PUF-8 negatively regulates RAS/MAPK signalling to promote differentiation of *C. elegans* germ cells

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SUMMARY

Signals that promote germ cell self-renewal by preventing premature meiotic entry are well understood. However, signals that control mitotic proliferation to promote meiotic differentiation have not been well characterized. In *Caenorhabditis elegans*, GLP-1 Notch signalling promotes the proliferative fate by preventing premature meiotic entry. The germline niche cell, which is the source of the ligand for GLP-1, spatially restricts GLP-1 signalling and thus enables the germ cells that have moved away from the niche to enter meiosis. Here, we show that the suppression of RAS/MAP kinase signalling in the mitotic and meiotic-entry regions is essential for the regulation of the mitosis-meiosis switch by niche signalling. We provide evidence that the conserved PUF family RNA-binding protein PUF-8 and the RAS GAP protein GAP-3 function redundantly to suppress the LET-60 RAS in the mitotic and meiotic entry regions. Germ cells missing both PUF-8 and GAP-3 proliferate in an uncontrolled fashion and fail to undergo meiotic development. MPK-1, the MAP kinase downstream of the LET-60 RAS, is prematurely activated in these cells; downregulation of MPK-1 activation eliminates tumours and restores differentiation. Our results further reveal that PUF-8 negatively regulates LET-60 expression at a post-transcriptional step. LET-60 is misexpressed in the *puf-8(–)* mutant germlines and PUF-8 physically interacts with the *let-60* 3′ UTR. Furthermore, PUF-8 suppresses *let-60* 3′ UTR-mediated expression in the germ cells that are transitioning from the mitotic to meiotic fate. These results reveal that PUF-8-mediated inhibition of the RAS/MAPK pathway is essential for mitotic-to-meiotic fate transition.

KEY WORDS: PUF-8, GAP-3, RAS, MAPK, *C. elegans*, Germ cells, Mitosis-meiosis decision

INTRODUCTION

Maintenance of a balance between proliferation and meiotic development is crucial for the continuous generation of gametes. Although uncontrolled proliferation leads to germline tumours, entry of all germ cells into meiosis depletes the self-renewing germline stem cells (GSCs). In both invertebrates and vertebrates, signals from adjacent somatic cells – called the GSC niche – initiate GSC self-renewal programmes (Kimble, 2011; Lehmann, 2012). Studies on different model organisms indicate that the primary function of GSC niche signal is to prevent premature entry into meiosis/differentiation. For example, in the absence of GLP-1/Notch signalling from the niche, *C. elegans* GSCs prematurely enter meiosis and form sperm (Crittenden et al., 2002; Kadyk and Kimble, 1998; Lamont et al., 2004). Similarly, DPP signalling from the *Drosophila* GSC niche inhibit the transcription of the differentiation-promoting factor Bam (Song et al., 2004; Xie and Spradling, 1998). These observations underscore the importance of blocking premature differentiation in the maintenance of GSCs. However, the signals that directly promote proliferation, and whether the downregulation of such signals is crucial for meiotic entry, are still poorly understood.

In *C. elegans*, removal of MAP kinase pathway components *lin-45* RAF, *mek-2* MEK or MPK-1 ERK enhances the premature meiotic entry phenotype of the partial loss of *glp*-1 function (Lee et al., 2007b). Thus, MAP kinase signalling promotes the mitotic proliferative fate. However, null mutations in any of the above three genes do not affect the mitotic-to-meiotic fate switch. Furthermore, the phosphorylated MPK-1, the active form, is not detected in germ cells until the mid-pachytene stage (Lee et al., 2007b). Thus, the role of RAS/MAPK signalling in the germ cell fate decision, under wild-type conditions, is not known.

The *C. elegans* PUF protein PUF-8 promotes both proliferation and differentiation. In the mitotic region, it functions redundantly with another RNA-binding protein called MEX-3 to promote GSC proliferation (Ariz et al., 2009). Germ cells lacking both these proteins do not proliferate even when the meiotic-entry block is intact. By contrast, PUF-8 promotes meiotic progression in primary spermatocytes (Subramaniam and Seydoux, 2003). In its absence, primary spermatocytes exit meiotic development and dedifferentiate. As one of our approaches to identify the downstream effectors of PUF-8, we performed a genetic screen and isolated a number of mutants that were sterile only in the absence of PUF-8. Significantly, these mutants group into two classes with opposite phenotypes – severely reduced number of germ cells in one class and germ cell tumour in the other. One of the latter category maps to *gap-3*, which encodes a *C. elegans* orthologue of the GTPase-activating protein (GAP) (Stetak et al., 2008). GAPs downregulate MAPK signalling by promoting the hydrolysis of GTP bound to RAS, an upstream activator of this signalling pathway. Our results reveal that PUF-8 suppresses expression of LET-60 RAS in the zone where germ cells switch from the proliferative mode to meiotic differentiation, and that the uncontrolled proliferation observed in the *puf-8(–) gap-3(–)* double mutant is due to LET-60-dependant ectopic activation of MPK-1 in this zone. Thus, these results identify PUF-8 as a crucial upstream regulator of MAPK signalling and highlight the importance of the negative regulation of this signalling in the mitosis-meiosis decision.

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MATERIALS AND METHODS

C. elegans strains

All worm strains were maintained at 20°C as described (Brenner, 1974), with the exception that all transgenic lines were kept at 25°C to avoid germline silencing of transgenes (Strome et al., 2001). Transgenes were introduced into different mutant backgrounds by standard genetic crosses. The strains used in this study are listed in supplementary material Table S2.

Mutagenesis

The design of the genetic screen used to isolate mutants that display synthetic phenotype with *puf-8* is shown in supplementary material Fig. S1. Mutagenesis was carried out as described earlier using EMS (Brenner, 1974). Briefly, about 200 L4 larvae of IT60 strain (see supplementary material Table S2 for genotype) were incubated in 4 ml M9 medium containing 50 mM EMS for 6 hours at 20°C. The worms were washed, and allowed to recover on OP50 *E. coli* lawns for about 12 hours at 20°C. They were then transferred to fresh lawns and embryos (F1 generation) were collected. About 300 F1 adults were cloned and the F2 embryos were collected for 24 hours. F2 progeny were examined for sterility.

Transgenic worms

The transgene construct pSV39, which expresses the GFP::GAP-3 fusion protein, was made as follows: GAP-3 ORF was amplified in two parts using primers KS3575 and KS3576 on wild-type cDNA and primers KS3575 and KS3102 wild-type genomic DNA (see supplementary material Table S3 for primer sequences), and cloned in pSV2 using a standard TA-cloning procedure (Mainpal et al., 2011). These two fragments were then combined in one construct pSV37 through restriction digestion and ligation. The 3018 bp GAP-3 ORF containing the first intron was amplified using primers KS3575 and KS3576, and introduced between *Spe*I and *Nar*I sites of pKS114 (Mainpal et al., 2011) to generate pSV39.

The transgene construct pAK9, which expresses PUF-8::9×HA::GFP was made as follows: KS3788 and KS3789 (see supplementary material Table S6 for primer sequences) were annealed, end-filled and the resulting fragment was cloned at the *Spe*I site in pPK44, a plasmid that expresses PUF-8::GFP fusion protein under the control of the *puf-8* promoter, to generate pAK8. KS3791 and KS3792 were annealed, end-filled and cloned at the *Cfr*9I site of pAK8 to generate pAK10. KS3794 and KS3795 were annealed, end-filled and cloned at the *Pau*I site of pAK10 to generate pAK9.

The GFP-3′ UTR transgene fusions were made as follows: respective 3′ UTR sequences were PCR-amplified from wild-type genomic DNA using the corresponding primers (see supplementary material Table S5 for primer sequences) and inserted at the *Bsp*120I site of pKS114 (Mainpal et al., 2011). Mutations in the *let-60* 3′ UTR sequences were introduced through PCR primers described in supplementary material Table S6.

Each transgene construct was generated in duplicate and were introduced into *unc-119(–)* strain by biolistic bombardment, as described earlier (Jadhav et al., 2008; Praitis et al., 2001). Results of transgene expression patterns presented here are representatives of at least eight independent lines; in each case, at least 20 worms per line were examined.

Immunostaining and fluorescence microscopy

Dissection, fixation of gonads and immunostaining were performed as described earlier (Ariz et al., 2009), except that for detecting LET-60, dissected gonads were fixed in 2% formaldehyde for 1 hour at room temperature, followed by methanol at −20°C for 5 minutes in a glass tube. We confirmed the specificity of this antibody by staining gonads depleted of LET-60 by RNAi (supplementary material Fig. S6). These gonads were not freeze-cracked. For detecting dpMPK-1, mouse monoclonal antibodies against dpMPK-1 (Sigma) was used at 1:500 dilution; for LET-60, goat polyclonal anti-LET-60 antibodies (Santa Cruz; catalogue number sc 9210) were used at 1:50 dilution. Anti-PH3 and anti-HIM-3 antibodies have been described previously (Ariz et al., 2009). Fluorescent images of immunostained gonads and transgenic worms expressing GFP reporters were examined using a fluorescence microscope (Zeiss Axioskop II mot plus) and images were acquired using a CCD camera (Axiocam HRm). All images presented are representative of at least 50 gonads per experiment; each immunostaining and DAPI-staining was repeated at least four times.

Electrophoretic mobility shift assay

Mobility shift experiments were carried out as described previously (Jadhav et al., 2008). Template DNA fragments for *in vitro* transcriptions were generated as follows: for fragment 1, fragment 2, fragment 3, fragment 4 and fragment 5 of *let-60* 3′ UTR RNA, respective sequences were PCR amplified from wild-type genomic DNA using the corresponding primers (see supplementary material Table S6 for primer sequences). These fragments were cloned into pSV2 using the standard TA cloning method (Mainpal et al., 2011). Each fragment was checked for orientation by using KS2808 and the fragment-specific reverse primer. From such positive clones, for each fragment, *in vitro* transcription template was made by PCR amplification using KS2808 and the fragment-specific reverse primer. The final plasmids thus generated were used to PCR-amplify the template for *in vitro* transcription using KS3432 and KS 3441 primers. Mutations in fragment 4 were introduced through PCR primers (see supplementary material Table S6 for primer sequences).

Co-immunoprecipitation and RT-PCR

All the steps for co-immunoprecipitation were carried out at 4°C. About 150,000 synchronized IT722 adult hermaphrodites (see supplementary material Table S2 for genotype) were homogenized using a dounce homogenizer in homogenization buffer [100 mM NaCl, 25 mM HEPES (pH 7.5), 2 mM MgCl$_2$, 0.5 mM EGTA, 10 mM EDTA, 1 mM DTT, 4 mM Na$_3$VO$_4$, 3 mM NaF, 0.1% IGEPAL C-30 (Sigma) supplemented with protease inhibitor (Sigma) and RNase inhibitor (Thermo Scientific)]. Homogenate was cleared by centrifuging at 14,000 *g* for 20 minutes. For immunoprecipitation, 100 μl of the homogenate was pre-cleared with Protein A beads (Genescript) and incubated for 3 hours with 1.5 μg of anti-HA antibody (Genescript). Again, Protein A beads were added and sample was incubated for 2 hours. Beads were collected by centrifugation and washed three times with homogenization buffer. For RT-PCR, RNA was isolated from the above beads, using TRI Reagent (Sigma), treated with DNase I for 30 minutes (Fermentas), extracted with phenol: chloroform and precipitated with ethanol. The pellet was washed with 70% ethanol and dissolved in 20 μl of RNase-free water. Ten microlitres of this total RNA was used for cDNA synthesis, and used for PCR amplification using mRNA-specific primers. RNA-mediated interference was performed by a feeding method described previously (Timmons et al., 2001).

RESULTS

puf-8 genetically interacts with several genes to control both mitosis and meiosis

Even null alleles of *puf-8* display a temperature-sensitive phenotype. At 20°C, all *puf-8(–)* worms are fertile, albeit they have a smaller germline and produce fewer progeny. However, at 25°C, 100% of the *puf-8(–)* worms are sterile (Ariz et al., 2009; Subramaniam and Seydoux, 2003). *C. elegans* germ cell development is significantly accelerated at the higher temperature. Processes involving multiple redundant factors are probably more sensitive to loss of any one of these factors when the rate is accelerated. We reasoned the temperature sensitivity of *puf-8(–)* phenotype is probably due to such a phenomenon, and decided to identify other genes that function redundantly with *puf-8*. Indeed, *puf-8* does function redundantly with *mex-3* to promote mitotic proliferation of germ cells, and *fbf-1* and *lip-1* in sperm-oocyte switch (Ariz et al., 2009; Bachorik and Kimble, 2005; Morgan et al., 2010). To identify such additional genes, we carried out a genetic screen that was designed to isolate mutants that display sterile phenotype only in the *puf-8(−/−)* genetic background at 20°C. This screen yielded 71 alleles, of which 37 belong to 29 complementation groups. These could be grouped into three phenotypic classes (see supplementary material Fig. S1 for details of the genetic screen). Class I mutant gonads had very few germ cells; class II mutants produced excess amounts of mitotic germ cells; and germ cells in the class III mutant hermaphrodites failed to switch from spermatogenesis to oogenesis (Fig. 1). Each class contained multiple
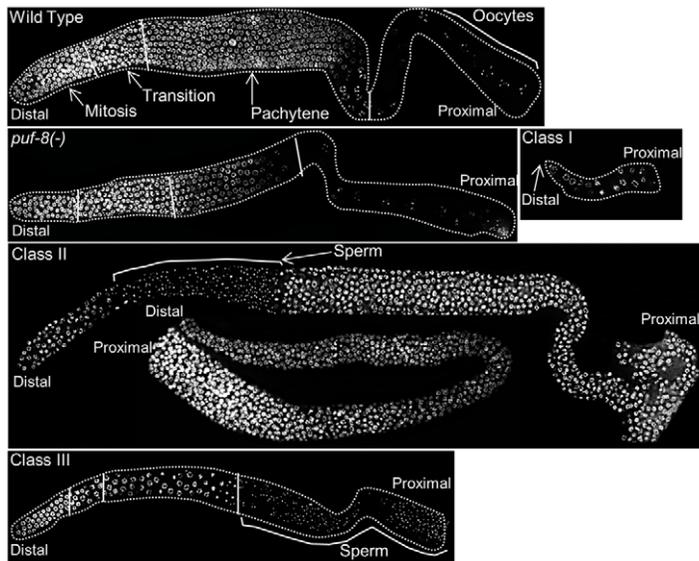
Fig. 1. Mutants synthetic sterile with *puf-8(–)* belong to three distinct classes. Dissected gonads, stained with the DNA-binding dye DAPI, are shown. Only the germline nuclei are visible. In this and subsequent figures, the distal mitotic region is oriented towards the left and the proximal region containing gametes to the right. Regions of the germline with germ cells at different stages of development are marked with solid white lines and labelled in the wild-type image. Class I mutants have very few germ cells. Class II mutants have tumourous germlines. This class has two subclasses. In one, the distal mitotic region is followed by a region with sperm, which is then followed by mitotically proliferating germ cells. In the second subclass, only mitotic germ cells were observed. Class III mutants fail to switch from spermatogenesis to oogenesis and make more sperm than do wild type.

complementation groups: 16 in class I, three in class II and 10 in class III (supplementary material Table S1). Within class II, two types of germ cell tumour were observed – in two alleles, *kp1* and *kp65* (belonging to one complementation group), proliferation was in the distal part of the gonad where GSCs normally reside. In the other two class II complementation groups, the tumourous proliferation was in the proximal part of the gonad, more like the dedifferentiation observed in the *puf-8(–)* single mutant grown at 25°C. These results show that *puf-8* genetically interacts with several genes to regulate proliferation as well as meiotic development, and suggest that PUF-8 plays a pivotal role in the mitosis-meiosis balance.

puf-8 functions redundantly with *gap-3* to suppress mitotic proliferation

We determined the chromosomal position of *kp1* and *kp65* using standard three-factor genetic crosses, RNAi, DNA sequencing and transgene rescue. Three-factor crosses using visible and SNP markers placed these mutations within the map positions −12.5 and −11.0 on chromosome I. There were 35 open reading frames (ORF) within this region. RNAi screening of these ORFs identified *gap-3*,

which encodes a RasGAP (Stetak et al., 2008), as a potential candidate for the complementation group represented by *kp1* and *kp65*. Sequencing the *gap-3* region of genomic DNA from *kp1* and *kp65* worms revealed point mutations within the GAP-3 coding sequence. In *kp1*, a C-to-T change converts the CAA codon for glutamine at amino acid position 516 into the TAA stop codon. In *kp65*, a T-to-G change converts the TTA codon for leucine at amino acid position 806 into the TGA stop codon. The shorter protein predicted for *kp65* completely eliminates the 250 amino acid GAP domain and *kp1* deletes the last 70 amino acids of this domain (supplementary material Fig. S2). To further confirm our mapping results, we tested whether a transgene carrying the GAP-3-coding region would rescue the *kp1* allele. For this, we generated transgenic lines in which GFP::GAP-3 fusion was expressed in the germline using *pie-1* promoter. This transgene is expressed in the germline, where GFP::GAP-3 preferentially localizes to the cell membrane of all germ cells except the oocyte that is about to ovulate (Fig. 2A). As shown in Table 1, this transgene was able to rescue the *kp1* mutation, thus indicating *kp1* is indeed an allele of *gap-3*. We conclude *kp1* and *kp65* are alleles of *gap-3*.
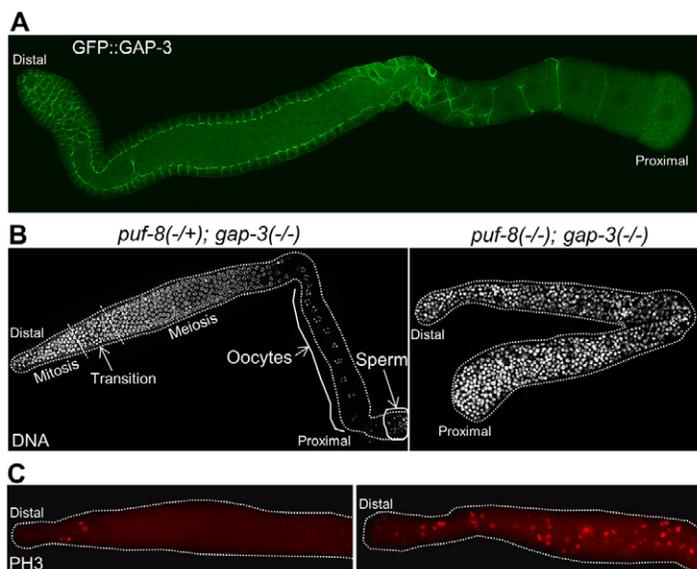

Fig. 2. Adult *puf-8(–/–); gap-3(–/–)* germline contain only mitotic germ cells. (**A**) Distribution pattern of GAP-3::GFP fusion protein expressed in the germline using the upstream and downstream sequences of *pie-1*. The fusion protein localizes predominantly to the cell membrane of germ cells. (**B**) Dissected, DAPI-stained gonads. Although the *puf-8(–/+); gap-3(–/–)* heterozygous germline contains oocytes in the proximal region, the germline homozygous for both mutations does not have oocytes. The broken lines in the left image mark the boundaries among the indicated stages. Only mitotic cells were observed in the double mutant gonad shown on the right. (**C**) Dissected gonads immunostained for the mitosis marker phosphohistone H3 (PH3). Unlike the *puf-8(–/+); gap-3(–/–)* heterozygous germline, which contains PH3-positive cells only in the distal part, the double mutant has PH3-positive cells throughout the germline. In addition, the number of PH-3-positive cells in the distal region in the double mutant is significantly more than the single mutant.

Table 1. *puf-8* and *gap-3* negatively regulate RAS/MAPK signalling to promote differentiation

Genotype	Percentage of germlines with gametes	Percentage of tumorous germlines	*n*
Wild-type	100	0	240
puf-8(zh17)	100	0	270
gap-3(kp1)	100	0	460
gap-3(kp65)	100	0	400
puf-8(zh17); gap-3(kp1)	0	100	380
puf-8(zh17); gap-3(kp1); kpIs(pSV39)	100	0	400
puf-8(zh17); gap-3(kp1); let-60(RNAi)	100	0	86
puf-8(–); gap-3(kp1); rom-1(RNAi)	54*	28	82
puf-8(–); gap-3(kp1); sur-6(RNAi)	62*	0	62
puf-8(–); gap-3(kp1); spe-6(h49)	0	100	46

*These genotypes produced only sperm.

As expected from the design of the genetic screen, neither *gap-3(kp1)* nor *gap-3(kp65)* single mutants displayed any observable defects. Both spermatogenesis and oogenesis were normal in these worms and they yielded viable fertile progeny at both 20°C and 25°C (Fig. 2B, Table 1). As reported earlier, the *puf-8(–)* single mutant was fertile at 20°C (Subramaniam and Seydoux, 2003) (data not shown). By contrast, both *puf-8(–); gap-3(kp1)* and *puf-8(–); gap-3(kp65)* double mutants were 100% sterile at this temperature. Although a small percentage of the double mutants formed a few sperm, they did not produce any oocytes. Instead, mitotically proliferating cells were observed throughout the gonad in 100% of these worms, including the few in which a few sperm were observed (Fig. 2C). These observations show that *puf-8* and *gap-3* function in a redundant fashion to inhibit ectopic germ cell proliferation.

PUF-8 and GAP-3 function redundantly to control RAS/MAPK signalling in germ cells

A tight control of RAS/MAPK signalling is crucial for normal levels of cell proliferation in different developmental contexts. Excessive signalling is responsible for several types of cancer in humans (Malumbres and Barbacid, 2003). RAS, the membrane-bound upstream activator of this signalling pathway, is a G protein and is active only in the GTP-bound form. Its intrinsic GTPase activity is stimulated by RAS GTPase-activating proteins (RasGAPs). Thus, RasGAPs act as negative regulators of RAS/MAPK pathway (Bernards, 2003). The *C. elegans* genome encodes three RasGAPs, namely GAP-1, GAP-2 and GAP-3 (Stetak et al., 2008). Each of these proteins controls the activity of the RAS protein LET-60 in different tissues. For example, GAP-3 has been shown to be the primary repressor of LET-60 in germ cells progressing through meiosis (Stetak et al., 2008). Therefore, we wanted to determine whether the RAS/MAPK signalling was altered in *puf-8(–) gap-3(–)* worms. For this, we immunostained dissected gonads with antibodies specific for the phosphorylated MPK-1 (dpMPK-1), which is the active form of the downstream kinase activated by RAS/MAPK signalling (Lee et al., 2007b). In the wild-type controls, dpMPK-1 expression could be observed in the mid-pachytene germ cells and oocytes, which is similar to the dpMPK-1 expression pattern reported earlier (Lee et al., 2007b). There were no noticeable variations in the levels or distribution pattern of dpMPK-1 in the *puf-8(–)* and *gap-3(–)* single mutants from the wild type. By contrast, in the *puf-8(–); gap-3(–)* double mutant gonads, dpMPK-1 was present throughout the gonad in all germ cells, and the immunofluorescence intensity was particularly higher in the region where germ cells exit proliferation and begin meiotic development (Fig. 3). These results show that PUF-8 and GAP-3 act redundantly to suppress MPK-1 activation in germ cells.
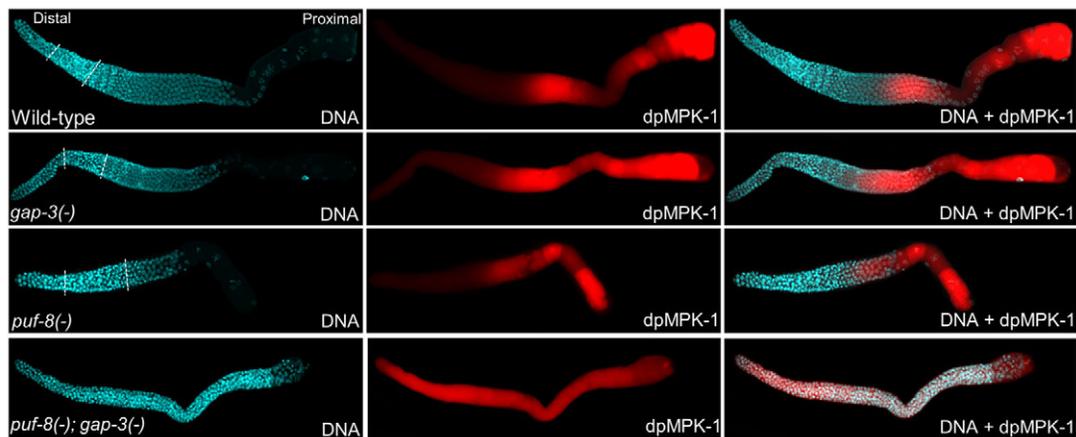
Fig. 3. MPK-1 is ectopically activated in the *puf-8(–); gap-3(–)* double mutant. Dissected adult gonads of the indicated genotypes stained with both DAPI (DNA) and anti-dpMPK-1 antibodies (dpMPK-1) to visualize the nuclear morphology and the active form of MPK-1, respectively. In the distal part of the germline, active MPK-1 level is significantly higher in the *puf-8(–); gap-3(–)* double mutant than the wild-type (WT) and both single mutants. Broken lines mark the boundaries among mitotic, transition and meiotic regions. In the double mutant, only mitotic germ cells were present throughout the gonad.

PUF-8- and GAP-3-mediated suppression of RAS/MAPK signalling is essential for the control of mitotic proliferation

To test whether the increased RAS/MAPK signalling is the cause of a tumour phenotype, we downregulated several RAS/MAPK pathway components (supplementary material Table S4) (Sundaram, 2006) in *puf-8(–); gap-3(–)* worms by RNAi. Although depletion of any one of these components reduced the tumourous proliferation, the extent of this reduction was significantly higher when LET-60, ROM-1 or SUR-6 was depleted by RNAi. In addition to tumour reduction, majority of these three RNAi gonads produced sperm. Strikingly, *let-60(RNAi)* arrested the tumour phenotype completely, and restored fertility (Table 1). Furthermore, immunostaining with anti-dpMPK-1 antibodies revealed a significant reduction in the levels of dpMPK-1 in these RNAi gonads (Fig. 4). These results show that the uncontrolled mitotic proliferation of *puf-8(–); gap-3(–)* worms is due to premature activation of RAS/MAPK signalling. We conclude PUF-8 and GAP-3 negatively regulate RAS/MAPK signalling to control mitotic proliferation.

PUF-8 and GAP-3 are not essential for the onset of meiosis, but are essential for the maintenance of mitosis-meiosis transition in the adult

Mitotic proliferation in the *puf-8(–); gap-3(–)* double mutant appeared to be uncontrolled as the gonads quickly filled up with mitotic germ cells and enlarged within 48 hours of L4-adult moult. Two types of germline tumours have been observed in *C. elegans*. The first type results from the failure of germ cells to switch from mitotic to meiotic fate. In this case, mitotically cycling GSCs present at the distal part of the gonad fail to enter meiosis and instead proliferate in an uncontrolled fashion (Berry et al., 1997; Hansen et al., 2004a). In the second type, the initial mitotic-to-meiotic cell fate switch is unaffected, but the germ cells exit meiosis and dedifferentiate into mitotically cycling cells (Francis et al., 1995a; Francis et al., 1995b; Subramaniam and Seydoux, 2003). As an initial attempt to determine the type of tumour observed in the *puf-8(–); gap-3(–)* worms, we blocked meiotic progression by

generating the *puf-8(–); gap-3(–); spe-6(–)* triple mutant. SPE-6 is essential for meiotic progression and the *spe-6(–)* mutant germ cells arrest at late prophase I (Varkey et al., 1993). As shown in Table 1, tumorous proliferation was also observed in this triple mutant, which suggests that the mutant germ cells either fail to switch from mitotic to meiotic fate or dedifferentiate after entering into meiosis but prior to the stage requiring SPE-6 activity.

Known mutations that cause meiotic exit and dedifferentiation do not alter the temporal and spatial positions of meiotic onset during larval development (Francis et al., 1995a; Francis et al., 1995b; Subramaniam and Seydoux, 2003). Therefore, we wanted to check whether the meiotic onset was normal in the *puf-8(–); gap-3(–)* double mutant. To do this, we immunostained the gonads at different developmental time points with antibodies against the meiotic marker HIM-3 (Zetka et al., 1999). In addition, we co-immunostained the same gonads with the anti-dpMPK-1 antibodies to identify the exact time and location of dpMPK-1 activation. In the wild-type *C. elegans*, meiosis begins at early L3 larval stage in the proximal part of the gonad (Hansen et al., 2004a). At the mid-L3 stage, HIM-3-positive cells were present at the proximal part of the wild type as well as the double mutant gonads (Fig. 5A). These results indicate that inhibition of LET-60 activity by *puf-8* and *gap-3* is not required for the initiation of entry into meiosis at the normal time during development. At this stage, no dpMPK-1 could be detected in the wild-type or the *puf-8(–); gap-3(–)* double mutant. By contrast, at the early L4 stage, dpMPK-1 expression was readily observable in the double mutant in all germ cells with the exception of a few at the distal end and most of the HIM-3-positive cells at the proximal end (Fig. 5B). Similar expression patterns also persisted at the mid-L4 stage (Fig. 5C). At both these later stages, although the zones of dpMPK-1 and HIM-3 expression were mutually exclusive for the large part, they overlapped in a few cells at the boundary between the two zones (Fig. 5B,C). However, whereas elevated levels of dpMPK-1 were observed throughout the *puf-8(–); gap-3(–)* adult germlines, no HIM-3-positive cells were detected in them (Fig. 5D). These results reveal that the activation of MPK-1 in the *puf-8(–); gap-3(–)* germline begins in cells at the proximal part of the distal proliferative zone, referred to as meiotic entry region or the transit-amplifying zone (Cinquin et al.,
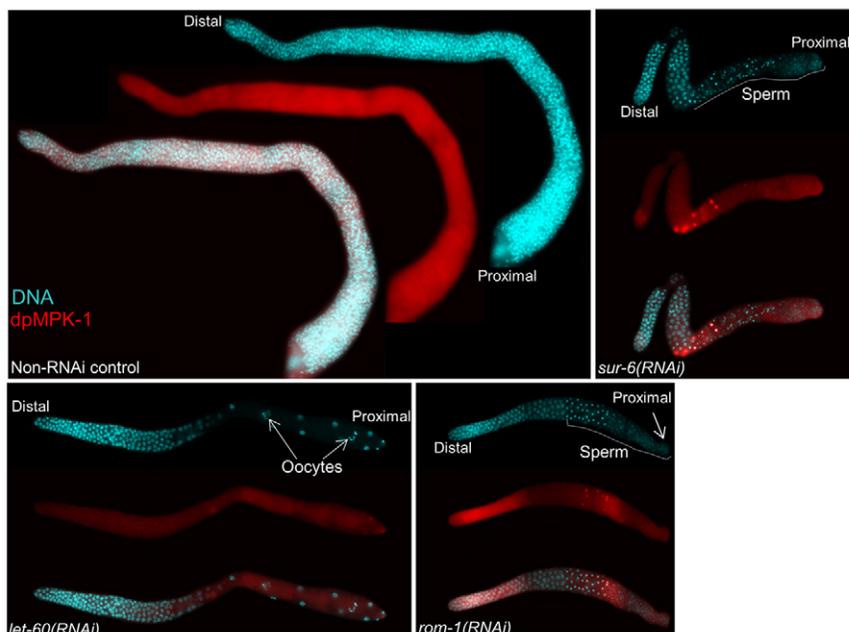
Fig. 4. Depletion of MAP kinase pathway components suppress *puf-8(–); gap-3(–)* phenotype. Dissected gonads of *puf-8(–); gap-3(–)* young adults following the indicated RNAi treatments stained as described in Fig. 3. Although *let-60(RNAi)* reduces dpMPK-1 levels considerably throughout the germline, *sur-6(RNAi)* and *rom-1(RNAi)* does so in the early pachytene region. In all three cases, tumourous proliferation is abolished and gametes are seen in the proximal region – oocytes in *let-60(RNAi)* and sperm in *sur-6(RNAi)* and *rom-1(RNAi)*. See text and Table 1 for additional information.

Fig. 5. Time-course analysis of MPK-1 activation and the onset of meiosis. (**A-D**) Gonads were extruded by dissecting worms at the indicated developmental stages and immunostained for the synaptonemal complex protein HIM-3 (green), which marks the meiotic cells, and dpMPK-1 (red), which is the active form of the MAP kin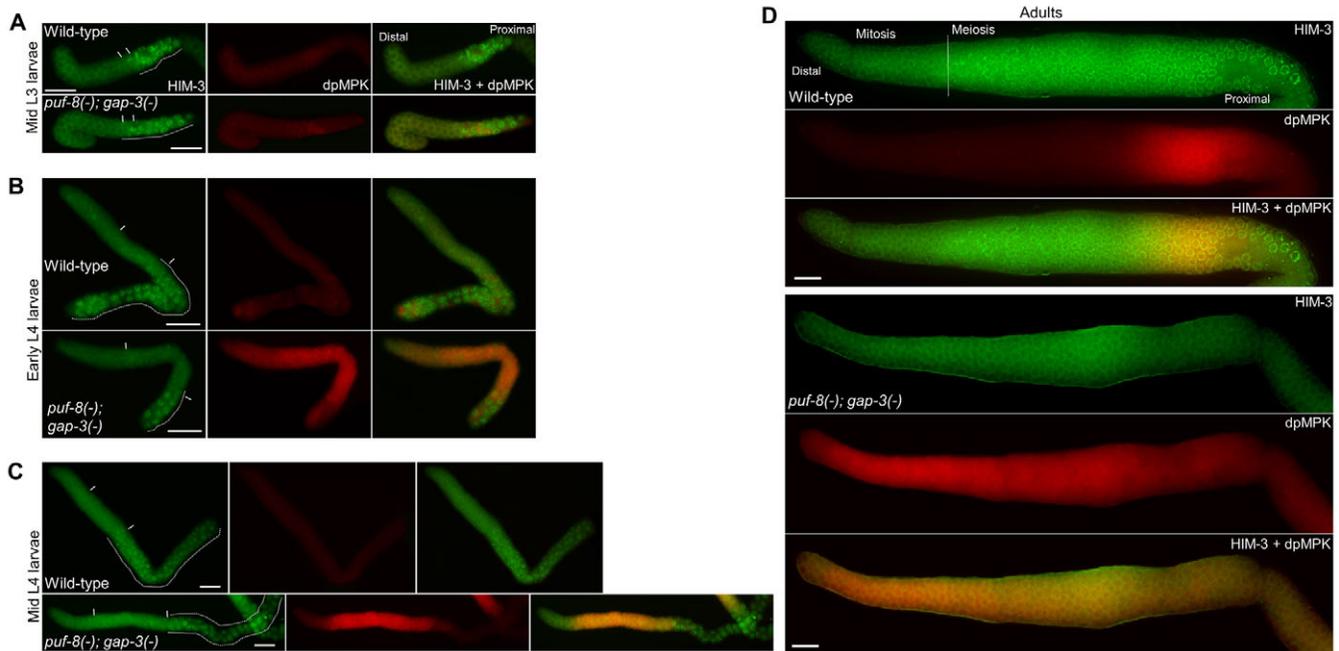ase MPK-1. In A-C, the broken lines indicate the area containing germ cells with HIM-3-positive chromatin. Vertical lines mark the approximate boundaries of the meiotic entry region. In D, the broken vertical line marks the boundary between the HIM-3-negative and HIM-3-positive cells in the wild type; no HIM-3-positive cells are seen in the *puf-8(–); gap-3(–)* gonad. Synchronous L1 larvae were fed on OP50 bacterial lawns for 36 hours (mid L3), 42 hours (early L4), 48 hours (mid L4) and 66 hours (adults) at 20°C before immunostaining. Scale bars: 20 μm.

2010; Hansen et al., 2004a), and that these cells, once the level of dpMPK-1 increases beyond a threshold, fail to differentiate into HIM-3-positive meiotic cells. This is consistent with the earlier observation that the downstream effectors of the RAS/MAPK pathway promote the mitotic fate (Lee et al., 2007b).

Another hallmark of the germ cell tumour resulting from dedifferentiation is that it is dependent on the germ cell sex (Francis et al., 1995a; Francis et al., 1995b; Subramaniam and Seydoux, 2003). Contradictory to this, *puf-8(–); gap-3(–)* males, as well as *puf-8(–); gap-3(–); fem-3(–)* and *puf-8(–); gap-3(–) fog-3(–)* feminized hermaphrodites, displayed germline tumour phenotype (supplementary material Fig. S3). Taken together, the data presented in this section indicate that the overproliferation phenotype of *puf-8(–); gap-3(–)* results from the failure to maintain the proliferative to the meiotic fate switch in the adult germline.

Germline tumour formation in *puf-8(–); gap-3(–)* depends on *glp-1*, but not on *fbf*, activity

GLP-1 signalling is essential for the mitotic fate of GSCs. In *glp-1(–)* worms, very few germ cells are made, and all these cells differentiate into sperm (Austin and Kimble, 1987). GLP-1 prevents premature entry into meiosis in part by promoting the expression of the PUF protein FBF, which suppresses the expression of the meiotic promoter GLD-1 (Crittenden et al., 2002). In addition, GLP-1 has been proposed to promote the expression of an unknown gene named gene X, which suppresses GLD-1 expression (Hansen et al., 2004b). To determine whether the overproliferation phenotype of *puf-8(–); gap-3(–)* germlines is dependent on *glp-1* and/or *fbf*, we performed epistasis analysis among these genes using loss-of-function alleles as well as RNAi. Germ cell proliferation in the *puf-*

8(–); gap-3(–); glp-1(RNAi) worms was reduced to the level observed in *glp-1(–)* single mutant worms, and the few cells present in the triple mutant differentiated into sperm (Fig. 6A). Similar results were obtained even when GLP-1 was depleted after ectopic MPK-1 activation (Fig. 6B). These results show that the tumorous proliferation observed in the *puf-8(–); gap-3(–)* germline is dependent on GLP-1, and suggest that *puf-8* and *gap-3* function either as upstream negative regulators of *glp-1*, or in a parallel pathway. In addition, these results reveal PUF-8 and GAP-3 are not essential for meiosis when the block of meiotic entry by GLP-1 is removed. The PUF protein FBF is encoded by two nearly identical paralogues *fbf-1* and *fbf-2* (Zhang et al., 1997). Therefore, we generated the *fbf-1(–) fbf-2(–) puf-8(–); gap-3(–)* quadruple mutants to test whether the *puf-8(–); gap-3(–)* tumour phenotype required FBF. Surprisingly, as shown in Fig. 6C, germ cells in this quadruple mutant proliferated in an uncontrolled fashion, just as they did in the *puf-8(–); gap-3(–)* double mutant worms, indicating that FBF is not required for the tumorous proliferation of the *puf-8(–); gap-3(–)* germ cells. Possibly, the ectopically activated MPK-1 blocks meiotic entry by promoting the expression and/or activity of gene X mentioned above, which is independent of FBF. Alternatively, the ectopic MPK-1 promotes proliferation directly; however, only when the block of meiotic entry is maintained either by FBF or gene X.

PUF-8 suppresses LET-60 expression in the proliferative and transition zones by direct 3′ UTR binding
Next, we wanted to investigate how PUF-8 and GAP-3 synergistically control the activation of MPK-1. As mentioned
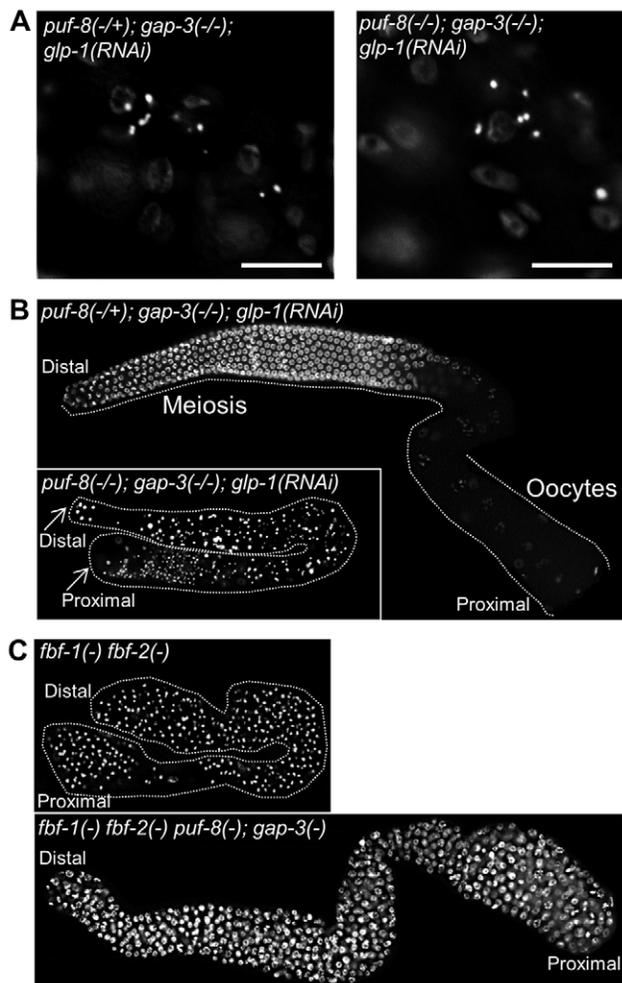
Fig. 6. _puf-8(–); gap-3(–)_ tumour phenotype is dependent on GLP-1, but not on FBF. (**A**) DAPI-stained worms of the indicated genotypes are shown. Only a part of the worm where the germline is present is seen here. Only a few sperm (bright white spots) are present in the germline. Fainter staining in the background is of somatic nuclei. (**B,C**) Dissected, DAPI-stained gonads of adult worms. GLP-1 was depleted either during early larval development, by allowing embryos to hatch and develop on _glp-1_ RNAi bacteria (A), or during L4 larval stage, by shifting larvae at late L3 to RNAi bacteria (B). In B, _puf-8(–/+)_ heterozygous germ cells have entered oogenesis, whereas the _puf-8(–/–)_ homozygotes (bottom) have differentiated as sperm, presumably owing to the lack of _puf-8_ and _glp-1(–)_. In C, whereas _fbf-1(–) fbf-2(–)_ gonads have only sperm, the _fbf-1(–) fbf-2(–) puf-8(–); gap-3(–)_ gonads have only mitotic germ cells.

above, GAP-3 is a RasGAP; it negatively regulates the LET-60 RAS to promote pachytene exit (Stetak et al., 2008). As PUF-8 is a post-transcriptional regulator, we tested whether PUF-8 controlled the expression of LET-60. First, we checked the levels of LET-60 protein in the germlines by immunostaining. In the wild-type hermaphrodite germline, no LET-60 signal could be detected in the proliferative, transition and in the distal part of the pachytene regions (Fig. 7). In the distal-proximal sense, LET-60 first appeared on punctate structures around the mid-pachytene region and became stronger where pachytene nuclei begin to cellularize and form oocytes. The levels of LET-60 dramatically increased in the maturing oocytes (supplementary material Figs S4, S5). By contrast, punctate-like LET-60 signals were readily observed in the distal

proliferative zone in the _puf-8(–)_ germlines; its expression was more pronounced in the transition zone, where the punctate structures appeared to be localized on the cell membrane (Fig. 7). These results show that PUF-8 suppresses the expression of LET-60 in the proliferative and transition zones.

PUF-8 has been shown to regulate its target mRNAs by binding to the 3′ UTR (Mainpal et al., 2011). Therefore, we tested whether PUF-8 can control _let-60_ 3′ UTR-mediated expression in the germline. For this, we generated GFP::H2B-_let-60_ 3′ UTR fusion transgene and expressed it in the germline using _pie-1_ promoter (D'Agostino et al., 2006). In the wild-type genetic background, the _let-60_ 3′ UTR fusion expressed GFP::H2B weakly in the proliferative as well as transition zones. By contrast, depletion of PUF-8 enhanced the expression of GFP::H2B in the crescent-shaped nuclei of the transition zone, which further increased in the early pachytene cells (Fig. 7). These results suggest PUF-8 negatively regulates _let-60_ mRNA in the transition zone and early pachytene germ cells. The slight proximal shift between the LET-60 misexpression detected by immunostaining and the GFP::H2B-_let-60_ 3′ UTR expression in the _puf-8(–)_ germline is probably due to the delay in the accumulation of sufficient levels of GFP::H2B.

To determine whether PUF-8 mediated its effect on _let-60_ 3′ UTR through direct RNA binding, we first tested its ability to interact with the _let-60_ 3′ UTR in electrophoretic mobility shift assays (EMSA). Earlier, a fusion protein containing the PUF domain (171-535 amino acids) of PUF-8 and the maltose-binding protein (MBP::PUF-8) has been used to demonstrate the interaction between PUF-8 and the 3′ UTR of one of its targets, _pal-1_ mRNA (Mainpal et al., 2011). Therefore, we used the same fusion protein in EMSAs with the _let-60_ 3′ UTR RNA. As the _let-60_ 3′ UTR is too long – 819 nucleotides – to be tested in EMSA, we divided it into five fragments and tested each of them individually. Of these five, MBP::PUF-8 retarded the mobility of fragment 4 in a sequence-specific manner (Fig. 8B and data not shown). We then substituted 60-nucleotide stretches of this 202-nucleotide fragment 4 with TG repeats to identify the specific sequence of RNA that is crucial for PUF-8 binding. As shown in Fig. 8C, substitution of SubC region completely abolished the mobility shift, indicating that this region is crucial for binding PUF-8.

Second, we investigated the importance of PUF-8-_let-60_ 3′ UTR interaction _in vivo_ using the GFP::H2B-_let-60_ 3′ UTR transgene assay described above. Transgenic worms with the wild-type _let-60_ 3′ UTR sequence showed a weak, uniform expression throughout the gonad (Fig. 7 and Fig. 8E). By contrast, the GFP::H2B expression was markedly elevated starting from the transition zone in worms carrying the transgene with SubC substitution (Fig. 8E). Thus, removal of PUF-8 or mutation of _let-60_ 3′ UTR both affect _let-60_ 3′ UTR-mediated expression in the germ cells of the transition zone. Third, we further validated the PUF-8-_let-60_ 3′ UTR interaction using the co-immunoprecipitation (co-IP) approach. There are no PUF-8-specific antibodies currently available; as the expression of PUF-8::GFP is very weak, anti-GFP antibodies have also not been successful, at least in our hands, for immunoprecipitation from worm extracts. Therefore, we generated a new transgene bearing nine repeats of the HA epitope tag fused to PUF-8::GFP. This transgene rescued _puf-8(–)_ worms, indicating that the fusion protein is functional (data not shown). Western blotting revealed that the anti-HA antibodies could specifically immunoprecipitate the 9×HA::PUF-8::GFP from worm lysates (data not shown). Reverse transcription-polymerase chain reaction (RT-PCR) on RNA templates extracted from the immunoprecipitated pellets using _let-60_-specific PCR primers
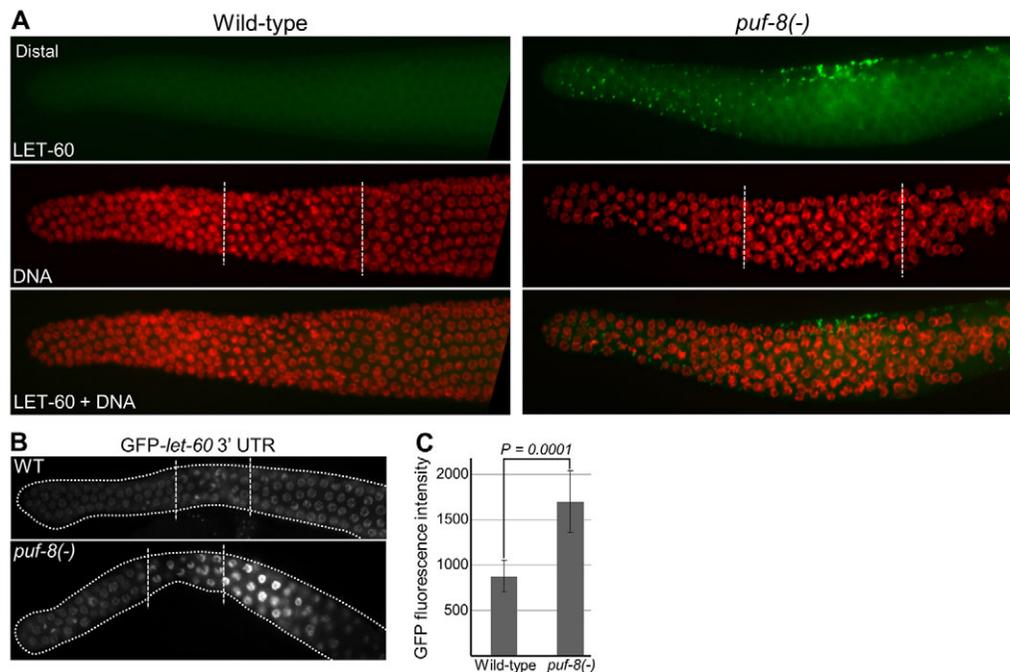
Fig. 7. PUF-8 negatively regulates LET-60 expression in the germline. (**A**) Dissected gonads stained with anti-LET-60 antibodies and DAPI. Genotypes are indicated on the top. In the transition zone, although LET-60 signal is seen only in a few cells at the top in this focal plane, it was observed in a several cells within this zone in other focal planes. The dots revealed by immunostaining signal correspond to cell membrane at the intersection of adjacent cells. See also supplementary material Figs S5 and S6. (**B**) Dissected gonads of live worms revealing the expression pattern of GFP::H2B reporter fusion expressed under the control of *pie-1* promoter and *let-60* 3′ UTR sequences. Only the distal germlines are shown. In the *puf-8(–)* genetic background, *let-60* 3′ UTR fusion shows significant upregulation in the transition and early pachytene region when compared with the wild type. In total, 20 worms each of 11 independent transgenic lines were examined, and representative images are shown here. broken lines in A and B mark the boundaries among the mitotic, transition and pachytene regions. (**C**) Comparison of the GFP fluorescence intensity (arbitrary units) per nucleus in the transition zone between the wild-type and *puf-8(–)* gonads. *n*=23 nuclei from five gonads; *P*=0.0001 (Student's *t*-test).

showed that the *let-60* mRNA specifically co-immunoprecipitated with the fusion protein (Fig. 8D). We conclude PUF-8 suppresses *let-60* expression in the transition zone germ cells by binding to the *let-60* 3′ UTR.

DISCUSSION

Results presented here identify the PUF protein PUF-8 as a crucial upstream regulator of the MPK-1 MAP kinase and explain why MPK-1 is not activated during the early pachytene stage, although MPK-1 activity is crucial for the meiotic progression (Church et al., 1995; Lee et al., 2007b; Ohmachi et al., 2002). Our results clearly show that the premature MPK-1 activation promotes mitotic fate, and as a result, germ cells fail to enter meiosis. Importantly, our findings reveal that the post-transcriptional control of *let-60* mRNA by PUF-8 and the protein-level of control of LET-60 RAS activity by the RasGAP GAP-3 constitute a redundant mechanism to maintain the MPK-1 activity at undetectable levels in the mitotic, meiotic-entry and the early pachytene regions. In *C. elegans*, the somatic distal tip cell regulates the mitosis-meiosis switch by spatially restricting GLP-1 signalling, which prevents premature meiotic entry (Kimble and Crittenden, 2007). In this context, the ectopic activation of a proliferation-promoting signal that is not spatially regulated will potentially interfere with the mitosis-meiosis transition. Results presented here show that PUF-8 and GAP-3 function redundantly to prevent one such proliferation-promoting signal – the ectopic activation of LET-60 RAS – from disrupting the regulation of the mitosis-meiosis switch mediated by the spatially restricted GLP-1 signalling.

Origin of the tumour-forming cells in the *puf-8(–); gap-3(–)* mutant

At 25°C, *puf-8(–)* single mutant primary spermatocytes exit meiosis, dedifferentiate and form a tumour (Subramaniam and Seydoux, 2003). Several observations indicate that the tumour phenotype of *puf-8(–); gap-3(–)* differs from this dedifferentiation-driven tumour of *puf-8(–)*. First, although meiotic block caused by the removal of SPE-6 arrests the *puf-8(–)* tumour, it does not do so in the case of the *puf-8(–); gap-3(–)* tumour. Second, although the onset of meiosis occurs normally in the double mutant, cells positive for the meiotic marker HIM-3 are absent at the adult stage. By contrast, HIM-3-positive cells are continuously present in the *puf-8(–)* mutant. Third, the double mutant tumour is not dependent on the germ cell sex, whereas the *puf-8(–)* tumour is dependent on the male germ cell sex (Subramaniam and Seydoux, 2003). Fourth, upon depletion of GLP-1 at the L4 larval stage – a stage when the removal of PUF-8 and GAP-3 results in elevated levels of active MPK-1 – the double mutant germ cells are able to differentiate and form sperm, which is unlikely if the uncontrolled proliferation is due to dedifferentiation. These observations strongly support that the tumorous proliferation in the *puf-8(–); gap-3(–)* double mutant is caused by the failure to switch from proliferative to meiotic fate, rather than by the dedifferentiation of cells that have already entered into meiosis.

MAPK pathway and meiotic entry

Signal transduction through the RAS/MAPK pathway promotes cell proliferation in diverse organisms. Increased signalling
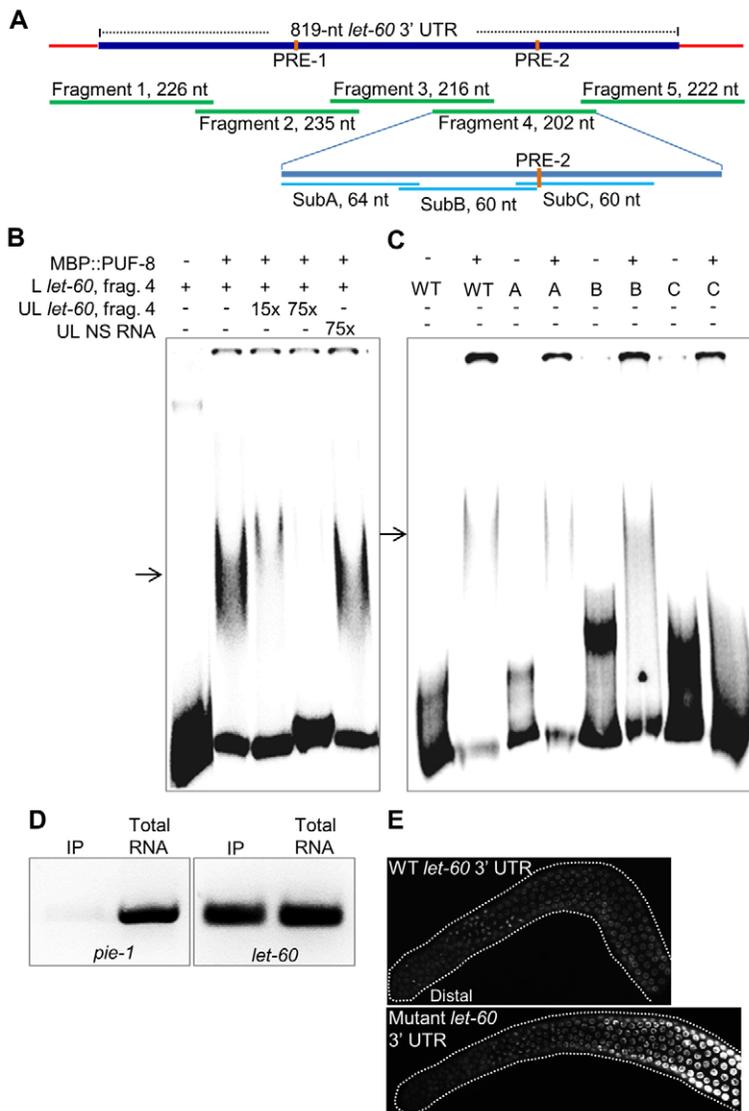
Fig. 8. PUF-8 interacts with *let-60* 3′ UTR. (**A**) Parts of *let-60* 3′UTR RNA tested in gel shift assays presented in B and C. Sequences of PRE-1 (UGUCAAAA) and PRE-2 (UGUCAAGU) match to the consensus PUF-8 recognition element (Opperman et al., 2005). SubA, SubB and SubC refer to regions substituted with TG repeats. (**B**) Electrophoretic mobility patterns of radiolabelled fragment 4 of *let-60* 3′ UTR RNA (L *let-60*, fragment 4) in the presence of indicated components. MBP::PUF-8, bacterially expressed fusion protein containing maltose-binding protein and the C-terminal (175-535 amino acids) PUF domain of PUF-8; UL, non-radiolabelled; NS, a non-specific RNA of about 200 nucleotides; 15× and 75×, 15 and 75 times molar excess when compared with the radiolabelled RNA, respectively. Arrow indicates the mobility-shifted band in both B and C. (**C**) Same assay as described in B except that the sequence of the radiolabelled RNA was substituted as indicated. Substitutions of the SubA, SubB and SubC regions are indicated as A, B and C, respectively. WT, no substitution. (**D**) Results of co-immunoprecipitation assay. PUF-8::9×HA::GFP fusion protein from transgenic worms was immunoprecipitated with anti-HA antibodies and the co-immunoprecipitation of the indicated RNAs was detected using RT-PCR. To determine the specificity of co-immunoprecipitation, *pie-1* mRNA was amplified as a negative control and the levels of both *pie-1* and *let-60* RT-PCR products from the immunoprecipitated pellet (IP) were compared with the total RNA. (**E**) Dissected gonads of live worms revealing the expression pattern of GFP::H2B reporter fusion expressed under the control of *pie-1* promoter and *let-60* 3′ UTR sequences; the bottom panel shows the expression pattern of the *let-60* 3′ UTR bearing SubC substitution. Images are representative of 20 worms from each of eight independent transgenic lines. The effect of *cis* mutation (SubC substitution) is not identical to that of the *trans* mutation (removal of PUF-8) presented in Fig. 7B, owing to other germline defects of *puf-8(–)* at 25°C (Subramaniam and Seydoux, 2003).

through this pathway is responsible for tumour growth in a variety of cancers. However, its role in the mitotic self-renewal of germ cells is poorly understood. In *C. elegans* germline, RAS/MAPK signalling is essential for meiotic progression, oocyte maturation and ovulation, all of which underscore the crucial role that this signalling pathway plays in the various cellular events involved in differentiation (Arur et al., 2011; Church et al., 1995; Lee et al., 2007b; Ohmachi et al., 2002). Despite its essential role in meiotic progression, we do not think RAS/MAPK pathway promotes the early events of meiotic entry for the following reasons. First, even in null *mpk-1* mutants, germ cells enter meiosis and arrest in pachytene (Church et al., 1995; Ohmachi et al., 2002). Rather, as mentioned in the Introduction, RAS/MAPK signalling promotes the proliferative fate (Lee et al., 2007b). Second, MPK-1 is not activated in germ cells just entering meiosis, and the downstream effectors of the RAS/MAPK pathway promote mitotic fate (Lee et al., 2007b). Third, our results show that, rather than promoting differentiation, an increase in the levels of dpMPK-1 in these cells actually promotes continued proliferation. Fourth, our epistasis analysis reveal that the tumour phenotype of *puf-8(–) gap-3(–)* is epistatic over *fbf-1(–) fbf-2(–)*, clearly indicating that, instead of enhancing the premature meiotic-entry phenotype of *fbf-1(–)*

fbf-2(–) (Crittenden et al., 2002), the elevated levels of active MPK-1 overcome the meiosis-promoting signals that are inappropriately activated in the absence of the FBFs and still supports proliferation.

Control of RAS/MAPK signalling by PUF proteins

PUF-8 is not the first PUF protein to be discovered as a regulator of the RAS/MAPK pathway. The FBF proteins have been shown to control the activity of MAPK phosphatase orthologue LIP-1, as well as MPK-1 itself, in sensitized backgrounds (Lee et al., 2006; Lee et al., 2007a). The net outcome of such a mutually opposite regulation, in terms of the levels of dpMPK-1 in the *fbf-1(–) fbf-2(–)* germ cells, is not known. Because of its negative effect on LIP-1 expression, FBF probably helps the maintenance of a basal level of MPK-1 activity in the distal mitotic zone. Existence of a basal level of RAS/MAPK signalling in the mitotic zone is consistent with the earlier observation that MPK-1 is essential for proliferation in a sensitized genetic background (Lee et al., 2007b). We propose PUF-8 diminishes this basal activity in the meiotic entry region and the transition zone to enable germ cells in this zone to exit the proliferative fate and enter meiosis.

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EMS

$$\frac{puf\text{-}8\ (zh17)\ unc\text{-}4(e120)}{mnC1}$$

Clone wild-type F1, collect embryos

$$\frac{puf\text{-}8\ (zh17)\ unc\text{-}4(e120)}{mnC1}\quad \frac{m}{+}$$

Screen for plates in which about 25% of the uncoordinated F2 worms are sterile

$$\frac{puf\text{-}8\ (zh17)\ unc\text{-}4(e120)}{mnC1}\quad \frac{m}{m}\qquad \frac{puf\text{-}8\ (zh17)\ unc\text{-}4(e120)}{puf\text{-}8\ (zh17)\ unc\text{-}4(e120)}\quad \frac{m}{m}$$

Clone wild-type F2s and select for the one whose uncoordinated progeny are all sterile

Fig. S1. The genetic screen. In total, 10,639 F1 worms, corresponding to 21,278 haploid genomes, were screened and 71 mutants, designated with allele numbers *kp-1* through *kp-71,* were isolated. Based on the germline phenotype, the mutants were broadly classified into three classes: class I, reduced germline; class II, tumourous germline; and class III, masculinized germline (mog) (see Fig. 1 and Table S1). So far, 37 of these alleles have been tested through complementation analysis; these 37 have been assigned to 29 different complementation groups. This screen also yielded new alleles of already known synthetic mutants of *puf-8*. The Hansen laboratory (University of Calgary, Canada) has isolated an allele of *puf-8* as an enhancer of the tumour phenotype observed in weak gain-of-function alleles of *glp-1* (Racher and Hansen, 2012). Both *glp-1* and three of our class II alleles, *kp16*, *kp31* and *kp43*, are on chromosome III. Therefore, we tested whether these three alleles map to *glp-1* by complementation. All three alleles failed to complement *ar202*, a known allele of *glp-1*, confirming that these three are indeed new alleles of *glp-1*. Mutations in *fbf-1* and *lip-1* have been shown to display synthetic phenotype with *puf-8(–)* (Bachorik and Kimble, 2005; Morgan et al., 2010). Complementation analysis confirmed that *kp8* and *kp12* are alleles of *fbf-1* and *lip-1*, respectively.

A

B
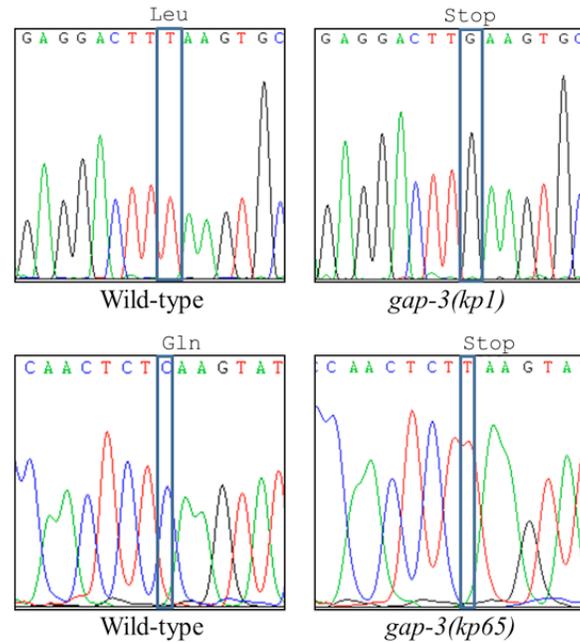
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SVFCADGCLFLSPSFPQQQPPTLYLCFRPYTAFLKWIRILRSRTIYQDVPPPMLQGTISVGEPNSQVSILSIEID
                                                                 kp65 *
KFRSELLKSDMFYSAQVSLNGVKIGVSNAFAPAGNKGPNEMPVVVIDSKFVIPCIPTCSTNIQLSMNSHSSIGKR
GRPCGVTVTICLNENNESICQSQADSTGFVFRAQRNRCPVLPLDRYKPLLDMIRECPSSILSWPSQVLPSHLKQF
MYTCISHLYALNPQFMSQVIRRIINDILVTSTAEDVFRKDSLATGIITQCLRHLFKTPFDEFLLENAQFSQSLKQ
QNLDSGGGAVELLVNFVDQRLLTIPLATRLLTIAAECAGCRFGDEQHEQHLIKRTLSALLILRVLNPIIFSTLNT
                                                             kp1 *
GIGSQIAKLVQISANSAASQTPTDSLTPAASTIRRMFDRIIFLVNQQQPAESPEDSEIGEVHTEWISCLTYMIGH
SLTIRSATSSEDVHLPLPVLELIRLHHL*
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Fig. S2. Mutations in *kp1* and *kp65* alleles disrupt the GTPase-activating domain (GAP) of GAP-3. (**A**) Electropherograms showing the wild-type and mutant sequences around the mutated nucleotide. (**B**) Amino acid sequence of GAP-3. The amino acids changed into STOP (*) codons is highlighted in red. The GAP domain sequence is underlined.
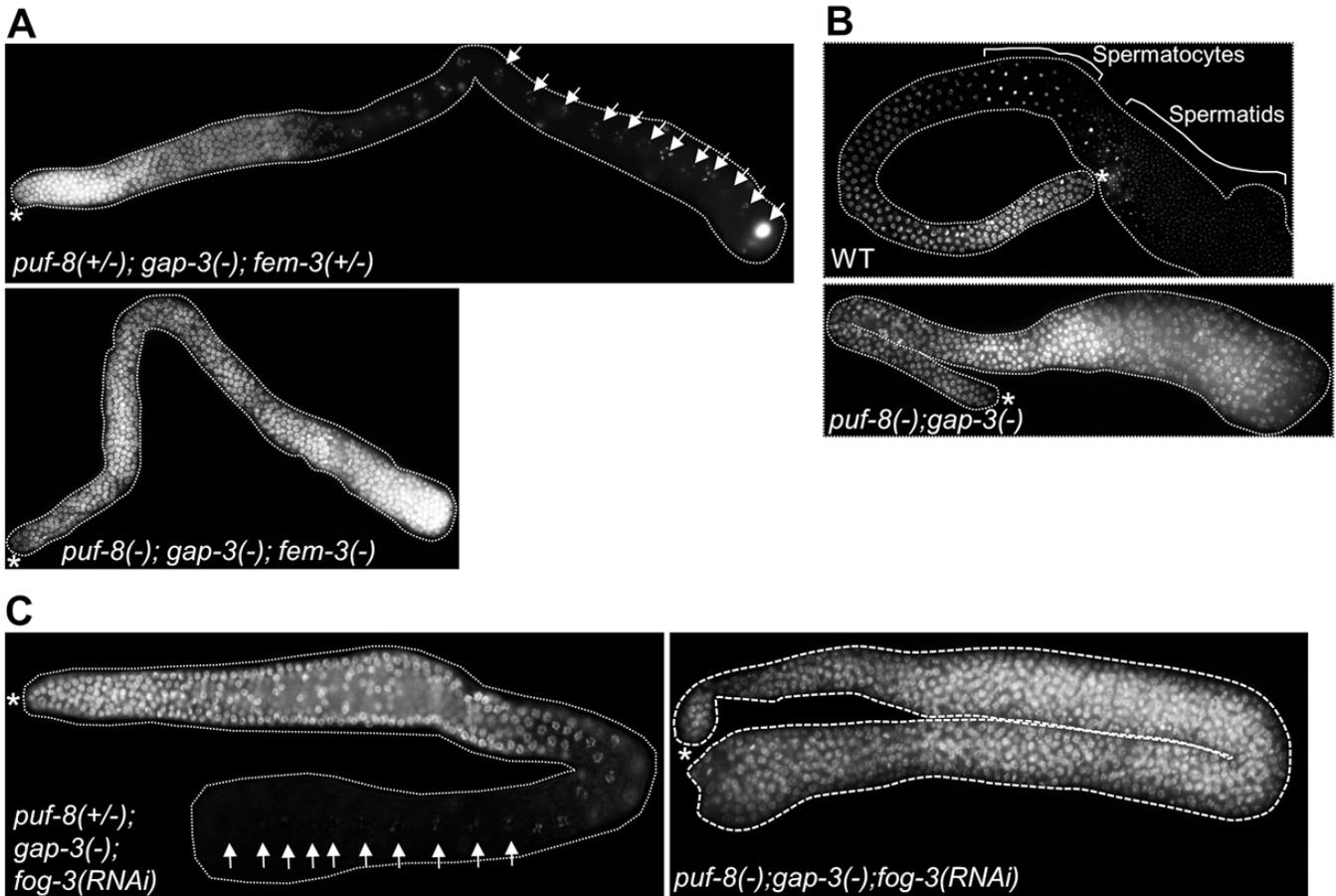
Fig. S3. Overproliferation phenotype of *puf-8(–); gap-3(–)* is not dependent on germ cell sex. (A-C) Dissected DAPI-stained gonads of the indicated genotypes are shown. Although the gonads in A and C are from hermaphrodite animals, the ones in B are from males. Arrows in A,C indicate oocyte nuclei. In this, as well as in Fig. S4, the distal part of the gonad is oriented towards the left; the distal end is indicated by an asterisk. Although gametes are present in the feminized [*fem-3(–)* and *fog-3(–)*] hermaphrodites and males heterozygous for *puf-8* [*puf-8(+/–)*], they are completely absent in the *puf-8(–/–)* homozygotes; instead, the proximal region of the *puf-8(–/–)* homozygous gonads are filled with mitotically proliferating cells. Hermaphrodites carrying loss-of-function in *fem-3* or *fog-3* fail to produce sperm, and make only oocytes. Hence these mutants are said to 'feminize' the hermaphrodite germline (Ahringer and Kimble, 1991; Chen et al., 2000).
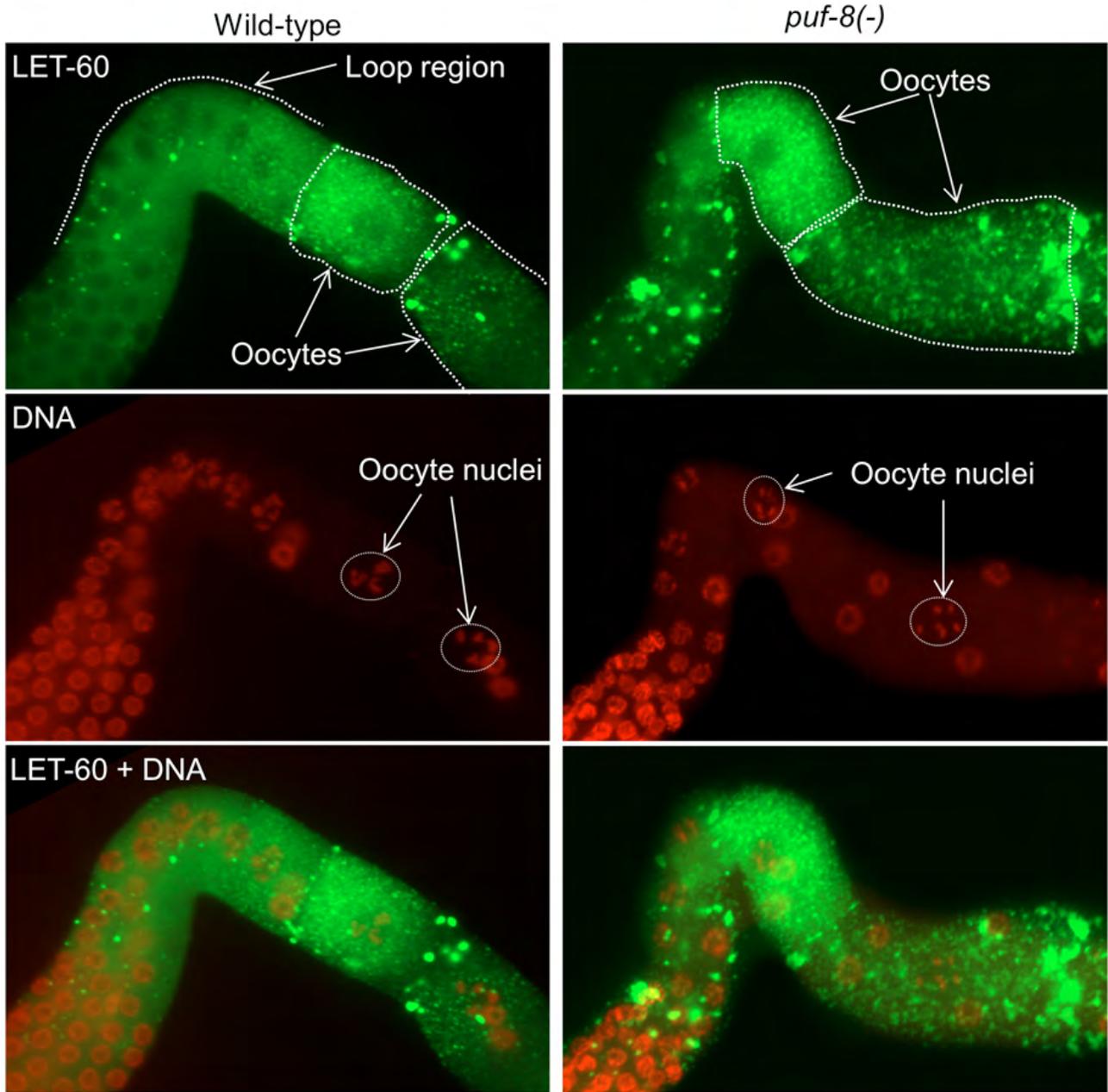
Fig. S4. Expression pattern of LET-60 in the wild-type and *puf-8(–)* gonads. Dissected gonads stained with anti-LET-60 antibodies and DAPI. Only the loop region and the first couple of oocytes are shown. Although the LET-60 level in the oocytes is approximately the same in the wild-type and *puf-8(–)*, the punctate structures stained by the anti-LET-60 antibodies are larger and more numerous in the loop region of *puf-8(–)* gonad when compared with the wild type. See Fig.7 for the LET-60 expression pattern in the distal part of the gonad. Owing to strong LET-60 signals in the region shown above, the duration of exposure for the acquisition of LET-60 images shown here was about one-third of that in the images in Fig. 7.
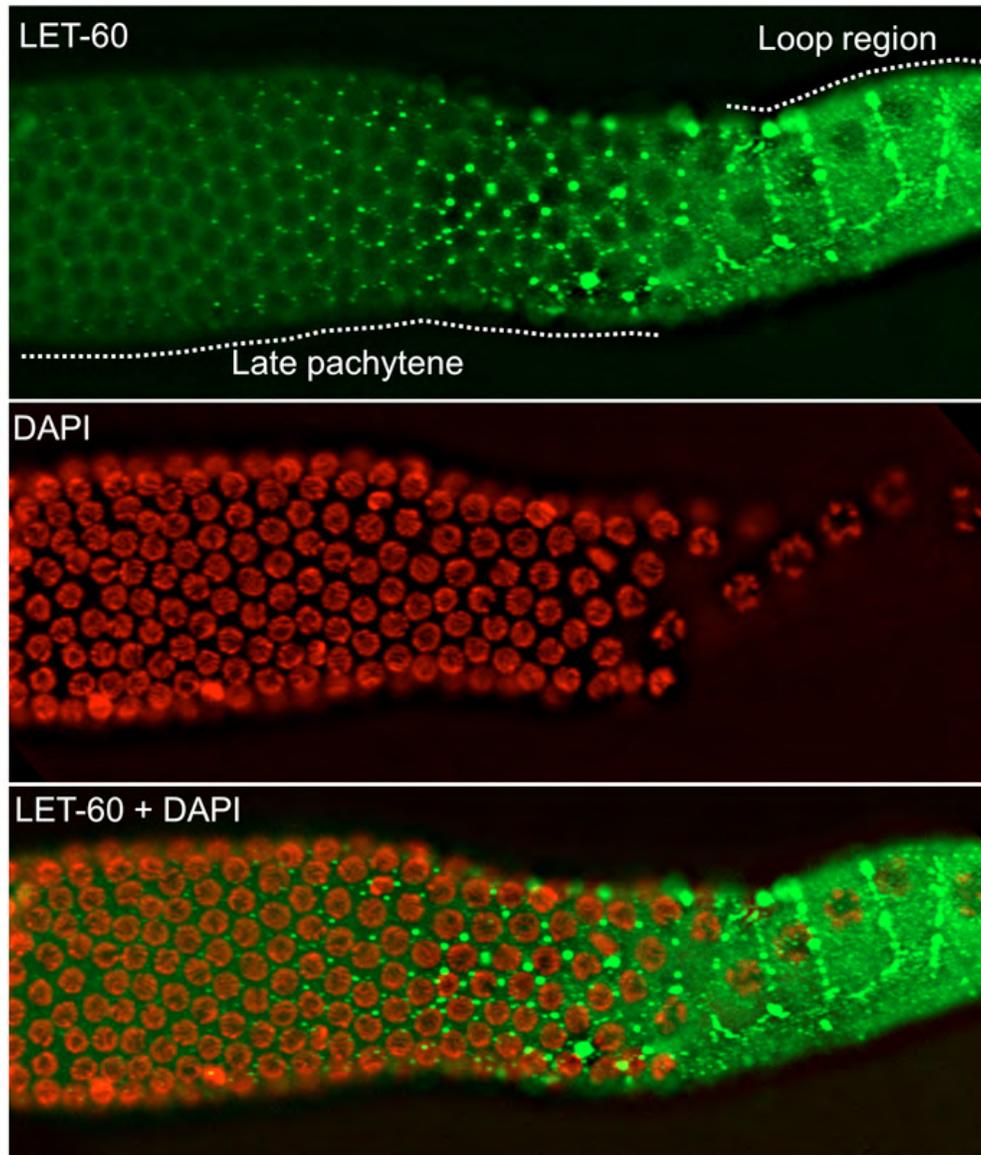
Fig. S5. Expression pattern of LET-60 in the pachytene region of wild-type gonads. Dissected gonad stained with anti-LET-60 antibodies and DAPI. Only the proximal half of the distal gonad is shown. Although the LET-60 signal is fainter in the distal (left) part of the late pachytene region, distinct puncta at the corners of the hexagonal germ cells are noticeable in most cells of the late pachytene region. Membrane localization of LET-60 is more readily observable in the rectangular immature oocytes at the loop region.
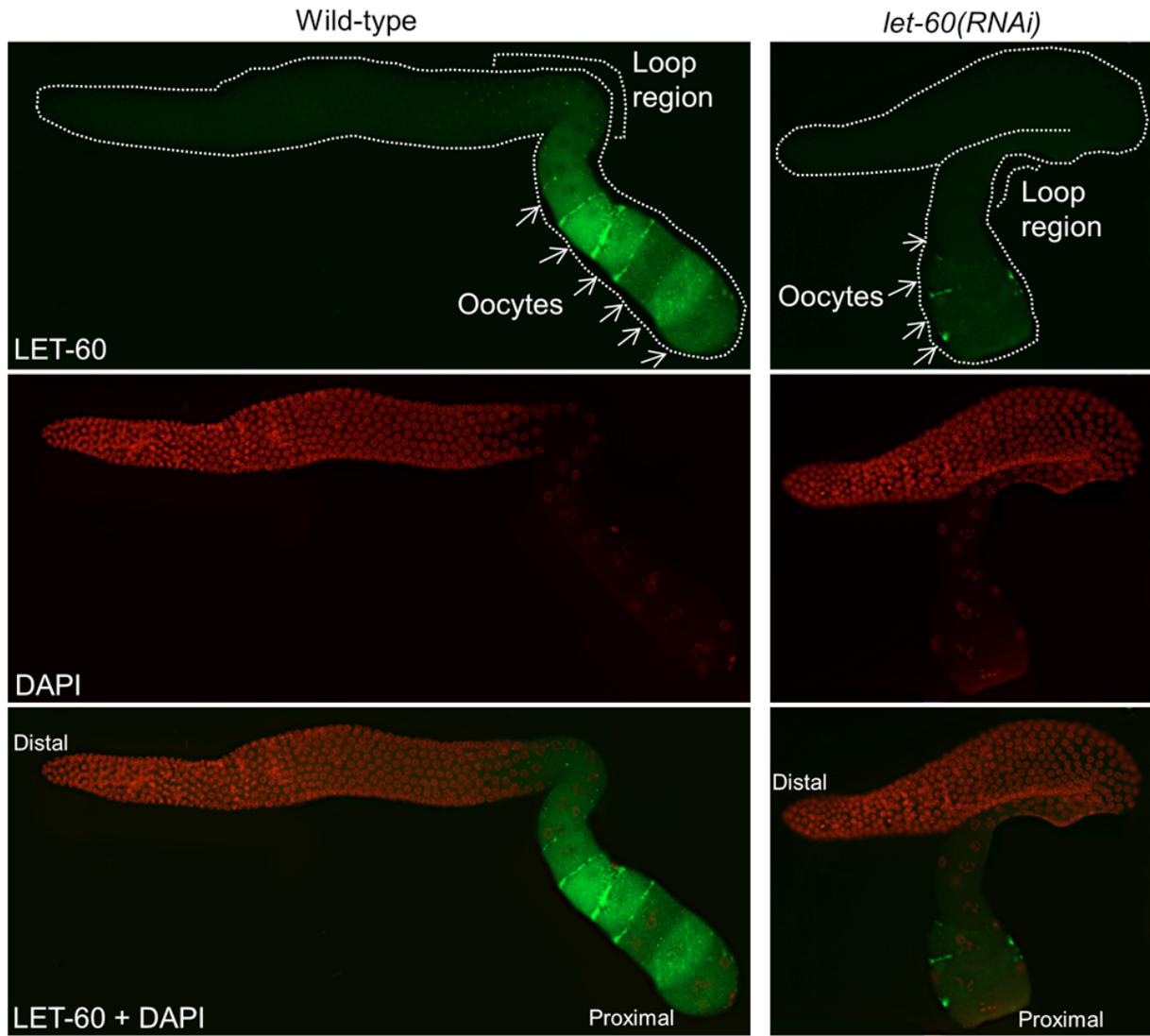
Fig. S6. Anti-LET-60 antibody specifically recognizes LET-60. Dissected gonads stained with anti-LET-60 antibodies and DAPI. Although LET-60 expression in the loop region and oocytes are readily visible in the wild-type gonad, LET-60 is undetectable in the loop region of *let-60(RNAi)* gonad; even in the oocytes, only a weak signal is visible in parts of the cell membrane.

Table S1. Classification of mutants that show synthetic sterility with *puf-8(–)*

Class	Germline defect	Number of mutants*	Penetrance
I	Reduced germline	20 (16)	~100%
II	Tumorous germline	7 (3)	20-100%
III	Mog (masculinization of germline)	10 (10)	15-100%

*Numbers in parentheses are the number of complementation groups for each class.

Table S2. *C. elegans* **strains used in this study**

3´UTR fusions			
Strain	**Transgene**	**Genotype**	**Reference**
IT285	*pie-1 prom:gfp:H2B:let-60 3'utr*	*unc-119(ed3) III; kpIs(pSV6)*	This study
IT734	*pie-1 prom:gfp:H2B:let-60 3'utr*	*puf-8(ok302) unc-4(e120) / mnC1* II	This study
IT460	*pie-1 prom:gfp:H2B:let-60 (SubC) 3'utr*	*unc-119(ed3) III; kpIs(pSV25)*	This study
ORF fusions			
IT543	*pie-1 prom:gfp:gap-3 ORF:hip-1 3'utr*	*unc-119(ed3) III; kpIs(pSV39)*	This study
IT654	*pie-1 prom:gfp:gap-3 ORF:hip-1 3'utr*	*dpy-5(e61) gap-3(kp-1) I*	This study
IT722	*puf-8 prom:puf-8 ORF:9xHA:gfp:puf-8 3'utr*	*unc-119(ed3) III; kpIs(pAK9)*	This study
Other strains			
Strain	**Genotype**		**Reference**
IT60	*puf-8(zh17) unc-4(e120) / mnC1* II		(Ariz et al., 2009)
JH1500	*puf-8(ok302) unc-4(e120) / mnC1* II		(Subramaniam and Seydoux, 2003)
IT540	*gap-3(kp1) I; puf-8(zh17) unc-4(e120) / mnC1* II		This study
IT770	*gap-3(kp65) I; puf-8(zh17) unc-4(e120) / mnC1* II		This study
IT124	*fbf-1(ok91) fbf-2(q704) puf-8(zh17) unc-4(e120)/mIn1[mIs14 dpy-10(e128)] II.*		This study
IT136	*fbf-1(ok91) fbf-2(q704) unc-4(e120)/mIn1[mIs14 dpy-10(e128)] II.*		This study
IT190	*dpy-5(e61)5 gap-3(kp-1) I; fbf-1(ok91) fbf-2(q704) unc-4(e120)/mIn1[mIs14 dpy-10(e128)] II.*		This study
IT191	*dpy-5(e61) gap-3(kp-1) I; fbf-1(ok91) fbf-2(q704) puf-8(zh17) unc-4(e120)/mIn1[mIs14 dpy-10(e128)] II*		This study
BA606	*spe-6(hc49) unc-25(e156)III; eDp6(III;f)*		(Varkey et al., 1993)
IT208	*dpy-5(e61) gap-3(kp-1)/hT2[dpy-18(h662)] I ; puf-8(zh17)unc-4(e120)/mnc1 II ; +/hT2; +/hT2[bli-4(e937)] III*		This Study
IT769	*gap-3(kp-1)/hT2[dpy-18(h662)] I ; puf-8(zh17)unc-4(e120)/mnc1 II ; spe-6(hc49) unc-25(e156)/hT2[bli-4(e937)] III*		This study

Table S3. Sequences of PCR primers used in this study: primers for generating GFP:GAP-3 fusion

Primer name	Sequence	Description
KS33575	tctggatccactagtatgcactcaagcgagaatac	Forward primer for upstream CDS fragment of *gap-3*
KS3102	cacagccagctgataggcatc	Reverse primer for upstream CDS fragment of *gap-3* from genomic DNA (to include first intron)
KS3576	tctggcgcccaaatgatgaagccgaatgagc	Reverse primer for downstream CDS fragment of *gap-3* from cDNA
KS2092	tttcctcaacaacagccgccg	To check orientation of *gap-3* cDNA fragment in pSV37

Table S4. List of the genes screened by RNAi for rescue of the germline tumour phenotype of *puf-8(–); gap-3(–)*

Sequence	Gene name	Functional description*
ZK1067.1	***let-23***	Encodes an EGF-receptor-family transmembrane tyrosine kinase
ZK792.6	***let-60***	Encodes a member of the GTP-binding RAS protooncogene family
C05D11.4	***let-756***	Encodes fibroblast growth factor (FGF)-like ligand required for progression through early larval development
F36H1.4	***lin-3***	Encodes a member of the EGF family of peptide growth factors
F43C1.2	***mpk-1***	Encodes a mitogen-activated protein (MAP) kinase an ERK ortholog
F26E4.1	*sur-6*	Encodes a regulatory (B) subunit of serine/threonine protein phosphatase 2A (PP2A-B)
F52C12.5	*elt-6*	Erythroid-Like Transcription factor family
F55A8.1	*egl-18*	Encodes a member of the GATA-family of transcription factors
C08C3.1	*egl-5*	Encodes a homeodomain transcription factor, orthologous to Drosophila Abd-B and the vertebrate Hox9-13 proteins
Y54G11A.10	*lin-7*	Encodes a protein that contains a PDZ domain and an L27 domain, two protein interaction domains that likely serve as organizational centers for large macromolecular complexes in polarized cells
F39B2.4	*sur-2*	Encodes a novel protein that is orthologous to the Drosophila and human MED23 mediator subunits
F26F4.3	*rom-1*	Rhomboid (drosophila) related
C48A7.1	*egl-19*	Encodes the pore-forming alpha1 subunit of a voltage-gated calcium channel orthologous to the alpha subunit of mammalian l-type calcium ion channels
M110.5	*dab-1*	Encodes an ortholog of the cytoplasmic adaptor protein DISABLED, required for normal molting and meiotic arrest;
F38H4.9	*let-92*	Encodes a homolog of PP2AC, the catalytic subunit of protein phosphatase 2A (PP2A)
AC7.2	*soc-2*	Encodes a leucine-rich repeat protein; soc-2 functions downstream in the let-60/Ras and egl-15/FGF receptor signaling pathways to positively and negatively regulate signaling through these pathways, respectively
C09H6.2	*lin-10*	Encodes a PDZ and PTB domain-containing protein that is homologous to mammalian Munc interacting proteins (Mint1, OMIM:602414) and is required for polarized protein localization
M01D7.7	*egl-30*	Encodes an ortholog of the heterotrimeric G protein alpha subunit Gq (Gq/G11 class)
R01H10.8	*cnk-1*	Encodes a protein that contains a SAM domain, a PDZ domain, and a PH domain.
C07H6.7	*lin-39*	Encodes a homeodomain protein homologous to the Deformed and Sex combs reduced family of homeodomain proteins

*www.wormbase.org

Core components of RAS/MAPK signaling are shown in bold, and the rest are positive regulators of RAS/MAPK signaling.

Table S5. Sequences of PCR primers used in this study: primers for generating GFP-3' UTR fusions

Primer name	Sequence	Description
KS2833	tctgggccctcagcatcgtgagcgtcacg	Forward primer for *let-60*
KS2834	tctgggccctcatgctccgatggcgaggtc	Reverse primer for*let-60*
KS3437	ggatcctgtgtgtgtgtgtgtgtgtgtgtgtaacggaacgaccggcagat	Reverse for upstream fragment for substitution C
KS3438	ggatcctgtgtgtgtgtgtgtgtgtgtgtgtgtatatagatacaattagacccc	Forward for downstream fragment for substitution C

Table S6. Sequences of PCR primers used in this study: primers used for testing PUF-8 : *let-60* **3' UTR interaction**

Primer name	Sequence	Description
Primers used for preparing in vitro transcription templates used in the mobility shift assays		
KS2808	ccaaggggttatgctaggaag	Common forward primer for synthesizing in-vitro transcription templates of the fragments of *let-60* 3'UTR
KS3295	tcgaagagtatgtagaggcgg	Reverse primer for fragment 1
KS3296	ccgcctctacatactcttcga	Forward primer for fragment 2
KS3098	ggccaattttggaacgagg	Reverse primer for fragment 2
KS3297	gcatccgaaccccctcctcg	Forward primer for fragment 3
KS3299	aacggaacgaccggcagatgc	Reverse primer for fragment 3
KS3298	tggcctcatctattttcag	Forward primer for fragment 4
KS3100	gaatgcaatacgagggagc	Reverse primer for fragment 4
KS3300	gctccctcgtattgcattc	Forward primer for fragment 5
KS3097	cctcatctttcgcttagccc	Common forward primer for upstream fragment for substitutions in fragment 4
KS3433	ggatcctgtgtgtgtgtgtgtgtgtgtgtgtcgaggaaggggaataaagcatc	Reverse primer for upstream fragment of substitution A
KS3434	ggatcctgtgtgtgtgtgtgtgtgtgtgtgttctgccggtcgttccgttt	Forward primer for downstream fragment of substitution A
KS3435	ggatcctgtgtgtgtgtgtgtgtgtgtgtgtcttctgaaaatagatgaggcc	Reverse primer for upstream fragment of substitution B
KS3436	ggatcctgtgtgtgtgtgtgtgtgtgtgtgtcctctcattttttgtcaag	Forward primer for downstream fragment of substitution B
KS3437	ggatcctgtgtgtgtgtgtgtgtgtgtgtgtaacggaacgaccggcagat	Reverse primer for upstream fragment of substitution C
KS3438	ggatcctgtgtgtgtgtgtgtgtgtgtgtgtatatagatacaattagacccc	Forward primer for downstream fragment of substitution C
KS3432	taatacgactcactatagggagaagatgctttattccccttcctcg	Common forward primer, with T7 promoter sequence, for synthesizing template for in-vitro transcription of substituted Fragment 4

KS3441	taaaatgtagaggaatagag	Common reverse primer for synthesizing template for in vitro transcription of substituted Fragment 4
Primers for PUF-8:9×HA:GFP transgene construct pAK9		
KS3788	tctactagttacccatacgacgtcccagactacgcctatccgtatgatgttccggattatgct	Forward primer amplify HA sequence for pAK8
KS3789	tctactagtcccggggggcgtagtctgggacgtcgtatgggtaagcataatccggaacatcatacggata	Reverse primer for HA sequence for pAK8
KS3791	tctcccgggtacccatacgacgtcccagactacgcctatccgtatgatgttccggattatgct	Forward primer for HA sequence for pAK10
KS3792	tctcccggggcgcgcggcgtagtctgggacgtcgtatgggtaagcataatccggaacatcatacggata	Reverse primer for HA sequence for pAK10
KS3794	tctgcgcgctacccatacgacgtcccagactacgcctatccgtatgatgttccggattatgct	Forward primer for HA sequence for pAK11
KS3795	tctgcgcgcagcgtagtctgggacgtcgtatgggtaagcataatccggaacatcatacggata	Reverse primer for HA sequence for pAK11
Primers for reverse transcription and RT-PCR		
KS2096	gcattagcggccgcgaaattaatacgactcactatagggaga(t)21 v	Anchored oligo dT primer
KS3297	gcatccgaaccccctcctcg	Forward for *let-60* mRNA
KS3299	aacggaacgaccggcagatgc	Reverse for *let-60* mRNA
KS1102	tctaagcttatggctcaaacaaagccgat	Forward for *pie-1* mRNA
KS2495	aatctgacgacgattcgagc	Reverse for *pie-1* mRNA