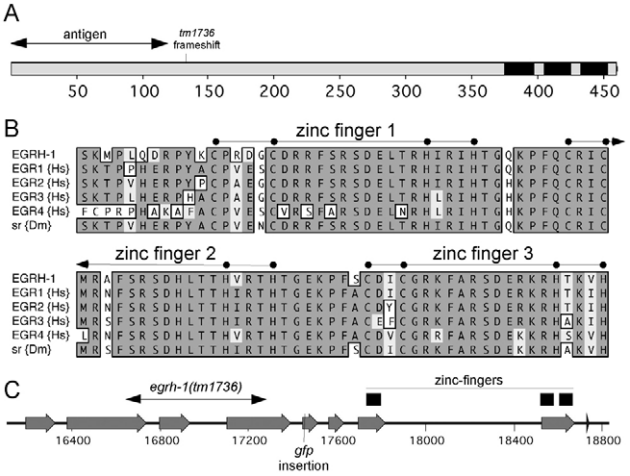

Fig. 1. Organization of the *egrh-1* gene and protein. (**A**) Schematic of the EGRH-1 protein indicating the positions of the zinc fingers (black boxes), the site of a frameshift mutation predicted in *egrh-1(tm1736)* and the antigen used to raise an EGRH-1 antibody. (**B**) ClustalW alignment of the zinc-finger regions of *C. elegans* EGRH-1 (Accession No. CAA93744), human EGR1 (Accession No. P18146), EGR2 (Accession No. P11161), EGR3 (Accession No. XP_944327) and *Drosophila* Stripe (Accession No. CG7847-PB). (**C**) Schematic of the *egrh-1* gene numbered according to the C27C12 cosmid sequence (Accession No. Z69883) with coding exons indicated as gray arrows. The regions encoding the zinc fingers, the site of *gfp* open reading frame insertion and the region deleted by *tm1736* (bp 16644-17282) are indicated.

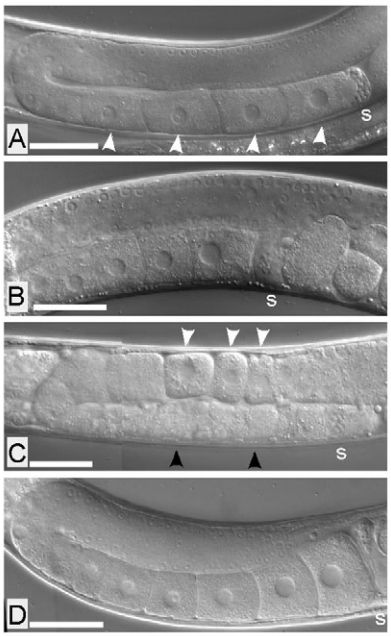

Fig. 2. *egrh-1(tm1736)* hermaphrodites accumulate degraded oocytes in the proximal gonad and differentiated oocytes in the distal gonad. DIC micrographs of adult hermaphrodite gonads: (**A**) wild type, 2 days post-L4; (**B**) *egrh1(tm1736)*, 1 day post-L4, prior to the onset of oocyte defects; (**C**) *egrh1(tm1736)*, 2 days post-L4, exhibiting degrading oocytes in the proximal gonadal arm and developing oocytes in the distal arm; and (**D**) *egrh-1(tm1736); cuEx503* carrying a wild-type *egrh-1* transgene, 2 days post-L4. In all cases, the distal arm is located on top and the proximal spermatheca (s) is located in the lower right. Differentiated oocytes in the distal and proximal gonadal arms are marked by white arrowheads (A,C), and degrading oocytes in the proximal gonadal arm are marked by black arrowheads (C). Images are composites from two images to include the entire gonad. Scale bars: 40 μm.

mammalian EGR-family factors. This motif is found at 469 distinct sites in the *C. elegans* genome (Markstein et al., 2002), although the function of these sites has not been explored.

egrh-1 mutants exhibit oocyte defects, small broods and embryonic lethality

To characterize EGRH-1 function in vivo, we obtained the deletion mutant *egrh-1(tm1736)* from the National BioResource Project. This deletion removes 639 bp of genomic DNA and is predicted to cause a frame-shift mutation upstream of the EGRH-1 zinc fingers after amino acid 135 (Fig. 1A,C). Based on the RNAi and rescue analyses described below, *egrh-1(tm1736)* appears to be a strong loss-of-function or null allele.

egrh-1(tm1736) mutants were morphologically normal and could be maintained as a homozygous, slow-growing strain but they exhibited fertility defects and partially penetrant embryonic lethality. Whereas wild-type hermaphrodites produced an average of 277 self progeny (n=3; range 253-298) that included only 1% arrested embryos (n=146), *egrh-1(tm1736)* hermaphrodites produced an average of 70 self progeny (n=5; range 14-169) that included 25% arrested embryos (n=97). The arrested *egrh-1(tm1736)* embryos exhibited a variable terminal phenotype and some exhibited a round eggshell phenotype (data not shown), which is observed in animals with ovulation defects (McCarter et al., 1997), but we have not characterized these embryonic phenotypes in detail.

The gonadal morphology and the number of sheath cells in the somatic gonad was normal in *egrh-1* mutants (data not shown). To understand the basis of the *egrh-1* mutant fertility defect, we examined the germline of adult *egrh-1(tm1736)* hermaphrodites. Immediately after molting to adults, the germline appeared normal, but by 20 hours after molting, 28% (n=64) of *egrh-1(tm1736)* animals contained abnormal and degrading oocytes in the proximal gonadal arm and developing oocytes in the distal arm, and these defects progressively worsened until 92% (n=63) of *egrh-1* animals exhibited these phenotypes by 40 hours after molting (Fig. 2; Table 1). The degrading proximal oocytes resembled the endomitotic oocytes described in mutants with defects in oogenesis and ovulation (Iwasaki et al., 1996; McCarter et al., 1997; Rose et al., 1997), and indeed we observed large, endomitotic nuclei in DAPI-stained animals (Emo phenotype; data not shown). The morphology of the distal oocytes in *egrh-1* mutants was similar to oocytes found in the proximal gonadal arm of wild-type animals but we never observed distal oocytes in wild-type hermaphrodites. We suggest that oocyte defects underlie both the low brood size and embryonic lethality in *egrh-1(tm1736)* mutants.

Because *egrh-1(tm1736)* might encode a truncated protein (Fig. 1A), we asked if this allele results in a loss of EGRH-1 function. We found that *egrh-1(+)* transgenes efficiently rescued *egrh-1* mutants, and that reducing *egrh-1* activity by RNAi produced oocyte defects nearly identical to *egrh-1(tm1736)* (Fig. 2D; Table 1). Importantly, we did not observe more severe phenotypes in *egrh-1(RNAi)* animals in either a wild-type or an RNAi

Table 1. Frequency of abnormal and distal oocytes

Genotype	% animals exhibiting abnormal or distal oocytes (*n*)*
+/+	2 (57)
egrh-1(tm1736)	92 (63)
egrh-1(tm1736); cuEx503[C27C12(+)]†,††	17 (89)
egrh-1(tm1736); cuEx542[egrh-1(+)]‡,††	18 (55)
egrh-1(tm1736); cuEx547[egrh-1::gfp]§,††	25 (53)
egrh-1(tm1736); cuEx531[C27C12 + sur-5::gfp]¶,††	36 (98)
*egrh-1(tm1736); cuEx562[ges-1::egrh-1(+)]***,††	52 (91)
egrh-1(RNAi)	42 (106)
egrh-1(RNAi); rrf-3(pk1426)	43 (203)
rrf-1(pk1417)	7 (60)
egrh-1(RNAi); rrf-1(pk1417)	12 (140)
fog-2(q71); egrh-1(tm1736)	82 (78)
*fog-2(q71); egrh-1(tm1736); cuEx562[ges-1::egrh-1(+)]***,††	45 (61)

*Hermaphrodites and females were scored 36-40 hours post-L4 (25°C).
†*cuEx503* contains cosmid C27C12 (encoding *egrh-1* and 6 additional transcripts) and pRF4.
‡*cuEx542* contains plasmid pOK236.01 (with bp 10647-19888 of C27C12 containing only *egrh-1*) and pRF4.
§*cuEx547* contains the rescuing *gfp* translational fusion in plasmid pOK240.01 (identical to pOK236.01 with the *gfp* open reading frame inserted in an *Nhe*I site in exon 5) and pRF4.
¶*cuEx531* contains cosmid C27C12; *sur-5::gfp* and pRF4.
***cuEx562* contains plasmid pOK262.03 (with the *ges-1* promoter driving expression of a cDNA) and pRF4.
††Rol transgenic animals were scored for the EGRH-1 phenotype.

hypersensitive *rrf-3(pk1426)* mutant background. Taken together, these results indicate that *egrh-1(tm1736)* is a strong loss-of-function or null allele.

egrh-1 mutant females exhibit constitutive MAPK activation and ovulation

In wild-type *C. elegans*, oocytes in the proximal gonad transiently arrest at the diakinesis stage of meiotic prophase I. In response to sperm-mediated signals, the proximal-most oocyte undergoes maturation, mitogen-activated protein kinase (MAPK) activation and ovulation (Lee et al., 2007; Miller et al., 2001; Page et al., 2001). MAPK is also activated in the distal gonadal arm as cells leave the pachytene stage, but this activation is sperm-independent during young adult stage (Lee et al., 2007). We hypothesized that EGRH-1 might be involved in oocyte arrest, and that the oocyte defects observed in *egrh-1* mutants result from inappropriate release from this arrest.

To test this hypothesis, we examined MAPK activation in the absence of sperm. Hermaphrodite sperm production is eliminated in *fog-2(q71)* and *fog-1(q253ts)* mutants, and these animals are functional females (Barton and Kimble, 1990; Schedl and Kimble, 1988). When unmated, these female oocytes do not undergo MAPK activation (Miller et al., 2001) and their ovulation is delayed, leading to a visible accumulation of oocytes stacked in the proximal gonad. We compared MAPK activation in the double mutant strains *fog-2(q71); egrh-1(tm1736)* and *fog-1(q253ts); egrh-1(tm1736)* (which we term *egrh-1* mutant females) with that in *fog-2(q71)* and *fog-1(q253ts)* single mutants (termed wild-type females). *egrh-1* mutant females exhibited MAPK activation in proximal oocytes far more frequently than wild-type females and, in some cases, activated MAPK was observed in more-distal oocytes in the proximal arm (Fig. 3; Table 2). Sperm-independent MAPK activation in the distal gonad occurred normally in both wild-type and *egrh-1* mutant females. Interestingly, *egrh-1* mutant females exhibited a delay in the accumulation of abnormal and distal oocytes compared with *egrh-1* mutant hermaphrodites and did not display these phenotypes until approximately 40 hours post-L4. However, when mated with males, this delay was eliminated, suggesting that sperm accelerate the onset of these oocyte defects.

We next asked if *egrh-1* mutant females ovulate in the absence of sperm-derived signals. We attempted to observe ovulations directly but, in contrast to wild-type animals, *egrh-1* mutant females did not ovulate when mounted for microscopy. However, we found that *egrh-1* females did not accumulate oocytes stacked in the proximal gonad (Fig. 4; Table 2), suggesting that *egrh-1*

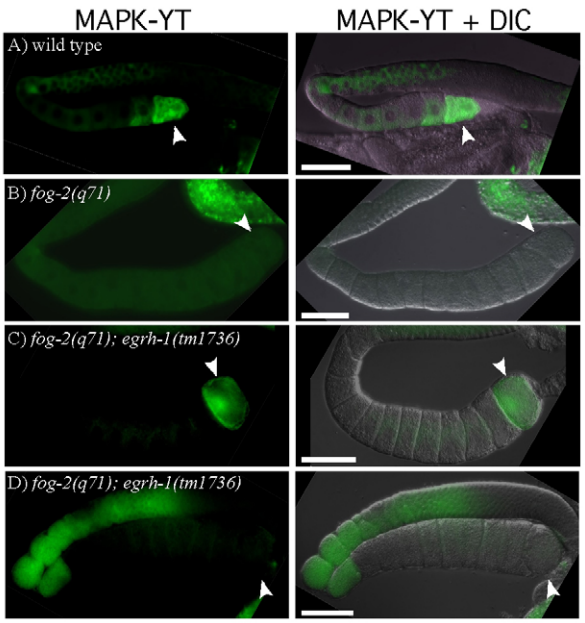

Fig. 3. *egrh-1(tm1736)* females activate MAPK in the absence of sperm. Fluorescence (left) and merged fluorescence and DIC (right) micrographs of dissected adult hermaphrodite gonads stained to detect the activated form of MPK-1 MAPK. Arrowheads mark the most proximal oocyte. (**A**) Wild-type hermaphrodite stained with MAPK-YT antibody shows activated MPK-1 MAPK in the most proximal oocytes. (**B**) Unmated *fog-2(q71)* females stained with MAPK-YT show no activated MAPK in oocytes. (**C**,**D**) Unmated *fog-2(q71); egrh-1(tm1736)* females show activated MAPK in the most proximal oocytes similar to wild-type hermaphrodites, as well as more distal oocytes not typically seen in wild-type hermaphrodites. Scale bars: 40 μm.

females ovulate in absence of sperm. Consistent with this conclusion, *egrh-1* mutant females contained more unfertilized oocytes ovulated into the uterus than wild-type females (Table 2).

When compared directly, the oocyte maturation rate for *fog-2(q71); egrh-1(tm1736)* females was significantly higher than that of *fog-2(q71); egrh-1(+)* females ($P<0.0001$; Table 2). This increase is comparable with that of mutants in some negative regulators of oocyte meiotic maturation (Govindan et al., 2006; Miller et al., 2003).

Together, these results indicate that EGRH-1 negatively regulates oocyte MAPK activation and ovulation.

egrh-1 mutant germ cells progress through normal developmental stages

Germ cells are continuously produced in adult hermaphrodites and a progression of distinct developmental stages can be recognized in DAPI-stained preparations along the distal-to-proximal axis (Hansen and Schedl, 2006; Kimble and Crittenden, 2005). Near the distal tip, a region of mitotic germ cells is termed the proliferative zone. As germline nuclei progress proximally within the distal arm, they exit the mitotic cell cycle and enter meiosis in the transition zone and subsequently enter the pachytene stage of meiosis. As nuclei exit the pachytene region, MAPK is activated and these cells enlarge in the bend region as they leave the distal arm and eventually differentiate as oocytes arrested in meiotic prophase I in the proximal gonad arm.

To determine if the progression of germ cell development is altered in *egrh-1* mutants, we compared the morphology and number of germ cell nuclei in different regions of the gonad in wild-type and *egrh-1* mutant females. In general, germ cell progression was normal in *egrh-1* mutants. The morphology of germ cell nuclei appeared normal in each of the regions in *egrh-1(tm1736)* mutants, and MAPK activation occurred near the end of the pachytene region. However, *egrh-1* mutants exhibited more variability in the number of nuclei in the transition zone and pachytene region, and the average number of pachytene nuclei was reduced (Table 3).

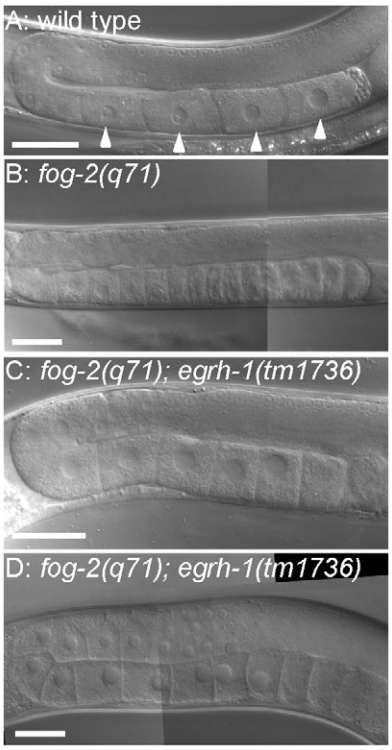

Fig. 4. *fog-2(q71); egrh-1(tm1736)* females ovulate in the absence of sperm. (**A**) Wild-type hermaphrodite with oocytes in the proximal gonadal arm (arrowheads mark oocyte nuclei). (**B**) *fog-2(q71)* female with many oocytes stacked in the proximal gonadal arm. (**C**) *fog-2(q71); egrh-1(tm1736)* female at 1 day post-L4. Unstacked oocytes appear similar to those of wild-type hermaphrodites. (**D**) *fog-2(q71); egrh-1(tm1736)* female at 2 days post-L4. The initial signs of degrading oocytes in the proximal gonadal arm and developing oocytes in the distal arm are visible. Images are composites from two images to include the entire gonad. Scale bars: 40 μm.

EGRH-1 is expressed in the distal tip cell and sheath cells of the somatic gonad

To identify *egrh-1*-expressing tissues, we first examined transgenic animals bearing a rescuing *egrh-1::gfp* translational fusion (Fig. 1C; Table 1). *egrh-1::gfp* was expressed in embryos through adult stages in most somatic tissues, including the gut, pharynx, body wall muscle, and nervous system (data not shown), suggesting that *egrh-1* is broadly expressed like mammalian Egr1 (McMahon et al., 1990; Watson and Milbrandt, 1990). To understand EGRH-1 function in regulating oocyte development, we examined *egrh-1::gfp* expression and endogenous EGRH-1 expression using an antibody targeting a unique region at the EGRH-1 N-terminus in dissected adult hermaphrodites. Using both techniques, EGRH-1 expression was observed in the sheath cells and distal tip cells of the somatic gonad, as well as in the intestine and sperm (Fig. 5). EGRH-1 was localized to nuclei in all tissues except sperm, where it appeared perinuclear. Importantly, no *egrh-1::gfp* expression or EGRH-1 antibody staining was observed in oocytes, suggesting that EGRH-1 functions in other tissues to regulate oocyte maturation and

Table 2. Frequency of oocyte MAPK activation and ovulation

Genotype	% animals with activated oocyte MAPK (*n*)	% animals with oocytes stacked in the proximal gonad (*n*)	% animals with 3 or more oocytes ovulated into the uterus (*n*)	Oocyte maturations per gonad arm per hour (*n*)
+/+	78 (49)	n.d.	n.d.	n.d.
fog-2(q71)	1 (70)	100 (39)	1 (87)	0.14±0.10 (14)
fog-2(q71); egrh-1(tm1736)	67 (45)	9 (45)	86 (125)	0.62±0.19 (18)
fog-2(q71); egrh-1(tm1736); cuEx562[ges-1::egrh-1(+)][*,†]	61 (97)	18 (33)	93 (27)	n.d.
fog-1(q253ts)	17 (24)	100 (48)	n.d.	n.d.
fog-1(q253ts); egrh-1(tm1736)	58 (90)	5 (43)	n.d.	n.d.

[*] *cuEx562* contains plasmid pOK262.03 (with the *ges-1* promoter driving expression of a cDNA) and pRF4.
[†] Rol transgenic animals were scored for the EGRH-1 phenotype.
n.d., not determined.

Table 3. Number of germline cell diameters in the different regions of the gonad

Genotype*	Proliferative zone (*n*)†	Transition zone (*n*)†	Pachytene region (*n*)†
fog-2(q71)	14±2.2 (5)	8.2±1.1 (5)	33.8±1.9 (5)
fog-2(q71); egrh-1(tm1736)	12.8±1.5 (5)	10.6±3.0 (5)	26.8±6.1 (5)‡

Cell diameters were measured by counting the number of nuclei in a line along the distal-proximal axis.
*Young adults 18-20 hours post-L4.
†The proliferative zone is distal to the transition zone. The transition zone begins where 80% of nuclei have a crescent-shaped morphology and ends where 80% of nuclei appear pachytene (Eckmann et al., 2004). The pachytene region is proximal to the transition zone and shows a thread-like appearance (Francis et al., 1995; MacQueen and Villeneuve; 2001).
‡This value is different from wild type at 95% confidence (*P*=0.04).

ovulation. To test if EGRH-1 antibody staining was specific, we stained *egrh-1(tm1736)* mutants, which are predicted to encode a truncated protein that would be recognized by our antibody, and *egrh-1(tm1736)* with *egrh-1* expression further reduced by RNAi. Staining was decreased in both cases, indicating that this antibody stain is specific for EGRH-1 (Table 4).

EGRH-1 function does not appear to be required in sperm. Wild-type and *egrh-1(tm1736)* males produce similar numbers of cross progeny when mated with *fog-1(q253)* females [+/+ males, average cross progeny: 206 (*n*=9); *egrh-1(tm1736)* males, average cross progeny: 143 (*n*=9)], and these cross progeny exhibit similar low levels of embryonic lethality [wild-type, 9.0% of embryos (*n*=556); *egrh-1(tm1736)*, 11.9% of embryos (*n*=616)].

EGRH-1 function is required in the gut and somatic gonad

Based upon the *egrh-1(tm1736)* mutant phenotype and EGRH-1 expression pattern, we hypothesized that EGRH-1 functions in the soma to inhibit maturation and ovulation. To test this hypothesis, we first examined the effect of *egrh-1(RNAi)* in an *rrf-1(pk1417)* mutant background. *rrf-1(pk1417)* mutants are specifically resistant to RNAi in the soma but remain sensitive to RNAi in the germline (Sijen et al., 2001). We found that *egrh-1(RNAi)* was less effective in *rrf-1(pk1417)* than in a wild-type background (Table 1). Indeed, *rrf-1(pk1417); egrh-1(RNAi)* animals exhibited only a slight increase in the frequency of oocyte defects when compared with untreated *rrf-1(pk1417)* mutants. Thus, we conclude that *egrh-1* functions in the soma.

We next examined the phenotype of *egrh-1(tm1736)* animals that were mosaic for a rescuing transgene (Mello and Fire, 1995) (see Materials and methods). Transformed DNAs in *C. elegans* recombine to form extrachromosomal arrays that are partially unstable in mitosis. We generated the strain *egrh-1(tm1736); cuEx531* containing the *egrh-1(+)* cosmid C27C12 and the cell-autonomous marker for somatic cells *sur-5::gfp* (Yochem and Herman, 2003). This transgene rescued *egrh-1(tm1736)* mutants (Table 1) but was lost at low frequency during early mitotic cell divisions. We first identified animals that specifically lost this transgene in the EMS blastomere, which is the precursor of the somatic gonad and gut but not the germline, and 95% of these animals exhibited abnormal and distal oocytes (Fig. 6). Thus, *egrh-1* function is required in somatic tissues derived from EMS. Although we were not able to find any animals that lost the transgene only in the germline precursor P2, we did find one animal that lost the transgene in P2 but had a second loss later in the E lineage. This animal exhibited a wild-type phenotype, suggesting that *egrh-1* function is not required in the germline. We next identified mosaic animals that lost the transgene only in MS or E, the descendants of EMS that give rise to the somatic gonad or gut, respectively. Loss in either MS or E resulted in the

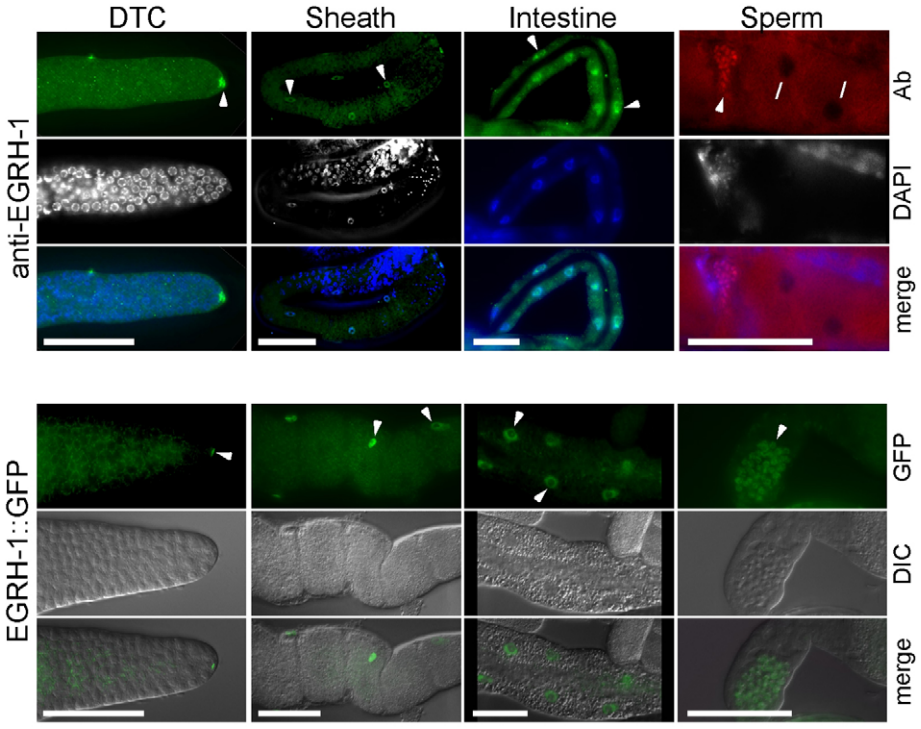

Fig. 5. EGRH-1 antibody staining and EGRH-1::GFP expression in the somatic gonad and intestine. EGRH-1 antibody staining (top panels) and EGRH-1::GFP expression (bottom panels) in nuclei of the distal tip cell (DTC) and sheath cells of the somatic gonad and intestinal cells. Arrowheads mark representative EGRH-1-containing nuclei. White lines point out oocyte nuclei lacking detectable EGRH-1 staining. For anti-EGRH-1, antibody-stained and DAPI-stained images are shown individually and merged. For EGRH-1::GFP, GFP fluorescence and DIC images are shown individually and merged. Distal tip cell staining with EGRH-1 antibody colocalized with *lag-2::gfp* expression, which marks this cell type (data not shown) (Siegfried and Kimble, 2002). Scale bars: 40 μm.

Table 4. Frequency of EGRH-1 antibody staining in dissected somatic gonads

Genotype	% distal tip cell staining (*n*)	% sheath cell staining (*n*)
+/+	93 (110)	91 (93)
*egrh-1(tm1736)**	69 (42)	60 (40)
egrh-1(tm1736); egrh-1(RNAi)	28 (43)	7 (70)

**egrh-1(tm1736)* is predicted to encode a truncated protein that would be detected by this targeted antibody (Fig. 1A).

egrh-1 phenotype, strongly suggesting that *egrh-1* function is required in both the somatic gonad and the gut for normal oocyte development.

We next expressed *egrh-1* in the E lineage using the *ges-1* promoter and asked if gut expression is sufficient to rescue *egrh-1* mutants. The *ges-1* promoter is active exclusively in the E lineage from early embryogenesis throughout the life of the worm (Kennedy et al., 1993). We found that *egrh-1* expressed using a *ges-1::egrh-1* fusion gene did not rescue sperm-independent ovulation and MAPK activation (Table 2). We suggest that *egrh-1* in the sheath cells of the somatic gonad negatively regulates oocyte MAPK activation and ovulation. However, we do not have a well-characterized sheath cell promoter to drive *egrh-1* expression in these cells to test this hypothesis. By contrast, *egrh-1* expression in the E lineage partially rescued the formation of abnormal and distal oocytes in both *egrh-1* mutant hermaphrodites and *egrh-1* mutant females (Table 1), although this result might reflect a role for *egrh-1* in other aspects of oocyte development.

DISCUSSION

In this paper, we characterize the role for the *C. elegans* EGR-family factor EGRH-1 during oogenesis. We find that *egrh-1* mutants exhibit ectopic oocyte development in the distal gonad and sperm-independent oocyte maturation and ovulation. EGRH-1 is broadly expressed both temporally and spatially, and tissue-specific RNAi analysis indicates that it functions in the soma to regulate oogenesis. Mosaic analysis indicates that EGRH-1 function is probably required in the somatic gonad, which is descended from the MS blastomere, and more surprisingly in the gut, which is descended from the E blastomere. Expression of *egrh-1* in the gut rescues the formation of ectopic oocytes in *egrh-1* mutants, but does not rescue sperm-independent oocyte maturation and ovulation. Together, these results suggest that EGRH-1 regulates cell-non-autonomous mechanisms controlling multiple aspects of oocyte development, and that EGRH-1 has distinct functions in the sheath cells and gut.

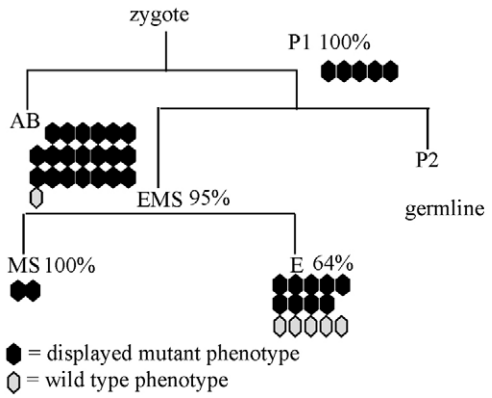

Fig. 6. Analysis of *egrh-1* genetic mosaics. Diagram of the early *C. elegans* cell lineage indicating the phenotypes of *egrh-1* genetic mosaics that lost a rescuing transgene in specific sublineages. Black indicates individual transgenic animals that lost GFP expression in the indicated lineages and displayed abnormal and degraded oocytes in the proximal and distal gonadal arms. Light gray indicates transgenic animals that lost GFP expression in these lineages and exhibited wild-type oocyte development. Numbers indicate the percentage of animals that lost the transgene in this sublineage that exhibited the mutant phenotype.

EGRH-1 negatively regulates oocyte maturation and ovulation

In wild-type *C. elegans* hermaphrodites, oocyte maturation and ovulation depend on signals between the oocytes, the sheath cells of the somatic gonad, and sperm (Greenstein, 2005). In the absence of sperm, sheath-cell-derived signals inhibit oocyte maturation and ovulation to preserve oocytes for fertilization. This inhibition depends on gap junctions between the sheath cells and the oocytes (Govindan et al., 2006; Whitten and Miller, 2007). When sperm are present, sheath cell inhibition is overridden by major sperm proteins (MSPs), which are secreted from sperm and bind the VAB-1/Eph receptor in oocytes and unidentified receptors in sheath cells, allowing oocyte maturation and ovulation (Govindan et al., 2006; Miller et al., 2001; Miller et al., 2003). Oocyte maturation coincides with the phosphorylation and activation of the MPK-1 MAPK protein in proximal oocytes and MAPK activation is necessary for maturation and ovulation. MAPK is typically highly activated in the most proximal oocyte, but this activation is rapidly lost as this oocyte completes maturation and is ovulated (Lee et al., 2007). Maturation leads to degradation of specific oocyte proteins allowing the transition from differentiated oocyte to totipotent zygote (Nishi and Lin, 2005; Shirayama et al., 2006; Stitzel et al., 2006).

egrh-1 mutants exhibit MPK-1/MAPK activation in proximal oocytes and ovulation independently of sperm signals, indicating that EGRH-1 negatively regulates oocyte maturation and ovulation. As in wild-type hermaphrodites, MPK-1/MAPK activation appeared transient and was occasionally observed in more distal oocytes. We suggest that this transient MAPK activation in *egrh-1* mutant oocytes inappropriately initiates the transition from oocyte to zygote, and this contributes to the partially penetrant embryonic lethal phenotype.

A number of other *C. elegans* mutants have been described that exhibit sperm-independent MAPK activation and ovulation. In particular, mutants affecting the CEH-18 POU-homeodomain transcription factor, which is expressed similarly to EGRH-1 in sheath cells, exhibit derepressed oocyte maturation (Greenstein et al., 1994; Rose et al., 1997). *ceh-18* is necessary for normal sheath cell differentiation and mutants exhibit disorganized myofilaments in sheath cells. We have not observed myofilament disorganization in the *egrh-1* mutant sheath cells (data not shown), suggesting that EGRH-1 and CEH-18 play distinct roles in inhibiting oocyte maturation.

EGRH-1 mutants accumulate differentiated oocytes in the distal gonad

In wild-type hermaphrodites, meiotic germ cells exit the pachytene stage in the distal arm of the gonad and form differentiated oocytes in the proximal arm. Exit from pachytene involves signals from the somatic sheath cells (McCarter et al., 1997), and oocyte growth depends on uptake of yolk secreted from the gut and proximal streaming of germline cytoplasm into enlarging oocytes (Grant and Hirsh, 1999; Wolke et al., 2007).

Our analyses indicate that *egrh-1* function is necessary and sufficient in the soma for normal oocyte development, and that *egrh-1* function is necessary in the descendents of both the MS blastomere, which includes the somatic gonad, and the E blastomere that gives rise to the gut. These conclusions are consistent with the expression of the rescuing *egrh-1::gfp* transgene and accumulation of EGRH-1 protein in somatic tissues but not in oocytes.

egrh-1 mutants accumulate differentiated oocytes in the distal gonadal arm and we hypothesize that, in gonadal sheath cells, EGRH-1 inhibits oocyte maturation and ovulation. This signal is overcome by sperm-dependent MSP signaling. Therefore, loss of *egrh-1* in the somatic gonad leads to oocyte degradation and blockage of the proximal arm that interferes with ovulation, and distal oocytes might form as a secondary consequence of defective ovulation. A similar phenotype can be observed in other Emo mutants at late times (D. Greenstein, personal communication). We do not understand how *egrh-1* loss in the gut leads to formation of distal oocytes. The gut produces complexes containing yolk proteins and precursors for signaling molecules involved in sperm recruitment that are transported into the psuedocoelom and are ultimately endocytosed by oocytes (Hall et al., 1999; Kimble and Sharrock, 1983; Kubagawa et al., 2006). *rme-2* mutants, which are defective in endocytosis of yolk complexes, exhibit ovulation defects (Grant and Hirsh, 1999). We have observed yolk accumulation in *egrh-1* mutants and perhaps EGRH-1 is necessary for synthesizing specific components of yolk complexes necessary for normal ovulation; loss of these components affects ovulation and leads indirectly to formation of distal oocytes. Similarly, polyunsaturated fatty acids synthesized in the gut are transported into oocytes, where they are believed to be precursors for signaling molecules that recruit sperm to the oocyte (Kubagawa et al., 2006). It is interesting in this regard that we have preliminary results indicating sperm recruitment defects in *egrh-1* mutants (data not shown).

Relationship to EGR family in other species

In mammalian cells, EGR-family expression is induced by a variety of stimuli, including growth factor and cytokine signaling, and expression is regulated in response to MAPK pathway signaling (O'Donovan et al., 1999). EGR proteins in turn regulate expression of a variety of transcriptional regulatory proteins and signaling molecules (Fu et al., 2003; Virolle et al., 2003). Thus, EGR-family proteins function at the convergence of signaling pathways. In *C. elegans*, MPK-1 is activated in the sheath cells of the somatic gonad (Lee et al., 2007) and *egrh-1* expression could be regulated in this tissue by activated MPK-1.

Mammalian EGR-family genes are broadly expressed, and mutants defective in EGR-family members exhibit a variety of defects (O'Donovan et al., 1999). Egr1 mutant mice have fertility defects and fail to ovulate owing to loss of luteinizing hormone β (LHβ) expression in the pituitary (Lee et al., 1996; Topilko et al., 1998), and its function is partially redundant with Egr4 (Tourtellotte et al., 2000). By comparison, Egr3 mutant mice do not have fertility defects but exhibit defects in muscle spindle stretch receptors (Tourtellotte et al., 2001; Tourtellotte and Milbrandt, 1998). Although *C. elegans* does not have an LHβ ortholog, EGRH-1 might function like mouse Egr1 by regulating expression of secreted factors that have non-autonomous effects on oocyte development.

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Competing interests statement

The authors declare no competing financial interests.

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