LARP-1 promotes oogenesis by repressing *fem-3* in the *C. elegans* germline

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Summary

LA-related protein 1 (LARP-1) belongs to an RNA-binding protein family containing a LA motif. Here, we identify LARP-1 as a regulator of sex determination. In *C. elegans* hermaphrodites, a complex regulatory network regulates the switch from sperm to oocyte production. We find that simultaneous depletion of *larp-1* and the *Nanos* homologue *nos-3* results in germline masculinization. This phenotype is accompanied by a strong reduction of the levels of TRA-1, a GLI-family transcription factor that promotes oogenesis. TRA-1 levels are regulated by CBC^{FEM-1}, a ubiquitin ligase consisting of the FEM proteins, FEM-1, FEM-2 and FEM-3 and the cullin CUL-2. We show that both the masculinization phenotype and the reduction of TRA-1 levels observed in *nos-3;larp-1* mutants require *fem-3* activity, suggesting that *nos-3* and *larp-1* regulate the sperm-oocyte switch by inhibiting the *fem* genes. Consistently, *fem-3* mRNA levels are increased in *larp-1* mutants. By contrast, levels of *fem-3* mRNA are not affected in *nos-3* mutants. Therefore, our data indicate that LARP-1 and NOS-3 promote oogenesis by regulating *fem-3* expression through distinct mechanisms.

Key words: Sex determination, LARP-1, TRA-1, NOS-3, La-motif, Nanos, FEM proteins, *Caenorhabditis elegans*

Introduction

Caenorhabditis elegans can develop as either self-fertile hermaphrodites or males. In the *C. elegans* hermaphrodite germline, sperm and oocytes are produced from a common pool of progenitor cells. Sperms are produced at the L3-L4 stage and oocytes at the adult stage. A complex regulatory network controls the switch from sperm to oocyte production. The final global sex-determination regulator TRA-1, a Gli-family transcription factor, specifies hermaphrodite fate by repressing male-specific genes (Ellis, 2008). TRA-1 protein levels are negatively regulated by a CUL-2/ElonginB/C-based ubiquitin ligase, referred to as CBC^{FEM-1} (Starostina et al., 2007). The CBC^{FEM-1} ubiquitin ligase contains the substrate-specific adaptor FEM-1, as well as two other FEM proteins: FEM-2, which is a phosphatase and FEM-3, which is a novel protein (Kamura et al., 2004; Starostina et al., 2007). The exact molecular function of FEM-2 and FEM-3 is not known, but both have been shown to stimulate CBC^{FEM-1} activity. Consistently, loss-of-function mutations in all three *fem* genes result in the feminization of the hermaphrodite germline (Kimble et al., 1984; Hodgkin, 1986).

FBF-1 and FBF-2 (FBF-1/2), two highly similar members of the *Pumilio* family, negatively regulate *fem-3* and thereby promote oogenesis (Zhang et al., 1997). FBF-1/2 bind to a regulatory element in the *fem-3* 3′UTR and have been suggested to repress *fem-3* translation (Zhang et al., 1997). In *Drosophila*, Pumilio forms a complex with Nanos and both are required for the translational repression of cyclin B and hunchback (Sonoda and Wharton, 1999; Kadyrova et al., 2007). Similarly, FBF-1/2 interact with one of the *C. elegans* homologues of Nanos, NOS-3, in yeast two-hybrid and in vitro binding assays (Kraemer et al., 1999), suggesting that NOS-3 represses *fem-3* translation with FBF-1/2. However, although the germline of *fbf-1/2* double mutants produces only sperm (Zhang et al., 1997), only 0.2% of the *nos-3(q650)* mutant hermaphrodites fail to switch from spermatogenesis to oogenesis (Kraemer et al., 1999).

Here, we show that *larp-1* acts redundantly with *nos-3* to control the sperm-oocyte switch in the hermaphrodite *C. elegans* germline. *larp-1* encodes a protein containing a LA motif (LAM) and a highly conserved C-terminal region named DM15/LARP1. LARP-1 is a member of the so-called LAM protein super-family, which is present throughout the eukaryotic kingdom from unicellular organisms to vertebrates (Nykamp et al., 2008; Bousquet-Antonelli and Deragon, 2009). The LAM protein super family is divided into five subfamilies based on their evolutionary and structural characteristics (Bousquet-Antonelli and Deragon, 2009). The genuine La family proteins contain a LAM and RNA recognition motif (RRM), localize to the nucleus and have been shown to have various functions, including RNA-chaperone activity and protection of small-nucleolar RNAs from degradation (Wolin and Cedervall, 2002). LARP1 belongs to LAM family 1 (Bousquet-Antonelli and Deragon, 2009). In *Drosophila*, loss of the LARP-1 homologue LARP1 causes several defects during male meiosis and embryogenesis, including defects in spindle-pole organization and cytokinesis (Ichihara et al., 2007; Blagden et al., 2009). Other members of the LAM family 1, including the human homologues LARP1 and LARP2/LARP1b, are uncharacterized.

In *C. elegans*, *larp-1* mutants have oogenesis defects similar to defects due to hyperactive Ras-MAPK signaling and have increased mRNA levels of several components of the MAPK-signaling pathway, suggesting that *larp-1* regulates Ras-MAPK signaling at several levels (Nykamp et al., 2008).

Here, we identify a role for *larp-1* in sex determination and show that *larp-1* is redundantly required with *nos-3* to allow the sperm-oocyte switch in the germline. We find that *larp-1*, in contrast to *nos-3*, is required to lower the abundance of *fem-3* mRNA in adult hermaphrodites. To our knowledge *larp-1* is the first gene identified that is shown to regulate levels of *fem-3* mRNA. Together with our genetic data, we propose that *larp-1* and *nos-3* act as distinct repressors of the CBC^{FEM-1} ubiquitin ligase, thereby promoting TRA-1 accumulation and oogenesis.

Results

LARP-1 and NOS-3 are redundantly required for the sperm-oocyte switch

We recently identified a role for NOS-3 in regulating cell polarity in the one-cell *C. elegans* embryo (Pacquelet et al., 2008). We showed that *nos-3(q650)* is a suppressor of *par-2(it5)* and that it regulates PAR-6 levels in a CUL-2-dependent manner (Labbe et al., 2006; Pacquelet et al., 2008). In an independent approach, we found that, similarly to *nos-3(q650)*, *larp-1(q783)* suppresses *par-2(it5)* lethality (E.Z., unpublished results). While generating *nos-3(q650);larp-1(q783)* double mutants to study the genetic interaction between these two *par-2(it5)* suppressors, we observed that a high proportion of *nos-3(q650);larp-1(q783)* worms were sterile. Closer examination of *nos-3(q650);larp-1(q783)* hermaphrodites showed that approximately half of them lacked oocytes and had an excess of sperm (Fig. 1 and Table 1). Similarly to the *nos-3(q650);larp-1(q783)* double mutant, *nos-3(q650); larp-1 (RNAi)* hermaphrodites lack oocytes and have an excess of sperm (Table 1). This phenotype indicates a failure of switching from spermatogenesis to oogenesis and is similar to the germline masculinization observed in animals carrying *fem-3* gain-of-function mutations (Barton et al., 1987). This phenotype was not observed in *larp-1(q783)* hermaphrodites (Nykamp et al., 2008) (Fig. 1 and Table 1) and was only observed in 0.2% of *nos-3(q650)* hermaphrodites (Kraemer et al., 1999). Thus, our data indicate that *nos-3* and *larp-1* are redundantly required in the hermaphrodite germline to regulate the switch from spermatogenesis to oogenesis.

LARP-1 and NOS-3 control oocyte arrangement and the length of the mitotic zone

Approximately half of the germlines in *nos-3(q650);larp-1(q783)* hermaphrodites were not masculinized. However, these germlines showed strong defects in the way oocytes were positioned in the gonad. In wild-type germlines, oocytes were aligned and formed a single row (Fig. 2Aa,B). By contrast, we found that in *larp-1(q783)* mutants, 30% of the germlines had two oocytes and 17% had three or more oocytes at the same proximal-distal position (Fig. 2Ab,B). This confirms previous results by Nykamp and co-workers, who reported that *larp-1(q783)* mutants have several rows of oocytes in the proximal germline as a result of hyperactive MAPK signaling (Nykamp et al., 2008). 18% of *nos-3(q650)* mutants also had several oocytes – in most cases, two oocytes – at the same proximal-distal position (Fig. 2B). *nos-3(q650); larp-1(q783)* mutants showed a strong increase in this phenotype: among the *nos-3(q650);larp-1(q783)* germlines that switched to oogenesis, 87% had two or more oocytes, and the majority (78%) had three or more oocytes at the same proximal-distal position (Fig. 2Ad,B). Altogether, our data indicate that LARP-1 and NOS-3 redundantly control oocyte arrangement.

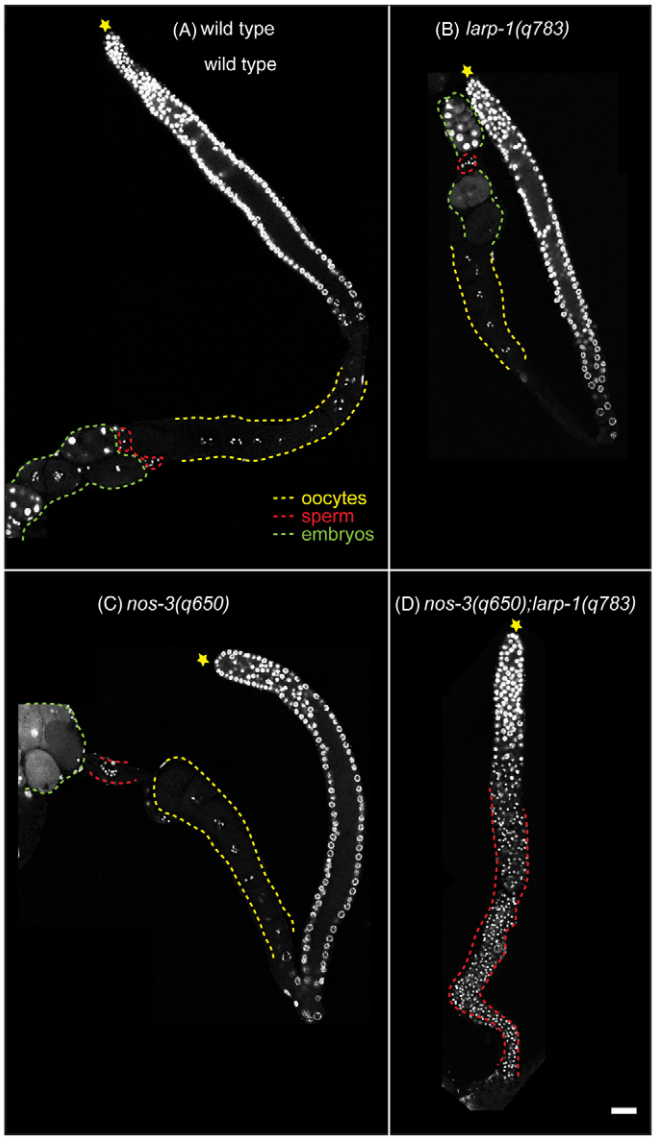

Fig. 1. *larp-1* and *nos-3* redundantly promote oogenesis. Adult hermaphrodite germlines of the indicated genotypes were dissected and stained with DAPI. *larp-1(q783)* and *nos-3(q650)* animals switch to oogenesis similarly to wild type, but 53% of *nos-3(q650);larp-1(q783)* double mutants do not switch to oogenesis and produce only sperm (see also Table 1). Yellow star indicates the distal tip of the germline and dashed lines highlight sperm (red), oocytes (yellow) and embryos (green). Scale bar: 50 µm.

We also observed that *larp-1(q783)*, *nos-3(q650)* and *nos-3 (q650);larp-1(q783)* germlines had a shorter mitotic zone compared with wild type. The distal end of the germline consists of mitotic cells. Following the mitotic zone is the 'transition zone', which contains both mitotic cells and cells that have entered meiosis. We measured the length of the mitotic zone in wild-type, *larp-1(q783)*, *nos-3(q650)* and *nos-3(q650);larp-1(q783)* germlines by counting the number of cells between the distal-tip cell (DTC) and the first

Table 1. Quantification of germline phenotypes

Genotype	*n*	Percentage of each phenotype				Conditions (temperature, RNAi)
		Wild type	Mog	Fem	No or L2/L3-like germline	
Wild type	150	100	0	0	0	25ºC
larp-1(q783)	306	99	0	0	1	25ºC
nos-3(q650)	125	100	0	0	0	25ºC
nos-3(q650);larp-1(q783)	253	47	53	0	0	25ºC
fem-3(e2006)	151	3	0	97	0	25ºC
larp-1(q783);fem-3(e2006)	139	4	0	96	0	25ºC
nos-3(q650);fem-3(e2006)	172	4	0	96	0	25ºC
nos-3(q650);larp-1(q783);fem-3(e2006)	286	5	0	95	0	25ºC
larp-1(RNAi)	20	100	0	0	0	25ºC, feeding
nos-3(q650)	60	100	0	0	0	25ºC, feeding
nos-3(q650);larp-1(RNAi)	102	59	41	0	0	25ºC, feeding
nos-1(RNAi)	94	100	0	0	0	25ºC, injection
nos-1(RNAi);larp-1(q783)	174	98	0	0	2	25ºC, injection
nos-2(RNAi)	152	73	0	0	27	25ºC, injection
nos-2(RNAi);larp-1(q783)	204	21	0	0	79	25ºC, injection
unc-5(e53)fem-3(q22)	215	59	41	0	0	15ºC
larp-1(q783);unc-5(e53)	58	100	0	0	0	15ºC
larp-1(q783);unc-5(e53)fem-3(q22)	105	18	82	0	0	15ºC
nos-3(q650);unc-5(e53)	77	100	0	0	0	15ºC
nos-3(q650);unc-5(e53)fem-3(q22)	99	13	87	0	0	15ºC

n, number of germlines. Germline phenotypes were determined by DAPI staining.

germ cells entering meiotic prophase (recognized by their 'half-moon' shape in DAPI-stained germlines, see supplementary material Fig. S1A). We found the mitotic region length to be 18.7 cell-diameters in wild-type germlines, but only 11.9, 11.7 and 9.8 cell-diameters in *larp-1(q783)*, *nos-3(q650)* and *nos-3(q650);larp-1(q783)* germlines, respectively (see supplementary material Fig. S1B).

To further validate these observations, we determined the size of the mitotic zone using markers specific for the proliferative or the meiotic zones. As a marker of the proliferative zone we used the cohesin REC-8 (Pasierbek et al., 2001) and as a meiotic prophase marker the synaptonemal complex protein HIM-3 (Zetka et al., 1999). Germ cells in the mitotic zone were stained only by REC-8 and not by HIM-3 (Fig. 3A) (Hansen et al., 2004a), whereas the 'transition zone' contained both REC-8- and HIM-3-positive germ cells (Fig. 3A). Using this method, we found that the mitotic region in wild-type germlines was 18.7 cell-diameters in size. Similarly to the results obtained by DAPI staining, we found that the mitotic region in *larp-1(q783), nos-3(q650)* and *nos-3(q650);larp-1(q783)* germlines was shorter than in wild type, ending 14.7, 11.8 and 11.6 cell-diameters from the DTC, respectively (Fig. 3B).

Finally, we counted the total number of germ cells occupying the mitotic region of the germline. Although wild-type germlines contained on average 243 germ cells in the mitotic region (Fig. 3C), *larp-1(q783)*, *nos-3(q650)* and *nos-3(q650);larp-1(q783)* germlines contained on average only 194, 188, and 152 mitotic germ cells, respectively (Fig. 3C). The total number of mitotic germ cells or the size of the mitotic region showed no correlation with the sex of the *nos-3(q650);larp-1(q783)* germline (data not shown). Therefore, as for other genes regulating sex determination (Zhang et al., 1997; Crittenden et al., 2002; Bachorik and Kimble, 2005; Thompson et al., 2005; Ariz et al., 2009), *nos-3* and *larp-1* also regulate the size of the proliferation zone in the germline. To conclude, LARP-1 and NOS-3 both have several functions in the germline, including regulation of the sperm-oocyte switch, oocyte arrangement and cell number in the mitotic zone.

larp-1 is redundantly required with *nos-2* for germline formation

In *C. elegans*, there are three homologues of *Drosophila* Nanos: NOS-1, NOS-2 and NOS-3 (Kraemer et al., 1999). All three single mutants have no or very mild defects in the sperm-oocyte switch but 12% of *nos-1(RNAi)nos3(q650)* and 24% of *nos-2(RNAi)nos-3(q650)* animals do not switch from spermatogenesis to oogenesis (Kraemer et al., 1999). *nos-1 nos-2* double mutants do not exhibit defects in the sperm-oocyte switch but *nos-1* and *nos-2* are redundantly required for germ-cell proliferation and survival at the second larval stage (Subramaniam and Seydoux, 1999). Since *nos-3* and *larp-1* redundantly control the sperm-oocyte switch, we tested whether *larp-1* also showed a genetic interaction with *nos-1* or *nos-2*. Depletion of *nos-1* by RNAi in *larp-1(q783)* did not result in a synthetic genetic interaction, as 98% of *nos-1(RNAi);larp-1(q783)* hermaphrodites had a wild-type germline (Table 1). Depletion of *nos-2* by RNAi in wild-type worms resulted in 27% of animals that either had no germline, or a small L2/L3-like germline (Table 1; supplementary material Fig. S2), which is similar to a previous report (Subramaniam and Seydoux, 1999). Depletion of *nos-2* by RNAi in *larp-1(q783)* resulted in 79% sterile animals. A closer examination of the sterile *nos-2(RNAi);larp-1(q783)* adult hermaphrodites, showed that they had either a small L2/L3-like germline or no germline (Table 1; supplementary material Fig. S2).

Our results therefore show that *larp-1* regulates the sperm-oocyte switch redundantly with *nos-3*, but not with the other Nanos homologues, *nos-1* and *nos-2*. Moreover, whereas we did not observe a genetic interaction with *nos-1*, we found that *larp-1* and *nos-2* were redundantly required for germline formation.

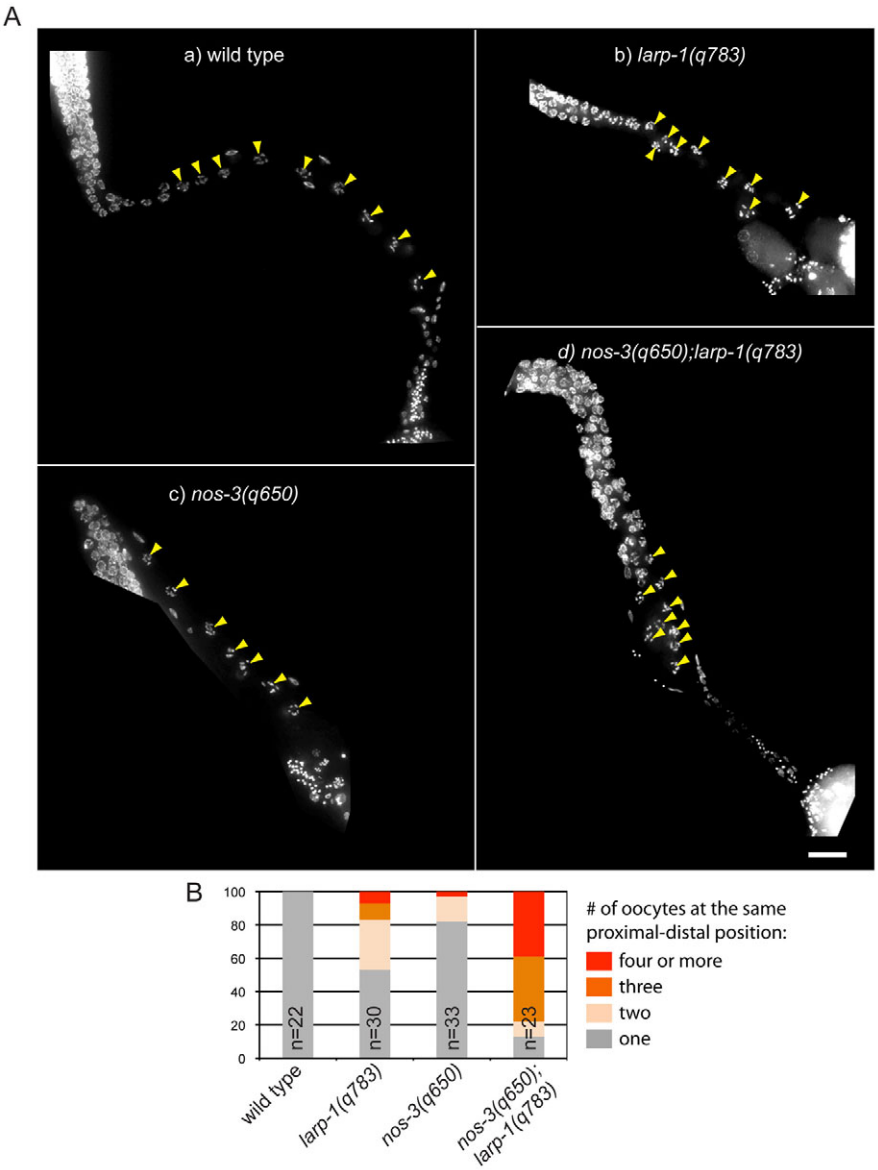

Fig. 2. *larp-1* and *nos-3* redundantly regulate oocyte arrangement in the gonad. (**A**) Germlines of the indicated phenotypes were stained with DAPI. Since oocytes are often found in different focal planes, maximum intensity projections are shown. In wild type (a) and most *nos-3(q650)* germlines (c), oocytes form a single row. In *larp-1(q783)* (b) and *nos-3 (q650);larp-1(q783)* (d) germlines, several oocytes are often found at the same proximal-distal position. Oocyte nuclei are indicated by yellow arrowheads. Scale bar: 20 µm. (**B**) Quantification of oocyte-arrangement defects. 47% of *larp-1(q783)* germlines have several oocytes at the same proximal-distal position: 30% have two (beige), 10% have three (orange) and 7% have four or more (red) oocytes at the same position. Germlines of the *nos-3(q650); larp-1(q783)* double mutant show a stronger phenotype than either single mutant: 9% had two, 39% had three and 39% had four or more of stacked oocytes. *n*=number of germlines analyzed.

The masculinization phenotype of *nos-3(q650);larp-1(q783)* germlines requires *fem*-3

We next focused on the masculinization phenotype of *nos-3 (q650);larp-1(q783)* double mutants. NOS-3 interacts with the *Pumilio* homologues FBF-1/2 (Kraemer et al., 1999), which bind to a regulatory element in the *fem-3* mRNA and have been proposed to repress *fem-3* translation at the L4 stage to allow oogenesis (Zhang et al., 1997). We therefore tested whether the sperm-oocyte switch defect due to simultaneous loss of *nos-3* and *larp-1* depends on *fem-3*. Contrary to *nos-3(q650);larp-1 (q783)* mutants and like *fem-3(e2006)* mutants, *nos-3 (q650);larp-1(q783);fem-3(e2006)* hermaphrodites had feminized germlines (Fig. 4 and Table 1, all genotypes in Fig. 1 and Fig. 4 were examined in parallel and under the same conditions). This indicates that the masculinization phenotype of the *nos-3(q650);larp-1(q783)* double mutant depends on *fem-3*. Consistent with the notion that both LARP-1 and NOS-3 promote oogenesis, we also found that inactivation of *larp-1* and *nos-3* enhanced the Mog (Masculinization of the germline) phenotype of a *fem-3* temperature-sensitive gain-of-function allele [*fem-3 (q22)* (Barton et al., 1987)] grown at the permissive temperature. Indeed, loss of *larp-1* or *nos-3* in *fem-3(q22)* doubles the number of germlines with a Mog phenotype [Table 1, compare *unc-5 (e53)fem-3(q22)* with *larp-1(q783);unc-5(e53)fem-3(q22)* and *nos-3(q650);unc-5(e53)fem-3(q22)*].

Therefore, masculinization of the germline in the *nos-3 (q650);larp-1(q783)* mutant depends on *fem-3,* which is consistent with NOS-3 and LARP-1 inhibiting *fem-3* to allow oogenesis to proceed.

NOS-3 and LARP-1 promote TRA-1 expression in the hermaphrodite germline

FEM-3 has been shown to function as a cofactor that enhances the activity of the CBC^{FEM-1} ubiquitin ligase and therefore promotes degradation of TRA-1 (Starostina et al., 2007), a transcription factor that inhibits spermatogenesis and promotes oogenesis (Hodgkin, 1987; Zarkower and Hodgkin, 1992). In wild-type hermaphrodite adult germlines, TRA-1 is predominantly localized

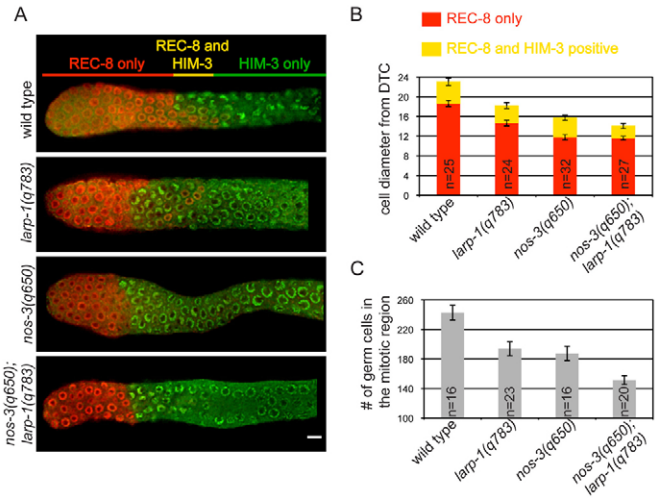

Fig. 3. *larp-1* and *nos-3* control the size of the mitotic region. (**A**) Surface view of REC-8- and HIM-3-stained dissected gonads of adult hermaphrodites of the indicated genotypes. Next to the distal tip of the germline is the mitotic zone (red line) where germ cells are stained only by REC-8 (red). Following the mitotic zone is the 'transition zone' (yellow line) where germ cells are stained with REC-8 and HIM-3 (yellow). The end of the 'transition zone' is defined by the last REC-8-only positive germ cell furthest from the DTC. After the 'transition zone', all germ cells enter meiosis and are only stained by HIM-3 (green line). Scale bar: 10 µm. (**B**) Quantification of the size of the mitotic region length and the 'transition zone'. The mitotic region is smaller in *larp-1(q783), nos-3(q650)* and *nos-3(q650);larp-1(q783)* than in wild type (all $P<0.001$). Error bars display s.e.m. *n*=number of germlines analyzed. (**C**) Quantification of the total number of germ cells in the mitotic zone. There are fewer germ cells in the mitotic region in *larp-1(q783), nos-3(q650)* and *nos-3(q650);larp-1(q783)* than in wild type (all $P<0.001$). Error bars display s.e.m. *n*=number of germlines analyzed.

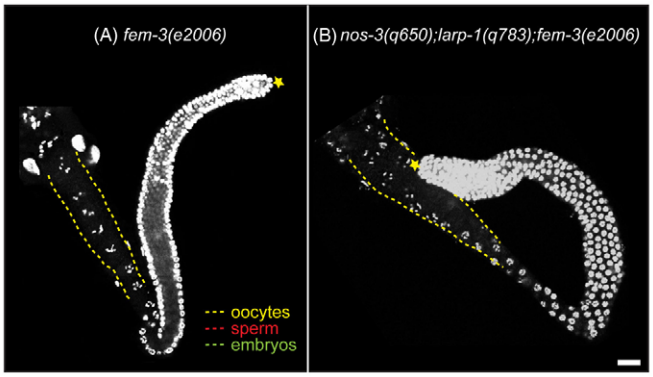

Fig. 4. *larp-1* and *nos-3* promote oogenesis in a *fem-3*-dependent manner. Adult hermaphrodite germlines of the indicated genotypes were dissected and stained with DAPI. Approximately 95% of *fem-3(e2006)* (**A**) as well as *nos-3 (q650);larp-1(q783);fem-3(e2006)* (**B**) hermaphrodite germlines are feminized (see also Table 1). Yellow star indicates the distal tip of the germline and dashed lines highlight sperm (red), oocytes (yellow) and embryos (green). Scale bar: 50 µm.

in the nucleus, with high expression in the distal region of the gonad (Schvarzstein and Spence, 2006; Starostina et al., 2007). We observed that TRA-1 levels were reduced in the distal region of *larp-1(q783)* and *nos-3(q650)* single-mutant germlines (Fig. 5Aa-c). Quantification of TRA-1 levels in the nuclei of the proliferation zone indicated that TRA-1 levels in *larp-1(q783)* and *nos-3(q650)* germlines corresponded to 76% and 67% of wild-type levels, respectively (Fig. 5B). TRA-1 levels were further reduced in *nos-3 (q650);larp-1(q783)* double-mutant germlines (Fig. 5Ad), corresponding to 34% of wild-type levels (Fig. 5B). This strong reduction in TRA-1 levels is consistent with their masculinization phenotype.

TRA-1 is also expressed in hermaphrodite somatic tissues where it inhibits all aspects of male somatic differentiation, including male anatomy, physiology and behavior (Hodgkin, 1987). TRA-1 is in particular expressed in the hermaphrodite intestine, where it localizes mostly in the nuclei. Notably, intestinal nuclear expression is lower in males than in hermaphrodites (Schvarzstein and Spence, 2006; Starostina et al., 2007). We found that TRA-1 levels in the intestinal nuclei of *nos-3(q650);larp-1(q783)* mutant were similar to those in the wild type (see supplementary material Fig. S3), indicating that *nos-3* and *larp-1* promote TRA-1 expression only in the germline and not in somatic tissues. This finding is consistent with our observation that the somatic tissues of *nos-3(q650);larp-1 (q783)* hermaphrodites have an apparently normal hermaphrodite morphology (data not shown).

We found that the decrease of TRA-1 levels in *larp-1(q783), nos-3(q650)* and *nos-3(q650);larp-1(q783)* mutant germlines required FEM-3. Indeed, there was no significant decrease of TRA-1 levels in either *larp-1(q783);fem-3(e1996)*, *nos-3 (q650);fem-3(e1996)* or *nos-3(q650);larp-1(q783);fem-3(e1996)* germlines compared with *fem-3(e1996)* (Fig. 5Ae-h,B). In conclusion, our data suggest that both *larp-1* and *nos-3* promote TRA-1 expression by inhibiting *fem-3*, and this function is specific to the germline.

LARP-1 lowers *fem-3* mRNA levels

LARP-1 binds poly-U and poly-G stretches of RNA in vitro and lowers the abundance of several mRNAs involved in the MAPK-signaling pathway in vivo (Nykamp et al., 2008). Since LARP-1 appears to promote oogenesis and regulate TRA-1 levels in a FEM-3-dependent manner, we tested whether LARP-1 regulates the abundance of *fem-3* mRNA. We measured mRNA levels of *fem-3* and *eft-3* (which encodes a translation elongation factor) with real-time PCR (rt-PCR) in *nos-3(q650)* and *larp-1(q783)* L4 hermaphrodites. As previously reported, we found that *eft-3* mRNA levels were not affected in *larp-1(q783)* mutants (Fig. 6) (Nykamp et al., 2008). In contrast to *eft-3*, *fem-3* mRNA levels were increased two- to threefold in *larp-1(q783)* and *nos-3(q650);larp-1(q783)* mutants in comparison with wild type (Fig. 6). No change in the level of the *fem-3* transcript was observed in *nos-3(q650)* mutants (Fig. 6). This finding indicates that LARP-1, in contrast to NOS-3, controls *fem-3* expression by regulating the levels of *fem-3* mRNA.

Discussion

In this work, we identify several roles for the LA-related protein LARP-1 during the development of the *C. elegans* germline. Interestingly, many of these roles are exacerbated by the simultaneous depletion of NOS-3 or NOS-2. We found that *nos-3 (q650);larp-1(q783)* double-mutant hermaphrodites have a masculinized germline and low TRA-1 protein levels. Note that *tra-1* mRNA levels are not reduced in the *nos-3(q650);larp-1(q783)* double mutant (data not shown). Consistent with TRA-1 being targeted to degradation by the CBC^{FEM-1} ubiquitin ligase (Starostina

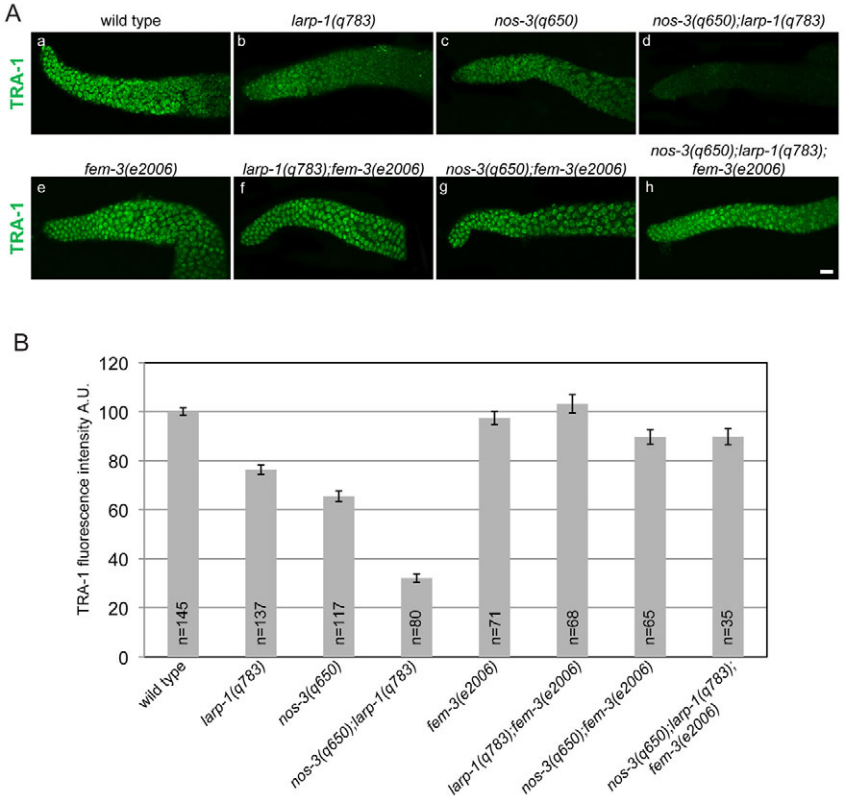

Fig. 5. *larp-1* and *nos-3* promote TRA-1 expression in the germline in a *fem-3*-dependent manner. (**A**) Adult hermaphrodite germlines of the indicated genotypes were dissected and stained with anti-TRA-1 antibody. Distal tips of the germlines point to the left. Representative images of the indicated genotypes are shown. Scale bar: 10 µm. (**B**) Quantification of TRA-1 levels. Mean fluorescence intensity (arbitrary units) was measured in nuclei of the mitotic zone. TRA-1 levels are reduced in *larp-1(q783)* and *nos-3(q650)* germlines compared with wild type and further reduced in *nos-3(q650);larp-1(q783)* (all $P<10^{-10}$) but are not reduced in *larp-1(q783);fem-3(e1996), nos-3 (q650);fem-3(e1996)* and *nos-3(q650);larp-1(q783);fem-3 (e1996)* germlines compared with *fem-3(e1996)* (all $P>0.05$). Error bars display s.e.m. *n*=number of analyzed germlines.

et al., 2007), we show that the regulation of the sperm-oocyte switch and of TRA-1 levels by *nos-3* and *larp-1* requires the activity of *fem-3*, suggesting that both NOS-3 and LARP-1 negatively regulate *fem-3* (Fig. 7). Indeed, LARP-1 negatively controls *fem-3* mRNA levels. LARP-1 also negatively regulates the abundance of mRNAs of the MAPK-signaling pathway (Nykamp et al., 2008). Taken together, these data suggest that LARP-1 regulates mRNAs, in particular *fem-3* mRNA, by regulating their stability. Consistently, *Drosophila* Larp physically interacts with PABP (poly-A-binding protein) and is proposed to affect mRNA stability by modulating the interaction between PABP and the poly-A tail of mRNAs (Blagden et al., 2009). We tested whether LARP-1 interacts with *fem-3* mRNA by performing immunoprecipitation experiments with a LARP-1 antibody. However, we found no enrichment of *fem-3* mRNA in LARP-1 immunoprecipitates (supplementary material Fig. S4). This result suggests that the interaction between LARP-1 and *fem-3* mRNA is too weak or too transient to be detected under these conditions or that LARP-1 affects *fem-3* mRNA levels indirectly by controlling an unidentified regulator of *fem-3* mRNA abundance.

In *nos-3* mutants, *fem-3* mRNA levels are not affected, indicating that NOS-3, in contrast to LARP-1, does not regulate the stability of the *fem-3* transcript. This is consistent with a model in which NOS-3 represses *fem-3* translation with FBF-1 and FBF-2 (Kraemer et al., 1999). Indeed, mutations in the *fem-3* 3′UTR that prevent the interaction between *fem-3* mRNA and FBF-1/2, result in an increase of *fem-3* mRNA poly-A tail length, but not in increased mRNA levels (Ahringer and Kimble, 1991; Zhang et al., 1997). FBF-1/2 physically interact with the CCF-1/Pop2 deadenylase in vitro and enhance the deadenylation activity of CCF-1/Pop2 on *gld-1* substrate mRNA in vitro (Suh et al., 2009). Whether FBF-1/2 also regulate the poly-A tail length of *fem-3* mRNA by recruiting and enhancing CCF-1/Pop2 deadenylating activity and how *nos-3* contributes to FBF-1/2-dependent repression of *fem-3* mRNA is currently unknown. The redundancy between *nos-3* and *larp-1* suggests that the repression of *fem-3* translation by FBF-1/2 is compromised in *nos-3* mutants, but is still sufficient to maintain low levels of FEM-3 and thereby high TRA-1 levels, to promote the sperm-oocyte switch. The concomitant increase of *fem-3* mRNA abundance as a result of *larp-1* depletion would then result in higher *fem-3* expression levels and thus in levels of TRA-1 which are too low to allow the sperm-oocyte switch in *nos-3;larp-1* mutants.

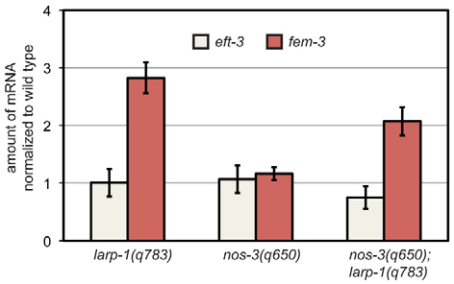

Fig. 6. *fem-3* mRNA levels are increased in *larp-1(q783)* and *nos-3 (q650);larp-1(q783)* mutants. *fem-3* and *eft-3* mRNA levels were measured by rt-PCR in wild type, *larp-1(q783), nos-3(q650)* and *nos-3(q650);larp-1 (q783)* L4 hermaphrodites. Ratio of *fem-3* and *eft-3* mRNA in *larp-1(q783), nos-3(q650)* and *nos-3(q650);larp-1(q783)* mutants relative to wild type (wild type=1). The average of three independent experiments is shown. Error bars display s.e.m. *fem-3* mRNA levels are reduced in *larp-1(q783)* compared with wild type ($P<0.005$), but not in *nos-3(q650)*. The difference in *fem-3* mRNA levels between *larp-1(q783)* and *nos-3(q650);larp-1(q783)* is statistically not significant ($P>0.1$).

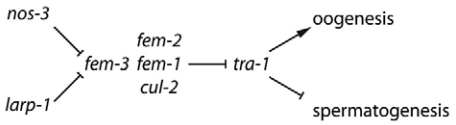

Fig. 7. LARP-1 and NOS-3 redundantly regulate sex determination by regulating *fem-3* expression. Scheme showing a simplified version of the hermaphrodite sex determination pathway, and the redundant role of *larp-1* and *nos-3* in *fem-3* regulation.

In addition to the FBF-1/2-dependent translational regulation of *fem-3* mRNA (Zhang et al., 1997), FEM-3 protein binds to and is inhibited by TRA-2 (Mehra et al., 1999). LARP-1 is the first gene identified that controls the abundance of *fem-3* mRNA, thereby revealing an additional mechanism for *fem-3* regulation. These multiple regulatory mechanisms emphasize the importance of controlling *fem-3* expression for proper sperm-oocyte switch. FEM-3 stimulates the activity of the CBC-1^{FEM-1} ubiquitin ligase, which degrades TRA-1; however, its exact molecular function is unknown. An understanding of its precise function might reveal why *fem-3* levels and activity have to be tightly regulated.

The regulation of TRA-1 by *nos-3* and *larp-1* occurs only in the germline and not in somatic tissues. LARP-1 is ubiquitously expressed (our unpublished results) (Nykamp et al., 2008) and *nos-3* mRNA was also detected in somatic tissues (Kraemer et al., 1999). This suggests that the repression of *fem-3* by NOS-3 and LARP-1 is restricted to the germline by a germline-specific factor that works with NOS-3 and/or LARP-1. FBF-1 and FBF-2 are good candidates because they are expressed only in the germline, interact with NOS-3 and regulate the sperm-oocyte switch (Zhang et al., 1997; Kraemer et al., 1999).

As previously shown by Nykamp and colleagues, we found that several oocytes can occupy the same proximal-distal position in *larp-1* single mutants. This phenotype is also observed in *nos-3 (q650)* mutants and strongly increased in *nos-3(q650);larp-1 (q783)* double mutants. In *larp-1* mutants the phenotype was shown to depend on hyperactivation of MAPK signaling and the mRNA abundance of several MAPK-signaling components, including *mpk-1*, was increased (Nykamp et al., 2008). Whether LARP-1 controls their mRNA abundance directly has not been addressed yet. Interestingly, FBF-1/2 have been shown to negatively regulate expression of MAPK (Lee et al., 2007). Altogether, this suggests that NOS-3 and LARP-1 might redundantly regulate MAPK signaling. As for *fem-3* regulation, it is plausible that in *nos-3;larp-1* double mutants the simultaneous decrease of translational repression and increase of mRNA levels result in increased expression of *mpk-1* and/or other components of the pathway, thereby leading to increased MAPK signaling.

LARP-1 and NOS-3 are also required to maintain the normal length of the mitotic region in the distal part of the germline. These results suggest that LARP-1 and NOS-3 are required either to promote mitosis in the proliferation zone or to inhibit entry into meiosis. Similarly, several other genes such as *puf-8*, *mex-3* and *fbf-1* regulate both mitosis-meiosis and sperm-oocytes decisions (Zhang et al., 1997; Crittenden et al., 2002; Bachorik and Kimble, 2005; Thompson et al., 2005; Ariz et al., 2009). Whether there is a direct link between the regulation of these two processes is currently not known. Although we found that *nos-3(q650)* mutants have a smaller mitotic region, it was also shown that *nos-3*, redundantly with *gld-2*, is required for entry into meiosis (Hansen et al., 2004b). This suggests that *nos-3*, probably depending on its binding partner, can promote both mitosis and entry into meiosis. Likewise, it was suggested that FBF-1, depending on its binding partner GLD-2 or CCF-1/Pop2, can either promote or inhibit GLD-1 expression, thereby explaining how FBF-1 can both maintain cells in mitosis and promote entry into meiosis (Suh et al., 2009).

Finally, we found that *larp-1*, contrary to *nos-3,* does not control the sperm-oocyte switch redundantly with *nos-1* and *nos-2* (Kraemer et al., 1999). Instead, *nos-2(RNAi);larp-1(q783)* double mutants have either no germ cells or a small L2/L3-like germline. Interestingly, *nos-2* was shown, with *nos-1*, to be required for germ-cell proliferation and survival during larval development (Subramaniam and Seydoux, 1999). Our observations therefore suggest that *larp-1* might function in the same pathway as *nos-1*.

In summary, we found that *larp-1*, with *nos-3* and *nos-2*, regulates several processes during germline development. Our results suggest that the regulation of both mRNA abundance and translation is a widespread mechanism to ensure tight regulation of several crucial steps of germline development.

Materials and Methods

Strains

All strains were maintained as described by Brenner (Brenner, 1974). The wild-type strain was the N2 (Bristol) strain. The alleles used in this study were: LGII, *nos-3 (q650)* (Kraemer et al., 1999); LGIII, *larp-1(q783)* (Nykamp et al., 2008); LGIV, *fem-3(e2006)* (temperature-sensitive loss-of-function allele) (Hodgkin, 1986), *fem-3 (e1996)* (null allele) (Hodgkin, 1986), *unc-5(e53)fem-3(q22)gf* (temperature-sensitive gain-of-function allele) (Barton et al., 1987).

RNAi and conditions used to determine germline phenotypes

larp-1(RNAi) construct covering bp3241 to 4771 of R144.7 (unspliced) was cloned into a Gateway compatible L4440 vector (gift from Simon Boulton, London Research Institute, Clare Hall, South Mimms, UK). The *nos-1(RNAi)* was made from cDNA and covered the *nos-1* coding region and *nos-2(RNAi)* construct was previously described (Kamath and Ahringer, 2003). *nos-1(RNAi)* and *nos-2(RNAi)* were both efficient as judged by the fact that *nos-1(RNAi)nos-2(RNAi)* double RNAi resulted in 91% of worms with a small or absent germline (n=248).

Germline phenotypes of *larp-1(q783), nos-3(q650)*, *nos-3(q650);larp-1(q783)* and *nos-3(q650);larp-1(q783);fem-3(e2006)* were determined after growing worms at 25°C and staining germlines with DAPI 24 hours after L4. Germline phenotypes of *nos-3(q650);larp-1(RNAi)* worms were examined by staining germlines with DAPI 52 hours after feeding L1 worms on 1 mM IPTG plates at 25°C. As a control, *nos-3 (q650)* worms were fed with the L4440 vector (Timmons and Fire, 1998). To determine the germline phenotype of *larp-1(q783);unc-5(e53)fem-3(q22), larp-1 (q783);unc-5(e53)*, *nos-3(q650);unc5(353)fem-3(q22), nos-3(q650);unc5(353)* and *unc-5(e53)fem-3(q22),* worms were grown at 15°C and germlines stained with DAPI 24 hours after L4. *nos-1* and *nos-2 RNAi* were injected into young adult worms, the injected animals were incubated at 25°C singled after 24 hours. The germline phenotype of F1 generation of the injected adults was determined by staining germlines with DAPI 18 hours after L4.

TRA-1 levels of *larp-1(q783)*, *nos-3(q650)*, *nos-3(q650);larp-1(q783)*, *larp-1 (q783);fem-3(e1996)*, *nos-3(q650);fem-3(e1996)*, *nos-3(q650);larp-1(q783);fem-3 (e1996)* and *fem-3(e1996)* were measured by growing worms at 25°C and staining germlines with anti-TRA-1 antibody 24 hours after L4. To determine the size of the mitotic region of *larp-1(q783)*, *nos-3(q650)* and *nos-3(q650);larp-1(q783)*, L1 worms were placed at 25°C and examined by DAPI, REC-8 and HIM-3 staining 18-24 hours after L4.

Quantitative real-time PCR

N2, *larp-1(q783), nos-3(q650)* and *nos-3(q650);larp-1(q783)* worms were grown at 25°C. Mid-to-late L4 larvae were harvested. RNA quantification was performed on L4 rather than adult worms in order to compare different genotypes with similar germlines (i.e. all producing sperms) and not worms producing both sperm and oocytes (N2, *nos-3* and *larp-1*) with worms producing only sperm (*nos-3;larp-1* mutants). Total RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel). Reverse transcription was done from 1 µg total RNA using random hexamers and Superscript III reverse transcriptase (Invitrogen). Real-time PCR was performed with Power SYBR Green Master Mix (Applied Biosystems) on a SDS 7900 HT instrument (Applied Biosystems). Each reaction was performed in three replicates. Raw C_t values obtained with SDS 2.2 (Applied Biosystems) were imported in Excel and normalization factor and fold changes were calculated using the GeNorm method (Vandesompele et al., 2002). *pgk-1* mRNA was used to normalize mRNA amounts. The following oligos

were used: 5′-AAGGCGGAACGGGAAAGT-3′ and 5′-ATCAAATCTTGCAACTC-GAATCG-3′ for *fem-3*, 5′-AAGAACATGATCACTGGAACATCC-3′ and 5′-GC-GACGACCAAGACGGC-3′ for *efl-3*, 5′-GCAAAATCACCAACAATCAACGT-3′ and 5′-CAGATTTGGCT CCGTTGCTAA-3′ for *pgk-1*.

Microscopy, immunofluorescence and image analysis

For TRA-1 (1/40) (Schvarzstein and Spence, 2006) immunofluorescence analysis, germlines and embryos were fixed in methanol and stained according to standard procedures. For HIM-3 (Zetka et al., 1999) and REC-8 (Pasierbek et al., 2001) staining, germlines were fixed for 5-10 minutes with 3% formaldehyde, 0.1 M K_2HPO_4, pH7.2, followed by a 5 minute fixation in 100% methanol (Hansen et al., 2004a). As a secondary antibody, we used Alexa-Fluor-488-coupled goat anti-rabbit (Molecular Probes) and Texas-Red-coupled anti-rat (Jackson ImmunoResearch). DAPI was used to stain DNA and images were acquired using a Leica SP2 confocal microscope (TRA-1) and DeltaVision system (Applied Precision) (REC-8 and HIM-3).

To quantify TRA-1 levels, germlines of the different mutants were stained in parallel under the same conditions and images were acquired using the same settings. For each analyzed germline, the mean fluorescence intensity per pixel was obtained by measuring and averaging the mean fluorescence intensity per pixel of 5 nuclei in the mitotic zone and subtracting the fluorescence background next to the observed germline. To quantify the total number of germ cells in the mitotic zone we acquired *z*-stacks of distal germlines and counted the germ cell using the ROI manager from ImageJ (http://rsb.info.nih.gov/ij/).

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http://jcs.biologists.org/cgi/content/full/123/16/2717/DC1

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