The Germline-Specific Factor OEF-1 Facilitates Coordinated Progression Through Germ Cell Development in *Caenorhabditis elegans*

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ABSTRACT The purpose of germ cells is to ensure the faithful transmission of genetic material to the next generation. To develop into mature gametes, germ cells must pass through cell cycle checkpoints while maintaining totipotency and genomic integrity. How germ cells coordinate developmental events while simultaneously protecting their unique fate is not well understood. Here, we characterize a novel nuclear protein, Oocyte-Excluded Factor-1 (OEF-1), with highly specific germline expression in *Caenorhabditis elegans*. OEF-1 is initially detected early in embryogenesis and is expressed in the nuclei of all germ cells during larval stages. In adults, OEF-1 expression abruptly decreases just prior to oocyte differentiation. In *oef-1* mutants, the developmental progression of germ cells is accelerated, resulting in subtle defects at multiple stages of germ cell development. Lastly, OEF-1 is primarily associated with the bodies of germline-expressed genes, and as such is excluded from the X chromosome. We hypothesize that OEF-1 may regulate the rate of progression through germ cell development, providing insight into how these critical maturation events are coordinated.

KEYWORDS germline; mitosis; meiosis; gametogenesis; checkpoints; *C. elegans*

As the only cells passed from generation to generation, the germline of an organism is critical to the survival of the entire species. Germ cells undergo specialized processes such as meiosis and gametogenesis to produce a totipotent zygote upon fertilization (Lesch and Page 2012). Thus, germ cells proceed through multiple stages of maturation while maintaining genomic integrity and preventing somatic differentiation (Robert *et al.* 2015). Deciphering the mechanisms that protect germ cells while permitting their development is critical to our overall understanding of how cell fates are specified.

In the nematode *Caenorhabditis elegans*, the germ lineage, known as the P lineage, is specified in the embryo via the asymmetric divisions of embryonic blastomeres (Wang and Seydoux 2013). The germline precursor cell P4 is formed at the 24-cell stage, and undergoes a single symmetric division to generate the two primordial germ cells (PGCs), Z2 and Z3,

at the 88-cell stage (Wang and Seydoux 2013). In adult worms, germ cells progress from proliferative progenitor cells to fully differentiated gametes within each of two gonad arms, with precise, multi-level molecular control at each cell fate juncture (Kimble and Crittenden 2007). In part, transcriptional repression of somatic transcripts through chromatin regulation maintains the unique germline fate throughout development (Robert *et al.* 2015). The entire X chromosome, for example, which houses few germline-expressed genes (Reinke *et al.* 2004), is held transcriptionally silent through most of germ cell development via the maintenance of repressive histone modifications (Kelly *et al.* 2002). Extensive post-transcriptional mechanisms function as another level of germ cell fate control. The inhibition or stabilization of specific transcripts by RNA-binding proteins enables rapid switching to different germ cell programs, such as the mitosis-to-meiosis transition or the sperm-to-oocyte switch (Kimble and Crittenden 2007). Finally, during these cell fate transitions, homologous chromosomes must pair, synapse, and recombine so that chromosomes can segregate properly (Hillers *et al.* 2017). If synapsis of any chromosome pair is delayed or fails, the synapsis checkpoint triggers apoptosis in these germ cells to prevent the formation of aneuploid gametes (Bhalla and Dernburg 2005).

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While some molecular mechanisms have been implicated in these transcriptional and checkpoint pathways, how critical events in germ cells are coordinated and interconnected remains poorly understood.

Here, we characterize the expression, regulation, and function of a novel, highly germline-specific nuclear factor that we have named **OEF-1** (Oocyte-Excluded Factor-1). We define spatial and temporal relationships between *oef-1* transcript and protein expression in the *C. elegans* germline throughout development, including exceedingly early protein expression in the P2 blastomere. **OEF-1** is expressed throughout germline development, but appears to be actively excluded from germ cells undergoing oogenesis. *oef-1* mutants exhibit faster progression of germ cells through multiple stages of development and differentiation, along with increased apoptosis due to activation of the synapsis checkpoint. Genome-wide binding site analysis demonstrates that **OEF-1** preferentially associates with the bodies of germline-expressed genes on autosomes, and is largely excluded from the X chromosome. We suggest that **OEF-1** might coordinate the timing of multiple germline processes as germ cells undergo critical regulatory transitions.

Materials and Methods

Strains

C. elegans strains were maintained by standard methods as described (Brenner 1974). Bristol **N2** was used as the wild-type reference strain. All growth was performed at 20°, except for **BA17**, **JK654**, and **YL312**, which were maintained at 15° and shifted to 25° to induce sterility.

OP383 *unc-119(tm4063)* III; *wgIs383* [*oef-1::TY1::EGFP::3xFLAG + unc-119(+)*] (Sarov *et al.* 2012).

YL465 *vrIs90* [*pmex-5::snpc-4::mCherry::snpc-4 3'UTR + unc-119(+)*] II; *unc-119(ed3)* III (Kasper *et al.* 2014).

LG I: **WS2277** *hus-1(op241)*.

LG II: **CA388** *pch-2(tm1458)*.

LG III: **YL312** *mpk-1(ga111)*; **RB869** *xnd-1(ok709)*; **qC1** [*dpy-19(e1259)* *glp-1(q339)* *qls26*]; **MT2547** *ced-4(n1162)*.

LG IV: **YL585** *oef-1(vr25)*; **YL527** *oef-1(tm4563)*; **VC30049** *oef-1(gk205699)*; **BA17** *fem-1(hc17)*; **JK654** *fem-3(q23)*; **CB1489** *him-8(e1489)*.

LG V: **DR466** *him-5(e1490)*.

Antibody generation

Complementary DNA (cDNA) corresponding to the first 100 amino acids of **OEF-1** was cloned into the pET-19b expression vector (Novagen) and transformed into BL21 cells. His-tagged **OEF-1** N-terminal fragments were purified over Nickle-NTA resin (QIAGEN, Valencia, CA) under denaturing conditions, and injected into rabbits (Pocono Rabbit Farm and Laboratory). Affinity purification of the bleeds was performed as in Porter and Koelle (2010). Briefly, purified **OEF-1**

N-terminal protein was run on an acrylamide gel and transferred onto nitrocellulose. The bleeds were incubated with the membrane overnight at 4°, and bound antibodies were eluted off the membrane with 100 mM glycine (pH 2.5).

Apoptosis assays

Worms 16 hr beyond larval stage 4 (L4) were picked into 1 ml of 33 μ M SYTO 12 (Molecular Probes, Eugene, OR) diluted in M9. After 4 hr of incubation at 23°, the worms were plated and fed for at least 30 min before visualization on agarose pads. The number of SYTO 12-positive cells per gonad arm was quantified. For checkpoint epistasis experiments, genotypes were blinded before quantification.

Brood size analyses

L4 worms were singly placed on plates seeded with the bacterial food source **OP50**, and moved to fresh plates each day until embryo production ceased. Unhatched embryos were scored as dead 24 hr after shifting. Larvae were counted 2 days after shifting and males were scored after 3 days. For *him-8* brood size analyses, wild-type and *him-8(e1489)* L1s were grown on **HT115** bacteria expressing empty L4440 vector or *oef-1* dsRNA in L4440 on RNA interference (RNAi) plates, as in Fraser *et al.* (2000). F1 L4s were singly placed on fresh RNAi plates and broods were analyzed as above.

Chromatin immunoprecipitation and sequencing (ChIP-seq)

ChIP-seq on **OEF-1::GFP** young adults was performed as part of the modENCODE consortium project (Araya *et al.* 2014), and was performed as described (Niu *et al.* 2011; Kasper *et al.* 2014). Target calling analysis was performed as in Kasper *et al.* (2014).

Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9

The following guide RNAs were designed to target the end of the second exon of *oef-1* utilizing the fact that a 3'-GG enhances editing efficiency (Farboud and Meyer 2015): 5'-GTTTGAAGACATCTGAATGG-3' and 5'-AGAAATATTAGAGAAATGGG-3'. The guides were cloned into p46149 (Addgene) as in Paix *et al.* (2014). Young adult worms were injected with Cas9 plasmid (Addgene p46168) at 50 ng/ μ l, each guide RNA at 75 ng/ μ l, and pRF4 *rol-6* injection marker at 50 ng/ μ l. F1 rollers were screened for heterozygous deletions by PCR using primers to amplify a 719-bp region around the second exon of *oef-1* (5'-AGACGAACAATCACTTGAATCAC-3' and 5'-CATGGTGATTTTCGACACAGG-3'). Sequencing confirmed a 56-bp deletion predicted to result in a stop codon after 134 amino acids. The resulting strain **YL585** was backcrossed 4 $\times$ prior to analysis.

DNA FISH

A probe to the 5S rDNA locus of chromosome V was prepared by PCR from genomic DNA as described (Dernburg *et al.* 1998). Probes were labeled using the FISH Tag DNA Green kit with Alexa Fluor 488 dye (Invitrogen, Carlsbad, CA)

according to the manufacturer's instructions, except that the isopropanol precipitation was performed overnight, the column purification steps were omitted, and the final labeled probe was resuspended in hybridization buffer. Dissection, fixation, and hybridization were performed as in Phillips *et al.* (2009). In the hybridization step, 100 ng of probe was used per slide. Images were acquired using a Zeiss Axioplan microscope with a 100× objective and a Zeiss AxioCam MRm camera (Zeiss, Thornwood, NY), and processed using Axiovision software.

5-ethynyl-2'-deoxyuridine (EdU) staining

The thymine-deficient *Escherichia coli* strain MG1693 was grown overnight at 37° in Luria broth, after which 2 ml of the overnight culture was added to 100 ml of M9 supplemented with 1% glucose, 1.25 µg/ml thiamine, 0.5 µM thymidine, 1 mM MgSO₄, and 20 µM EdU. The 100 ml culture was grown for at least 24 hr at 37°, then was pelleted and resuspended in 1 ml of M9. Plates lacking peptone and containing 60 µg/ml carbenicillin were seeded with 250 µl of the resuspended bacteria and allowed to dry overnight. Worms 16 hr post-L4 were washed off plates seeded with OP50, and were washed once with M9 before being placed on MG1693-seeded plates. Worms were washed off MG1693-seeded plates after a 30-min incubation, washed twice with M9, and placed on OP50-seeded plates for 10 hr before dissection and fixation in 3% formaldehyde. For the L4 time point, arrested L1s were grown on OP50 for 36 hr before being placed on MG1693 for 30 min, and were chased on OP50 for 8 hr. Slides were freeze-cracked on a metal block embedded in dry ice and placed in -20° methanol. The next day, the slides were washed in PBST (1× PBS with 0.1% Tween) and stained with DAPI. EdU-positive cells were labeled using the Click-iT Plus EdU Alexa Fluor 488 Imaging Kit (Invitrogen) according to the manufacturer's instructions, except that two sequential 250 µl reactions were performed per slide as in Crittenden *et al.* (2017). 0.375 µm Z stacks were acquired using a 40× objective, and the number of nuclei from the distal tip to the most proximal EdU-positive nucleus was counted. The file names were blinded before quantification.

Immunostaining

Adult worms 16–20 hr post-L4 were dissected to release gonads in 1.1× egg salts on silanized coverslips, and fixed with 1% formaldehyde for most antibodies. For anti-phospho-H3, anti-mCherry, and anti-SP56/anti-RME-2 staining, 3.7% formaldehyde was used. Poly-prep slides (Sigma-Aldrich, St. Louis, MO) were freeze-cracked and immediately placed in -20° methanol. Blocking was performed using 0.5–1% BSA in PBST for 30 min–1 hr at room temperature. Embryo samples were fixed with methanol and acetone, and washed with PBS as described (Strome and Wood 1983). All primary and secondary antibody incubations were performed overnight at 4°. The following antibodies and dilutions were used: 1:50 anti-OEF-1; 1:50 anti-OMA-1/2 (a gift of C. Eckmann); 1:50 anti-RME-2 (a gift of B. Grant); 1:50 SP56 (a gift of S. Strome); 1:100

anti-HIM-3 (a gift of M. Zetka); 1:200 anti-SYP-1 (a gift of A. Villeneuve); 1:1000 anti-H3K36me3 (ab9050; Abcam); 1:2000 anti-GFP (ab13970; Abcam); 1:200 anti-phospho-H3 (3H10; Upstate); 1:20 anti-PGL-1 (OIC1D4; Developmental Studies Hybridoma Bank); 1:100 anti-mCherry (PA5-34974; Pierce); and 1:500 anti-rabbit Alexa Fluor 488 or 568, anti-chicken Alex Fluor 488, anti-mouse Alexa Fluor 568, and anti-guinea pig Alexa Fluor 596 (Molecular Probes). Images were acquired as for DNA FISH experiments, except that a 40× objective was sometimes used.

L1 PGC feeding assay

Gravid adult worms were bleached and the resulting embryos were hatched overnight in M9 in the absence of food. The synchronized L1s were plated on OP50 for 7 hr. The fed larvae were collected, washed, then freeze-cracked on slides pre-treated with a 10 µl drop of 0.01% poly-L-lysine (Sigma). The larvae were fixed with -20° methanol for 10 min followed by -20° acetone for 5 min, and then stained for PGL-1 as above, except PBST was used for washes and the secondary antibody incubation was performed for 1 hr at room temperature.

Small molecule FISH (smFISH)

A set of *Stellaris* FISH probes to *oef-1* coupled to CAL Fluor Red 610 was generated by Biosearch Technologies. Fixation and hybridization were performed on embryos and dissected adults as in Ji and van Oudenaarden (2012) and Campbell and Updike (2015).

SNPC-4 foci quantification

Dissection, fixation, and staining were performed as above using the mCherry antibody, and 0.375 µm Z stacks were taken of germ cells post-transition zone. The file names were blinded, and the number of pachytene nuclei with one SNPC-4:mCherry focus vs. two SNPC-4:mCherry foci was quantified as in Kasper *et al.* (2014).

Sperm counts

L4s were aged for 8–9 hr before fixation in Carnoy's solution (300 µl 100% ethanol, 150 µl chloroform, and 50 µl acetic acid). Next, 2 µl of 1 mg/ml DAPI was added to 1 ml of M9, and 12 µl of the DAPI dilution was added to the slides. 0.375 µm Z stacks were acquired for 10 spermathecae per genotype. Sperm nuclei were counted using the Cell Counter plugin in Image J.

Statistical analyses

The statistical and graphing program GraphPad Prism (version 7) was used to perform Student's *t*-tests and Fisher's exact tests, as well as to calculate SD. For gene set overlaps, hypergeometric probability tests were calculated using the web-based tool http://nemates.org/MA/progs/overlap_stats.html.

Data availability

Strains and reagents generated during this project are available upon request. The mutant alleles of *oef-1* (F49E8.2) will

also be deposited in the *Caenorhabditis* Genetics Center database. The *OEF-1* ChIP-seq data generated by modENCODE/modERN are publicly available at the Gene Expression Omnibus under accession number GSE107190, and at <http://encodeproject.org/> and <http://epic.gs.washington.edu/modERN>.

Results

OEF-1 is a novel, germline-specific factor

While screening transgenic lines of GFP-tagged transcription factors generated for the modENCODE consortium project, we identified *F49E8.2* as an uncharacterized nuclear protein with highly germline-specific expression (Sarov *et al.* 2012; Araya *et al.* 2014). *F49E8.2* is conserved in *Caenorhabditis* but has no known homologs in other species. It contains a single C₂H₂ zinc finger (Supplemental Material, Figure S1 in File S2), which is typically a DNA-binding domain (Wolfe *et al.* 1999). Examination of the GFP-tagged strain (referred to as *F49E8.2::GFP*) revealed nuclear expression restricted to the P lineage in embryos beginning in the P4 PGC (Figure 1A). In larval stages, *F49E8.2::GFP* is present in all germ cells (Figure 1, B and C). In adults, *F49E8.2::GFP* localizes to the nuclei of germ cells in the mitotic zone through entry into meiosis (Figure 1D). Strikingly, *F49E8.2::GFP* expression abruptly disappears in the late pachytene region and is absent from developing oocytes (Figure 1D). Based on these features and additional observations described below, we named *F49E8.2 OEF-1*.

OEF-1 is expressed early in embryogenesis and is maternally loaded as a transcript

To examine endogenous *OEF-1* expression, we generated a polyclonal antibody to *OEF-1*. The endogenous antibody recapitulates the expression pattern of the *OEF-1::GFP* transgene in L4 and adult dissected gonads (Figure 3, A and B and Figure S2 in File S2), and is lost in animals lacking *OEF-1* expression (Figure S2 in File S2). In embryos, endogenous *OEF-1* protein expression is first visible even earlier than *OEF-1::GFP*, in the P2 blastomere rather than P4 (Figure 2A). The P lineage is transcriptionally silent in embryos (Seydoux *et al.* 1996), which makes it unlikely that *oef-1* is newly transcribed during embryogenesis. However, *OEF-1* protein expression is not detectable in maturing oocytes, implying that little to no *OEF-1* is maternally loaded (Figure 1D and Figure 3B). Thus, we wondered whether *oef-1* transcript was maternally loaded to explain this remarkably early protein expression in P2. Using smFISH, we detected *oef-1* transcripts throughout wild-type dissected gonads, including in oocytes, as well as in early embryos prior to *OEF-1* protein expression (Figure 2, B and D). In addition, *oef-1* transcripts become entirely restricted to the P lineage by the formation of Z2 and Z3 (Figure 2E). Taken together, these results indicate that *oef-1* is maternally loaded as a transcript, with post-transcriptional regulation accounting for the delay in protein expression until the 4-cell embryonic stage.

OEF-1 exhibits distinct expression patterns in spermatogenesis and oogenesis

OEF-1 expression is detected in the proximal germ cells of L4 animals undergoing spermatogenesis, but not in the proximal germ cells of adult hermaphrodites undergoing oogenesis (Figure 1, C and D). Because wild-type hermaphrodites switch from spermatogenesis to oogenesis at the L4/adult transition, we hypothesized that *OEF-1* expression is affected by gamete fate. We first costained dissected L4 gonads with *OEF-1* and the sperm marker SP56 (Ward *et al.* 1986), and observed coexpression in the proximal gonad, indicating that *OEF-1* is expressed throughout spermatogenesis, with the exception of mature sperm (Figure 1D and Figure 3A). By contrast, costaining of *OEF-1* and the oocyte marker OMA-1/2 (Nousch *et al.* 2013) in adults confirmed that *OEF-1* is not expressed during oogenesis (Figure 3B). Thus, *OEF-1* is specifically excluded from oogenic germ cells, potentially through a protein degradation pathway.

To determine whether entry into oogenesis triggers loss of *OEF-1* expression, we crossed the *OEF-1::GFP* line into gamete fate mutant backgrounds. *fem-1(hc17)* is a loss-of-function mutant resulting in feminized gonads that produce oocytes during L4 (Nelson *et al.* 1978), while *fem-3(q23)* is a gain-of-function mutant resulting in masculinized germlines that continue to produce sperm into adulthood (Barton *et al.* 1987). Notably, the *fem-1; OEF-1::GFP* strain showed a premature loss of *OEF-1::GFP* expression in proximal germ cells at L4 compared with wild-type hermaphrodite L4s undergoing spermatogenesis ($n = 50/50$; Figure 3C, compare to Figure 1C). Conversely, *fem-3; OEF-1::GFP* adults exhibited extended *OEF-1::GFP* expression past the gonad bend in presumptive primary spermatocytes ($n = 44/50$; Figure 3D, compare to Figure 1D). Finally, we utilized *mpk-1(ga111)* mutants, in which germ cells arrest at the late pachytene stage of meiosis I and do not initiate oogenesis (Church *et al.* 1995). In *mpk-1; OEF-1::GFP* adults, *OEF-1::GFP* was present in pachytene-arrested germ cells, indicating that reduction of *OEF-1* expression occurs upon or after pachytene exit (Figure 3E). Together, these data indicate that the abrupt loss of *OEF-1* expression at the pachytene-to-diplotene transition depends upon progression into oogenesis, and not on other temporal or spatial gonadal signals.

oef-1 mutants have an accelerated rate of germ cell progression

We next examined mutants of *oef-1* to understand its potential function in the germline. Two alleles were previously available from consortia, and we generated a third using CRISPR-Cas9 gene editing (Figure S1 in File S2). The *tm4563* allele contains a complex substitution (a 304-bp deletion plus a 19-bp insertion) spanning almost the entirety of exon 1 to the beginning of exon 2, while the *gk205699* allele contains a point mutation that alters a proline to a serine in the sole zinc finger of *OEF-1* (Figure S1 in File S2). The CRISPR allele, *vr25*, contains a 56-bp frameshift deletion in

Figure 1 OEF-1::GFP exhibits germline-specific expression throughout development. (A) The strain OP383 expresses OEF-1 tagged with GFP beginning in ~28-cell embryos in the nucleus of the primordial germ cell P4 (center), with no expression seen in the 4-cell stage embryo (left). OEF-1::GFP expression is seen in the nuclei of Z2 and Z3 in a ~100-cell embryo (right). Merged DIC and GFP channels are shown. (B) OEF-1::GFP is present in the nuclei of Z2 and Z3 in a newly hatched L1 larva. White box indicates inset. Arrows indicate Z2 and Z3. DIC, GFP, and merged channels are shown. (C) OEF-1::GFP is present in all L4 germ cell nuclei, and (D) in mitotic and meiotic germ cell nuclei through late pachytene in adults. Arrows indicate maturing oocytes. Bar, 10 μ m. L, larval stage; Sp, sperm.

Figure 2 OEF-1 is expressed early in embryogenesis and is maternally loaded as a transcript. (A) A 4-cell stage wild-type embryo immunostained with OEF-1 (green) and the germline-specific P granule component PGL-1 (red). OEF-1 is detected in the nucleus of the P2 blastomere. DAPI (DNA) is in blue. (B) *oef-1* small molecule FISH (smFISH) probes hybridized to wild-type dissected adult gonads. *oef-1* transcript is detected throughout the gonad, including in oocytes. White square indicates inset at right. (C) Dissected *oef-1(vr25)* adult gonads lose *oef-1* signal. White square indicates inset at right. (D) Two-cell wild-type embryo with *oef-1* smFISH signal (right) in both blastomeres. DAPI, left. (E) smFISH signal of an ~100-cell wild-type embryo showing *oef-1* (right) largely restricted to Z2 and Z3. DAPI, left. Bar, 10 μ m.

exon 2. The OEF-1 antibody could not detect OEF-1 in the *tm4563* or the *vr25* mutant background (Figure S2 in File S2). Notably, the epitope targeted by the OEF-1 antibody overlaps almost completely with the deleted amino acids in *tm4563*, but not entirely in *vr25* (Figure S1 in File S2). Therefore, because we could be confident that *vr25* was a protein null, we subsequently used it as the primary mutant for phenotypic

analysis. Intriguingly, OEF-1 was detected in the *gk205699* background (Figure S2 in File S2), suggesting that this point mutant retains normal expression.

As OEF-1 is expressed early in embryonic development (Figure 1A and Figure 2A), we first examined *oef-1(vr25)* PGCs for potential defects. After P4 divides into Z2 and Z3 during late embryogenesis, the two PGCs arrest in G2 until

Figure 3 OEF-1 exhibits distinct expression patterns in spermatogenesis and oogenesis. (A) Dissected L4 gonad stained with OEF-1 (green) and the spermatogenesis marker SP56 (red). DAPI is in blue. (B) Dissected adult gonad stained with OEF-1 (green) and the oogenesis marker OMA-1/2 (red). DAPI, blue. (C) *fem-1(hc17)*; OEF-1::GFP L4s show a precocious loss of OEF-1::GFP expression in proximal germ cells ($n = 50/50$). Arrows indicate germ cells without OEF-1::GFP expression. (D) *fem-3(q23)*; OEF-1::GFP adults exhibit extended OEF-1::GFP expression past the gonad bend ($n = 44/50$). (E) OEF-1::GFP is detected in pachytene-arrested germ cells in *mpk-1(ga111)* mutants. Bar, 10 μ m. 1°, primary spermatocytes; L, larval stage; Sp, sperm.

hatched L1s are exposed to food (Fukuyama *et al.* 2006). After 7 hr of feeding, only 11.1% of PGCs in wild-type larvae had undergone an initial cell division (Figure 4A). However, in 42.4% of *oef-1(vr25)* mutant larvae, PGCs had divided once or even twice (Figure 4A). This result was reproducible in the two other *oef-1* mutant alleles (Figure S3A in File S2),

and introduction of the OEF-1::GFP transgene into the *oef-1(vr25)* mutant background rescued early PGC divisions to wild-type levels (Figure S3B in File S2).

We then assayed *oef-1(vr25)* adult hermaphrodites to ask whether germ cells continued to exhibit any cell cycle defects at later developmental stages. We did not observe any difference

Figure 4 *oef-1* mutants have an accelerated rate of germ cell progression. (A) Starved and synchronized wild-type and *oef-1(vr25)* L1s were fed for 7 hr, then fixed and stained with PGL-1 to mark primordial germ cells (PGCs). The percentages of larvae with one, two, three, or four PGCs are shown. $n \geq 63$ L1s per genotype. (B) Wild-type and *oef-1(vr25)* adult worms were fed 5-ethynyl-2'-deoxyuridine (EdU)-labeled bacteria followed by a 10-hr chase on unlabeled bacteria. The number of germ cell diameters from distal tip to the most proximal EdU-labeled nucleus was quantified. $n \geq 16$ gonad arms per genotype. **** $P < 0.0001$, Student's *t*-test. DTC, distal tip cell. Error bars represent SD. (C) Brood sizes of wild-type and *oef-1(vr25)* mutants at 20°. $n \geq 13$ parental animals. **** $P < 0.0001$, Student's *t*-test. Error bars represent SD. (D) The number of sperm in wild-type and *oef-1(vr25)* spermathecae. $n = 10$ spermathecae per genotype. **** $P < 0.0001$, Student's *t*-test. Error bars represent SD.

in the length of the proliferative or transition zones in *oef-1(vr25)* mutants by DAPI staining (Figure S4, A and B in File S2), suggesting no changes in the number of cells in mitosis or entering meiosis. We did detect a slight increase in the number of germ cells in M phase in *oef-1(vr25)* adults by phospho-H3 staining (8.65 vs. 7.22 positive nuclei, $n \geq 37$ gonad arms per genotype, $P < 0.01$, Student's *t*-test) (Figure S4C in File S2). These conflicting observations suggested that *oef-1* mutant germ cells either exhibit a slight delay in M phase or an increased rate of progression through mitosis and into meiosis.

We therefore determined whether overall germ cell progression was altered in *oef-1(vr25)* mutants. We performed

pulse-chase experiments using the thymidine analog EdU. EdU is incorporated into germ cells undergoing S phase, and thus labels proliferative cells (Crittenden *et al.* 2006). Wild-type and *oef-1(vr25)* mutants were exposed to EdU-labeled bacteria for 30 min followed by a 10-hr chase on unlabeled bacteria. Relative to wild-type, *oef-1(vr25)* mutants exhibited a significant increase in the number of cell diameters from the distal tip to the most proximally EdU-labeled germ cell, suggesting that *oef-1* mutant germ cells progress at a faster rate (44.1 vs. 36.1 cell diameters, $n \geq 16$ gonad arms per genotype, $P < 0.0001$, Student's *t*-test) (Figure 4B and Figure S5A in File S2). A similar difference was also observed at the L4 stage (Figure S5B in File S2).

Consistent with an accelerated rate of germ cell progression, *oef-1(vr25)* mutants exhibit a 33% increase in brood size relative to wild-type (Figure 4C) (340.0 vs. 255.3, $n \geq 13$ animals per genotype, $P < 0.0001$, Student's *t*-test). The other two *oef-1* mutant alleles also had increased brood sizes (Figure S6 in File S2). Since brood size is limited by sperm number, we quantified the number of sperm present in the spermathecae of young adults. We found that *oef-1(vr25)* mutants had a significant increase in the number of sperm generated per gonad arm compared to wild-type (Figure 4D) (156.7 vs. 113.4, $n = 10$ spermathecae per genotype, $P < 0.0001$, Student's *t*-test). A faster rate of germ cell progression could explain an increase in brood size as more germ cells would be specified to sperm during the spermatogenesis window. Increased sperm production might also occur if spermatogenesis is extended due to a delay in the transition to oogenesis (Hodgkin and Barnes 1991). However, we saw no detectable alteration in the onset of oogenesis between L4 and adulthood in *oef-1(vr25)* mutants compared to wild-type by staining with the oogenesis marker *RME-2* (Grant and Hirsh 1999) (Table S1 in File S2).

Finally, we asked whether the rate of germ cell progression was altered in maturing oocytes. We allowed wild-type and *oef-1(vr25)* adults to lay eggs for 4 hr and quantified the number of eggs laid. We found that more eggs were laid by *oef-1(vr25)* mutants, suggesting that oocyte progression is also accelerated relative to wild-type (Figure S5C in File S2). We conclude that germ cells progress more rapidly through all stages of development and differentiation in *oef-1* mutants.

***oef-1* mutants exhibit increased germline apoptosis that depends upon the synapsis checkpoint**

To ask whether a faster rate of germ cell progression affected cell viability, we stained wild-type and *oef-1* mutant adults for apoptotic germ cells using the dye SYTO 12. We detected a significant increase in the number of positively-stained germ cells in *oef-1(vr25)* mutants compared to wild-type (11.0 vs. 6.91, $n = 23$ gonad arms per genotype, $P < 0.0001$, Student's *t*-test) (Figure 5A). This defect was also present in the other two *oef-1* mutant alleles (Figure 5B), and was rescued by the *OEF-1::GFP* transgene (Figure S7 in File S2). The activation of either of two meiotic checkpoints can increase apoptosis above levels of physiological germline apoptosis: the synapsis checkpoint or the DNA damage checkpoint (Gartner *et al.* 2008). In *hus-1(op241)* mutants, the DNA damage checkpoint does not occur (Hofmann *et al.* 2002), whereas the synapsis checkpoint is not activated in *pch-2(tm1458)* mutants (Bhalla and Dernburg 2005). We performed an epistasis test to determine whether one of these pathways accounted for the increased apoptosis in *oef-1* mutants. Apoptosis levels were restored to wild-type in *pch-2; oef-1* mutants, but remained high in *hus-1; oef-1* mutants (Figure 5C). This result indicates that the increased apoptosis in *oef-1* mutants is due to activation of the synapsis checkpoint.

To determine whether *oef-1* mutants had defects in chromosome synapsis, we used two chromosomal markers to assess pairing, a DNA locus near the pairing center (PC) on chromosome V (Phillips and Dernburg 2006) and a transcription factor that localizes to a domain at the opposite end from the PC on chromosome IV (Kasper *et al.* 2014) (see *Materials and Methods*). Intriguingly, the marker on chromosome IV showed delayed pairing in 40.5% of *oef-1(vr25)* pachytene nuclei compared to 28.2% of wild-type pachytene nuclei ($n \geq 440$ nuclei per genotype, $P < 0.0001$, Fisher's exact test) (Figure 5D), while the marker on chromosome V did not exhibit any detectable pairing defect (Figure S8 in File S2). Consistent with this latter result, we did not detect defects in the colocalization of the meiotic axis protein *HIM-3* and the synaptonemal complex component *SYP-1* (Figure S9 in File S2), and did not observe univalent chromosomes during diakinesis in *oef-1* mutants (data not shown), indicating completed chromosome synapsis (Dombecki *et al.* 2011). Moreover, when we eliminated all germline apoptosis using *ced-4(n1162)* mutants, we did not observe an increased incidence of male offspring or embryonic lethality in *ced-4; oef-1* double-mutants (Table S2 in File S2), as would be expected if pairing or synapsis were defective (Bhalla and Dernburg 2005). We suggest that a faster rate of progression through the proliferative zone in *oef-1* mutants results in germ cell nuclei entering meiosis before they are fully prepared to undergo pairing and synapsis, and/or that accelerated progression through pachytene slightly perturbs the fidelity of these meiotic events (see *Discussion*).

***OEF-1* associates with germline-expressed genes and localizes to autosomes**

To probe the molecular function of *OEF-1*, we analyzed ChIP-seq data sets for the *OEF-1::GFP* strain in young adults that were generated by the modENCODE project (Araya *et al.* 2014). Significant *OEF-1*-binding sites were assigned to 1998 protein-coding target genes (File S1). Of these, 86.5% were germline-expressed genes (Ortiz *et al.* 2014) (1729 genes; 1.7 $\times$ enriched, $P < 6.32 \times 10^{-253}$, hypergeometric probability test) (Figure 6A). Protein-coding targets were also enriched for genes with germline-biased expression (the combined data set of germline-intrinsic and oogenesis-enriched genes) (Reinke *et al.* 2004) (748 genes; 2.7 $\times$ enriched, $P < 1.74 \times 10^{-184}$, hypergeometric probability test) (Figure 6A). Notably, individual gene targets display unusual *OEF-1*-binding profiles, with prominent association with gene bodies rather than promoter or intergenic regions (Figure 6B). In particular, target genes often displayed *OEF-1* peaks at the 5' and 3' ends, with distributed binding above input signal along gene bodies (Figure 6B).

Few germline-expressed genes reside on the X chromosome (Reinke *et al.* 2004), and accordingly *OEF-1* targets were markedly depleted for X-linked genes (28 genes; 0.1 $\times$ enriched, $P < 1.585 \times 10^{-95}$, hypergeometric probability test) (Figure 6C). Indeed, even the few X-linked genes that are expressed in the germline show reduced *OEF-1*

Figure 5 *oef-1* mutants exhibit increased germline apoptosis depending on the synopsis checkpoint. (A) Wild-type and *oef-1(vr25)* adults were stained with SYTO 12 to mark apoptotic germ cells and the number of positively-stained germ cells per gonad arm was quantified. $n = 23$ gonad arms per genotype. **** $P < 0.0001$, Student's t -test. Error bars represent SD. (B) SYTO 12 quantification in other *oef-1* mutant alleles. $n \geq 19$ gonad arms per genotype. *** $P < 0.001$ and **** $P < 0.0001$, Student's t -test. Error bars represent SD. (C) SYTO 12 quantification in apoptosis checkpoint mutants. $n \geq 35$ gonad arms per genotype. **** $P < 0.0001$, Student's t -test. Error bars represent SD. (D) Tagged SNPC-4 foci (green) were counted in pachytene nuclei in wild-type (left) and *oef-1(vr25)* (right) dissected gonads. SNPC-4 binds strongly to a specific region of chromosome IV in the germline and forms visible foci, making it a highly specific chromosomal marker (Kasper *et al.* 2014). Arrows indicate nuclei with two foci in *oef-1(vr25)*. DAPI, blue. The percentages of nuclei with one focus or two foci are shown. $n = 10$ germlines per genotype, at least 20 nuclei per germline (total $n \geq 440$ nuclei per genotype). Bar, 10 μm . n.s., not significant.

binding relative to autosomal germline-expressed genes, as exemplified by *meg-1* (Figure 6B). To determine whether this autosomal-specific binding pattern was distinguishable *in vivo*, we examined dissected gonads costained with *OEF-1* and the

autosomal-specific histone modification H3K36me3 (Rechtsteiner *et al.* 2010; Gaydos *et al.* 2012). A DAPI-stained chromosome that did not stain for H3K36me3 or *OEF-1* was indeed distinguishable in pachytene nuclei (Figure 6D). We conclude that

Figure 6 OEF-1 associates with germline-expressed genes and localizes to autosomes. (A) The overlap between OEF-1::GFP coding targets, the set of germline-expressed genes (Ortiz *et al.* 2014), and the combined set of germline-intrinsic/oocyte-enriched genes (Reinke *et al.* 2004). $P < 6.32 \times 10^{-253}$ and $P < 1.74 \times 10^{-184}$, respectively, hypergeometric probability test. (B) OEF-1::GFP ChIP-seq signal along the germline-expressed genes *csr-1*, *pgl-1*, and *rme-2*. OEF-1 associates with the 5' and 3' ends of genes, as well as with gene bodies. *meg-1* is an example of an X-linked germline-expressed gene for which OEF-1 signal is reduced. OEF-1::GFP ChIP-seq signal is in green, control "input" signal is in black. (C) A genome-wide view of OEF-1::GFP ChIP-seq signal (green) and input control (black). OEF-1 ChIP-seq signal is reduced along the X chromosome compared to autosomes. (D) Pachytene nuclei of the OEF-1::GFP strain stained with GFP (green) and H3K36me3 (red). Arrows indicate DAPI (blue) regions not stained with GFP or H3K36me3. Bar, 10 μ m. ChIP-seq, chromatin immunoprecipitation and sequencing; Chr, chromosome.

Table 1 *oef-1* partially rescues *him-5* percentage of males

Genotype	Percentage of males (%)	Total progeny
Wild-type	0.00	2576
<i>oef-1(vr25)</i>	0.07	4238
<i>him-5(e1490)</i>	37.53	2775
<i>oef-1; him-5</i>	30.24****	2811

n = 13–14 parental animals per genotype. **** *P* < 0.0001, Fisher's exact test.

OEF-1 is an autosomal-enriched factor with preferential association with germline-expressed loci.

***oef-1* partially rescues the *Him* phenotype of *him-5*, *xnd-1*, and *him-8* mutants**

Because of this striking chromosomal bias, we asked whether **OEF-1** functioned in the same pathway as two other autosomal-specific factors that also lose expression in late pachytene: **HIM-5** (High Incidence of Males-5) and **XND-1** (X chromosome Nondisjunction-1) (Wagner *et al.* 2010; Meneely *et al.* 2012). These two germline-specific proteins act in the same genetic pathway to regulate crossover frequency on the X chromosome (Wagner *et al.* 2010; Meneely *et al.* 2012). Reduced crossover events in *him-5* and *xnd-1* mutants lead to broods with high percentages of embryonic lethality and males (Wagner *et al.* 2010; Meneely *et al.* 2012). Since **HIM-5** and **XND-1** exhibit the same autosomal localization and loss at late pachytene as **OEF-1**, we analyzed the broods of *oef-1*; *him-5* and *xnd-1*; *oef-1* double-mutants. Given that *xnd-1* mutants have pleiotropic germline defects and show stochastic sterility in later generations (McClendon *et al.* 2016), we focused our analyses on the broods of *xnd-1* M+Z- parents. We found that *oef-1* partially rescued the *him-5* percentage of males from 37.53 to 30.24% (*P* < 0.0001, Fisher's exact test) (Table 1). In *xnd-1*; *oef-1* double-mutants, we found that *oef-1* again partially rescued the percentage of males from 6.31 to 4.11% (*P* = 0.0045, Fisher's exact test) (Table 2).

To determine whether the effect of *oef-1* on the **HIM-5**/**XND-1** pathway was specific, we analyzed *him-8* mutants. **HIM-8** works independently of **HIM-5**/**XND-1** to mediate pairing and synapsis specifically of the X chromosome, and localizes to the X chromosome PC (Phillips *et al.* 2005). Thus, if the effect of *oef-1* were specific to *him-5* and *xnd-1*, *oef-1* would not have an effect on the *him-8* percentage of males. However, when we performed *oef-1* RNAi on *him-8(e1489)* mutants and examined the percentage of males in the progeny, we found that *oef-1* also partially rescued the percentage of males in *him-8* broods (*P* < 0.0001, Fisher's exact test) (Table 3). We conclude that **OEF-1** alters the frequency of nondisjunction through a mechanism that affects both the **XND-1**/**HIM-5** and **HIM-8** pathways, and that is genetically distinct from both pathways.

Discussion

Here, we have characterized the expression of the highly tissue-specific novel factor **OEF-1** and gained insight into its

Table 2 *oef-1* partially rescues *xnd-1* percentage of males

Genotype	Percentage of males (%)	Total progeny
Wild-type	0.06	3574
<i>oef-1(vr25)</i>	0.00	4420
<i>xnd-1(ok709)</i> M+Z-	6.31	1474
<i>xnd-1</i> M+Z-; <i>oef-1</i>	4.11**	1945

n = 13–21 parental animals per genotype. ** *P* < 0.005, Fisher's exact test.

role in the *C. elegans* germline. **OEF-1** is detectably expressed only in the germline, and is continually expressed from the P2 blastomere until the onset of oogenesis, with the exception of mature sperm. *oef-1* mutants exhibit accelerated germ cell progression throughout germ cell development, which likely leads to increased apoptosis due to activation of the synapsis checkpoint. Finally, we found that **OEF-1** associates broadly with many germline-expressed genes and predominantly localizes to autosomes. Together, these observations suggest that **OEF-1** might play a subtle but important role in coordinating germline-specific events, and ensuring robust or buffered progression through germ cell development.

***OEF-1* is expressed early and specifically in the germline**

We identified **OEF-1** as a germline-specific protein with strikingly early embryonic expression restricted to the P lineage. We detect endogenous **OEF-1** in the nucleus of the P2 blastomere (Figure 2A) and GFP-tagged **OEF-1** in the nucleus of P4 (Figure 1A). This delay between endogenous and transgene protein expression could be due to the additional time required for folding of the GFP tag (Heim *et al.* 1994). Because the *oef-1* transcript is present throughout the gonad and in early embryonic stages (Figure 2, B and D), we suggest that a post-transcriptional repression mechanism prevents **OEF-1** protein expression during oogenesis and early embryogenesis. **OMA-1** and **OMA-2** are two RNA-binding proteins that are critical for the oocyte-to-embryo transition and are expressed during oogenesis (Detwiler *et al.* 2001). Previously, the *oef-1* transcript was found to immunoprecipitate with **OMA-1/2** by RNA immunoprecipitation and sequencing (RIP-seq) (Spike *et al.* 2014), suggesting that **OMA-1/2** may bind to and inhibit the translation of *oef-1* during oogenesis. The transcripts of *xnd-1* and *him-5* also immunoprecipitated with **OMA-1/2** in the same study (Spike *et al.* 2014), suggesting a potentially common translational repression mechanism between factors with oocyte-excluded protein expression.

Gamete fate program and the link to pachytene

Notably, we showed that the loss of **OEF-1** protein expression in the late pachytene region in adult germlines occurs at and depends upon the onset of oogenesis (Figure 3). During spermatogenesis, **OEF-1** is detected in primary spermatocytes, and expression does not cease until late in differentiation, when many proteins are eliminated *en masse* from maturing sperm (L'Hernault 2006) (Figure 1D and Figure 3A). By contrast, in the oogenic germline, **OEF-1** expression abruptly becomes undetectable in late pachytene, concomitant with

Table 3 *oef-1* partially rescues *him-8* percentage of males

Genotype	RNAi treatment	Percentage of males (%)	Total progeny
Wild-type	Empty vector	0.12	1691
	<i>oef-1</i>	0.21	1933
<i>him-8(e1489)</i>	Empty vector	40.77	1869
	<i>oef-1</i>	34.79****	2018

n = 6–7 parental animals per genotype. **** *P* < 0.0001, Fisher's exact test. RNAi, RNA interference.

the onset of protein expression of early oogenesis factors and before any morphological signs of differentiation (Figure 1D and Figure 3B). The loss of other proteins (like *HIM-5* and *XND-1*) at pachytene during oogenesis may also depend on the gamete fate program. We hypothesize that a proteasomic pathway degrades nonmaternally-loaded proteins at the pachytene stage. This degradation pathway is likely tied to the proper exit from pachytene, as inhibition of pachytene exit prevents the degradation of *OEF-1* (Figure 3E). This transition point in germ cells may be critical to the proper development of oocytes; the consequence of continued expression of oocyte-excluded factors is not known. Attempts to drive *OEF-1* in the proximal germline through 3'-UTR regulation (Merritt *et al.* 2008) did not result in detectable *OEF-1* expression (data not shown), which is consistent with regulation of *OEF-1* at the protein level.

***OEF-1* may limit the rate of germ cell progression through development**

Germ cells in *oef-1* mutants progress faster than in wild-type, beginning with a precocious first division of PGCs (Figure 4A). In wild-type L1 larvae, the Z2/Z3 division occurs when a prolonged G2 arrest is released (Fukuyama *et al.* 2006). This release is triggered by the presence of food and depends upon activation of the genome-wide transcriptional program (Butuci *et al.* 2015). The checkpoint kinase *CHK-1* is implicated in the timing of Z2/Z3 cell cycle reentry; in *chk-1* mutants, the Z2/Z3 division occurs precociously (Butuci *et al.* 2015), similarly to *oef-1* mutants, suggesting that *OEF-1* may act in or affect this checkpoint. Even as late larvae and adults, *oef-1* mutants continue to show accelerated germ cell progression, as shown by EdU labeling (Figure 4B), likely manifesting in more sperm generation during the spermatogenesis window and an increase in brood size (Figure 4, C and D). The observation that *oef-1(vr25)* mutants lay eggs at a faster rate (Figure S5C in File S2) suggests that even differentiated oocytes progress faster compared to wild-type.

In addition to changes in the rate of germ cell progression, *oef-1* mutants exhibited an increase in germ cell death, which was eliminated upon removal of the synapsis checkpoint by *pch-2* mutation (Figure 5). This checkpoint is triggered by delays or defects in synapsis (Bhalla and Dernburg 2005). Synapsis occurs progressively, originating from a specific site, the PC, on one end of each chromosome, followed by elongation of the synaptonemal complex down chromosome pairs (Rog and Dernburg 2015). We observed defective pairing in

oef-1 mutants when tracking a locus far from a PC (Figure 5D), but not for a locus close to a PC (Figure S8 in File S2), suggesting that pairing along the length of homologous chromosomes is mildly delayed. However, synapsis ultimately occurs successfully in *oef-1* mutants, as we found no defects in the colocalization of *HIM-3* and *SYP-1* (Figure S9 in File S2), bivalent chromosome number in oocytes (data not shown), or embryonic viability and incidence of males even if apoptosis is blocked (Table S2 in File S2).

From these phenotypes, we hypothesize that *oef-1* mutant germ cells exhibit accelerated transit through the proliferative zone and enter meiosis before pairing can be completed, leading to a slight delay in completed pairing and synapsis of homologous chromosomes, and subsequent activation of the synapsis checkpoint. Additionally, the accelerated rate of progression seems to continue through pachytene in *oef-1* mutants, and may directly affect meiotic events and thus trigger the synapsis checkpoint. Because apoptosis only occurs during oogenesis (Gartner *et al.* 2008), we propose that in the L4 stage, spermatogenic germ cells rapidly progress unchecked through meiosis, leading to increased sperm number and brood sizes in *oef-1* mutants. In *oef-1* mutant adults, the elevated apoptosis might eliminate more germ cells with unsynapsed X chromosomes upon loss of *him-5*, *xnd-1*, or *him-8* activity, leading to fewer XO male progeny (Table 1, Table 2, and Table 3). Consistent with this possibility, we found that apoptosis levels in *oef-1*; *him-5* mutants remain elevated, as in *oef-1* mutants, relative to wild-type and *him-5* (data not shown). Together, these phenotypes are consistent with a possible role of *OEF-1* in limiting or controlling the rate at which germ cells progress through various stages of development or differentiation.

The molecular function of *OEF-1*

How might *OEF-1* have such a subtle but influential effect on coordinating germ cell processes? Through analysis of mod-ENCODE ChIP-seq data, we found that *OEF-1* primarily localizes to germline-expressed genes (Figure 6A). However, it does not bind like most “point-source” transcription factors; *OEF-1* peaks are distributed over gene bodies, rather than localized to promoters. Similar distributed binding profiles have been observed for splicing factors (Fontrodona *et al.* 2013) and for histone modifications (Evans *et al.* 2016). Moreover, we did not detect statistically significant changes in transcript abundance of *OEF-1* ChIP-seq targets upon loss of *oef-1* in preliminary analyses of RNA-seq data on dissected gonads (data not shown). We therefore theorize that *OEF-1* does not act to modulate transcription of specific target genes, but might have some other molecular role, perhaps in RNA processing or chromatin biology. Notably, even though C₂H₂ zinc fingers are most commonly associated with DNA binding, they can also bind RNA or mediate protein–protein interactions (Hall 2005; Gamsjaeger *et al.* 2007). The C₂H₂ zinc finger is clearly critical to *OEF-1* function, as a single damaging point mutation in the zinc finger domain is sufficient to phenocopy a protein null (Figure 5B, Figure S3A,

and Figure S6 in File S2). In the future, additional studies will further delineate the molecular mechanisms that underlie the enhanced progression of germ cells in *oef-1* mutants.

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Author contributions: C.E.M. and V.R. conceived and designed the experiments. C.E.M. performed the experiments. C.E.M. and V.R. analyzed the data. C.E.M. and V.R. wrote the paper.

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