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MPK-1 ERK controls membrane organization in *C. elegans* oogenesis *via* a sex determination module

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Abstract

Tissues that generate specialized cell-types in a production line must coordinate developmental mechanisms with physiological demand, although how this occurs is largely unknown. In the *C. elegans* hermaphrodite, the developmental sex-determination cascade specifies gamete sex in the distal germline, while physiological sperm signaling activates MPK-1/ERK in the proximal germline to control plasma membrane biogenesis/organization during oogenesis. We discovered repeated utilization of a self-contained negative regulatory module, consisting of NOS-3 translational repressor, FEM-CUL-2 (E3 ubiquitin ligase) and TRA-1 (Gli transcriptional repressor), which acts both in sex-determination and in physiological demand control of oogenesis, coordinating these processes. In the distal germline, where MPK-1 is not activated, TRA-1 represses the male fate as NOS-3 functions in translational repression leading to inactivation of the FEM-CUL-2 ubiquitin ligase. In the proximal germline, sperm-dependent physiological MPK-1 activation results in phosphorylation-based inactivation of NOS-3, FEM-CUL-2 mediated degradation of TRA-1 and the promotion of membrane organization during oogenesis.

INTRODUCTION

A number of tissues are polarized production lines involved in the generation of highly specialized cell types. Examples include oogenesis within the gonad of many vertebrates and invertebrates and the crypt-villus axis of the mammalian gut (Simon and Gordon 1995; Ko et al. 1997). Oogenesis involves the constant production of oocytes (which are very large totipotent cells rich in cellular machinery, information molecules and nutrients) in a stepwise fashion for reproduction (Blumenfeld and Amit 1994; Matova and Cooley 2001). The generation of differentiated cells within such polarized tissue production-lines involves both

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developmental and physiological control mechanisms. Central to understanding polarized tissue function and homeostasis is uncovering the spatially integrated regulatory pathways that co-ordinate the developmental and physiological control of differentiated cell type production.

The germline of the adult *C. elegans* hermaphrodite gonad is a polarized assembly line for the production of oocytes (Figure 1; (Hirsh et al. 1976)). In the presence of sperm, major sperm protein (MSP) induces oocyte maturation/ovulation every ~23 min (McCarter et al. 1999; Miller et al. 2001) achieving continuous oocyte production, a process that requires the biogenesis and organization of plasma membranes and cytoplasmic constituents, regulating gene expression as well as progression of chromosomes through meiotic prophase. Conversely, in middle-aged adult hermaphrodites (which have exhausted their sperm) or mutant females that lack male germ cells, oocytes already produced are arrested in late meiotic prophase and oocyte production is dramatically downregulated.

The RTK-RAS-ERK pathway relays physiological and developmental extracellular signals through a conserved kinase cascade that results in phosphorylation and activation of the extracellular-signal regulated kinase (ERK) (Sundaram et al. 1996). Active ERK, in turn, controls biological processes through phosphorylation of substrate proteins (Chang and Karin 2001). The *C. elegans* ERK ortholog, MPK-1, controls at least seven different processes in hermaphrodite germline development, including membrane organization during oogenesis and progression of germ cell nuclei through pachytene of meiotic prophase (Lee et al. 2007). Each of the seven processes is mediated by multiple MPK-1 substrates, with additional substrates likely remaining to be identified (Arur et al. 2009). Activation of MPK-1 is (a) induced by the MSP signal and (b) spatially restricted to the medial and proximal regions of the oogenesis production line (Figure 1a, b) where MPK-1 dependent processes are executed (Miller et al. 2001; Lee et al. 2007).

An essential prerequisite for oogenesis is the developmental specification of oocytes / female fate. In the hermaphrodite germline, the male fate (sperm) is specified during larval development and female fate (oocyte) is specified throughout adulthood. Germline sexual fate in *C. elegans* is determined through an elaborate pathway involving more than 30 genes (Meyer 2005; Zarkower 2006; Ellis and Schedl 2007), part of which is shown in Figure 1c. Important for this study is a multi-step negative regulatory module (NOS-3/FEM-CUL-2/ TRA-1 module) necessary for oocyte fate specification. NOS-3, a homolog of the Nanos RNA binding protein, binds to FBF-1 and FBF-2 (FBF), two nearly identical Pumilio RNA binding protein homologs, which together repress translation of the *fem-3* mRNA (Zhang et al. 1997; Kraemer et al. 1999). FEM-3 along with the sex determination proteins FEM-1 and FEM-2, forms a subunit of the CUL-2-based E3 ubiquitin ligase complex (Starostina et al. 2007). As regulation of this CUL-2-based E3 ubiquitin ligase in the germline appears to be largely accomplished through FEM-3 levels and FEM-1, which is the substrate specificity subunit of the uniquitin ligase complex, we refer to it as the FEM-CUL-2 complex. The FEM-CUL-2 complex negatively regulates the TRA-1 Gli transcriptional repressor through ubiquitin mediated degradation (Starostina et al. 2007). In the adult hermaphrodite, NOS-3 and FBF repress the translation of *fem-3* mRNA resulting in the FEM-CUL-2 E3 ubiquitin ligase complex being inactive, leading to stabilization of nuclear TRA-1 Gli. For the specification of the oocyte fate, TRA-1 represses the transcription of *fog-1* and *fog-3*, genes that specify the sperm fate (Chen et al. 2000; Jin et al. 2001). Sexual fate is specified in the distal part of the germline, in the region of the germline stem cells (Crittenden et al. 2006; Hansen and Schedl 2006) determining the cell type generated within the gonadal production-line.

Here, we report an unexpected function of the NOS-3/FEM-CUL-2/TRA-1 module in regulation of plasma membrane organization during oogenesis. We demonstrate that MPK-1 promotes membrane organization by phosphorylation and inactivation of NOS-3, which through the negative regulatory module leads to degradation of nuclear TRA-1. We show that physiological control through MPK-1 and the NOS-3/FEM-CUL-2/TRA-1 module occurs in the medial and proximal germline where oocytes are being generated. By contrast, developmental control of sexual fate specification occurs in the distal germline. Thus, developmental and physiological mechanisms are integrated within the polarized germline tissue through spatially restricting the regulation and output of the NOS-3/FEM-CUL-2/TRA-1 module.

RESULTS

nos-3 functions downstream of *mpk-1* during oogenesis to regulate plasma membrane organization

The adult hermaphrodite germline displays a honeycomb pattern of pachytene germ cells on the surface of the gonadal tube, with an interior rachis functioning in cytoplasmic transport, followed proximally by a single file row of growing oocytes (Figure 1a). Plasma membranes of pachytene cells and oocytes are visualized by GFP::PH(PLCδ) (Figure 2; (Audhya et al. 2005), while the interior surfaces of pachytene cells and growing oocytes are visualized by anilin ANI-2 staining, where non-staining 'windows' represent the opening in pachytene cells for deposition of cytoplasm and the opening in growing oocytes for cytoplasm uptake (Figure S1 (Maddox et al. 2005; Wolke et al. 2007). MPK-1 signaling is required for the organization of plasma membranes and the cytoskeleton in pachytene germ cells and growing oocytes (Lee et al. 2007). In *mpk-1* null (*mpk-1(0)*) adults, the honeycomb organization of pachytene cells and the interior rachis is completely lost; pachytene arrested nuclei, largely devoid of plasma membranes, are present in clumps and surrounded by large nuclei free regions (Figures 2, S1). We serendipitously found that the cellular disorganization of *mpk-1(0)* was significantly suppressed by a null allele of *nos-3*, which encodes a Nanos RNA binding protein homolog. *nos-3(0);mpk-1(0)* germlines show restoration of plasma membranes around nuclei in a semi-honeycomb pattern on the surface of the gonadal tube (Figure 2c) and restoration of the interior rachis, with 'windows' observed connecting small and large pachytene cells to a common cytoplasm (Figure S1c). *nos-3(0)* was also found to suppress the plasma membrane/ interior rachis disorganization phenotypes of *lin-45* Raf null and *mek-2* Mek null mutants (data not shown), genes immediately upstream of MPK-1 in the ERK signaling cascade. The restoration of plasma membranes by loss of *nos-3* suggests that inappropriate NOS-3 activity is responsible, at least in part, for the membrane disorganization phenotype of *mpk-1(0)*. Thus, a simple hypothesis is that MPK-1 phosphorylates and inactivates NOS-3.

In *nos-3(0);mpk-1(0)* germlines, restoration of the plasma membranes is incomplete (e.g. some cells contain multiple nuclei) and other MPK-1 dependent processes (Lee et al. 2007; Arur et al. 2009) are not restored (e.g. nuclei remain arrested in pachytene; Figure 2c). We previously identified six distinct substrates that function to promote membrane organization (Arur et al. 2009), and it is likely that one or more of these genes function redundantly to regulate membrane organization, consistent with the partial suppression by *nos-3(0)* of membrane organization.

NOS-3 is an *in vitro* and *in vivo* substrate of MPK-1

Our genetic data raised the possibility that NOS-3 is an MPK-1 substrate. Characterized MPK-1 substrates harbor one or more identified ERK docking and phospho-acceptor sites (Jacobs et al. 1999; Fantz et al. 2001; Galanis et al. 2001; Barsyte-Lovejoy et al. 2002; Arur et al. 2009). We scanned the NOS-3 sequence and found one putative ERK docking site (a D-domain) and six potential phospho-acceptors (Figure 3a). Through *in vitro* kinase assays we determined that full length NOS-3 is a robust substrate of active ERK2 (Figures 3, S2). Additional *in vitro* kinase analysis of an N-terminal (aa 1-520) and a C-terminal fragment (aa 520-871, containing the D-domain and RNA binding domain) indicated that active ERK2 phosphorylates the C-terminal fragment. We then used site-specific mutagenesis of the C-terminal NOS-3 fragment, generating unphosphorylatable sites (Serine (S) or Threonine (T) to Alanine (A)) to identify S644 and T648 as the major phospho-acceptors utilized by ERK2 to phosphorylate NOS-3 *in vitro* (Figures 3b, S2a-e). Thus, our *in vitro* biochemical and genetic data together support the model that MPK-1 phosphorylates and inactivates NOS-3 in order to promote membrane organization during oogenesis.

To assess whether NOS-3 is phosphorylated *in vivo* in an *mpk-1* dependent manner, we generated antibodies that specifically recognize NOS-3 when phosphorylated on S644 and/ or T648 (pNOS-3, Methods, Supplement). Western blot analysis of worm lysates identified a single band of an appropriate size in wild-type but not *nos-3(0)*, demonstrating specificity (Figure 4a). In addition, no signal was present in *mpk-1(0)* lysates, demonstrating MPK-1 dependence (Figure 4a). In contrast, an antibody that specifically recognizes NOS-3 when neither S644 nor T648 are phosphorylated (non-pNOS-3, Methods) identifies the NOS-3 band in wild-type and *mpk-1(0)* whole worm lysates, but not in *nos-3(0)* lysates (Figure 4b). We conclude that NOS-3 is phosphorylated *in vivo* on S644 and/or T648 in an *mpk-1* dependent manner.

To determine the spatial pattern of pNOS-3 in the germline, we dissected and co-stained wild-type hermaphrodite gonads with the anti-pNOS-3 antibody and an antibody specific for the diphosphorylated active form of MPK-1 (dpMPK-1). dpMPK-1 displays a dynamic pattern of activation in the germline (Miller et al. 2001; Lee et al. 2007) being first detected in the proximal pachytene region, followed by partial down-regulation in the loop region, and then robust activation in late stage oocytes (Figures 1a, 4c). The pattern of pNOS-3 largely mirrors that of dpMPK-1 activation; pNOS-3 is first detected in proximal pachytene coincident with the appearance of dpMPK-1, remains present during the rest of oogenesis, with a slight decrease in accumulation in the final oocyte (Figure 4c). pNOS-3 is not detected in *nos-3(0)* germlines, demonstrating specificity of the antibody (Figure S3a). Furthermore, pNOS-3 is not detected in *mpk-1(0)* germlines or in regions of the germline where MPK-1 is not active, such as the distal germline of wild-type hermaphrodites, or in adult females that lack MSP signal and thus MPK-1 activation (Figures 4c, S3b, data not shown). Together, these results demonstrate that NOS-3 phosphorylation is dependent on MPK-1 activation. In contrast, the non-pNOS-3 antibody shows a reciprocal pattern of accumulation compared to pNOS-3. Non-pNOS-3 accumulates from the distal end through mid-pachytene in wild-type germlines (Figure 4d). In addition, non-pNOS-3 accumulates throughout in the *mpk-1(0)* germlines (Figure S3d) due to lack of MPK-1 activity. Non-pNOS-3 is not observed in proximal pachytene and the rest of oogenesis in wild-type germlines where we detect dpMPK-1 and pNOS-3. The combined accumulation of pNOS-3 and non-pNOS-3 is consistent with total NOS-3 being present cytoplasmically throughout the adult hermaphrodite germline (Figure 4e). These results indicate, within the limits of detection by antibody staining, that phosphorylation of NOS-3 may go to completion in proximal pachytene and the remainder of oogenesis containing active MPK-1.

The presence of active MPK-1 switches NOS-3(S644/T648) from unphosphorylated to phosphorylated as germ cells transition from mid to late-pachytene, the precise region where plasma membrane defects arise in *mpk-1(0)* mutant germlines. Together with the genetic analysis these results suggest that MPK-1 regulates the organization and integrity of plasma membranes during oogenesis by phosphorylating and inactivating NOS-3.

How does MPK-1 phosphorylation of NOS-3 regulate membrane organization?

As described in the Introduction, NOS-3 functions in the NOS-3/FEM-CUL-2/TRA-1 module as a part of the larger sex determination cascade in the regulation of hermaphrodite germline sexual fate, which occurs in the distal region of the tissue (Figure 1). However, phosphorylated NOS-3 along with active MPK-1 are found in more proximal germ cells whose sex is already specified and are now part of the oogenesis production line. We propose that in response to MSP/sperm signaling, MPK-1 in the proximal germline controls the activity of the NOS-3/FEM-CUL-2/TRA-1 module by phosphorylating NOS-3 to regulate a distinct essential function in germline development, plasma membrane organization during oogenesis (Figure 1). Our model predicts the following: 1) Physiological MSP signal promotes oogenesis and activates MPK-1, which in turn phosphorylates and inactivates NOS-3. Inactive NOS-3 can no longer repress *fem-3* mRNA translation, which would then allow for the formation of a functional FEM-CUL-2 E3 ubiquitin ligase complex that degrades nuclear TRA-1. The absence of TRA-1 Gli family repressor from proximal pachytene nuclei would then lead to transcription of genes that promote membrane organization during oogenesis. 2) In the absence of the MSP signal oocyte production is arrested, MPK-1 is inactive and unphosphorylated NOS-3 can now repress *fem-3* mRNA. The lack of *fem-3* mRNA translation results in a failure to form a functional FEM-CUL-2 E3 ubiquitin ligase complex, which stabilizes nuclear TRA-1 and the repression of genes that promote membrane organization. Below, we describe our evaluation of this model through examination of NOS-3-FBF-1 binding, analysis of FEM-3 and TRA-1 accumulation in *mpk-1(0)* and NOS-3/FEM-CUL-2/TRA-1 module mutant backgrounds, and testing these mutants for interactions with *mpk-1(0)* for membrane organization phenotype.

NOS-3 phospho-mimetic mutations reduce its ability to bind FBF-1 *in vitro*

fem-3 mRNA has been shown to be regulated by NOS-3 and FBF, where binding of NOS-3 to FBF-1 appears critical for the function of NOS-3 in translational repression (Zhang et al. 1997; Kraemer et al. 1999). The minimal region of NOS-3 required for binding to FBF-1 is aa 429-681 (Kraemer et al. 1999), which includes the residues phosphorylated by MPK-1 (S644 & T648). Since genetic analysis indicates that MPK-1 phosphorylation inactivates NOS-3, one possibility is that phosphorylated NOS-3 may be impaired in FBF-1 binding. To examine this possibility, we conducted *in vitro* pull-down assays with S^{35} labeled wild-type FBF-1 (produced by *in vitro* transcription/translation) combined with wild-type and mutant versions of bacterially generated recombinant FLAG::NOS-3. Wild-type and unphosphorylatable FLAG::NOS-3 (S644A,T684A) bind strongly to FBF-1 (Figure 5a). In contrast, the phospho-mimetic FLAG::NOS-3 mutant (S644, T648 to Glutamic Acid (E)) binds poorly to FBF-1 (Figure 5a), showing that mimicking the MPK-1 phosphorylation state (by a charged residue) reduces the ability of NOS-3 to bind FBF-1 *in vitro* and, by extension, supporting the model that NOS-3 phosphorylation reduces its binding to FBF-1 and thus its function in translational repression of *fem-3* mRNA *in vivo*.

Regulation of FEM-3 levels by MPK-1 and NOS-3

Genetic and molecular data indicate that NOS-3-FBF-1/-2 repress *fem-3* mRNA translation (Barton et al. 1987; Zhang et al. 1997; (Kraemer et al. 1999) however, there is no information on FEM-3 accumulation *in vivo*. The germline sex determination pathway predicts that FEM-3 should be low in the distal germline of adult hermaphrodites where the oocyte fate is specified, while in the model for physiological control of membrane organization during oogenesis FEM-3 should be high in the presence of active MPK-1 and pNOS-3 in the proximal end (Figure 1). Using anti-FEM-3 antibodies we find that FEM-3 is absent from the distal wild-type adult germline, a pattern consistent with its function in the sex determination cascade. In physiological control, FEM-3 accumulates in proximal pachytene and diplotene/diakinesis oocytes, mirroring the accumulation of dpMPK-1 and pNOS-3 (Figures 5b, S4). Furthermore, FEM-3 accumulation depends on *nos-3* and *mpk-1* activity. In *nos-3(0)*, FEM-3 accumulates throughout the distal germline, supporting a role for NOS-3 mediated repression of *fem-3* translation in this region (Figure 5c). In *mpk-1(0)* germlines, FEM-3 fails to accumulate, consistent with active NOS-3 mediated translational repression throughout. Finally, in the *nos-3(0);mpk-1(0)* double mutant germlines FEM-3 accumulates through the germline, consistent with NOS-3 functioning downstream to MPK-1 to regulate FEM-3 levels (Figure S4d). Importantly, no staining is observed in *fem-3(0)* germlines (Figure S4c). These results confirm the genetic interpretation that MPK-1 phosphorylation inactivates NOS-3, allowing *fem-3* mRNA to be translated during oogenesis.

Regulation of nuclear TRA-1 levels by MPK-1, NOS-3 and the FEM-CUL-2 Complex

FEM-3 functions as part of a CUL-2 E3 ubiquitin ligase complex to regulate TRA-1 accumulation during sex determination (Starostina et al. 2007). In the wild-type adult hermaphrodite high levels of nuclear TRA-1 are observed in the distal germline, consistent with TRA-1 promoting the oocyte fate (Starostina et al. 2007). However, for physiological control of membrane organization during oogenesis, our model predicts that when MPK-1 is active, TRA-1 levels will be low due to high levels of the FEM-CUL-2 E3 ubiquitin ligase complex, owing to the absence of NOS-3 mediated translational repression (Figure 1). This is exactly what we observe; in wild-type hermaphrodite germlines high distal nuclear TRA-1 levels fall dramatically as MPK-1 is activated (Figure 6). We next assayed *fem-3(0)*, *fem-1(0)* and *cul-2(RNAi)* germlines, from mated animals to activate MPK-1, and find that nuclear TRA-1 persists much further proximally until the end of pachytene, indicating that nuclear TRA-1 level is regulated by FEM-CUL-2 complex in this region (Figure 6, data not shown, also see below). Consistent with the activity of the FEM-CUL-2 E3 ubiquitin ligase complex being regulated by FEM-3 levels, we do not detect nuclear TRA-1 in the germline of *nos-3(0)* (which has high FEM-3 levels), while nuclear TRA-1 accumulates throughout in *mpk-1(0)* germlines (Figure 6).

NOS-3 phospho-mimetic mutations disrupt *fem-3* mRNA translational repression *in vivo*

The above results support the model that NOS-3 is inactivated by MPK-1 phosphorylation, leading to *fem-3* mRNA translation, which in turn targets nuclear TRA-1 degradation (Figure 1). As a direct test of the effect of NOS-3 phosphorylation status on regulation of FEM-3 and TRA-1, we generated transgenic worms carrying wild-type, phospho-mimetic and unphosphorylatable NOS-3 mutant proteins (Methods and Supplement). These transgenes were assayed for function in *nos-3(0)*, where FEM-3 accumulates throughout the germline while nuclear TRA-1 is not detected (Figures 5c, 6). The wild-type NOS-3 transgene rescues *nos-3(0)*, restoring *fem-3* translational repression and nuclear TRA-1 accumulation in the distal germline, with high FEM-3 accumulation and the absence of nuclear TRA-1 in proximal pachytene (Figures 7, S5). The phospho-mimetic NOS-3(S644/T648>E) mutant is predicted to be inactive throughout the germline, mimicking continuous phosphorylation by MPK-1, while the unphosphorylatable NOS-3(S644/T648>A) mutant is predicted to be constitutively active for *fem-3* translation repression, being refractory to downregulation by MPK-1. Consistent with these predictions, the phospho-mimetic NOS-3 mutant is unable to rescue *nos-3(0)*, failing to restore *fem-3* translational repression and nuclear TRA-1 accumulation, while the unphosphorylatable NOS-3 mutant displays *fem-3* translational repression and nuclear TRA-1 accumulation throughout the germline (Figures 7, S5). These data show that mimicking MPK-1 mediated phosphorylation by a charged residue disrupts NOS-3 function in translational repression of *fem-3* mRNA *in vivo*.

nos-3(0) adult hermaphrodites display only a very low level of germline masculinization (Kraemer et al. 1999; data not shown). This is surprising as we observe high cytoplasmic FEM-3 (Figures 5c, 7b, S4) and low nuclear TRA-1 levels (Figures 6, S5) in the distal germlines of *nos-3(0)* and *nos-3(0)* containing the phospho-mimetic *nos-3* mutant transgene. The reasons that *nos-3(0)* adult hermaphrodite germlines are not masculinized despite the high FEM-3 and low TRA-1 levels is unclear. One speculative possibility is that NOS-3 also acts as a translational repressor of a gene that promotes the oocyte fate, downstream or in parallel to TRA-1, which in *nos-3(0)* is overexpressed and counteracts the high FEM-3 / low TRA-1 levels for germline sex determination.

tra-1 and *fem-3* function in plasma membrane organization during oogenesis, downstream of *mpk-1*

While the protein accumulation patterns of FEM-3 and TRA-1 suggest a role for these proteins in oogenesis downstream to *mpk-1*, we directly investigated their function in this process. So far, we have shown that in *mpk-1(0)*, nuclear TRA-1 levels are high in pachytene cells because FEM-3 levels are low, while in *nos-3(0)* and *nos-3(0);mpk-1(0)* nuclear TRA-1 levels are low because of high FEM-3 (Figures 5b, 6, S7). These findings lead to two predictions. First, that suppression of the disorganized plasma membrane phenotype of *mpk-1(0)* by *nos-3(0)* is due to high FEM-3 accumulation (Figure S4) and thus high FEM/CUL-2 E3 ubiquitin ligase activity. Second, that the *mpk-1(0)* disorganized plasma membrane phenotype is caused, in part, by high nuclear TRA-1 in pachytene cells. If the first prediction is correct, then loss of FEM-CUL-2 E3 ubiquitin ligase activity should reverse the suppression of *mpk-1(0)* by *nos-3(0)*. We find that reduction or elimination of *fem-1*, *fem-3* or *cul-2* activity reverses the suppression of *mpk-1(0)* by *nos-3(0)* (Table 1, lines 12-14). We note that *fem-1(0)*, *fem-3(0)* and *cul-2(RNAi)* alone do not show a significant disorganized membrane phenotype; this is likely because there are MPK-1 substrates, in addition to NOS-3, that function in plasma membrane organization (Arur et al. 2009). If the second prediction is correct, that high nuclear TRA-1 contributes to the disorganized plasma membrane phenotype, then loss of *tra-1* activity should suppress the *mpk-1(0)* membrane disorganization phenotype. However, *tra-1* loss-of-function results in masculinization of the soma, precluding analysis of oogenesis phenotypes within the hermaphrodite gonad. We therefore performed *tra-1* RNAi in *mpk-1(0)* that is also mutant for *rrf-1*, which causes RNAi to only function in the germline. *rrf-1* encodes an RNA-dependent RNA polymerase necessary for RNAi in the soma but not the germline (Sijen et al. 2001). In such somatically female animals, the plasma membrane disorganization phenotype of *mpk-1(0)* was significantly suppressed following *tra-1* RNAi (Table 1, line 9), similar to the *nos-3(0);mpk-1(0)* mutant (Figure 2). Analysis of additional mutant combinations (Table 1) indicates (a) that NOS-3 functions solely through TRA-1 (the extent of suppression of *mpk-1(0)* membrane disorganization is equivalent in doubles with *nos-3(0)* or *tra-1(RNAi)* or in the triple with *nos-3(0)* and *tra-1(RNAi)*, lines 8, 9 and 15) and (b) that FEM-3 acts solely on TRA-1 (restoration of suppression in *nos-3(0);mpk-1(0);fem-3(0)*, following *tra-1(RNAi)*, lines 12 and 17) for plasma membrane organization during oogenesis. The genetic and molecular results together demonstrate that MPK-1, through phosphorylation of NOS-3, regulates TRA-1 accumulation in the control of membrane organization during oogenesis.

In germline sex determination, TRA-1 promotes the female fate through transcriptional repression of *fog-1* and *fog-3*, genes that repress the sperm fate (Figure 1). In principle, membrane organization could have been mediated by *fog-1* and *fog-3*, where the absence of TRA-1 in proximal pachytene cells would lead to their expression. However, *fog-1* and *fog-3* are not expressed in the oogenic germline (Chen and Ellis 2000; Thompson et al.

2005). Thus, TRA-1 likely regulates currently uncharacterized gene(s) for membrane organization during oogenesis (see Discussion).

Physiological regulation of the NOS-3/FEM-CUL-2/TRA-1 module for membrane organization

The adult hermaphrodite germline has two physiological states: in the presence of sperm and the MSP signal, MPK-1 is activated and the production line generates oocytes while in the absence of sperm and the MSP signal, MPK-1 is not activated, oocytes are arrested in late prophase, and the production line is dramatically slowed. To assess the role of the absence of MSP signaling on the control of FEM-3 and TRA-1 levels, we examined *fog-1(0)* females and middle-aged adult hermaphrodites that had exhausted their self sperm. As predicted by the model when oogenesis is arrested (Figure 1), we observed nuclear TRA-1 throughout pachytene while FEM-3, pNOS-3 and dpMPK-1 levels are low (Figure S6, data not shown). Mating restores the pattern to that observed in wild-type adult hermaphrodites; low nuclear TRA-1 and high FEM-3, pNOS-3 and dpMPK-1 in proximal pachytene. (In contrast, in *fem-1(0)* or *fem-3(0)* mutants the observed pattern of TRA-1 accumulation, high nuclear throughout, is not altered in the presence or absence of sperm and activated MPK-1 because the FEM-CUL-2 complex is defective.) These results indicate that under conditions where the generation of oocytes is limited (no sperm), nuclear TRA-1 in proximal pachytene represses the transcription of genes that promote membrane organization. By contrast, in adult hermaphrodites with sperm and where oocytes are actively produced, TRA-1 is degraded, permitting genes that promote membrane organization to be transcribed.

Is MPK-1 regulation of the NOS-3/FEM-CUL-2/TRA-1 module for membrane organization evolutionarily conserved?

In the sibling species *C. briggsae*, *fem-1, fem-3* and *tra-1* orthologs have conserved functions in somatic sex determination. However, for germline sex determination *Cb-fem-1* and *Cb-fem-3* are not required. For example, *Cb-fem-3(0)* animals are self-fertile hermaphrodites (Haag et al. 2002; Hill et al. 2006). To begin to examine if membrane organization during oogenesis in *C. briggsae* is controlled by MPK-1 and the NOS-3/FEM-CUL-2/TRA-1 module we examined *Cb-mpk-1* function in germline development and examined dpMPK-1, pNOS-3 and TRA-1 accumulation in the germline using cross-species reacting antibodies.

RNAi of *Cb-mpk-1* results in a spectrum of adult germline phenotypes that are very similar to those observed in *C. elegans mpk-1* loss-of-function and null mutants (Table S1, Lee et al. 2007). Specifically, the honeycomb organization of pachytene cells on the surface and the interior rachis are completely lost with clumped nuclei arrested in pachytene (Figures S8, S9). Thus MPK-1 orthologs regulate membrane organization, as well as the progression through pachytene, during oogenesis in both *C. briggsae* and *C. elegans*.

Staining for dpMPK-1, pNOS-3 and TRA-1 in adult *C. briggsae* germlines gives essentially identical patterns as observed for the *C. elegans* orthologs (Figures S8, S9). Cb-pNOS-3 and Cb-dpMPK-1 are coincident, rising to a high level in proximal pachytene and extending through diakinesis oocytes, while nuclear Cb-TRA-1 accumulates from the distal end to mid-pachytene and then is not detected in proximal pachytene nuclei. To determine if the absence of TRA-1 from proximal pachytene was due to FEM-CUL-2 E3 ubiquitin ligase activity, we examined Cb-TRA-1 in the *Cb-fem-3* null mutant *nm63* (Hill et al. 2006). We find that Cb-TRA-1 is observed in proximal pachytene nuclei in *Cb-fem-3(0)* hermaphrodites (Figure S8), analogous to what is observed in *C. elegans fem-1(0)* or *fem-3(0)* mutants. These results support the idea that MPK-1 regulates the NOS-3/FEM-CUL-2/TRA-1 module to control membrane organization during oogenesis *in C. briggsae* as well as in *C. elegans*. It is currently unclear if the ancestor to *C. elegans* acquired *fem* function for germline sex determination, in which case the role the NOS-3/FEM-CUL-2/TRA-1 module in membrane organization during oogenesis is ancestral, or if the ancestor to *C. briggsae* lost *fem* function for germline sex determination. However, since these results indicate that *Cb-fem-3* regulates Cb-TRA-1 stability in proximal pachytene, it is not obvious why similar *Cb-fem-3* regulation of Cb-TRA-1 does not also have a function in *C. briggsae* sex determination.

DISCUSSION

Here we report an unexpected role for a module within the *C. elegans* sex determination cascade, NOS-3/FEM-CUL-2/TRA-1, to regulate plasma membrane organization during oogenesis. MPK-1 ERK regulates this multi-step negative regulatory module by phosphorylating and inactivating NOS-3 function which allows translation of *fem-3* mRNA. FEM-3 then participates in the CUL-2 E3 ubiquitin ligase complex to help degrade nuclear TRA-1 Gli, resulting in the transcription of genes that promote membrane organization during oogenesis (Figure 1). The function of the NOS-3/FEM-CUL-2/TRA-1 module in membrane organization is spatially distinct from its role in germline sex determination and has different transcriptional targets for output. Furthermore, it is likely that the MPK-1 regulation of this module for membrane organization is conserved in the related nematode *C. briggsae*, even though the function of the module in germline sex determination is not (Haag et al. 2002; Hill et al. 2006). Below we discuss our findings.

Regulation of NOS-3 function by MPK-1 ERK phosphorylation

Reversible phosphorylation is an important mechanism for regulating protein function. Examples of ERK phosphorylation affecting substrate function include changing protein activity (e.g. activating TIF-1A, (Zhao et al. 2003)), or protein function (e.g. converting LIN-1 Ets from a repressor to a transcriptional activator, (Leight et al. 2005)) and changing subcellular localization (EPLIN translocating from stress fibers to peripheral ruffles, (Han et al. 2007)). We show that MPK-1 phosphorylation of NOS-3 inactivates its function in *fem-3* translational repression based on three lines of evidence: 1) genetic suppression of *mpk-1(0)* by *nos-3(0)*, 2) failure of a phospho-mimetic NOS-3 version to translationally repress *fem-3* mRNA *in vivo*, and 3) substantial reduction of *in vitro* binding of a phospho-mimetic version of NOS-3 to co-factor FBF-1, which binds the *fem-3* mRNA.

Phosphorylation and inactivation of NOS-3 is restricted to the region of the oogenesis production line where *mpk-1* dependent membrane organization is occurring based on staining with phospho-specific antibodies. Phosphorylation did not noticeably alter the cytoplasmic subcellular localization of NOS-3. We found, within the limits of sensitivity of antibody staining, that all detectable NOS-3 above background is converted to the phosphorylated form in regions of the germline containing active MPK-1, dividing the germline into zones of unphosphorylated and phosphorylated NOS-3. This conversion of NOS-3 to the inactive pNOS-3 form is likely important to relieve *fem-3* translational repression and thus producing sufficient FEM-3 to efficiently degrade nuclear TRA-1.

TRA-1 and regulation of oogenesis membrane organization genes

During the physiological control of oocyte production, we propose that TRA-1 represses the transcription of genes that promote membrane organization under conditions where the production line is slowed down (absence of sperm and loss of MPK-1 activation), but is degraded to allow transcription of these genes when the production line is active (presence of sperm and MPK-1 activation). *fog-1* and *fog-3*, two genes whose germline expression is repressed by TRA-1, for sex determination, are unlikely to function as TRA-1 transcriptional targets for membrane organization as they are not expressed in the oogeneic germline (Chen and Ellis 2000; Lamont et al. 2004). As a preliminary approach to assess genes that may be repressed by TRA-1 to modulate membrane organization during oogenesis we scanned two sets of potential target genes for the presence of the TRA-1 DNA binding sites (Methods). First, we examined a set of genes whose germline expression is MPK-1 responsive (Leacock and Reinke 2006). This analysis identified *meg-1* and *meg-2* as containing the TRA-1 site in their 5′ intergenic regions. *meg-1* and *-2* encode P-granule components required maternally for fertility (Leacock and Reinke 2008) but are not known to function in membrane organization; however, these genes have not been examined in the appropriate sensitized backgrounds to overcome redundancy among genes that function downstream of MPK-1 in membrane organization (Arur et al. 2009). Importantly, additional MPK-1 responsive genes, which may contain the TRA-1 binding sequence, likely remain to be identified as the temperature sensitive *mpk-1* mutant used in the original expression study (Leacock and Reinke 2006) is not fully mutant at the restrictive temperature and not fully wild-type at the permissive temperature (Lee et al. 2007) thus reducing the sensitivity of the microarray analysis. This finding, although preliminary, supports the proposal that the MPK-1 transcriptional output is, in part, through indirect regulation of TRA-1 via the action of NOS-3/FEM-CUL-2. We next asked whether genes that encode MPK-1 substrates known to function in membrane organization during oogenesis (Arur et al. 2009) contain TRA-1 binding sites. These genes did not contain TRA-1 binding sites suggesting that are not direct transcriptional targets of TRA-1, although they could be indirect TRA-1 transcriptional targets.

NOS-3 may function with other substrates in MPK-1 mediated control of membrane organization

We previously found that the seven MPK-1 controlled biological processes are mediated by multiple substrates and that different processes are executed by distinct sets of substrates (Arur et al. 2009). The membrane disorganization phenotype of *mpk-1(0)* is only partially suppressed by *nos-3(0)*. We identified six distinct substrates of MPK-1 that function to promote membrane organization of pachytene cells (Arur et al. 2009), suggesting that there may be redundancy among a number of genes governing membrane organization during oogenesis downstream to MPK-1. Furthermore, since TRA-1 regulation does not appear to be acting directly through known membrane organization MPK-1 substrates as transcriptional targets (see above), the NOS-3/FEM-CUL-2/TRA-1 module is likely acting through a distinct gene(s), in parallel to the known substrates, to control membrane organization. Additionally, since *nos-3(0)* (and *tra-1* RNAi) does not suppress other phenotypes in *mpk-1(0)* (e.g. progression of nuclei through pachytene), function of NOS-3, and thus the NOS-3/FEM-CUL-2/TRA-1 module, may be limited to membrane organization.

Integration of developmental and physiological control mechanisms in tissue function

The NOS-3/FEM-CUL-2/TRA-1 module is a multi-step negative regulatory cassette that is utilized in the *C. elegans* hermaphrodite germline to integrate developmental and physiological mechanisms for function during oocyte production. At the distal end, the module acts in developmental control as part of the sex determination pathway to specify the oocyte fate, when NOS-3 and TRA-1 are active. The generation of oocytes, in the medial and proximal parts of the germline tissue, is controlled through a physiological mechanism: when the oogenesis production line is active in the presence of the MSP sperm signal, active MPK-1 ERK phosphorylates and inactivates NOS-3, leading to inactivation/degradation of TRA-1 and expression of genes that promote membrane organization necessary for generating oocytes. When the oogenesis production line is inactive in the absence of the MSP sperm signal, MPK-1 is inactive, NOS-3 and TRA-1 are active leading to repression of oogenesis membrane organization genes as part of downregulation of oocyte production. Spatial control of the NOS-3/FEM-CUL-2/TRA-1 module within the germline occurs at two levels. First, the physiological control mechanism, MPK-1 activation, is restricted to the medial/proximal proximal region and does not occur in the distal germline where the oocyte sexual fate is specified. Second, the transcriptional output from TRA-1 is distinct, for sex determination it is repression of *fog-1* and *fog-3* while for oogenesis it is repression of as yet to be identified membrane organization genes.

METHODS

Germline dissection and antibody staining

Wild-type and each of the corresponding mutant germlines were dissected from animals that were synchronized at L4/ adult molt and allowed to develop for 24 hours, except in some cases where deviation from this were dictated by the experimental design. Dissected germlines were then processed as described before (Lee and Schedl 2001; Lee et al. 2007; Arur et al. 2009) and treated with the respective primary antibody at 4°C overnight and secondary for 2 hours at room temperature.

Image capture and processing

All images were taken on a Zeiss compound microscope using AxioPlan 2.0 imaging software and Hamamatsu camera. Each dissected and stained gonad was captured as a montage, with overlapping cell boundaries and processed as described earlier (Lee and Schedl 2001; Lee et al. 2007; Arur et al. 2009).

In vitro kinase analysis using activated murine ERK2

Purified proteins were dialyzed into the kinase assay buffer for 2-4 hours (50 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM DTT, 1 mM EGTA, 0.01 % Brij 35 at pH 7.5). Purified ERK2 kinase was purchased from NEB (cat # P6080L) and reactions carried out as described previously (Jacobs et al. 1998; Jacobs et al. 1999; Fantz et al. 2001; Arur et al. 2009). Kinetic analysis was performed using seven concentrations of protein ranging from 0.2μM to 2μM, with results an average of 5 separate experiments. Km and Vmax were calculated from the Lineweaver-Burke plot and in each case the data closely approximated a straight line (Figure S2). Vmax was determined by assaying the amount of total phosphate incorporation per unit time/ enzyme amount (usually calculated to around 0.03pm). Total phosphate incorporation was calculated by using the measured CPM, the specific activity of P^{32}, and the reaction volume. Relative acceptor ratio is Vmax/ Km and was normalized by setting value for myelin basic protein to 1.0.

NOS-3, FBF-1 GST binding

Wild-type and mutant Flag-tagged NOS-3 were generated as described in the supplement. Purified FLAG::NOS-3 (wt / mutant) was then bound to Flag M2 beads (Sigma) for 1 hour at room temperature in binding buffer (20mM Tris, pH 7.5, 500 mM NaCl, 0.5% NP 40). The beads were then washed extensively in wash buffer (10mM Tris 7.5, 150mM NaCl). FBF-1 was generated in parallel using Ambion *in vitro* transcription translation coupled system with S^{35} labeled methionine. The S^{35} labeled FBF-1 extract was then combined with FLAG::NOS-3 beads for 2 hours at room temperature and the extensively washed in the wash buffer. Each FBF-1, FLAG::NOS-3 tube also contained 100 μM *fem-3* 3′ UTR (supplement). The bound protein, were then eluted with 3x Flag peptide as described earlier (Lee and Schedl, 2001) and run out on a 10% SDS-PAGE gel, dried and exposed to the autoradiograph. Quantitation of binding was determined by densitometry of the autoradiograph.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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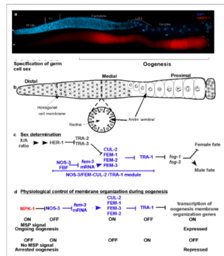

Figure 1. Summary of adult hermaphrodite germline development and the functions of the NOS-3/FEM-CUL-2/TRA-1 regulatory module

(a) Dissected adult hermaphrodite gonad arm stained for activated MPK-1 (dpMPK-1, red) and germ cell nuclear morphology (DAPI, blue). For all Figures, distal (marked with *) is to the left, proximal to the right. In the distal region, germ cell sexual fate is specified (male/sperm or female/oocyte) while in the medial and proximal germline, activated MPK-1 is necessary for physiological control of the oogenesis production-line, including membrane organization. (b) Schematic representation of the adult hermaphrodite germline, showing a surface view of hexagonally organized cells and their membranes from the distal tip through end pachytene (medial-to-loop), and an internal view of growing diplotene to diakinesis oocytes in the proximal region. Cross-section show cells on the surface of the gonadal tube, each with an interior opening to the Rachis that provides cytoplasm to growing oocytes; the interior surface can be visualized by anilin staining with non-staining windows representing the interior openings. (c) Part of the pathway for germline sex determination is shown, with the NOS-3/FEM-CUL-2/TRA-1 module highlighted in blue. (d) Model for physiological control of membrane organization during oogenesis. The NOS-3/FEM-CUL-2/TRA-1 module is regulated by MPK-1 ERK, active (ON) during ongoing oogenesis in the presence of sperm/MSP signaling and inactive (OFF) in the absence of sperm/MSP signaling when oogenesis is arrested. (See also Figure S6, S8, and S9; Table S1)

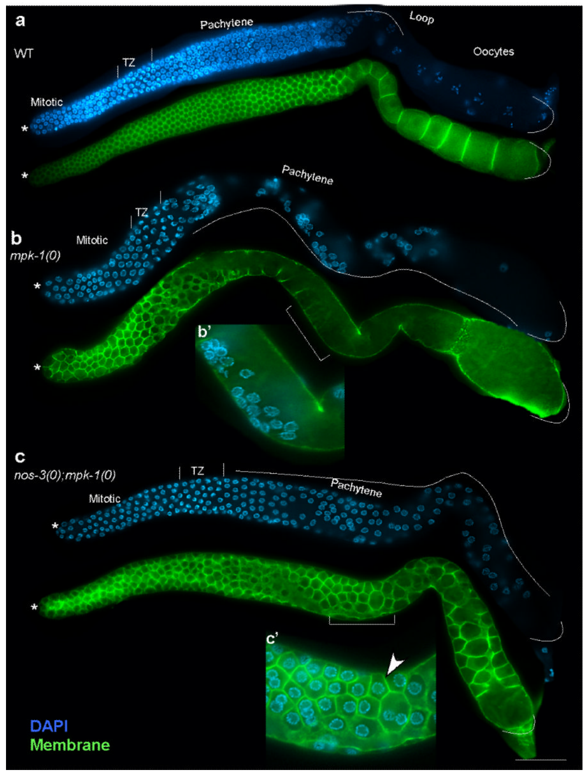

Figure 2. NOS-3 partially restores membrane organization of oogeneic pachytene cells in *mpk-1* null adult hermaphrodites

Dissected gonads from strains expressing GFP::PH(PLCd), stained with anti-GFP, to visualize cell membranes (green) and DAPI to show nuclear morphology (blue). (a) Wild-type adult hermaphrodite germline, with hexagonally arranged germ cells on the surface from the distal end to the loop and growing oocytes proximally. (b) *mpk-1(ga117)* null mutant germline with massive disruption of membrane organization in the region of pachytene arrested nuclei. (c) *nos-3(oz231); mpk-1(ga117)* germline displaying substantial restoration of cell membranes around pachytene arrested nuclei. Insets (b') pachytene arrested nuclei lacking organized membranes; (c') organized membranes around individual or multiple pachytene nuclei. Scale bar, 20 microns, for all figures. (See also Figure S1)

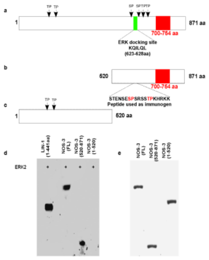

Figure 3. NOS-3 is phosphorylated *in vitro* by active murine ERK2 at Serine 644 and Threonine 648

(a-c) A schematic protein domain structure of NOS-3. Green box marks the ERK docking site and the red box marks the Nanos domain. The arrows indicate the phospho-acceptors. (b) and (c) indicate the two truncated versions of the protein that were tested in *in vitro* kinase assays. (d) Autoradiograph of kinase assays with full length and the two truncated versions of NOS-3 reveals that the C-terminal domain of NOS-3 is phosphorylated by active murine ERK2 *in vitro*. (e) An anti-HIS western blot of the proteins tested in (d). (See also Figure S2)

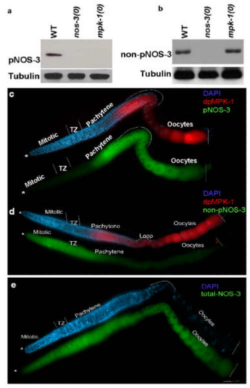

Figure 4. NOS-3 is phosphorylated *in vivo* at S644 and T648 in an *mpk-1* dependent manner

(a) Western blot analysis of pNOS-3 from lysates of wild-type, *mpk-1(ga117)* and *nos-3(oz231)* null mutant worms reveals that NOS-3 is phosphorylated on S644 and/or T648 as detected by the phospho-epitope specific antibody; in wild-type a band at about 90kDa is detected, but not in *nos-3(0)*, showing specificity for NOS-3, and not in *mpk-1(0)*, indicating *mpk-1* dependence. (b) Western blot analysis of non-pNOS-3 on lysates from wild-type, *mpk-1(0)* and *nos-3(0)* mutant worms shows that unphosphorylated NOS-3 accumulates in *mpk-1(0)* and wild-type worms at about 90kDa. The absence of signal in *nos-3(0)* indicates specificity of the non-pNOS-3 antibody. (c), (d) and (e) Dissected gonads from wild-type adult hermaphrodites stained for pNOS-3 (c, green), non-p-NOS-3 (d, green) and total NOS-3 (e, green), dpMPK-1 (red) and nuclear morphology (blue). pNOS-3, non-pNOS-3 and total NOS-3 are largely cytoplasmically localized. (c) pNOS-3 accumulation mimics dpMPK-1, detected from mid-pachytene through the end of oogenesis. (d) non-pNOS-3 (green) accumulates in a manner reciprocal to dpMPK-1 (red) and pNOS-3, detected from the distal tip through mid-pachytene. Non-pNOS-3 was not observed in proximal pachytene and in diplotene/ diakinesis oocytes, where residual staining was equivalent to the background observed in *nos-3(0)* (Figure S3c). (e) Total NOS-3 (green) shows homogeneous accumulation throughout the oogenic germline. (See also Figure S3)

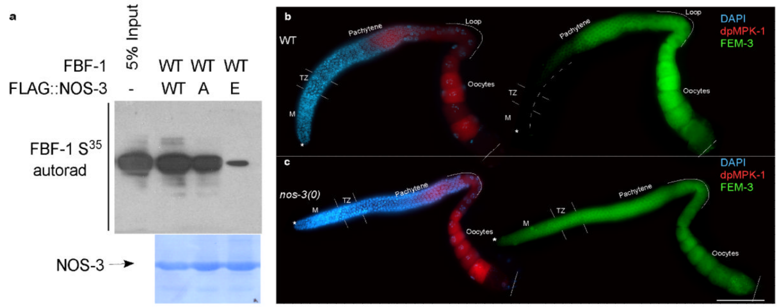

Figure 5. Phospho-mimetic NOS-3 mutant Ser644Glu and Thr648Glu reduces FBF binding *in vitro* and FEM-3 accumulates during oogenesis and is repressed in the distal germline by NOS-3

(a) Autoradiograph (top) showing binding analysis of recombinant Flag-tagged wild-type, S644/T648 sites mutated to alanine (phospho-null) or S644/T648 sites mutated to glutamic acid (phospho-mimetic) NOS-3 with S^{35} labeled FBF-1 (Supplemental Methods). Wild-type and phospho-null NOS-3 bind FBF-1 very robustly (lanes 2 and 3), while phospho-mimetic NOS-3 results in a reduction of FBF-1 binding (lane 4) by 14 fold compared to wild-type. In replicate experiments, FBF-1 binding was reduced 17 fold or great than 20 fold in two additional trials (data not shown). Photo (bottom) shows Coommassie Blue staining of the input recombinant NOS-3 proteins used. (b and c) Adult hermaphrodite gonads stained for FEM-3 (green) and dpMPK-1 (red). (b) In wild-type, FEM-3, which is largely cytoplasmic, accumulates from mid-pachytene through the end of oogenesis mimicking that of dpMPK-1. FEM-3 is not detected in the distal germline. (c) In *nos-3(oz231)*, FEM-3 accumulates throughout the germline, consistent with NOS-3 functioning in translational repression of *fem-3* in the distal germline of wild-type. Dashed line marks the region of *fem-3* translation repression by NOS-3. (See also Figure S4)

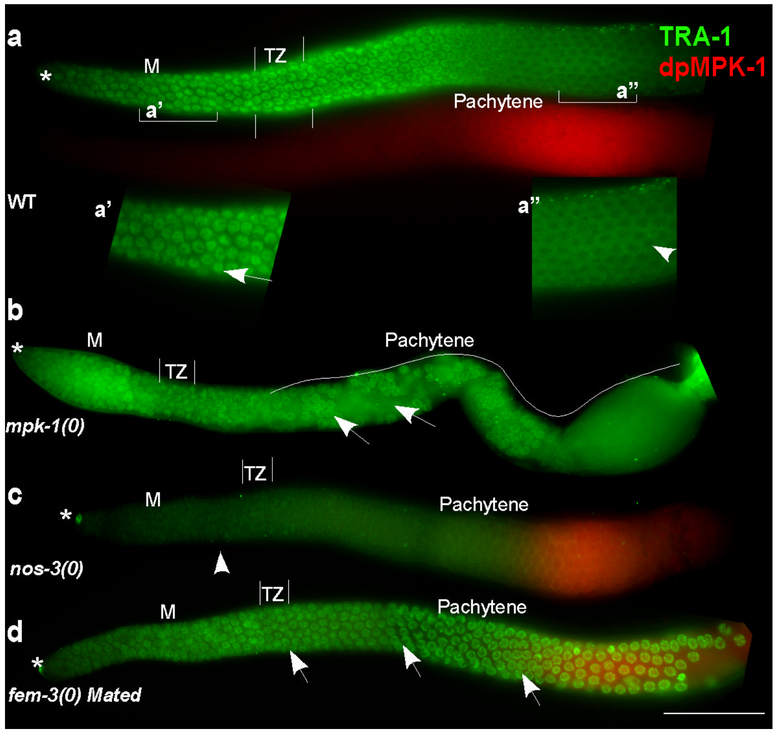

Figure 6. TRA-1 nuclear accumulation during MPK-1 activation

Dissected adult hermaphrodite gonads stained for TRA-1 (green) and dpMPK-1 (red). (a) In wild-type, TRA-1 is detected in nuclei (inset a', arrow) from the distal tip until mid-pachytene, a region that lacks dpMPK-1. From mid-pachytene through proximal pachytene, a region containing high dpMPK-1, nuclear TRA-1 is not detected (inset a", arrowhead). (b) In *mpk-1(ga117)*, nuclear TRA-1 (arrows) is detected throughout the germline including all proximal pachytene arrested nuclei. (c) In *nos-3(oz231)* nuclear TRA-1 is not detected from the distal tip through the end of pachytene, although the pattern of MPK-1 activation is normal. (d) In *fem-3(e1996)* null mated with wild-type males, resulting in the normal pattern of MPK-1 activation and ongoing oogenesis, nuclear TRA-1 (arrows) is observed throughout, including proximal pachytene containing dpMPK-1. (See also Figure S5)

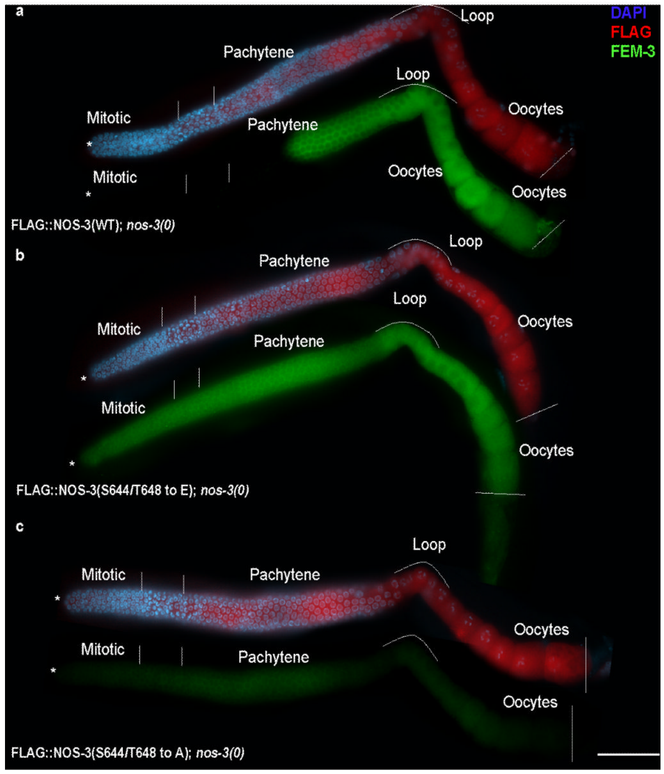

Figure 7. Phospho-mimetic NOS-3 mutant Ser644Glu and Thr648Glu is unable to mediate translational repression of *fem-3* mRNA *in vivo*

Dissected gonads from *nos-3(oz231)* strains containing wild-type or mutant NOS-3 low-copy integrated transgenes stained for FEM-3 (green), FLAG-tagged NOS-3 (red) and nuclear morphology (blue)(also see Figure S5). (a) The wild-type NOS-3 transgene (*ozIs27*) rescues the *nos-3(0)* background restoring distal repression of *fem-3* mRNA translation. (b) The NOS-3 transgene containing the phospho-mimetic site changes (S644/T648>E; *ozIs29*) results in FEM-3 accumulation throughout the germline, indicating that phospho-mimetic NOS-3 is unable to translationally repress *fem-3* mRNA. (c) The NOS-3 transgene containing the non-phosphorylatable site changes (S644/T438>A; *ozIs28*) fails to accumulate FEM-3 throughout the germline, consistent with continuous translational repression of *fem-3* mRNA by this mutant form of NOS-3. For all three transgenes, driven by the *nos-3* promoter, FLAG::NOS-3 accumulates throughout the germline similar to endogenous NOS-3 and the level of protein accumulation is similar (Figure 4e, data not shown).

Table 1

nos-3, *fem-3*, and *tra-1* Function Genetically Downstream to *mpk-1* to Regulate Plasma Membrane Morphogenesis during Oogenesis

Line Number	Genotype	% Germlines with *mpk-1(0)* Membrane Disorganization	n
1	N2	WT[a]	80
2	*mpk-1(ga117)*	100[b]	80
3	*rrf-1(pk1417); mpk1(ga117)*	100[b]	80
4	*nos-3(oz231)*	WT[a]	80
5	*fem-3(e1996)*	WT[c]	80
6	*fem-1(e1991)*	WT[c]	80
7	*cul-2(RNAi)*	WT[c,d]	27
8	*nos-3(oz231); mpk-1(ga117)*	0[e]	80
9	*rrf-1(pk1417); mpk1(ga117); tra-1(RNAi)*	0[e]	11[f]
10	*mpk-1(ga1117);fem-3(e1996)*	100[b]	80
11	*mpk-1(ga117);fem-1(e1991)*	100[b]	75
12	*nos-3(oz231); mpk-1(ga117); fem-3(e1996)*	100[g]	80
13	*nos-3(oz231);mpk-1(ga117);fem-1(e1991)*	100[g]	67
14	*nos-3(oz231);mpk-1(ga117);cul-2RNAi*	100[g]	54
15	*rrf-1(pk1417); nos-3(oz231); mpk-1(ga117); tra-1(RNAi)*	0[h]	43[f]
16	*rrf-1(pk1417); nos-3(oz231); mpk-1(ga117); fem-3(e1996)*	100[g]	80
17	*rrf-1(pk1417); nos-3(oz231); mpk-1(ga117); fem-3(e1996); tra-1(RNAi)*	0[i]	13[f]

[a] Wild-type (wt) adult hermaphrodite germline pattern of sexual fate specification and plasma membrane organization during oogenesis (see Figures 1A, 1B, and 2A; Figure S1A).

[b] Germline displays the adult mpk-1(ga117) null severe disorganization of plasma membranes and pachytene arrest phenotype (see Figure 2B) (Lee et al., 2007).

[c] Germline is feminized, failing to specify the male (sperm) fate but otherwise displays normal organization of membranes during oogenesis.

[d] Additional phenotypes are observed including cell cycle arrest in the distal proliferative zone.

[e] Suppression of the mpk-1(ga117) null severe membrane disorganization phenotype, but not the pachytene arrest phenotype (see Figure 2C; Figure S1C).

[f] tra-1 RNAi, even following injection of dsRNA, is inefficient. Only those germlines showing strong reduction in anti-TRA-1 antibody staining are included.

[g] Suppression of mpk-1(ga117); nos-3(oz231) is reversed (suppression of the suppression) back to the mpk-1(ga117) severe membrane disorganization phenotype.

[h]The extent of suppression of mpk-1(ga117) null is equivalent in lines 8, 9, and 15, indicating that NOS-3 is acting solely through TRA-1.

[i]tra-1(RNAi), in line 17, suppresses the disorganized plasma membrane phenotype of mpk-1(ga117); nos-3(oz231) germlines whose suppression has been reversed by fem-3(e1996) null. This indicates that fem-3 acts upstream and through tra-1 to promote membrane organization.

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