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Dedifferentiation of Primary Spermatocytes into Germ Cell Tumors in *C. elegans* Lacking the Pumilio-like Protein PUF-8

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Summary

PUF proteins are a conserved family of RNA binding proteins that regulate RNA stability and translation by binding to specific sequences in 3′-untranslated regions. *Drosophila* PUMILIO and *C. elegans* FBF are essential for self-renewal of germline stem cells, suggesting that a common function of PUF proteins may be to sustain mitotic proliferation of stem cells [1]. Here, we show that PUF-8, the *C. elegans* PUF most related to PUMILIO, performs a different function in germ cells that have begun meiosis: in primary spermatocytes, *puf-8* is required to maintain meiosis and prevent the return to mitosis. Primary spermatocytes lacking PUF-8 complete meiotic prophase but do not undergo normal meiotic divisions. Instead, they dedifferentiate back into mitotically cycling germ cells and form rapidly growing tumors. These findings reveal an unexpected ability for germ cells that have completed meiotic prophase to return to the mitotic cycle, and they support the view that PUF proteins regulate multiple transitions during germline development.

Results

puf-8, a *pumilio*-Related Gene, Is Expressed in the Germline of *C. elegans*

Among the 11 PUF proteins in the *C. elegans* genome, *puf-8* and *puf-9* are the most related to *Drosophila pumilio* (49% identity, 68% similarity [1]). Northern analysis revealed that *puf-8* is expressed primarily in the germline, whereas *puf-9* is expressed primarily in the soma (Figure S1A in the Supplementary Material available with this article online). Consistent with germline expression, a *puf-8:gfp* fusion was expressed strongly in sperm (Figure S1B).

To analyze the consequences of loss of *puf-8* expression, we obtained a *puf-8* deletion allele (*puf-8(ok302)*) from the *C. elegans* Gene Knockout Consortium (Figure S1C). We also used RNA-mediated interference [2] to reduce *puf-8* expression (Figure S1A). Both methods yielded identical phenotypes, albeit with different frequencies (Table 1).

puf-8 Is Required for Faithful Meiotic Divisions in Spermatocytes

At 20°C, *puf-8(ok302)* hermaphrodites were subfertile (brood size: 150 compared to 240 in wild-type) and segregated a significant number of dead embryos (53%) and males (11%), as is typical of mutants that affect chromosome segregation [3]. To determine whether the lethality was due to defective sperm, oocytes, or both, we mated *puf-8(ok302)* hermaphrodites with wild-type males. Mated *puf-8(ok302)* hermaphrodites gave rise to 91.3% live progeny compared to 47% for unmated hermaphrodites, indicating that most of the embryonic lethality was due to defective sperm (see the Supplementary Material).

Consistent with this finding, staining of *puf-8(ok302)* spermatids with DAPI frequently revealed the absence of DNA (20%) or extra masses of DNA (32%) ($n = 100$; Figure 1C). In contrast, no defects were seen in oocytes and in maternal pronuclei in embryos (polar bodies were formed as in wild-type, data not shown), confirming that *puf-8* is not essential for oocyte development.

DAPI staining of the germlines of *puf-8(ok302)* adult males revealed no obvious abnormalities as germ cells progressed from mitosis to metaphase of meiosis I (data not shown and Figure 1B). Abnormal DAPI patterns were first seen during and after the meiotic divisions. In wild-type, secondary spermatocytes and spermatids appear as uniformly sized, round cells, which often remain attached to a residual body (Figure 1B). In contrast, *puf-8(ok302*) meiotic products were frequently misshapen, had no recognizable residual bodies, and had DNA masses of varying sizes (Figure 1B). We conclude that *puf-8* is required for meiotic divisions in spermatocytes.

At 25°C, 44% of *puf-8(ok302)* Germlines Develop Tumors

When raised at 25°C, *puf-8(ok302)* hermaphrodites became 100% sterile. DAPI staining revealed two predominant phenotypes: small germlines with fewer cells than wild-type, and tumorous germlines with abnormally high numbers of cells (Table 1; Figure 2). We have shown previously that *puf-8* is required redundantly with other PUF proteins to maintain germ cell viability during larval stages [4]. The occasional small germline phenotype of *puf-8* mutants, therefore, may reflect an early function essential for proliferation and/or viability that *puf-8* shares with other PUFs. In this study, we focus on the tumorous phenotype, since this phenotype has not yet been described for other PUFs and is most related to the meiotic defects seen in *puf-8* mutants raised at 20°C (see below).

DAPI staining of the *puf-8* tumorous germlines revealed that the distal to proximal order of mitosis to meiosis was not affected, except in the most proximal region (Figure 2). This region is normally filled with gametes, which include oocytes and sperm in hermaphrodites and only sperm in males. In *puf-8(ok302)* and *puf-8(RNAi)*, oocytes were present in hermaphrodites (data not shown), but sperm were absent or present in reduced numbers, in both hermaphrodites and males (Figure 2 and data not shown). In their place, numerous, apparently undifferentiated, cells filled the most proxi-

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Table 1. Formation of *puf-8* Tumors Requires Spermatogenesis and Progression through Meiotic Prophase

Genotype	Gametogenesis	% Small Germline	% Tumorous Germline (n)
puf-8(ok302) XX	Spermatogenesis/oogenesis	56%	44% (82)
puf-8(ok302) XO	Spermatogenesis only	39%	61% (100)
puf-8(RNAi) XX	Spermatogenesis/oogenesis	10%	86% (89)
puf-8(RNAi) XO	Spermatogenesis only	12%	87% (95)
puf-8(RNAi) fem-1(hc17) XX	Oogenesis only	ND	0% (100)
puf-8(RNAi) tra-2(q122) XX	Oogenesis only	ND	0% (100)
puf-8(RNAi) tra-2(q122) XO	Spermatogenesis only[a]	ND	90%(100)
puf-8(RNAi) mek-2(q425) XX	Spermatogenesis/oogenesis: all arrest at pachytene	ND	0% (46)
puf-8(RNAi) spe-6(hc49) XX	Spermatogenesis/oogenesis: sperm arrest at diakinesis	ND	0% (48)
puf-8(ok302);spe-6(hc92)/ + XX	Spermatogenesis/oogenesis	ND	50% (18)[b]
puf-8(ok302);spe-6(hc92) XX	Spermatogenesis/oogenesis: sperm arrest at diakinesis	ND	13% (15)[b]

All genotypes were grown at 25°C. ND: not determined.
[a] The *tra-2(q122)* mutation eliminates sperm production in XX animals but has no effect on spermatogenesis in XO animals.
[b] *spe-6* mutations block spermatocytes in diakinesis but do not affect oocytes. The 2 out of 15 putative *puf-8(ok302);spe-6(hc92)* XX hermaphrodites that developed a tumor are likely non-Spe recombinants; see the Supplementary Material.

mal region (Figure 2). These cells increased in number over time, from about 20 in L4 larvae to over 1000 in 2-day-old adults (Figure 2). We verified that the cells in the tumors were germ cells, by staining with three germ cell-specific markers (PGL-1, GLH-1, and GLP-1, [5–7], Figure 3A, and data not shown) and that they were proliferating, by staining with the mitosis-specific marker phospho-H3 ([8]; Figure 3B).

puf-8 Tumors Arise from Primary Spermatocytes that Have Completed Meiotic Prophase but Fail to Undergo Normal Meiotic Divisions

We next asked whether the *puf-8* tumors were derived from cells destined to become oocytes, sperm, or both. The presence of the tumors in males (which only make sperm) indicated that spermatogenic germ cells can contribute to the tumor (Table 1). To test whether oogenic germ cells were also competent, we examined the effect of removing *puf-8* in *fem-1(hc17)* and *tra-2(q122)*, two mutants that cause hermaphrodites to produce only oocytes [9, 10]. We found that *puf-8(RNAi);fem-1(hc17)* and *puf-8(RNAi);tra-2(q122)* "females" formed oocytes, but no tumors (Table 1). These oocytes could be fertilized by wild-type sperm and form viable embryos (data not shown). We conclude that the *puf-8* tumors are derived only from spermatogenic germ cells.

We next asked whether *puf-8* tumors were derived from germ cells that had initiated meiosis. *mek-2(q425)* and *spe-6(hc49)* are mutations that arrest germ cells during early (pachytene; *mek-2*) or late (diakinesis; *spe-6*) prophase of meiosis I [11, 12]. We found that *puf-8(RNAi);mek-2(q425)* and *puf-8(RNAi);spe-6(hc49)* males failed to develop tumors (Table 1) and instead resembled *mek-2* and *spe-6* single mutants (data not shown). Similar results were obtained when examining *puf-8(ok302);spe-6(hc92)* double mutant hermaphrodites (Table 1). We conclude that the *puf-8* tumors are derived from germ cells that have progressed through prophase of meiosis I.

DAPI staining of the *puf-8* tumorous germlines suggested that germ cells progress normally through meiotic prophase and to metaphase of meiosis I (Figures 2, 3, and 4). Staining with meiosis and spermatogenesis markers confirmed this assessment. HIM-3 is a synaptonemal complex protein that decorates chromosomes throughout meiotic prophase and comes off chromosomes during the meiotic divisions (Figures 4A and 4C; [13]). In *puf-8* tumorous germlines, HIM-3 was present on chromosomes throughout meiotic prophase and became cytoplasmic during the divisions, as in wild-type (Figures 4B and 4D). In most cells of the tumor, HIM-3 remained cytoplasmic (Figure 4D), although cells with nuclear HIM-3 were also detected (Figure 4B). Markers for spermatogenesis (MO and MSP, [14, 15]) also appeared during meiotic prophase, as in wild-type, in *puf-8(RNAi)* tumorous germlines (Figures 4E and 4F and data not shown). In wild-type, MO and MSP persist during the meiotic divisions and in differentiating spermatids (Figure 4E and data not shown). Remarkably, MO and MSP were absent from the *puf-8* tumors. This finding is consistent with cells in the tumors having returned to a less-differentiated state (Figure 4F and data not shown).

To investigate the transition from primary spermatocytes to tumor, we dissected *puf-8* tumorous germlines and stained the dissociated germ cells with DAPI and anti-tubulin (Figures 1 and 4G–4H). Primary spermatocytes that had budded off from the germline syncytium displayed an apparently normal metaphase arrangement of chromosomes and centrosomes (Figures 1, 4D, and 4H). The ensuing division products, however, were highly aberrant, often consisting of large cells (similar in size to budded primary spermatocytes) with disorganized spindles and two or more masses of partially decondensed DNA (Figures 1, 4D, and 4H). These aberrant intermediates were reminiscent of those seen in *puf-8* germlines grown at 20°C (Figure 1). However, unlike those germlines, which yield aneuploid sperm, *puf-8* tumorous germlines gave rise to many rounded cells with decondensed DNA (Figures 1C and 4D). These cells resembled the stem cells found in the distal region of the germline, with one important difference. Whereas distal germ cells exist in a syncytium connected to a central core (rachis), cells in the *puf-8* tumors could easily be separated from one another (Figures 1 and 4). This difference is consistent with *puf-8* tumors arising from cells that have completed meiotic prophase, since

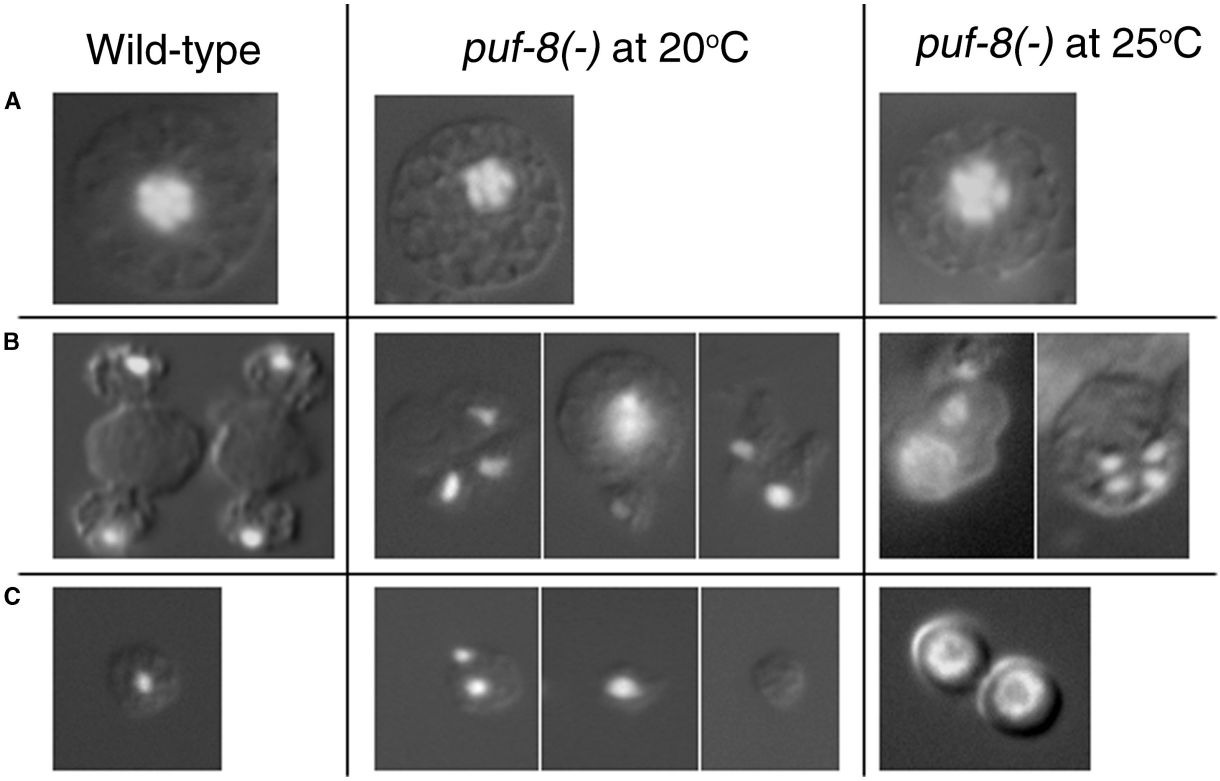

Figure 1. *puf-8* 1° Spermatocytes Undergo Aberrant Meiotic Divisions

(A) DAPI-stained 1° spermatocytes in metaphase of meiosis I from wild-type males, *puf-8(ok302)* males grown at 20°C, and *puf-8(RNAi)* males grown at 25°C. *puf-8* 1° spermatocytes are indistinguishable from wild-type.

(B) DAPI-stained division intermediates. In wild-type, four spermatids with condensed DNA surround residual bodies. In *puf-8* mutants, aberrant intermediates are seen.

(C) DAPI-stained division products. Wild-type sperm have one highly condensed mass of DNA. Sperm in *puf-8* males grown at 20°C have extra DNA masses, a single partially decondensed DNA mass, or lack DNA altogether. *puf-8* males grown at 25°C give rise to individualized, round cells with decondensed DNA.

primary spermatocytes are known to bud off the rachis just before the meiotic divisions [16].

To determine whether cells in the tumor had undergone a reductional division (meiosis I), we hybridized them to a probe specific for the X chromosome (which in male germ cells segregates asymmetrically during meiosis I). We found that all cells in the *puf-8* tumors hybridized to the X-specific probe ($n = 50$; Figure 3C). Although we cannot exclude that cells lacking an X chromosome may have been underrepresented in the tumor, our results are most consistent with *puf-8* tumors being derived from primary spermatocytes that have not completed a reductional division. We conclude that, at elevated temperatures, *puf-8* is required both to complete meiotic divisions and to prevent primary spermatocytes (and/or their division products) from returning to the mitotic cycle.

Discussion

We reported previously that a subset of PUF proteins (FBF-1/2, PUF-5/6, PUF-8) function together to regulate primordial germ cell development in embryos [4]. FBF-1 and FBF-2 also function redundantly in adults to promote the sperm-to-oocyte switch [17] and to maintain the self-renewing potential of germline stem cells [18]. We report here that PUF-8 performs yet another function in meiotic germ cells. In the absence of PUF-8, primary spermatocytes exit meiosis and return to the mitotic cycle. Together, these data demonstrate that PUF proteins regulate several transitions during germ cell development and can affect the development of both premeiotic and meiotic germ cells. What target RNAs are involved, and are some targets involved in more than one transition? Two direct targets have been identified so far for FBF: *gld-1*, which promotes entry into meiosis [18–20] (see below), and *fem-3*, which promotes entry into spermatogenesis [17, 21]. Neither are known to function during meiotic divisions, suggesting that PUF-8 regulates yet another target(s) in spermatocytes.

This complexity is unlikely to be specific to nematode PUFs, as the single *Drosophila* PUF, Pumilio, also has been implicated in several aspects of germline development. During embryogenesis and larval stages, Pumilio is required for primordial germ cell migration and growth [22–24]. Later in the adult, Pumilio is required in germline stem cells for self-renewal and to promote oogenesis [24–26]. Pumilio also has functions outside of the germline, in the embryo [27, 28], the somatic gonad [24], and the nervous system [29]. Thus, Pumilio, like the *C.*

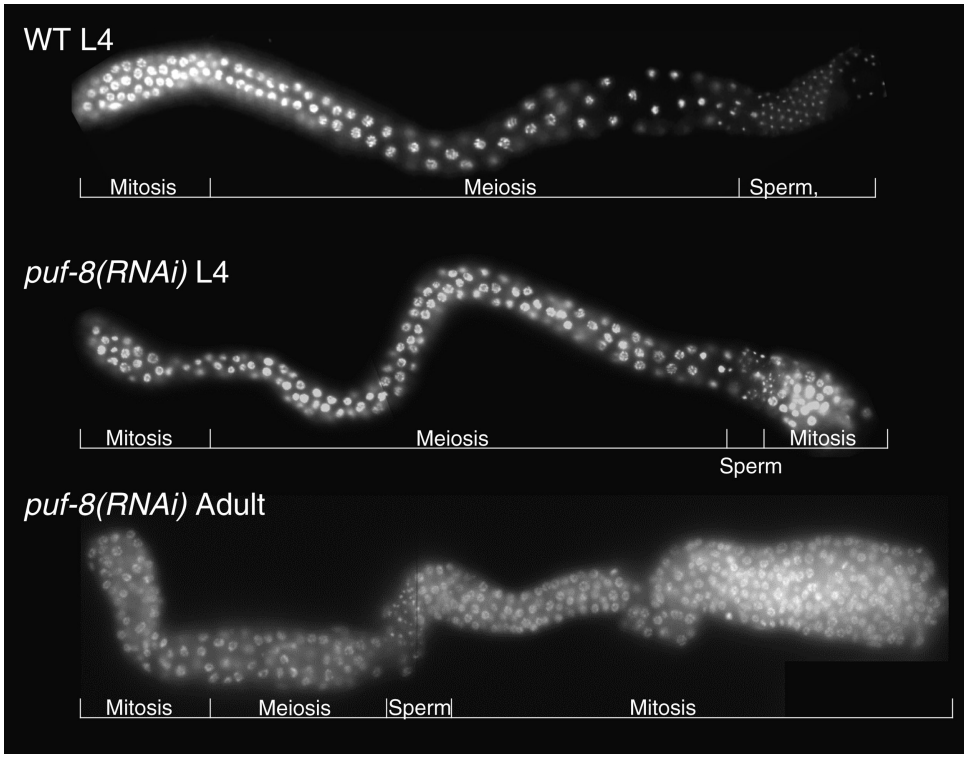

Figure 2. *puf-8* Males Raised at 25°C Form Tumors that Grow into Adulthood

Dissected germlines stained with DAPI; the distal end of the germlines is oriented toward the left, and the proximal end is oriented toward the right. Wild-type germlines accumulate sperm at the proximal end, whereas *puf-8(RNAi)* germlines develop proximal tumors that grow into adulthood. L4: last larval stage before the adult stage.

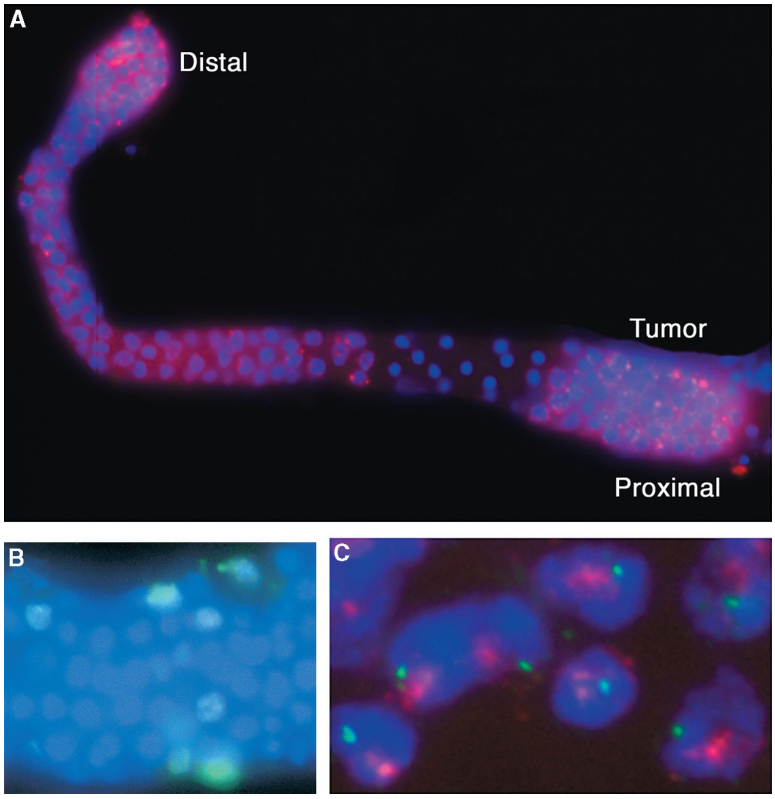

Figure 3. Cells in *puf-8* Tumors Are Mitotically Cycling Germ Cells that Have Not Undergone a Reductional Division

(A) Dissected *puf-8(RNAi)* tumorous germline stained with DAPI (blue) and with anti-PGL-1 (pink), a germ cell-specific marker. PGL-1 is present in granules in distal mitotic germ cells and in germ cells progressing through early meiotic prophase (pachytene). PGL-1 granules are absent from primary spermatocytes that have progressed past the pachytene stage (as is true in wild-type, [34]), but they return in the tumor, confirming that cells in the tumor are germ cells. Tumor cells also stained with two other germ cell markers, GLH-1 and GLP-1 (data not shown).

(B) Close-up of a *puf-8(RNAi)* tumor stained with DAPI (blue) and anti-phospho-H3 (green).

(C) *puf-8(RNAi)* tumor dissected from an XO animal, stained with DAPI (blue), and hybridized to DNA probes specific for the X chromosome (green) and chromosome I (red, ribosomal DNA locus). All nuclei stain with both probes, suggesting that all cells in the tumor retain an X chromosome.

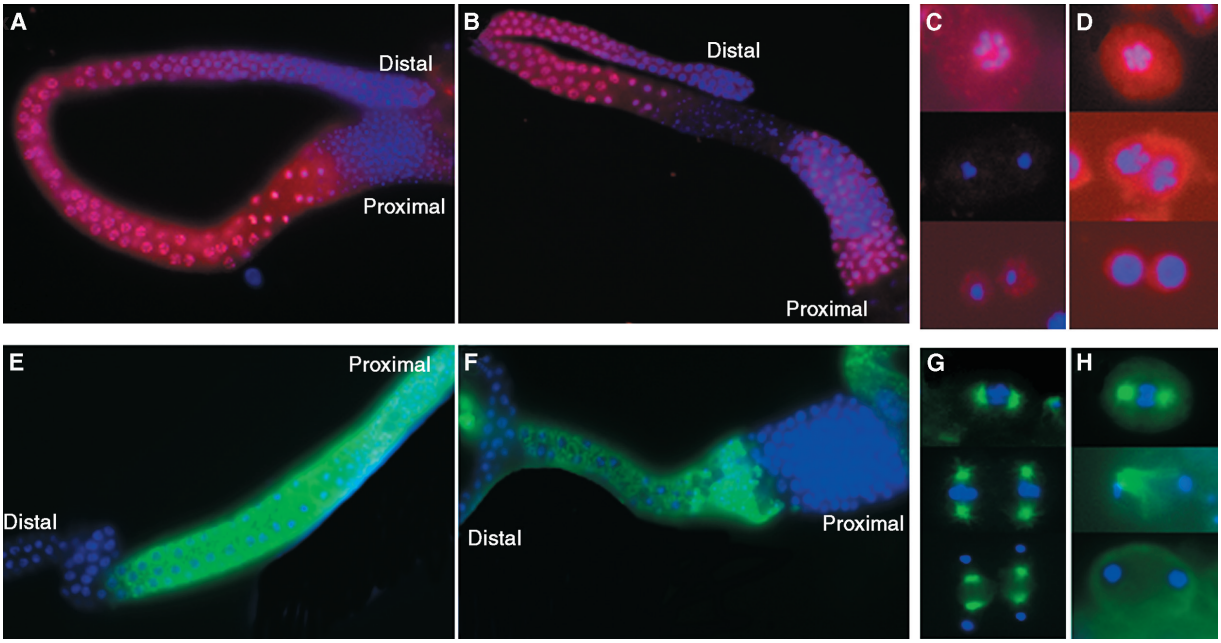

Figure 4. Meiotic Prophase and the Onset of Spermatogenesis Proceed Normally in *puf-8* Tumorous Germlines

(A and B) (A) Wild-type and (B) *puf-8(RNAi)* tumorous germlines costained with DAPI (blue) and with anti-HIM-3 (red).

(C and D) (C) Wild-type and (D) *puf-8(RNAi)* primary spermatocytes in metaphase of meiosis I (top); division intermediates (middle) and division products (bottom) costained with DAPI (blue) and with anti-HIM-3 (red).

(E and F) (E) Wild-type and (F) *puf-8(RNAi)* tumorous germlines costained with DAPI (blue) and the spermatogenesis marker MSP (green).

(G and H) (G) Wild-type and (H) *puf-8(RNAi)* primary spermatocytes in metaphase of meiosis I (top); division intermediates (middle) and division products (bottom) costained with DAPI (blue) and anti-tubulin (green).

In wild-type, primary spermatocytes undergo two divisions in quick succession to give rise to four spermatids with highly condensed DNA surrounding a residual body (or cystoblast) where spindle remnants are discarded (Figure 4G, bottom). *puf-8* primary spermatocytes set up a spindle (top) and, at least occasionally, appear to successfully separate the DNA into two masses (middle), but they fail to form a residual body (bottom).

elegans PUFs, is likely to regulate many different RNAs. The redundancy of the *C. elegans* PUFs during embryogenesis suggests that they regulate common targets in primordial germ cells and have acquired unique targets in later stages. The recurring requirement for PUFs in the germline likely reflects a high dependency for this tissue on posttranscriptional regulation [30, 31].

In *puf-8* mutants, 1° spermatocytes dedifferentiate into mitotically cycling germ cells, as evidenced by the loss of spermatogenesis markers (MO and MSP), the return of early germ cell markers (P granules and GLP-1), and reentry into mitosis and tumor formation. We do not yet know how 1° spermatocytes transition from metaphase of meiosis I to mitosis, but one possibility is that sister chromatid cohesion fails, causing at least some cells to undergo a division that is more akin to an equational division (meiosis II) than a reductional division (meiosis I). This possibility is supported by the high frequency of aneuploidy in *puf-8* mutants grown at 20°C, and by the equational segregation of the X in XO tumors. A return to mitosis by cells that initiate but fail to complete the meiotic program has also been observed in *gld-1* mutants. GLD-1 is an RNA binding protein required for progression through meiotic prophase [19]. In the absence of *gld-1*, germ cells exit meiosis during pachytene (prophase of meiosis I) and return to mitosis, resulting in a tumorous germline. Unlike *puf-8* mutants, *gld-1* germ cells never complete meiotic prophase and do not initiate gametogenesis. Furthermore, whereas *puf-8* affects only cells undergoing spermatogenesis, *gld-1* affects only cells destined to become oocytes. These differences notwithstanding, in both the *gld-1* and *puf-8* mutants, failure to complete meiosis is linked to tumor formation. In humans, germline tumors are most commonly thought to be derived from premeiotic germ cells. An exception, however, may exist in the male germline. Spermatocytic seminomas are testicular tumors derived from germ cells that have initiated spermatogenesis and may also have initiated meiosis, although this last point is still under debate [32, 33]. Our findings demonstrate that cells that have progressed past meiotic prophase can give rise to tumors, and they raise the possibility that PUF proteins could function as tumor suppressors in the germlines of other organisms.

Supplementary Material

Supplementary Material including Experimental Procedures and information regarding the *puf-8* locus is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

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