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The Ras-ERK MAPK regulatory network controls dedifferentiation in *Caenorhabditis elegans* germline

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ABSTRACT

How a committed cell can be reverted to an undifferentiated state is a central question in stem cell biology. This process, called dedifferentiation, is likely to be important for replacing stem cells as they age or get damaged. Tremendous progress has been made in understanding this fundamental process, but its mechanisms are poorly understood. Here we demonstrate that the aberrant activation of Ras-ERK MAPK signaling promotes cellular dedifferentiation in the *Caenorhabditis elegans* germline. To activate signaling, we removed two negative regulators, the PUF-8 RNA-binding protein and LIP-1 dual specificity phosphatase. The removal of both of these two regulators caused secondary spermatocytes to dedifferentiate and begin mitotic divisions. Interestingly, reduction of Ras-ERK MAPK signaling, either by mutation or chemical inhibition, blocked the initiation of dedifferentiation. By RNAi screening, we identified RSKN-1/P90$_{RSK}$ as a downstream effector of MPK-1/ERK that is critical for dedifferentiation: *rskn-1* RNAi suppressed spermatocyte dedifferentiation and instead induced meiotic divisions. These regulators are broadly conserved, suggesting that similar molecular circuitry may control cellular dedifferentiation in other organisms, including humans.

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1. Introduction

Cell fate reprogramming manipulates cellular differentiation and allows its redirection, a process critical for regenerative medicine [1,2]. One mechanism often inherent to reprogramming is dedifferentiation. In this process, a cell reverts from a differentiated and restricted state to a more undifferentiated and multipotent state. Moreover, tumor-initiating cells (sometimes called cancer stem cells) may arise from the dedifferentiation of more differentiated cell types [3]. Although cellular dedifferentiation has been observed in tissue culture cells and in organisms [4–7], the mechanism is still poorly understood.

Normally, germ cells differentiate to produce either sperm or eggs, which maintain the potential to create an entirely new organism. In the nematode *Caenorhabditis elegans* (*C. elegans*), germ cells progress from germline stem cell (GSC) at the distal end, through meiotic prophase as they move proximally to differentiated gamete at the proximal end (Fig. 1A); they rely on conserved regulators to control their state of differentiation [8]. In particular, PUF (*Pu*milio and *F*BF) RNA-binding proteins are required for GSC self-renewal in worms [9], flies [10,11], and have been implicated in this role in mammals [12,13]. *C. elegans* has multiple PUF proteins with specialized roles [14]. Among them, FBF-1 and FBF-2 (collectively called FBF) and PUF-8 proteins regulate GSC self-renewal [9,15] and cell fate specification [16–18]. Interestingly, a previous report found that PUF-8 maintains commitment to the meiotic cell cycle and prevents dedifferentiation of spermatocytes into germline tumors [19]. However, it has been unclear how PUF-8 inhibits dedifferentiation.

Here we investigate the molecular and cellular bases of dedifferentiation in the nematode *C. elegans* germline. We demonstrate that PUF-8 and LIP-1, a dual specificity phosphatase and inhibitor of MPK-1/ERK MAPK signaling [20], work together in the *C. elegans* germline to repress dedifferentiation and that they do so by inhibiting MPK-1/ERK MAPK signaling. Moreover, activation of RSKN-1 (P90$_{RSK}$, P90 Ribosomal S6 Kinase homolog) by MPK-1/ERK in *puf-8; lip-1* mutant spermatocytes disturbs microtubule organization and leads to germ cell dedifferentiation and formation of proximal germline tumors. Importantly, ERK2 MAPK

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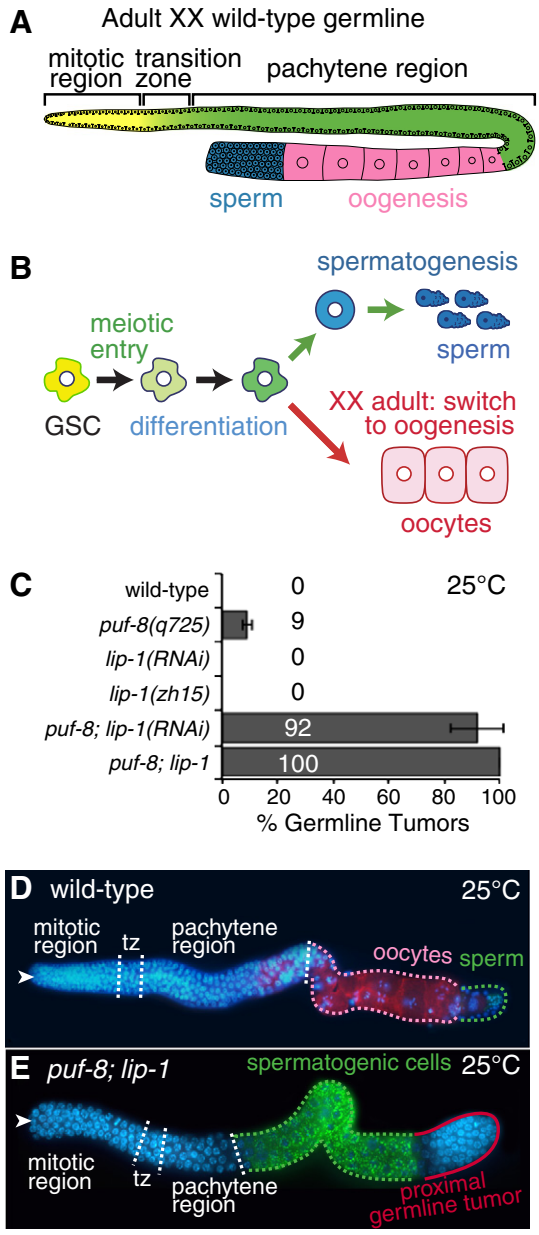


Fig. 1. PUF-8 and LIP-1 normally repress proximal germline tumors. (A) Schematic of normal adult hermaphrodite germline. This organization is typical of wild-type animals, most *puf-8* single mutants, and most *lip-1* single mutants. Yellow, germ cells in mitotic cell cycle, including germline stem cells (GSCs); green, germ cells progressing through meiotic prophase I; mature sperm were made in larvae whereas oocytes are made in adults. (B) Schematic of germ cell progression. Above, larval gametogenesis generates sperm; below, adult gametogenesis generates oocytes. The switch from spermatogenesis to oogenesis occurs during the fourth larval stage (L4). (C) Graph showing the percentage of animals with proximal germline tumors. All strains were grown at 25 °C. Germline tumors were determined by cellular morphology and DAPI staining of dissected gonads. Standard deviation bars were calculated from at least three independent experiments. (D and E) Adult hermaphrodite germlines were extruded and co-stained with SP56 (sperm-specific marker; green), RME2 (oocyte-specific marker; pink) and DAPI (DNA; blue). (D) Wild-type. (E) *puf-8; lip-1*. Arrows indicate the distal end of the gonad arm.

signaling has also been implicated in cellular dedifferentiation of Sertoli cells [21], myoblasts [22], and islet cells [23] in mammals. Therefore, the regulatory circuitry controlling *C. elegans* germ cell dedifferentiation has important parallels with the control of cellular dedifferentiation in other organisms, including humans.

2. Materials and methods

2.1. C. elegans strains

All strains were maintained at 25 °C as described unless otherwise noted [24]. We used the wild-type Bristol strains N2 as well as the following mutants: *LGI: rrf-1(pk1417)*; *LGII*: *fbf-1(ok91)*, *fbf-2(q738)*, *puf-8(q725)*, *puf-8(ok302)*; *LGIII*: *glp-1(q224)*, *mpk-1(ga111)*; and *LGIV*: *lip-1(zh15)*, *let-60(n1046)*, *let-60(ga89)*, *fem-3(q20)*. All compound mutants and their representative phenotypes are summarized in Table S1.

2.2. RNA interference (RNAi)

RNAi experiments were performed by feeding bacteria expressing double strand RNAs corresponding to the gene of interest [25]. Briefly, five young adult worms were plated onto RNAi plates and allowed to lay embryos for 1 day at 25 °C before removal. Germline phenotypes of F1 progeny were determined by staining dissected gonads with specific markers and DAPI. For *mpk-1b* isoform-specific RNAi, the unique region (exon 1; 1–240 nt) of the *mpk-1b* gene was amplified by PCR from C. *elegans* genomic DNA and cloned into the pPD129.36 (L4440) vector containing two convergent T7 polymerase promoters in opposite orientations separated by a multi-cloning site. Other RNAi bacteria were from *C. elegans* RNAi feeding library (Source Bioscience LifeSciences) and *C. elegans* ORF-RNAi library (Open Biosystems).

2.3. Germine immunohistochemistry

For antibody staining, dissected gonads were fixed in 3% paraformaldehyde with 100 mM K_2HPO_4 (pH 7.2) for 10–60 min at room temperature followed by 100% cold methanol for 5 min at −20 °C [26]. After blocking for 1 h with 0.5% BSA in 1× PBS (+0.1% Tween 20), fixed gonads were incubated for 2 h at room temperature with primary antibodies followed by 1 h at room temperature with secondary antibodies. SP56 (sperm marker—a gift from S Ward), RME-2 (oocyte marker—a gift from B. Grant), REC-8 (mitosis marker—a gift from Josef Loidl) and α-tubulin (Sigma) were used as primary antibodies. For DP-MAPK (YT) antibody (Sigma) staining, all procedures were performed as described [27]. For Phospho-Histone H3 (Upstate Biotechnology) staining, fixed gonads were incubated overnight at room temperature. DAPI staining followed standard methods.

2.4. U0126 treatments

Small-molecule inhibitor (U0126) of MEK was performed using a slightly modified method of the protocol previously described [18]. Briefly, *puf-8; lip-1* double mutants were synchronized by the alkaline hypochlorite method and arrested in M9 media at the first larval or L1 stage. L1 larvae were then plated onto NGM plates containing mixture of 100 μM U0126 and OP50 *E. coli*, and grown at 25 °C for 68 h, corresponding to day one of adult life. Fertility was observed using a dissecting microscope and germline phenotypes were determined by staining dissected gonads with DAPI.

2.5. Western blots

Blots were prepared by standard procedures. Protein samples were separated on 4%–20% gradient gels (Cambrex), and the blot was probed with 1:20,000 rabbit polyclonal anti-ERK-1/2 antibody (Sc94; Santa Cruz Biotechnology), followed by washing and incubation with 1:10,000 HRP-anti-Rabbit (Jackson ImmunoResearch). Blots were re-blocked and re-probed with 1:10,000 Mouse monoclonal anti-α-tubulin (Sigma-Aldrich) and 1:10,000 HRP-conjugated anti-mouse (Jackson ImmunoResearch).

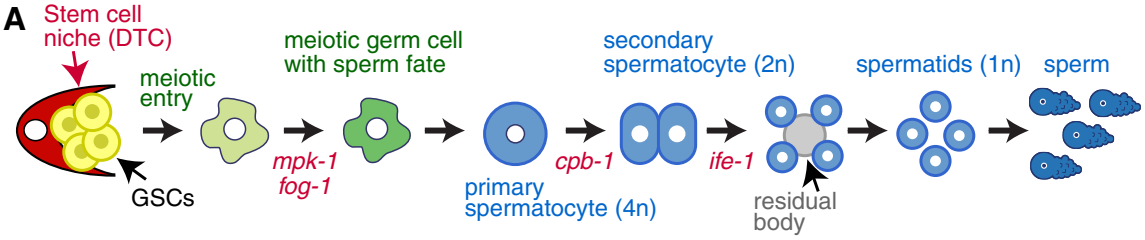
3. Results

3.1. PUF-8 and LIP-1 normally repress the formation of germline tumors

Wild-type *C. elegans* hermaphrodites make sperm during larval stages and switch to oogenesis as adults, which are therefore self-fertile (Fig. 1A and B). Similar to wild-type, most hermaphrodites homozygous for *puf-8(q725)*, henceforth called *puf-8(0)*, make sperm and oocytes, and are self-fertile at permissive temperature (20 °C) [17]. However, at restrictive temperature (25 °C), 9% of *puf-8(0)* mutants develop germline tumors in the proximal gonad (Fig. 1C and Table S1), as shown previously for a different allele [19]. Importantly, a previous work showed that cells in *puf-8* proximal germline tumors derive from primary spermatocytes via dedifferentiation [19]. We have found that the proximal germline tumor phenotype of *puf-8* single mutants is dramatically enhanced by the additional loss of LIP-1, a dual specificity ERK/MAPK phosphatase (Fig. 1C and Table S1). On their own, *lip-1(RNAi)* treated animals or *lip-1(zh15)* null mutants, henceforth called *lip-1(0)*, produce both sperm and oocytes at both 20 °C and 25 °C (Fig. 1C and Table S1) [28,29]. However all *puf-8; lip-1* double mutants generate proximal germline tumors at 25 °C (Fig. 1C and Table S1). This tumor phenotype differs from the *puf-8; lip-1* Mog (*M*asculinization *o*f *G*ermline: no oocyte and excess of sperm) phenotype seen at 20 °C (Table S1) [18]. To visualize the *puf-8; lip-1* defects, we used DAPI to stain DNA in all cells and SP56 (sperm-specific marker) and RME-2 (oocyte-specific marker) to stain gametes. Wild-type germlines stained positively for both gamete-specific markers (Fig. 1D), but the *puf-8; lip-1* mutant stained only with the SP56 sperm marker and also had a proximal germline tumor (Fig. 1E). Immunohistochemistry using anti-REC-8 (mitotic cell marker) [30] and Phospho-Histone H3 (metaphase marker) antibodies showed that germ cells in the proximal tumor were actively cycling (Fig. S1). We conclude that PUF-8 and LIP-1 proteins normally act to inhibit the formation of proximal germline tumors.

3.2. puf-8; lip-1 proximal germline tumors may arise from secondary spermatocytes via dedifferentiation

A previous report showed that PUF-8 prevents primary spermatocytes from dedifferentiating and generating proximal germline tumors [19]. To test if the proximal germline tumors of *puf-8; lip-1* double mutants are also derived from primary spermatocytes, we blocked key steps of germline development by RNAi to specific genes (Fig. 2A). In wild-type males or Mog mutants, spermatogenesis produces sperm continuously from GSCs, a process tightly regulated by signaling (e.g., MPK-1/ERK MAPK signaling) and a variety of RNA regulators (Fig. 2A) [31]. Loss of these regulators arrests germ cells at the specific stages of meiosis or spermatogenesis (Fig. 2B). As depicted in Fig. 2A, *mpk-1* (ERK homolog) RNAi arrests germ cells in pachytene [27]. Two cytoplasmic polyadenylation element binding (CPEB) proteins, *fog-1* and *cpb-1*, have distinct functions in spermatogenesis: *fog-1* RNAi blocks sperm specification [32] and *cpb-1* RNAi arrests germ cells as primary spermatocytes [33]. Last, RNAi directed against *ife-1*, one of five *C. elegans* mRNA cap-binding eIF4E proteins, arrests germ cells as secondary spermatocytes [34]. For this study, RNAi treatment was started in synchronized L1 larvae at 25 °C and germline phenotypes were analyzed by DAPI staining of dissected gonads after they reached adulthood (approximately 1.5 days after L4). Indeed, RNAi of either *mpk-1*, *fog-1*, or *cpb-1* dramatically suppressed *puf-8; lip-1* germline tumors (Fig. 2C). For example, no proximal germline tumors were found in *puf-8; lip-1; mpk-1(RNAi)* germlines at 25 °C; instead germ cells arrested in pachytene (Fig. 3E). These germlines also showed massive disruption of membrane



B

Gene	Homologous	RNAi phenotypes	Ref.
mpk-1	ERK MAPK	arrest germ cells in pachytene	27
fog-1	Cytoplasmic polyadenylation element binding protein (CPEB)	switch germ cells from spermatogenesis to oogenesis	32
cpb-1	CPEB	arrest germ cells in 1° spermatocytes	33
ife-1	Cap-binding protein eIF4E	arrest germ cells in 2° spermatocytes	34

C

Strains	°C	% Germline Tumors	n
puf-8; lip-1; vector(RNAi)	25	100	61
puf-8; lip-1; mpk-1(RNAi)	25	0	56
puf-8; lip-1; fog-1(RNAi)	25	18	17
puf-8; lip-1; cpb-1(RNAi)	25	29	84
puf-8; lip-1; ife-1(RNAi)	25	100	71

Fig. 2. Proximal germline tumors are formed by the dedifferentiation of secondary spermatocytes. (A) Schematic of *C. elegans* spermatogenesis (see [31]). In wild-type male and Mog mutant germlines, spermatogenesis produces sperm from GSCs. Regulatory genes are shown at stages when they are likely to work, but the temporal order of *mpk-1* driving pachytene exit before *fog-1* drives initiation of spermatogenesis is not known. (B) Table of specific regulators critical for meiotic progression (MPK-1/ERK MAPK signaling), sperm fate specification (FOG-1), first meiotic cell division from primary to secondary spermatocyte (CPB-1), and second meiotic cell division from secondary spermatocyte to spermatozoan (IFE-1). (C) The percentage of germline tumors. All RNAi experiments were performed at 25 °C and germline phenotypes were analyzed by staining dissected gonads with DAPI.

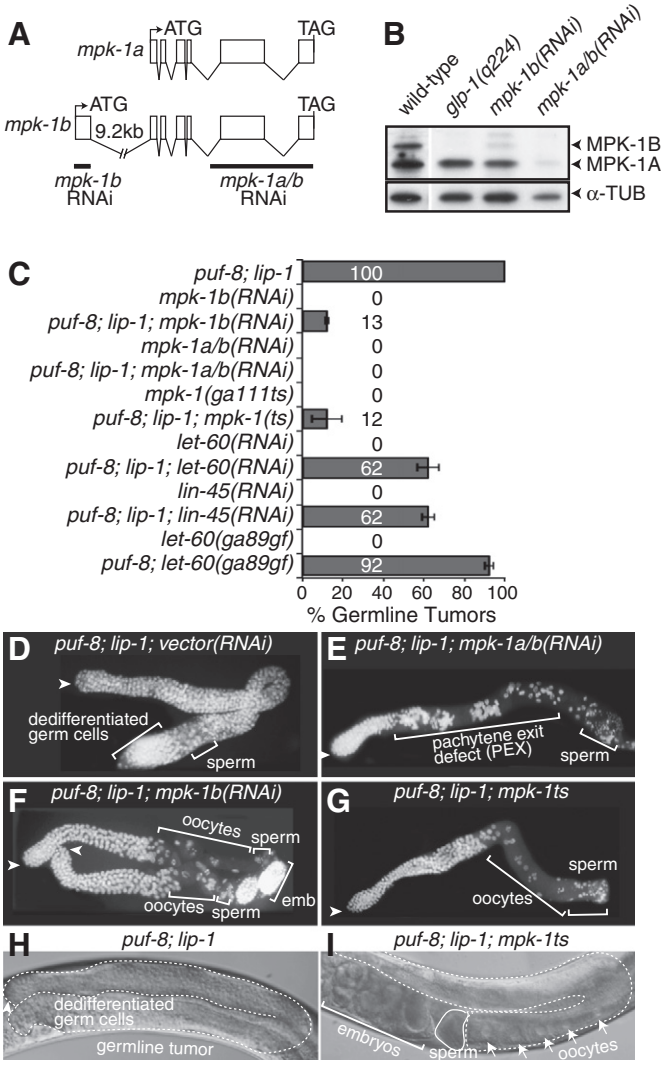


Fig. 3. Lowering MPK-1/ERK activity prevents dedifferentiation in *puf-8; lip-1* at 25 °C. (A) Schematics of *mpk-1a* and *mpk-1b* mRNA isoforms. The *C. elegans mpk-1* gene encodes two major transcripts, *mpk-1a* and *mpk-1b*. Box, exon; connecting line, intron; ATG, initiation codon; TAG, termination codon. Below schematics: thick lines indicate the region of RNAi targeting *mpk-1b* and *mpk-1a/b*. (B) Western blot. MPK-1A protein is ~45 kDa, MPK-1B is ~55 kDa, and α-TUB is α-tubulin. *mpk-1b* is likely expressed predominantly in the germline, because MPK-1B was not detected in animals without a germline [*glp-1(q224)* grown at 25 °C] [36]. *mpk-1b(RNAi)* specifically targets *mpk-1b* isoform. (C) Graph showing the percentage of animals with proximal germline tumors. All strains were grown at 25 °C. Germline tumors were determined by staining dissected gonads with DAPI. Standard deviation bars were calculated from at least three independent experiments. (D–G) Adult hermaphrodite germlines were extruded and stained with DAPI. (D) *puf-8; lip-1; vector(RNAi)*. (E) *puf-8; lip-1; mpk-1a/b(RNAi)*. (F) *puf-8; lip-1; mpk-1b(RNAi)*. (G) *puf-8; lip-1; mpk-1ts*. Arrowheads indicate distal end. Nomarski micrograph of (H) *puf-8; lip-1* and (I) *puf-8; lip-1; mpk-1ts* at 25 °C. Dashed lines indicate the boundary of gonad. Arrows indicate oocytes.

organization as seen in *mpk-1(ga117)* null mutants (not shown) [27]. The *fog-1(RNAi)* and *cpb-1(RNAi)* also significantly suppressed formation of *puf-8; lip-1* germline tumors (Fig. 2C), indicating that the proximal germline tumors arise after sperm fate specification and after the primary spermatocyte stage. Therefore, the tumors likely arise via dedifferentiation of secondary or later stage spermatocytes. To test this idea, we blocked the transition from secondary spermatocytes to spermatozoa using *ife-1(RNAi)*. In contrast to the earlier blocks, all *puf-8; lip-1; ife-1(RNAi)* animals generated proximal germline tumors (Fig. 2C). Therefore, the *puf-8; lip-1* germline tumors likely arise via dedifferentiation of secondary spermatocytes.

3.3. MPK-1/ERK MAPK signaling promotes dedifferentiation in the germline

We previous reported that manipulation of MPK-1/ERK MAPK signaling can reprogram germ cell fate in *puf-8; lip-1* mutants at 20 °C [18]. To test whether the process of dedifferentiation also depends on MPK-1/ERK MAPK signaling in the *C. elegans* germline, we depleted expression of core MPK-1/ERK MAPK signaling genes using RNAi in *puf-8; lip-1* mutant at 25 °C. The *mpk-1* gene encodes two major transcripts, *mpk-1a* and *mpk-1b*, which produce MPK-1A and MPK-1B proteins, respectively (Fig. 3A) [35]. *mpk-1a* mRNA accumulates predominantly in somatic cells and *mpk-1b* is expressed specifically in the germline [27,36]. We used Western blot analysis to assay depletion of these *mpk-1* isoforms (Fig. 3B). Depletion of both isoforms, henceforth called *mpk-1a/b(RNAi)*, caused a defect in pachytene exit (Pex) and massive disruption of membrane organization in *puf-8; lip-1* mutants at 25 °C (Fig. 3C,E and S2A). However, depletion of the germline-specific *mpk-1b* isoform using *mpk-1b(RNAi)* rescued *puf-8; lip-1* germline tumors (Fig. 3C,F and S2A). To test the possibility that the *mpk-1b* RNAi represents partial *mpk-1* suppression rather than *mpk-1b*-specific suppression, *puf-8; lip-1* mutants were placed on RNAi plates with serially diluted *mpk-1a/b(RNAi)* bacteria. However, weak *mpk-1a/b(RNAi)* failed to rescue *puf-8; lip-1* germline tumors. Next, to ask if MPK-1/ERK MAPK signaling acts within the germline to drive dedifferentiation, we performed germline-specific RNAi using an *rrf-1(pk1417)* mutant, which is largely defective for somatic but not germline RNAi [37]. Indeed, RNAi directed against either *mpk-1b* or *mpk-1a/b* dramatically suppressed generation of germline tumors in *puf-8; lip-1; rrf-1* triple mutants (Fig. S3) as in *puf-8; lip-1* double mutants (Fig. 3E and F). This suggests that MPK-1/ERK MAPK signaling is required in the germline tissue to promote the dedifferentiation in *puf-8; lip-1* mutants. To confirm the role of MPK-1 on dedifferentiation, we took advantage of an *mpk-1(ga111)* temperature-sensitive (ts) mutant, henceforth called *mpk-1(ts)*, that only has germline defects (Table S1) [35]. Consistent with RNAi results, the inactivation of MPK-1 in *mpk-1(ts)* mutants rescued the *puf-8; lip-1* germline tumor phenotype (Fig. 3C, G and Table S1). In addition, RNAi of either *lin-45* (a Raf homolog) or *let-60* (a Ras homolog) also dramatically suppressed *puf-8; lip-1* germline tumors (Fig. 3C and Fig. S2). Importantly, the sterility typical of *puf-8; lip-1* germline tumor mutants was also rescued by *lin-45(RNAi)* by 50% (Fig. S2A, S2B). Using the SP56 and RME2 markers, these *puf-8; lip-1; mpk-1(ts)* germlines made both sperm and oocytes. Moreover, whereas *puf-8; lip-1* animals were sterile with germline tumors (Fig. 3H), *puf-8; lip-1; mpk-1(ts)* animals were fertile with both sperm and oocytes (Fig. 3I). The importance of MPK-1/ERK activity in dedifferentiation was further confirmed using a small-molecule MEK inhibitor (U0126). U0126 inhibited *puf-8; lip-1* germline tumors sufficiently to render them fertile. Finally, we tested whether the aberrant activation of MPK-1/ERK MAPK signaling is sufficient to promote dedifferentiation using *let-60(n1046)* and *let-60(ga89)*, two gain-of-function (gf) mutants henceforth, called *let-60(gf)*, which have much higher MPK-1/ERK activity than wild-type [27]. Neither *let-60(gf)* mutants generated proximal germline tumors in males (XO) or hermaphrodites (XX) at either 20 °C or 25 °C (not shown, Fig. 3C). However, dedifferentiation was induced in 67% of the *let-60(gf)* mutants using RNAi against *puf-8*. To confirm this, we generated *puf-8(0); let-60(ga89gf)* double mutants and assayed their germlines by DAPI staining of dissected gonads. Remarkably, 92% of *puf-8; let-60(gf)* mutants had germline tumors (Fig. 3C). All together, we conclude that activation of MPK-1/ERK in the *puf-8* mutant is critical for dedifferentiation in the *C. elegans* germline.

3.4. Active MPK-1/ERK may control initiation of dedifferentiation, but not maintenance of proximal germline tumors

To investigate whether MPK-1/ERK is activated in *puf-8; lip-1* mutant germlines, we stained dissected gonads with MAPK (YT) monoclonal

antibody, which recognizes the dual-phosphorylated, active form of *C. elegans* MPK-1/ERK MAPK (Fig. 4) [27,36], and we quantitated levels with ImageJ software (Fig. S4D). In wild-type germlines, activated MPK-1/ERK was not detected in the distal mitotic region (Fig. S4A, B), but became abundant in the proximal pachytene region and in maturing oocytes (Fig. 4A–C), as seen before [27]. A similar distribution was seen in both *puf-8* and *lip-1* single mutants. In contrast, activated MPK-1/ERK was detected in the proximal part of the mitotic region and in the early meiotic region of *puf-8; lip-1* at 20 °C (Fig. 4D–F and S4C, D). Also in these *puf-8; lip-1* mutants at 25 °C, activated MPK-1/ERK was abundant in both nuclei and cytoplasm of proximal germ cells (Fig. 4G–I). Interestingly, after the dedifferentiated germ cells began their mitotic cell cycles, activated MPK-1/ERK was only present at a very low level (Yellow line in Fig. 4H). Based on this result, we hypothesized that MPK-1/ERK MAPK signaling may not be required for the maintenance of germline tumors once germ cells had dedifferentiated. To test this idea, *puf-8; lip-1* animals were grown at 25 °C on a normal NGM plate until adulthood (1 day after L4), and then transferred to either *vector(RNAi)*, *mpk-1b(RNAi)* or *mpk-1a/b(RNAi)* plates after verifying that their siblings all had proximal germline tumors (Fig. 5A). Remarkably, these germlines retained their proximal germline tumors, as assayed using DAPI staining (Fig. 5C–E). We confirmed loss of MPK-1 activity with the MAPK (YT) monoclonal antibody (Fig. 5F–H): 68% of *mpk-1b(RNAi)* and 89% of *mpk-1a/b(RNAi)* animals showed very weak or no activated MPK-1/ERK in their germlines. Therefore, depletion of either *mpk-1b* or *mpk-1a/b* was no longer able to suppress *puf-8; lip-1* germline tumors once established (Fig. 5B). We suggest that MPK-1/ERK activity initiates dedifferentiation and tumor formation, but that it is not required for the maintenance of the tumorous state. Which factors are required to maintain proximal germline tumors? We do not know but suggest that GLP-1/Notch signaling may be involved, because the GLP-1/Notch receptor was expressed in proximal germline tumors ([19], not shown).

3.5. *fbf-1* and *fbf-2* mutants enhance *puf-8* dedifferentiation

We reported previously that FBF-1 and FBF-2 bind the *mpk-1* 3′UTR and repress *mpk-1* expression in the distal germlines [36]. Moreover, MPK-1 is activated in the distal germline of *fbf-1; lip-1* double mutants as it is in *puf-8; lip-1* at 20 °C (not shown) [36]. To ask whether the aberrant activation of MPK-1 in an *fbf-1* and/or *fbf-2* background promotes dedifferentiation, we examined the germlines of (1) *fbf-1* and *fbf-2* single mutants, (2) *fbf-1 fbf-2*, *fbf-1; lip-1* and *fbf-2; lip-1* double mutants and (3) *fbf-1 fbf-2; lip-1* triple mutants, all grown at 25 °C and all assayed by staining with mitosis markers and DAPI. None of these strains showed any proximal germline tumors (Fig. 6A and Table S1), suggesting that MPK-1/ERK activation alone does not initiate dedifferentiation. We next tested whether *fbf* mutations enhance dedifferentiation of *puf-8* mutants at 25 °C. To this end, we examined dissected adult gonads of *puf-8 fbf-1* double mutants, *puf-8 fbf-2* double mutants, and *puf-8 fbf-1 fbf-2* triple mutants. Indeed, 71% of *puf-8 fbf-1* and 76% of *puf-8 fbf-2* animals had proximal germline tumors, which are likely to derive from dedifferentiation (Fig. 6A–C and Table S1). However no *puf-8 fbf-1 fbf-2* triple mutants had germline tumors (Fig. 6A, D and Table S1).

Why do *puf-8 fbf-1 fbf-2* triple mutants fail to develop proximal germline tumors? We do not know, but suggest two possibilities. Perhaps dedifferentiation must be programmed at a stage of germline development lacking in *fbf-1 fbf-2* mutants (e.g. adult GSCs) [17]. Alternatively dedifferentiation may be repressed by the aberrant de-repression of meiosis-promoting genes in *fbf-1 fbf-2* mutants (e.g., *gld-1* [9]). Distinguishing between these possibilities is beyond the scope of this work but will be an important challenge for the future.

The three strains that induced dedifferentiation at 25 °C (*puf-8; lip-1*, *puf-8 fbf-1*, and *puf-8 fbf-2*) have no proximal germline tumors at 20 °C, but instead produce excess sperm and no oocytes, the Mog phenotype (Table S1) [17]. We therefore tested whether a different mutant driving excess sperm production might enhance dedifferentiation when placed in the *puf-8* mutant background. To this end, we used a *fem-3(q20)* gain-of-function (gf) mutant, henceforth called *fem-3(gf)*, which makes sperm continuously as an adult and fails to switch into oogenesis at both 20 °C and 25 °C (Fig. 6E, F and Table S1) [16]. We first treated *fem-3(gf)* with *puf-8(RNAi)*, but generated no germline tumors in the *fem-3(gf); puf-8(RNAi)* animals (Fig. 6E and Table S1). To confirm this result, we scored both *puf-8(q725); fem-3(gf)* and *puf-8(ok302); fem-3(gf)* double mutants for germline tumors at 25 °C. Consistent

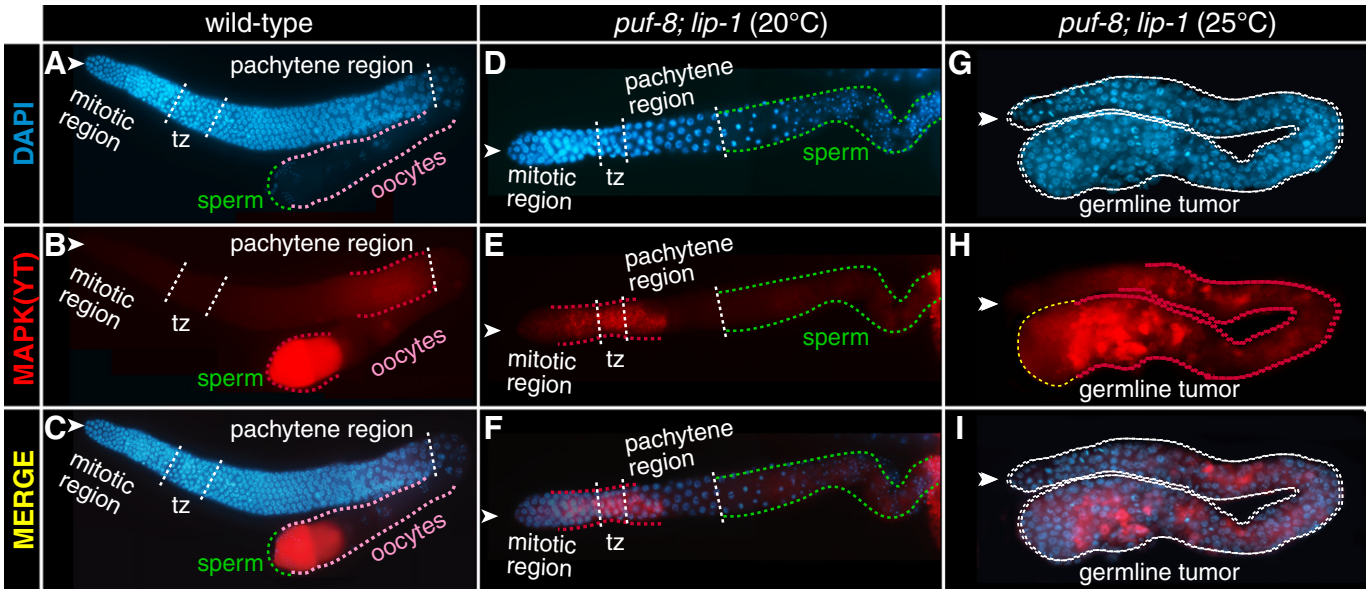
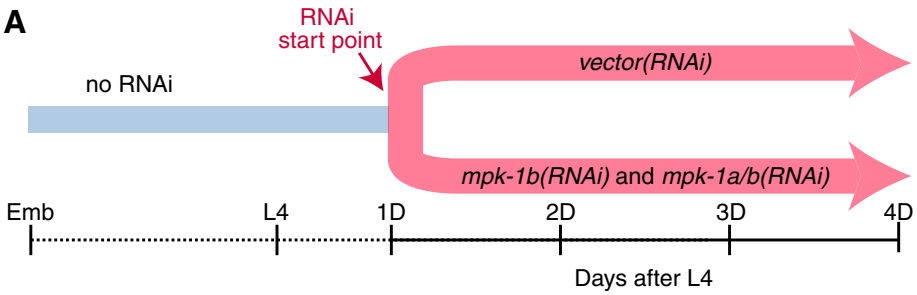


Fig. 4. Localization of activated MPK-1/ERK in *puf-8; lip-1* germlines at 20 °C and 25 °C. Dissected adult gonads stained with DAPI (A,D,G), anti-DP-MAPK (YT) antibody (B,E,H), and merge images (C,F,I). Wild-type (A–C). *puf-8; lip-1* grown at either 20 °C (D–F) or 25 °C (G–I). Activated MPK-1/ERK (dashed red lines) was detected in the proximal pachytene region and oocytes of the wild-type germline (B); it is found in the proximal part of the mitotic region and extend into the early pachytene region of *puf-8; lip-1* at 20 °C (E); it is found throughout the *puf-8; lip-1* germline at 25 °C but decreases most proximally (yellow line) (H). Dashed lines mark the boundaries of mitotic region, transition zone (tz), pachytene region, and gametogenesis (white in A–F) as well as sperm (green), oocytes (pink), and outline of gonad (white in G, I).



B

Strains \ Days after L4	% Germline Tumors (n) 1D	2D	3D	4D
puf-8; lip-1	100% (32)	nd	nd	nd
puf-8; lip-1; vector(RNAi)	nd	100% (10)	100% (26)	100% (10)
puf-8; lip-1; mpk-1b(RNAi)	nd	100% (12)	100% (14)	100% (9)
puf-8; lip-1; mpk-1a/b(RNAi)	nd	100% (9)	100% (37)	100% (7)

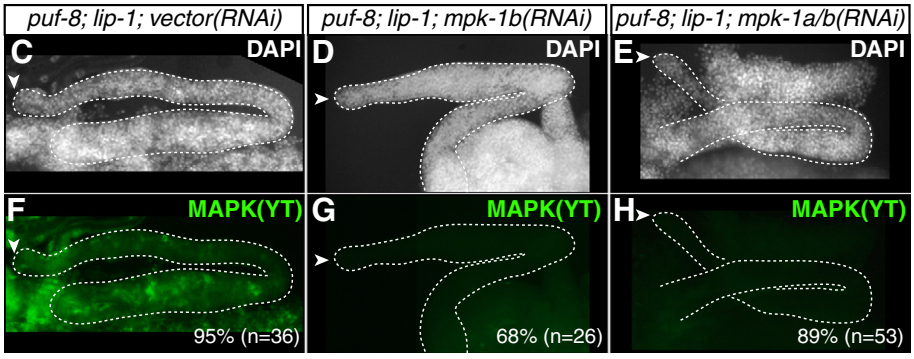


Fig. 5. MPK-1/ERK activation is required for initiation of dedifferentiation, but probably not for maintenance of proximal germline tumors. (A) Experimental design to test the requirement of MPK-1/ERK in maintenance of proximal germline tumors. *puf-8; lip-1* embryos were placed on normal NGM (Nematode Growth Media) plates with OP50 bacteria and grown to adulthood at 25 °C. Adults that were one day after L4 were transferred to plates seeded with bacteria carrying the RNAi vector or plasmids directing RNAi against either *mpk-1b*, or *mpk-1a/b*. Germline phenotypes were determined by DAPI staining of dissected gonads on consecutive days (1–4) after the L4 stage. (B) Percentage of animals with proximal germline tumors. All data were obtained from three independent experiments. (C–H) Dissected adult (3 days after L4) gonads stained with anti-MAPK(YT) (green) and DAPI (white). (C,F) Dissected *puf-8; lip-1; vector(RNAi)* gonad. (D,G) *puf-8; lip-1; mpk-1b(RNAi)* gonad. (E,H) *puf-8; lip-1; mpk-1a/b(RNAi)* gonad. 95% of *puf-8; lip-1; vector(RNAi)* germlines expressed activated MPK-1/ERK, but 68% of *puf-8; lip-1; mpk-1b(RNAi)* (n = 28) and 89% of *puf-8; lip-1; mpk-1a/b(RNAi)* (n = 53) germlines did not express activated MPK-1/ERK. Arrows indicate the distal end of the gonad arm.

with the RNAi results, only a low percentage of the double mutants produced germline tumors, which was also typical of *puf-8* single mutants (Fig. 6E, G and Table S1). Therefore, excess sperm does not necessarily lead to dedifferentiation. We conclude that PUF-8 and the two FBFs work together to inhibit germline dedifferentiation.

3.6. RSKN-1/P90$_{RSK}$, a downstream effector of MPK-1/ERK, is critical for germline dedifferentiation

Which ERK/MAPK targets are required for dedifferentiation in *puf-8; lip-1* germline? Recently, Schedl and colleagues identified about 30 conserved ERK/MAPK substrates by an integrated bioinformatics, genetic, and biochemical analysis [38]. Based on this report and our ideas of other possible substrates, we conducted an RNAi-based genetic screen to identify candidate genes that promote dedifferentiation in the *puf-8; lip-1* germline at 25 °C (Fig. S5). 26 out of 28 genes affected *puf-8; lip-1* germline tumors at 25 °C (Fig. S5). Interestingly, three genes (*rskn-1*, *ttbk-2*, and *toe-3*) significantly suppressed the *puf-8; lip-1* germline tumor and reverted it to a Mog (excess sperm) germline with no dramatic proliferation defects (Fig. 7A and S5B, 5G). These three genes therefore likely promote dedifferentiation in the *puf-8; lip-1* germlines at 25 °C.

Among them, we focus on *rskn-1* for two reasons: vertebrate P90$_{RSK}$ (P90 Ribosomal S6 Kinase) has been identified as a direct ERK substrate and it also appears to be an effector of ERK-induced transition through meiotic cell division [39,40]. We therefore investigated the effect of RSKN-1 on nematode meiotic cell divisions by staining dissected gonads with an anti-α-tubulin antibody and DAPI. In wild-type L4 hermaphrodite and adult male germlines, primary and secondary spermatocytes stain for α-tubulin during meiotic cell divisions (not shown). By contrast, the vast majority (96%) of spermatocytes in *puf-8; lip-1* mutants grown at 25 °C displayed no α-tubulin staining (Fig. 7C, F, I and K), although mitotic germ cells still stained for α-tubulin (not shown). To test whether RSKN-1 might be required for meiotic cell divisions and microtubule organization, we used RNAi to deplete *rskn-1* from *puf-8; lip-1* mutants, starting at the L1 stage, and stained their adult germlines with the α-tubulin antibody. Remarkably, *rskn-1(RNAi)* in *puf-8; lip-1* at 25 °C restored α-tubulin staining and allowed meiotic divisions (Fig. 7D, G, J and K); the staining and divisions were comparable to those in wild-type males (not shown) and *puf-8; lip-1* Mog germlines at 20 °C (Fig. 7B, E, H and K). From this result, we suggest that the aberrant RSKN-1 activation by MPK-1/ERK in *puf-8; lip-1* mutants prevents meiotic divisions and promotes germ cell dedifferentiation. Consistent with that idea, mammalian P90$_{RSK}$ activation by ERK/MAPK affects meiotic cell cycle progression and disturbs microtubule organization in mouse oocytes [41].

3.7. Conclusion

In this report, we demonstrate that PUF RNA-binding proteins (PUF-8 and FBFs) and Ras-ERK MAPK signaling regulates dedifferentiation in the

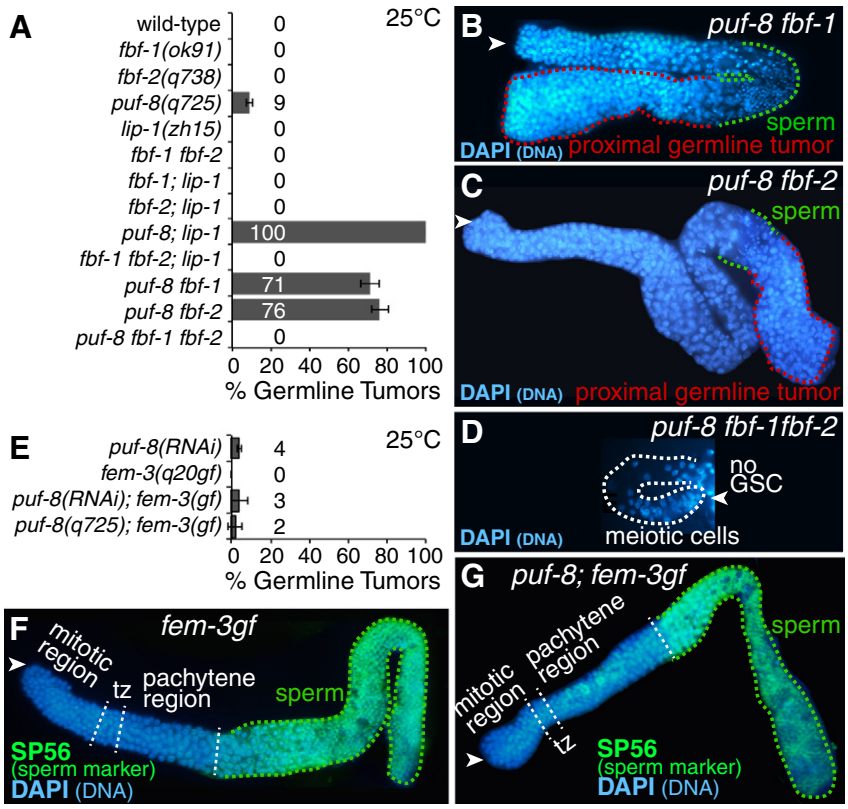


Fig. 6. Redundant control of dedifferentiation in *C. elegans* germline. (A,E) Graph showing the percentage of animals with proximal germline tumors. Standard deviation bars were calculated from at least three independent experiments. All animals were grown at 25 °C. Germline phenotypes were determined by cellular morphology and DAPI staining of dissected gonads. (B) *puf-8 fbf-1* germline. (C) *puf-8 fbf-2* germline. (D) *puf-8 fbf-1 fbf-2* germline. Dashed lines mark sperm (green) and proximal germline tumors via dedifferentiation (red) in B and C. *puf-8 fbf-1 fbf-2* germline has only a few meiotic cells (dashed white line in *D*) and does not maintain GSCs [17]. (F,G) Adult hermaphrodite germlines were extruded and co-stained with SP56 (sperm-specific marker; green) and DAPI (DNA; blue). (F) *fem-3(q20gf)* and (G) *puf-8(q720); fem-3(q20gf)* germlines at 25 °C. Arrows indicate the distal end of the gonad arm.

C. elegans germline (Fig. 8). Specifically, the combination of *puf-8* loss and MPK-1/ERK activation by the removal of negative regulators (e.g., *fbf-1*, *fbf-2*, or *lip-1*) induces germ cell dedifferentiation in spermatocytes, probably as a result of abnormal meiotic cell divisions (Figs. 3, 6, and 8). Importantly, RSKN-1/P90$_{RSK}$, a putative MPK-1/ERK target [38], functions as a key regulator for dedifferentiation in the *puf-8; lip-1* germlines (Figs. 7 and 8).

We previously reported that the LIP-1 dual specificity phosphatase normally promotes germline mitoses by inhibiting MPK-1/ERK MAPK signaling [28,36]. In addition, Ariz and colleagues reported that PUF-8 functions redundantly with MEX-3 to promote GSC mitoses [15]. Here we show that PUF-8 and LIP-1 proteins also promote meiotic cell divisions, in this case by inhibiting MPK-1/ERK MAPK signaling after GSC differentiation. Importantly, their roles in mitotic germ cells appear to be coupled to differentiation, whereas their role in meiotic divisions is coupled to dedifferentiation. How do we explain these opposite effects? This is not the first case of opposite effects: *C. elegans* FOG-3 (Tob/BTG ortholog) can either promote or inhibit germline proliferation, depending on gene dosage and genetic context [42]. Similarly, the various effects of MPK-1/ERK signaling depend on genetic and cellular context. For example, MPK-1/ERK MAPK signaling promotes differentiation in mitotic region [28], meiotic progression in pachytene region [27], oocyte maturation during oogenesis [27]. In addition, it promotes sperm fate specification in male and *puf-8; lip-1* Mog germlines at 20 °C [18,27], and dedifferentiation in *puf-8; lip-1* proximal germline at 25 °C (this work). In mammals, activated ERK2 MAPK signaling promotes differentiation of embryonic stem cells [43], but in differentiated cells such as Sertoli cells [21], myoblasts [22], and islet cells [23], activated ERK2 MAPK signaling promotes dedifferentiation and proliferation [44,45]. Therefore, our findings have striking parallels in vertebrates. *C. elegans* provides a powerful model for analysis of molecular mechanisms controlling cellular dedifferentiation *in vivo*. These findings may also have implications for regenerative medicine and cancer therapy in humans, since all regulators studied here are highly conserved in all eukaryotes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.07.006.

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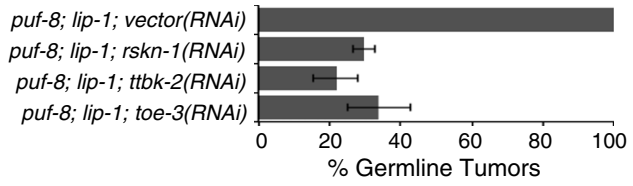
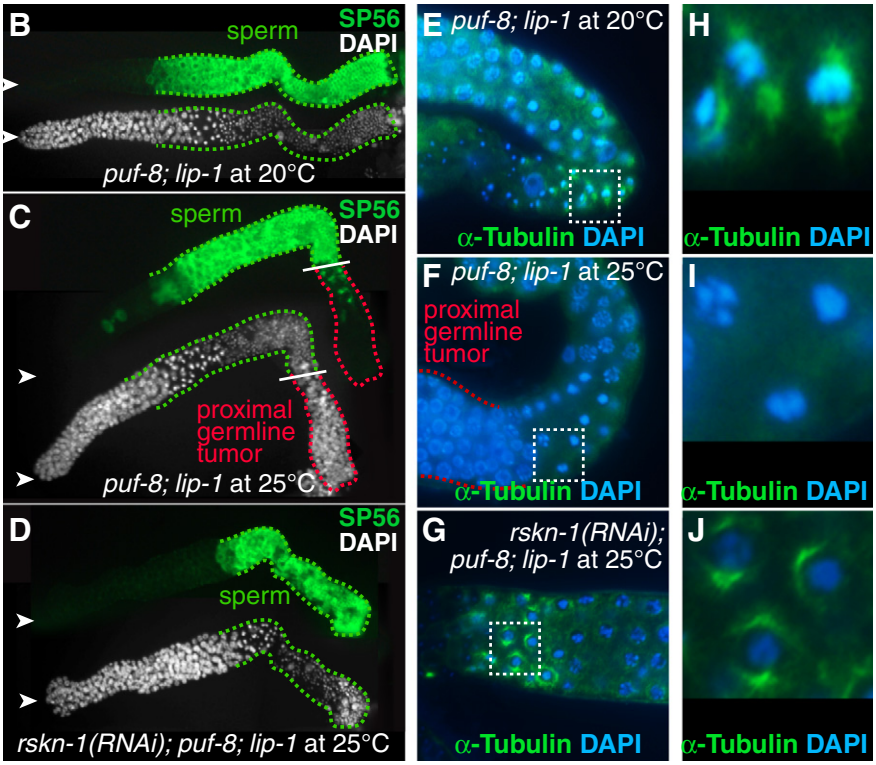
A

Gene	Human orthologs	Molecular Identity
rskn-1	RSK4	Ribosomal protein S6 kinase
ttbk-2	TTBK2	Ser-Thr Kinase/ Casein Kinase
toe-3	USP46	Ubiquitin Protease





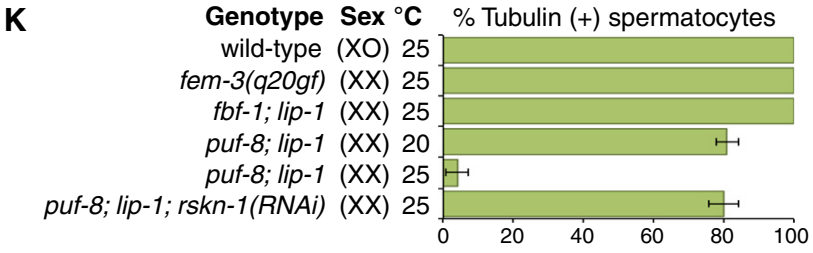


Fig. 7. RSKN-1/P90$_{RSK}$ as a promoter of *puf-8; lip-1* dedifferentiation. (A) Summary of regulators and the percentage of animals with proximal germline tumors. Standard deviation bars were calculated from at least three independent experiments. (B–D) Upper image, dissected adult gonads were stained with SP56 (sperm-specific marker; green); Lower image, same germline stained with DAPI (white). Arrows indicate the distal end of the gonad arm. (E–G) Dissected adult gonads were stained with α-tubulin (green) and DAPI (Blue). (H–J) Magnified pictures of inset in E–G. Dashed lines mark sperm (green) and proximal dedifferentiated germ cells (red). Genotypes are labeled in the figure. (K) Graph showing the percentage of α-tubulin-positive (+) spermatocytes. Standard deviation bars were calculated from at least three independent experiments.

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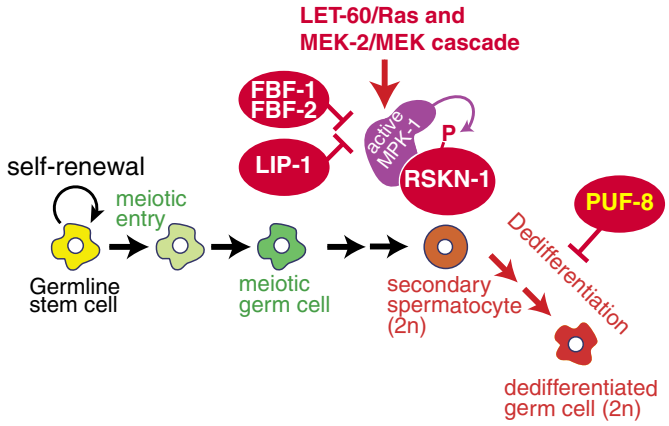

Fig. 8. Model for molecular control of dedifferentiation. In a Mog germline, the germline stem cells (GSCs) enter into the meiotic cell cycle to produce sperm. Activation of MPK-1/ERK in *puf-8* mutant background at 25 °C results in abnormal meiotic cell division of spermatocytes and promotes the initiation of germ cell dedifferentiation, probably through RSKN-1 phosphorylation.

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