EGG-4 and EGG-5 link events of the oocyte-to-embryo transition with meiotic cell cycle progression in *Caenorhabditis elegans*

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Summary

The molecular underpinnings of the oocyte-to-embryo transition are poorly understood. Here we show that two protein tyrosine phosphatase-like (PTPL) family proteins, EGG-4 and EGG-5, are required for key events of the oocyte-to-embryo transition in *Caenorhabditis elegans*. The predicted EGG-4 and EGG-5 amino acid sequences are 99% identical and their functions are redundant. In embryos lacking EGG-4 and EGG-5 we observe defects in meiosis, polar body formation, the block to polyspermy, F-actin dynamics, and eggshell deposition. During oogenesis, EGG-4 and EGG-5 assemble at the oocyte cortex with the previously identified regulators or effectors of the oocyte-to-embryo transition EGG-3, CHS-1 and MBK-2 [1,2]. All of these molecules share a complex interdependence with regards to their dynamics and subcellular localization. Shortly after fertilization, EGG-4 and EGG-5 are required to properly coordinate a redistribution of CHS-1 and EGG-3 away from the cortex during meiotic anaphase I. Therefore EGG-4 and EGG-5 are not only required for critical events of the oocyte-to-embryo transition but also link the dynamics of the regulatory machinery with the advancing cell cycle.

Results and Discussion

The *egg-4* and *egg-5* genes encode redundant protein tyrosine phosphatase-like proteins

The *egg-4* (T21E3.1) and *egg-5* (R12E2.10) genes were discovered through a search for oocyte enriched expressing genes with protein tyrosine phosphatase-like (PTPL) domains similar to *egg-3* [1] (Figures 1A and 1B) (see Supplemental Text and Experimental Procedures available online). PTPL family proteins have been implicated in the regulation of cell growth and differentiation and are defined by a region of homology to protein tyrosine phosphatases

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lacking key active site amino acids (Figure 1A) [3]. These molecules are predicted to bind to phosphorylated substrates but do not catalyze substrate dephosphorylation. Other PTPL family proteins and EGG-4 have been shown to lack phosphatase activity [4] (G. Seydoux, personal communication). The *egg-4* and *egg-5* genes have a high level of sequence identity, sharing 747 of 753 amino acids (99.2% identical) (Figures 1A and 1B). The *egg-4* and *egg-5* genes are functionally redundant (see below) and we will refer to the pair of genes as *egg-4/5*.

Loss of *egg-4/5* function leads to maternal-effect lethality

In order to determine the function of *egg-4/5*, we obtained deletion alleles of both genes from the National Bioresource Project for the Nematode (Japan) and the North American *C. elegans* Gene Knockout Consortium. The various alleles available are shown in Figure 1B. For our studies, we used the *egg-4(tm1508)* and *egg-5(ok1781)* alleles and/or RNAi treatment that knocks down both genes simultaneously (Figure S1). Viable progeny counts showed that each single mutant had a reduced number of hatching larvae compared to wild-type hermaphrodites (Table S1). RNAi treated and *egg-4/5* double mutant hermaphrodites produce no viable progeny. Crossing wild-type males to *egg-4/5* hermaphrodites did not result in the production of viable progeny. This data supports the conclusion that *egg-4/5* are largely redundant and that loss of function of both genes leads to maternal-effect lethality.

Loss of *egg-4/5* leads to defects in the oocyte-to-embryo transition

To determine the nature of the maternal-effect lethality associated with loss of *egg-4/5* we examined the oocytes produced by RNAi treated and double mutant animals. Germline development, gametogenesis, positioning of gametes, ovulation, and fertilization all appeared to occur normally in *egg-4/5(RNAi)* and double mutant hermaphrodites [5] (Figures 1C and 1D; Table S2). However, we found a number of important abnormalities in embryos produced by *egg-4/5* loss of function animals.

The block to polyspermy is considered a hallmark of egg activation in many species. DAPI stained maternal DNA and sperm DNA can be distinguished based on morphology [6]. In the majority of embryos produced by *egg-4/5(RNAi)* or double mutant animals, the block to polyspermy was intact and only a single sperm entered each oocyte (Figure 1D). However, in 16% of *egg-4/5(RNAi)* eggs and 25% in *egg-4/5* double mutants, DAPI staining reveals what appear to be polyspermic embryos (Figure 1D).

Some of the most striking features of oocyte-to-embryo transition are associated with changes at the cell surface. In *C. elegans*, a chitinous eggshell is secreted after sperm entry and its formation is required to support embryonic development [7]. Embryos produced by *egg-4/5* hermaphrodites lack any visible rigid eggshell and are indistinguishable (except for sperm entry and the completion of meiosis, see below) from unfertilized oocytes produced by other types of mutants (Figure 1C) [8,9]. Further, *egg-4/5(RNAi)* oocytes lack the defining chitin containing layer of the wild-type eggshell (Figure 1E). Therefore, we conclude that EGG-4/5 proteins are required for the production of the eggshell after fertilization.

We examined filamentous actin (F-actin) dynamics using a GFP-moesin fusion (GFP:MOE, Figure 1F) [1]. In wild-type oocytes, an actin cap forms in the presumptive posterior of the egg cortex several minutes after moving into the spermatheca and marks the site of sperm entry. This actin cap then disperses in a fashion that is limited to the posterior half of the embryo as it moves from the spermatheca to the uterus. This actin cap and its rearrangement are aberrant in *egg-4/5(RNAi)* animals and are not restricted to the posterior half of the one cell embryo during dispersal (Figure 1F). Therefore, this polarized cytoskeletal rearrangement requires EGG-4/5 function.

We observed oocyte chromosomes during meiosis by using mCherry:histone and GFP:tubulin in *egg-4/5(RNAi)* animals [1] (Figure 1G). Meiotic spindle formation, translocation and rotation were indistinguishable from wild-type through anaphase I [9] (Figure S2B). Chromosomes segregated normally until after anaphase I (Figure 1G). In unfertilized oocytes, meiosis fails to progress beyond this point [9]. In *egg-4/5(RNAi)* embryos, meiosis progresses beyond anaphase I but no polar bodies were formed and 12 univalents could be seen at metaphase II (Figure 1G). The degradation of CYB-1:GFP (cyclin B) in *egg-4/5(RNAi)* animals (Figure S2C) suggest that there is no significant meiotic delay. Therefore, EGG-4/5 are required for normal meiosis and polar body formation. The meiotic defects seen in *egg-4/5(RNAi)* animals are very similar to those seen in *egg-3, chs-1*, paternal effect lethal *spe-11* mutants, and oocytes depleted in F-actin [1,9].

EGG-4/5 localize to the cortex of developing oocytes and then disperse in embryos

We employed fusion protein and antibody (supplemental text) approaches to determine the subcellular localization of EGG-4 and EGG-5. Transgenic worm strains were created carrying integrated *gfp* fusions driven by the germline-specific *pie-1* promoter [10]. Multiple independently derived transgenic lines (4 lines for GFP:EGG-4 and 2 lines for GFP:EGG-5) all had the same distribution pattern, dynamics and genetic interactions (see below). Both GFP:EGG-4 (Figure 2A, G) and GFP:EGG-5 (Figure 2B, H) were associated with the cortex of developing oocytes and newly fertilized embryos. In oocytes and newly fertilized embryos GFP:EGG-4 (Figure 2D-F) and GFP:EGG-5 (not shown) colocalize with mCherry:EGG-3. Therefore, by extension they also colocalize with the other egg activation molecules CHS-1 and MBK-2 during meiotic maturation [1,2]. We created a strain of worms carrying both GFP:EGG-4 and mCherry-histone to determine the dynamics of EGG-4 localization relative to the cell cycle. By anaphase I, GFP:EGG-4 and GFP:EGG-5 was degraded or dispersed away from the cortex (Figure 2I-J). This movement from the cortex is in sharp contrast to the movement of other oocyte-to-embryo transition molecules (compare embryos in Figures 2A and 2B to 2C). For instance mCherry:EGG-3 moves to cortical foci at anaphase I (Figure 2C) [1]. We have never seen any of our GFP:EGG-4 or GFP:EGG-5 fusion molecules form foci in embryos (Figures 2A and 2B). Rather, when we follow the movement of these GFP fusions in live animals, the GFP signal abruptly leaves the cortex just before or at early anaphase I. This timing is similar to the timing of when EGG-3, CHS-1 and MBK-2 move from a uniform cortical distribution to cortical foci [1,2].

The redistribution of EGG-4/5 did not depend on fertilization. Localization and the dynamics of GFP:EGG-4 was normal in *spe-9(hc52)* mutants and in old hermaphrodites that had completely depleted their sperm (not shown). However, the redistribution does depend on meiotic progression. GFP:EGG-4 stayed associated with the cortex in *mat-1(RNAi)* animals where oocytes were arrested at metaphase I [11] (Figure 3B). The differences in the redistribution of EGG-4/5 from the cortex after fertilization compared to other molecules that moved to cortical foci may reflect important differences in the roles of these molecules during the oocyte-to-embryo transition.

The localization of EGG-4/5 to the oocyte cortex in developing oocytes depends on EGG-3 and CHS-1 but not MBK-2

We checked EGG-4/5 for interactions with previously identified molecules involved in the oocyte-to-embryo transition. Like EGG-4/5, EGG-3 is a PTPL family protein that is required for the oocyte-to-embryo transition [1,2] and mCherry:EGG-3 colocalizes with GFP:EGG-4 and GFP:EGG-5 in developing oocytes (Figures 2D-2F). In *egg-3(RNAi)* worms, GFP:EGG-4 (Figure 3C) and GFP:EGG-5 (not shown) do not accumulate at the cortex. Therefore, EGG-3 is required for the proper localization of EGG-4/5 in developing oocytes.

CHS-1 is a chitin synthase that is required for eggshell formation and the oocyte-to-embryo transition [1]. CHS-1 was also shown to colocalize with EGG-3 and the proper localization of these two proteins was shown to be interdependent [1]. In *chs-1(RNAi)* animals, GFP:EGG-4 (Figure 3D) and GFP:EGG-5 (not shown) do not localize to the cortex of developing oocytes. Therefore, like EGG-3, CHS-1 is also required for the proper subcellular localization of EGG-4/5.

The *mbk-2* gene encodes a DYRK kinase that is required for the oocyte-to-embryo transition and marks maternal proteins for timely degradation [12]. The localization pattern of MBK-2 is identical to EGG-3 and CHS-1. Further, the proper subcellular localization patterns of MBK-2 depend on EGG-3 and CHS-1 but not the other way around [1,2,12]. We found that *mbk-2(RNAi)* has no effect on the localization pattern of GFP:EGG-4 (Figure 3E) and GFP:EGG-5 (not shown).

EGG-4/5 are required for the proper localization of MBK-2

Although MBK-2 is not required for the localization pattern of EGG-4/5 we wanted to check if EGG-4/5 were required for the localization of MBK-2. In *egg-4/5(RNAi)* animals GFP:MBK-2 was indeed mislocalized (Figures 4A and 4B). Rather than accumulating at the cortex of developing oocytes, GFP:MBK-2 remains diffusely cytoplasmic and it also does not localize to cortical foci after fertilization. Next we wanted to check if the EGG-4/5 dependent mislocalization of MBK-2 altered its ability to regulate the degradation of its known target proteins OMA-1 and MEI-1 [12-14]. In *egg-4/5(RNAi)* animals, we observed no obvious changes in the degradation patterns of GFP:MEI-1 and OMA-1:GFP (Figure S3A).

EGG-4/5 are required for the movement of EGG-3 and CHS-1 from the cell cortex to the cytoplasm during meiotic progression

We wanted to further examine the interactions of EGG-4/5 with EGG-3 and CHS-1. We find that *egg-4/5(RNAi)* did not alter the cortex localization of GFP:EGG-3 or GFP:CHS-1 in developing oocytes (Figures 4C-4F). However, in embryos, GFP:EGG-3 (Figure 4D), mCherry:EGG-3 (Figure S3B) and GFP:CHS-1 (Figure 4F) remained associated with a uniform cortex distribution rather than moving to cortical foci at anaphase I. It is important to note that the localization patterns of GFP:EGG-3 and GFP:CHS-1 in *egg-4/5(RNAi)* embryos resembles GFP:EGG-3 [1] in a *mat-1* mutant background where the meiotic cell cycle is arrested and embryos do not reach anaphase I (Figure S3B). In *egg-4/5(RNAi)* animals the meiotic cell cycle progresses past anaphase I (Figure 1G and S2C) yet GFP:EGG-3 and GFP:CHS-1 remain uniformly associated with the cortex. We conclude that EGG-4/5 are required to couple the dynamics of EGG-3 and CHS-1 with meiotic cell cycle progression.

Physical interactions between EGG-3, EGG-4 and MBK-2

We used the yeast-two-hybrid assay to determine if the various cytoplasmic oocyte-to-embryo transition molecules could physically interact. Interactions were detected between EGG-3 and EGG-4, MBK-2 and EGG-4, and MBK-2 and EGG-3 (Figure 4G). The interaction results for MBK-2 and EGG-3 are consistent with previously reported binding studies for these two proteins [2]. Although the interactions are weaker than our strong positive control, interactions in vivo could potentially be strengthened by phosphorylation of the various molecules. These results support the idea that direct physical interactions are the mechanism for the co-localization of these proteins.

A cell cortex associated complex

Loss of EGG-3, EGG-4, EGG-5, CHS-1 and MBK-2 all lead to many defects in the oocyte-to-embryo transition [1,2,8]. Our epistasis studies indicate that there is a complicated

interdependence of these maternally provided molecules with regards to their subcellular localization and dynamics [1,2]. Many of these molecules can physically interact and we propose that they form a complex at the cell cortex during oogenesis. This cortex-associated complex would need to spatially and temporally regulate the output of different effectors relative to the needs of the developing oocyte or embryo and in the context of cell cycle progression. For instance, MBK-2 activity must be properly regulated during oogenesis and during meiotic progression after fertilization [2]. Therefore, it makes sense that different components of this cellular machinery will have some specialization of function. For instance, only EGG-4/5 are known to be required for the block to polyspermy. Like EGG-3, loss of EGG-4/5 can alter F-actin dynamics and polarization after fertilization [1]. Unlike EGG-3, loss of EGG-4/5 can also alter the F-actin cap at the fertilization site. After fertilization EGG-4/5 move from the cortex to the cytoplasm or could be degraded rather than forming cortical foci like EGG-3, CHS-1 and MBK-2. It remains unclear how the unique dynamics of EGG-4/5 are related to their differential effects on the oocyte-to-embryo transition. Certainly their unique localization pattern just after anaphase I strongly suggest that EGG-4/5 are no longer binding to effectors such as MBK-2 and CHS-1 at this critical moment of early development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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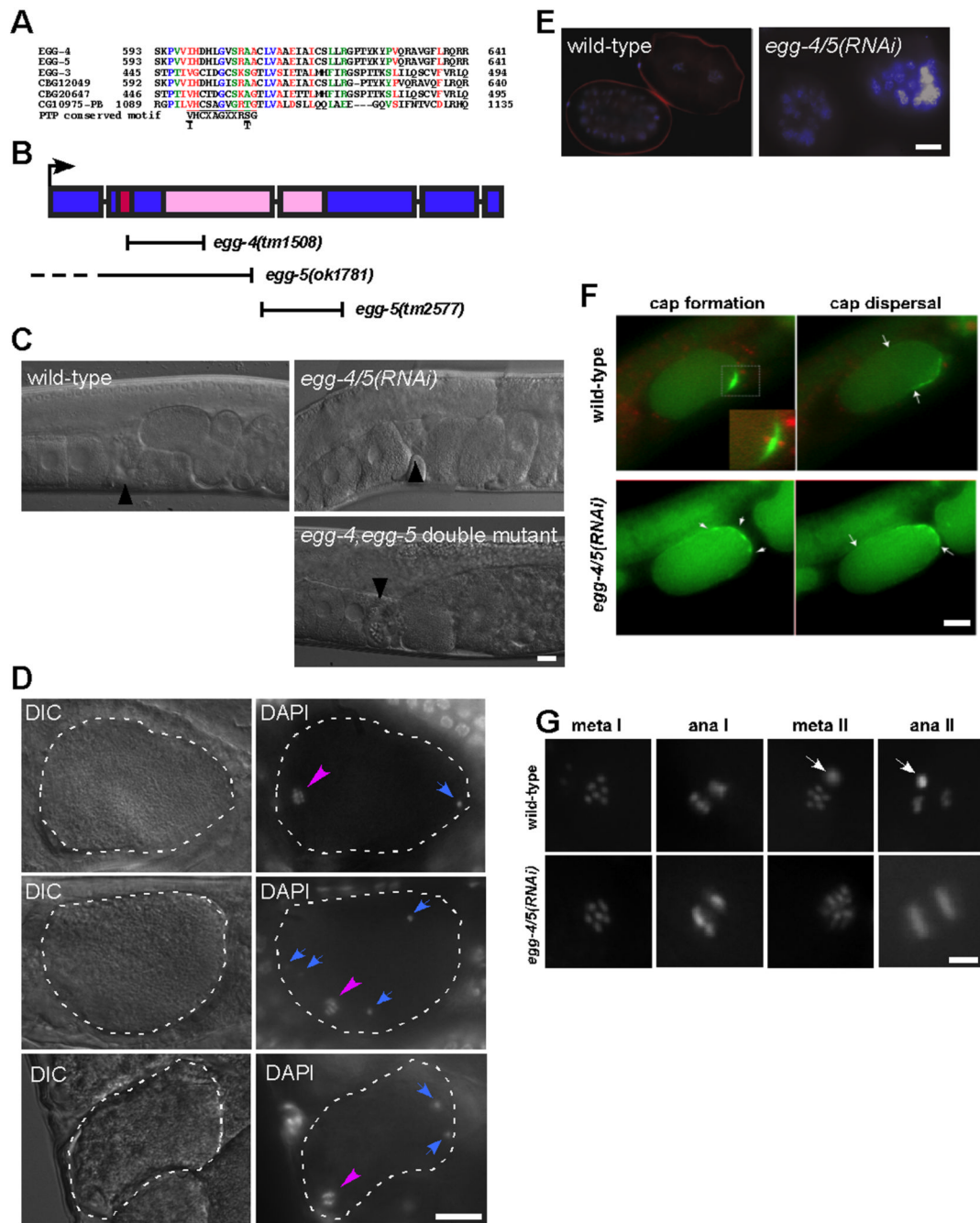
Figure 1.

EGG-4/5 Phenotypes

(A) Alignment of the phosphatase domains of PTPL family proteins. The EGG-4/5 ortholog in *C. briggsae* (CBG12049), the EGG-3 ortholog in *C. briggsae* (CBG20647) and a *Drosophila melanogaster* phosphatase (CG10975-PB). Blue, identical; red, conserved; green, semiconserved. (B) The gene structure of EGG-4 and EGG-5 are identical and therefore depicted with a single schematic. PTP domain, Pink. Amino acid sequences used to generate antisera, red. The sequences that are deleted by various alleles are indicated as brackets. (C) DIC images of wild-type, *egg-4/5(RNAi)* and *egg-4/5* double mutant hermaphrodites in the region of the spermatheca. Arrowheads indicate the position of the spermatheca. Maturing

oocytes are to the left of the spermatheca and fertilized eggs are in the uterus to the right for each panel. Properly developing embryos can only be seen in the uterus of wild-type animals. Scale bar represents 10 μm. (D) DIC images of a newly fertilized *egg-4/5(RNAi), egg-4/5* double mutant embryos and DAPI staining of the same cells showing oocyte and sperm DNA. Polyspermy is seen in about 16% of RNAi treated embryos (middle 2 panels, n=49) and 25% in double mutants (bottom 2 panels, n=29). Pink arrowheads indicate oocyte DNA and blue arrows indicate sperm DNA. Scale bar represents 10 μm. (E) Chitin staining of wild-type and *egg-4/5(RNAi)* embryos. Red staining is chitin; blue staining is DAPI. Scale bar represents 10 μm. (F) Compared to wild-type, actin cap formation and the polarized dispersal of F-actin is defective in *egg-4/5(RNAi)* embryos. A robust actin cap does not form in *egg-4/5(RNAi)* embryos. The dispersal of the F-actin in *egg-4/5(RNAi)* crossed 50% egg length as denoted by the arrows marking the edges of F-actin localization. Wild-type controls 15/15 embryos displayed wild-type actin dynamics. *egg-4/5(RNAi)*; 4/5 embryos had abnormal cap formation and 3/6 had abnormal polarized dispersal. Scale bar represents 10 μm. (G) mCherry-histone in wild-type and *egg-4/5(RNAi)* embryos during meiosis. meta I, metaphase I; ana I, anaphase I; meta II, metaphase II; ana II, anaphase II. Arrows indicate the position of polar bodies. Scale bar represents 5 μm.
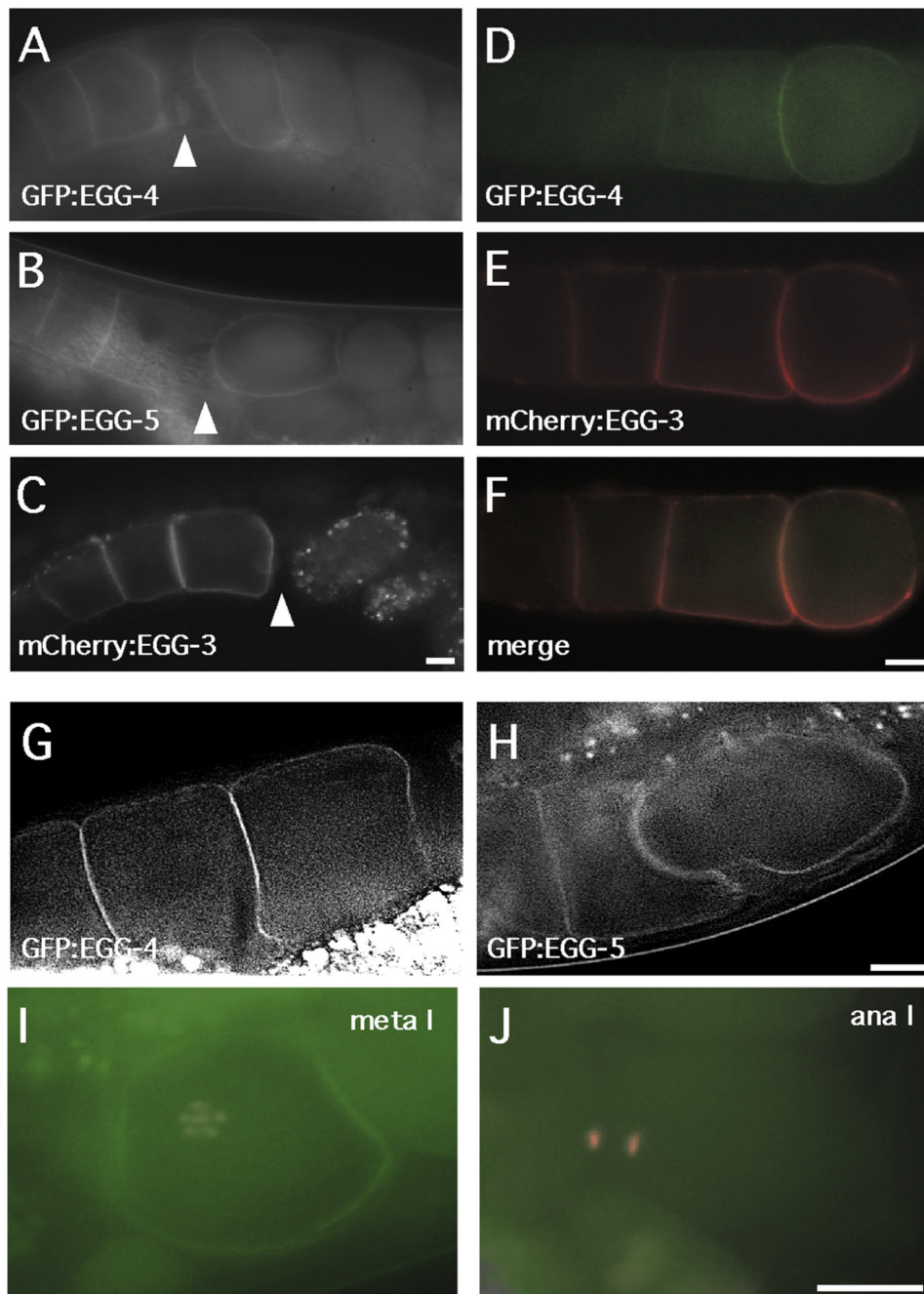
Figure 2.
EGG-4 and EGG-5 Are Associated With the Cell Cortex in Developing Oocytes and Become Cytoplasmic in Embryos. (A-C) The arrowheads indicate the position of the spermatheca. Oocytes can be seen to the left of the spermatheca and embryos to the right. mCherry:EGG-3 moves to cortical foci in embryos at anaphase I. In contrast, at this stage GFP:EGG-4 and GFP:EGG-5 become diffusely cytoplasmic. (A) GFP:EGG-4. (B) GFP:EGG-5. (C) mCherry:EGG-3. Scale bar represents 10 μm. (D-F) GFP:EGG-4 and mCherry:EGG-3 colocalize in oocytes. (D) GFP:EGG-4. (E) mCherry:EGG-3. (F) Merge. Scale bar represents 10 μm. (G-H) Deconvolved images showing the subcellular localization of (G) GFP:EGG-4 and (H) GFP:EGG-5. Scale bar represents 10 μm. (I-J) The dynamics of GFP:EGG-4.

GFP:EGG-4 is in green and mCherry-histone is in red. (I) At metaphase I, GFP:EGG-4 is at the cortex. (J) By anaphase I, GFP:EGG-4 is no longer at the cortex. Scale bar represents 10 μm.
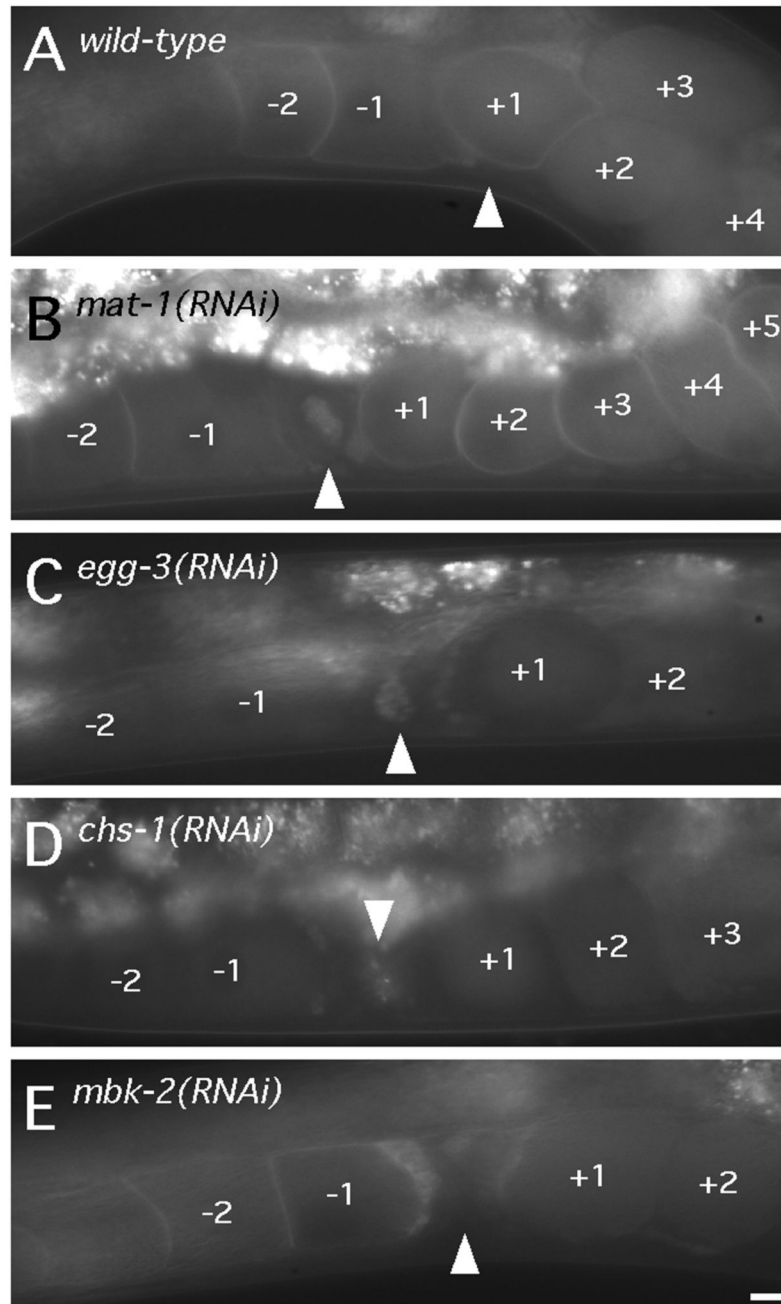
Figure 3.
Genetic Interactions. In all panels the arrowheads indicate the position of the spermatheca. Oocytes are to the left of the spermatheca and embryos are to the right. Oocytes are labeled with negative numbers towards the spermatheca and embryos are labeled with positive numbers from youngest (+1) to oldest. (A) In wild-type animals shown for comparison. GFP:EGG-4 accumulates at the cortex of developing oocytes but is redistributed at anaphase I. (B) The redistribution of GFP:EGG-4 to the cytoplasm is dependent on cell cycle progression. In *mat-1 (RNAi)* embryos GFP:EGG-4 remains associated with the cortex. (C) The localization of GFP:EGG-4 is dependent on EGG-3. In *egg-3(RNAi)* animals, no GFP:EGG-4 is seen accumulating at the cortex of developing oocytes or embryos. (D) The localization of

GFP:EGG-4 is dependent on CHS-1. In *chs-1(RNAi)* animals, no GFP:EGG-4 is seen accumulating at the cortex of developing oocytes. (E) The localization of GFP:EGG-4 is not dependent on MBK-2. In *mbk-2(RNAi)* animals, the localization of GFP:EGG-4 is indistinguishable from wild-type. Scale bar represents 10 μm.
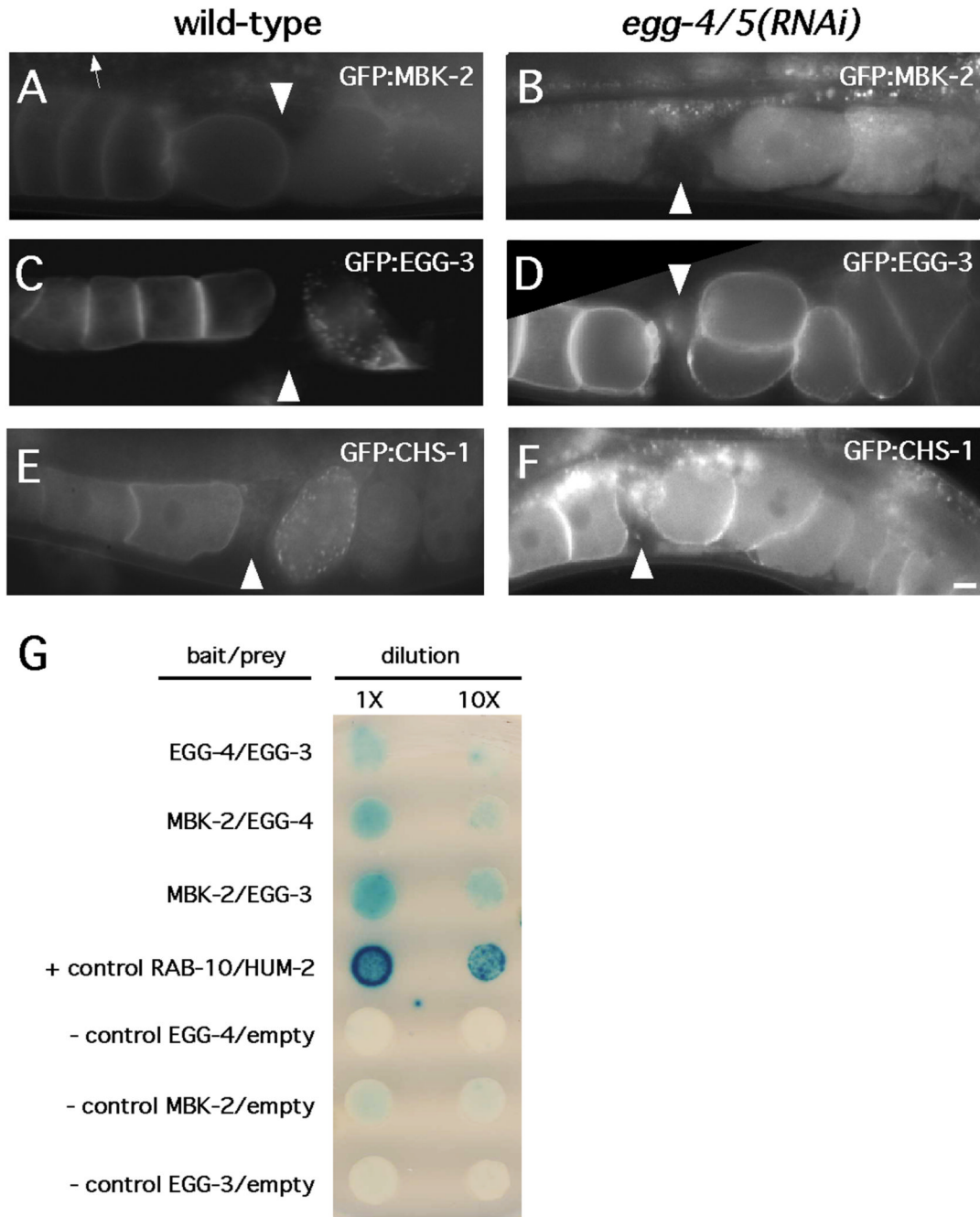
Figure 4.
EGG-4/5 Are Required For the Proper Localization of MBK-2, EGG-3 and CHS-1. (A-F) In all panels the arrowheads indicate the position of the spermatheca. Oocytes are to the left of the spermatheca and embryos are to the right. (A-B) Rather than accumulating at the cortex and forming cortical foci in embryos, GFP:MBK-2 is diffusely cytoplasmic. (C-D) In *egg-4/5 (RNAi)* animals, rather than forming foci at anaphase I, GFP:EGG-3 remains associated with the cortex despite meiotic progression. An identical experiment with the same result using mCherry:EGG-3 can be seen in Figure S3B. (E-F) In *egg-4/5(RNAi)* animals, rather than forming foci at anaphase I, GFP:CHS-1 remains associated with the cortex despite meiotic progression. Scale bar represents 10 μm. (G) Binding of EGG molecules in the yeast-two-

hybrid assay as indicated by stimulation of the blue X-gal color reaction. The RAB-10/HUM-2 combination serves as a strong positive control. Negative controls show that each molecule alone cannot activate transcription of the reporter. Equivalent numbers of cells were plated in each panel.