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Article

The *C. elegans* Glycopeptide Hormone Receptor Ortholog, FSHR-1, Regulates Germline Differentiation and Survival

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Summary

Background: The mammalian glycopeptide hormone receptors (GPHRs) are key regulators of reproductive development, and their homologs are widely distributed throughout the animal kingdom. The *C. elegans* genome encodes a single GPHR family member, FSHR-1, which shares equal identity to the FSH, LH, and TSH receptors from mammals.
Results: Because loss of *fshr-1* function does not produce a visible phenotype in *C. elegans*, we conducted a genome-wide RNAi-feeding screen to identify genes that perform functions that overlap with those of *fshr-1*. This approach led to the identification of the PUF family members *fbf-1* and *fbf-2* (the *fbfs*). Whereas a weak reduction in *fbf* activity caused little or no discernable effect in the wild-type, an equivalent loss in the *fshr-1(0)* mutant background resulted in a highly penetrant germline-masculinization phenotype. Furthermore, many *fshr-1(0);fbf(RNAi)* animals failed to maintain a germline stem cell niche. We also show that *fshr-1* and the *fbfs* promote germline survival and prevent apoptosis with *fog-1* and *fog-3* and that simultaneous loss of *fshr-1* and the *fbfs* can override the canonical requirement for *fog-1* and *fog-3* in the execution of the male-germline fate. Finally, we provide evidence that FSHR-1 controls germline processes nonautonomously via the soma and that FSHR-1 acts through a canonical signaling pathway involving $G\alpha_s$ and adenyl cyclase.
Conclusions: Our results indicate a conserved role for GPHR family receptors in controlling germline development and fertility. Our data suggest a model whereby FSHR-1 signaling acts in parallel to the known sex-determination pathway to control multiple aspects of germline development.

Introduction

The mammalian glycopeptide hormone receptors are members of a conserved subfamily of rhodopsin-like G protein-coupled receptors [1–4]. These include the receptors for the gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), as well as the thyroid regulator thyrotropin (TSH). Signaling through these receptors is crucial for ensuring proper development, fertility, and metabolic health in mammals. In particular, extensive work has demonstrated that signaling through the FSH and LH receptors is critical for normal sexual development and for the proper maturation and differentiation of both oocytes and sperm [5, 6]. Despite the presence of structural orthologs for these receptors throughout the animal kingdom, to date little or no analysis has been carried out to determine the functions of these receptors in model organisms other than the mouse.

The genome of *C. elegans* encodes a single glycopeptide hormone-like receptor, FSHR-1. FSHR-1 is approximately 30% identical and 50% similar to the FSH, LH, and TSH receptors from humans (Figures S1 and S2 in the Supplemental Data available online) [7]. Somewhat surprisingly, strains carrying a homozygous-null deletion in *fshr-1* (*ok778*) are viable and fail to display an overt phenotype (this work). As described below, we have genetically probed for the functions of *fshr-1* in *C. elegans* through the use of a genome-wide RNAi screen to uncover genes that carry out functions that overlap with those of *fshr-1*. This approach has led to the finding that *fshr-1* coordinately regulates several germline processes, including the specification of germline sex-specific differentiation, in conjunction with other genes.

In *C. elegans*, sexual identity is regulated by a hierarchical pathway that is ultimately responsive to the ratio of the sex chromosome (X) to the autosomes; males contain a single X chromosome (XO), whereas hermaphrodites contain two X chromosomes (XX) [8, 9]. Mutations in this pathway generally result in animals that are either masculinized or feminized. Masculinized animals produce excess sperm at the expense of oocytes, and feminized animals produce oocytes at the expense of sperm. Mutations in certain components of the sex-determination pathway lead to transformations that affect both somatic and germline sex-specific cells [10]. In contrast, other pathway components function exclusively in germline sex control [8]. In wild-type hermaphrodites, the dual sexual nature of the germline is accomplished through a temporal separation of germline fates. During late larval development, the hermaphrodite germline assumes a male fate, during which time sperm are produced. This is followed by a switch to the female fate at around the time of the adult molt, after which hermaphrodites exclusively produce oocytes.

In this work, we describe a role for FSHR-1 in promoting oocyte development in conjunction with the PUF family proteins FBF-1 and FBF-2 (referred to as the FBFs) [11]. PUF proteins bind to the 3′-untranslated regions (UTRs) of their target mRNAs to mediate transcriptional degradation or translational inhibition [12]. The FBFs have been implicated as negative regulators of a number of distinct targets within the *C. elegans* sex-determination pathway; such targets include *fem-3* [11], *gld-1* [13], and *fog-1* and *fog-3* [14]. In addition, the FBFs mutually inhibit one another's expression and are likely to have autoregulatory activities [15]. Whereas *fbf-1* and *fbf-2* single-mutant hermaphrodites are largely

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aphenotypic, double mutants are sterile and exhibit a germline-masculinization phenotype [11, 13, 15]. In addition, *fbf* double mutants are defective at maintaining a germline stem cell population beyond the L4 stage, leading to a diminutive and nonproliferative adult germline [13, 15].

Our studies have uncovered an apparent functional redundancy between the FBFs, which act exclusively in the germline [15, 16], and FSHR-1, which functions through the soma. In addition to mutually controlling germline sexual fates, FSHR-1 and the FBFs function to inhibit germline apoptosis and promote the survival of germ cells. We also demonstrate that the simultaneous loss of *fshr-1* and the *fbfs* can to a large extent override the canonical requirement for *fog-1* and *fog-3* in the expression of the male-germline fate.

Results

FSHR-1 and the FBFs Redundantly Promote Oogenesis and Germline Proliferation

Using a genome-wide RNAi-feeding screen to uncover genes that carry out functions that overlap with those of *fshr-1*, we identified 114 clones that produced little or no effect on the development or growth of the wild-type but that generated strong synthetic phenotypes in an *fshr-1*(*ok778*)-null-deletion mutant [*fshr-1(0)*] background (S.C. and D.S.F, unpublished data). Among these were 14 clones that led to fertility defects suggesting that *fshr-1* may control one or more aspects of germline development or physiology (Table S1). One of the identified clones directly targeted the *C. elegans* PUF family member *fbf-1* [11, 17] and is the focus of this study. Because *fbf-1* is 93% identical at the nucleotide sequence level to its close paralog *fbf-2* [11], this clone is expected to efficiently target both genes [11, 18, 19] and is hereafter referred to as *fbf(RNAi)* (also see below). Whereas *fbf(RNAi)* feeding produced minimal effects in the N2 (wild-type) background, similar to the findings of previous reports [17], this treatment led to a highly penetrant germline-masculinization phenotype in the *fshr-1(0)* mutant background (Table 1). These effects were strongly suppressed in transgenic *fshr-1(0)* mutants carrying wild-type-rescuing copies of *fshr-1* via an extrachromosomal array (*fdEx31*), indicating that the genetic interaction with the *fbfs* is specific to the *fshr-1(0)* deletion and is not due to a separate mutation in the background (Table 1 and Figure S3). Furthermore, the effect of *fbf(RNAi)* on *fshr-1(0)* mutants was far greater then that observed for several bona fide RNAi-hypersensitive mutants (Table 1) [20, 21], and results from our genome-wide screen demonstrate that *fshr-1(0)* mutants are not constitutively RNAi hypersensitive (data not shown).

The induction of germline masculinization in *fshr-1(0);fbf(RNAi)* hermaphrodites took several distinct forms (Figure 1). In some of the affected animals, oocytes were still present although distally displaced, whereas in others only sperm were detectable. In common among all masculinized animals was the substantial overproduction of sperm at the expense of oocytes. In many independent experiments, we found that both the penetrance and spectrum of germline-masculinization phenotypes could be modulated if the potency of the *fbf(RNAi)* feeding plates was carefully controlled (Table S2; also see Supplemental Experimental Procedures). Thus, for the experiments described below, strains were processed in parallel on identically prepared *fbf(RNAi)* plates of uniform strengths. Regardless of *fbf(RNAi)* potencies, however, *fshr-1(0)* mutants always displayed a dramatic enhancement of the induced masculinization phenotypes in comparison with the wild-type (Table 1 and Table S2). In no cases were effects on somatic cells observed, indicating that the masculinization phenotype of *fshr-1(0);fbf(RNAi)* animals is confined to the germline.

Table 1. Genetic Interactions of *fshr-1* and the *fbfs*

Genotype	Percent Masculinized (n)
N2	0 (250)
fshr-1(ok778)[a]	0 (242)
fbf(RNAi)[b]	4 (356)
fshr-1(ok778); fbf(RNAi)[b]	96 (333)
fshr-1(ok778); fbf(RNAi); fdEx31[c]	5 (130)
rrf-3;fbf(RNAi)[d]	19 (124)
lin-35;fbf(RNAi)[d]	12 (116)
fbf-1(ok224)	1 (182)
fbf-2(q738)	0 (187)
fbf-1(ok224); fshr-1(ok778)	0 (74)
fbf-2(q738);fshr-1(ok778)	0 (136)
fbf-1(ok91);fbf-2(q738)	100 (89)
fshr-1(ok778);fbf-1(ok91); fbf-2(q738)	100 (62)

[a] Identical results were obtained for *fshr-1* mutants grown on vector-only control (pPD129.36) RNAi-feeding plates (n = 104).
[b] Percentages were derived from the averages of three independent experiments.
[c] In parallel experiments, 87% of sibling array-minus segregants (n = 106) and 95% of *fshr-1* mutants (n = 104) were masculinized.
[d] In parallel experiments, 2% of N2 (n = 126) and 98% of *fshr-1* mutants (n = 102) were masculinized.

Given that *fbf(RNAi)* is predicted to target both *fbf* family members, it was possible that the synthetic interaction with *fshr-1* was specific to either *fbf-1* or *fbf-2* or required both family members. To test this, we constructed double mutants with *fshr-1* and deletion alleles of the individual *fbf* genes. In contrast to *fshr-1(0);fbf(RNAi)* animals, most or all double mutants were fertile and showed no evidence of germline masculinization, demonstrating that the observed synthetic interaction requires the simultaneous inhibition of all three genes (Table 1). From these data, we conclude that *fshr-1* most likely functions redundantly with the *fbfs* either to promote oogenesis directly or to inhibit spermatogenesis.

As described in the Introduction, the FBFs redundantly both regulate germline sex and promote maintenance of the germline stem cell niche [11, 13, 15]. Consistent with this, we observed that a substantial percentage of *fshr-1(0);fbf(RNAi)* animals displayed pronounced defects in germline proliferation (the Glp phenotype) and failed to maintain robust populations of undifferentiated mitotic germ cells as adults (Figure 1E). For example, of the 381 *fshr-1(0);fbf(RNAi)* masculinized adults observed to contain sperm only (from five separate experiments), 98 (26%) were strongly Glp. In contrast, Glp animals were never observed in corresponding *fbf(RNAi)* experiments carried out in the wild-type background, including in 111 sperm-only masculinized animals. Taken together, these results indicate that *fshr-1* acts redundantly with the *fbfs* to control both germline sexual fate

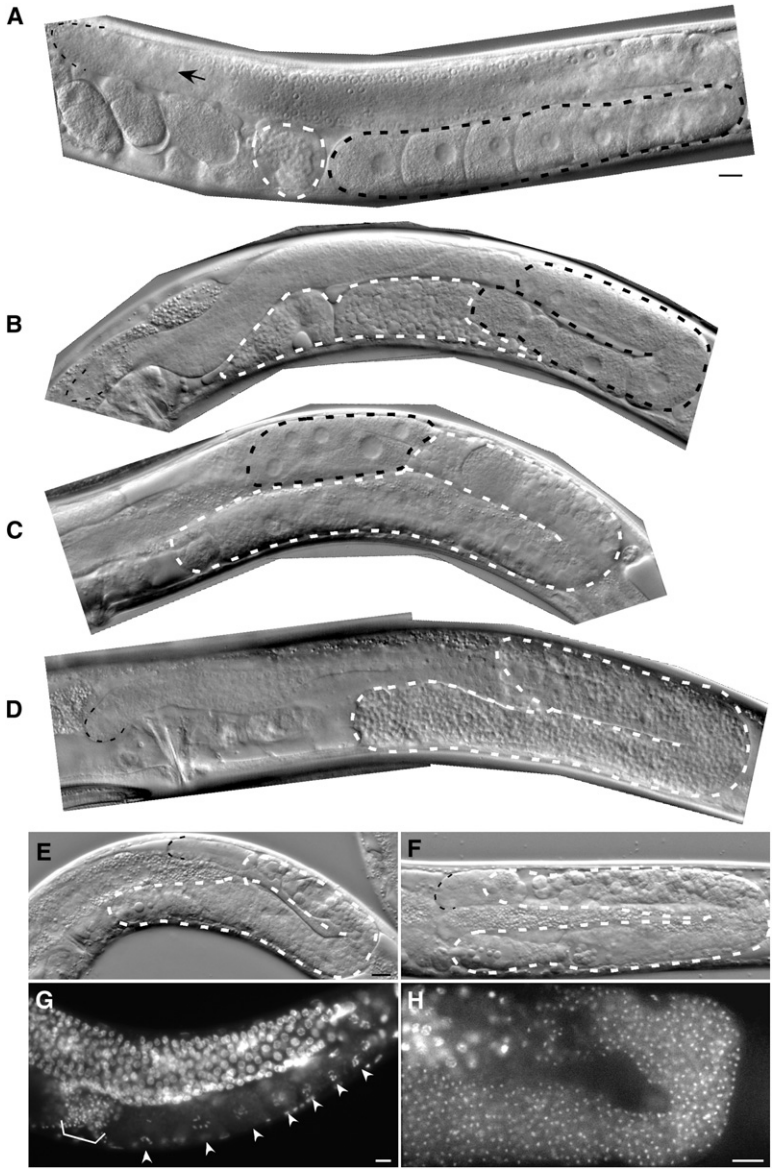


Figure 1. *fshr-1; fbf(RNAi)* Germline Phenotypes

(A–F) DIC images of (A) wild-type, (B–E) *fshr-1(0);fbf(RNAi)*, and (F) *fbf-1(0);fbf-2(0);fshr-1(0)* adult hermaphrodites. The regions containing sperm and oocytes are outlined in thick white and black dashed lines, respectively. Thin dashed lines indicate the distal terminus of the germline (out of focal plane in panel [C]). The black arrow in (A) indicates the approximate proximal border of the germline stem cell niche. (B–E) shows the range of masculinized phenotypes observed in *fshr-1(0);fbf(RNAi)* animals. Also note the strong reduction in germline size and the virtual absence of nondifferentiated germ-cell nuclei in (E) and (F).

(G and H) DAPI-stained images of (G) wild-type and (H) *fshr-1(0);fbf(RNAi)* adult hermaphrodites. The white bracket in G indicates the location of the spermatheca, which contains sperm; white arrowheads indicate oocytes in the diakinesis stage of meiotic prophase I. Note the absence of oocytes and the increased number of sperm nuclei in *fshr-1(0); fbf(RNAi)* mutants.

The scale bar in (A) represents 10 μm for (A–D); that in (E) represents 10 μm for (E) and (F); and those in (G) and (H) each represent 10 μm.

and maintenance of germline stem cell populations. We also note that inactivation of *fshr-1* via either the deletion mutation or RNAi in the *fbf-1(0);fbf-2(0)* double-mutant deletion background failed to exacerbate the Glp phenotype of *fbf-1(0);fbf-2(0)* double mutants further (Figure 1F and data not shown). This suggests that similar to the *fbfs*, *fshr-1* most likely promotes germline proliferation during late larval development and adulthood [13].

FSHR-1 and the FBFs Promote Germ-Cell Survival with FOG-1 and FOG-3

To gain insight into how FSHR-1 may integrate its functions with the known sex-determination pathway in *C. elegans*, we carried out genetic epistasis analyses. Two genes, *fog-1* and *fog-3*, have been previously shown to act at the downstream terminus of the known *C. elegans* germline sex-determination pathway [8]. Mutations in *fog-1* and *fog-3* result in a highly penetrant feminization phenotype, whereby hermaphrodites and males produce oocytes at the expense of sperm [22, 23]. *fog-1* encodes a cytoplasmic polyadenylation-element-binding (CPEB) protein [24, 25], whereas *fog-3* encodes a Tob family member [26]. *fog-1* and *fog-3* are both individually epistatic to *fbf-1(0) fbf-2(0)* double mutants (i.e., triple mutants are feminized) [14]. Consistent with this, we found that *fog-1(0);fbf(RNAi)*, *fog-1(0);fbf-1(0) fbf-2(0)*, and *fog-3;fbf(RNAi)* animals were strongly feminized, as were *fog-1(0);fshr-1(0)* and *fog-3;fshr-1(0)* double mutants (Figure 2 and Table S3). Interestingly, animals that were compromised for *fshr-1*, the *fbfs*, and either *fog-1* or *fog-3* (e.g., *fog-1(0);fshr-1(0) fbf(RNAi)*, *fog-1(0);fbf-1(0) fbf-2(0);fshr-1(0)*, and *fog-3;fshr-1(0);fbf(RNAi)*; referred to as compound mutants) displayed a germline phenotype that was unexpected and categorically distinct from all other mutant combinations analyzed. Specifically, compound mutant adult hermaphrodites contained germlines that were of ambiguous sexual identity, and these germlines uniformly underwent a precipitous degeneration beginning in early adulthood (Figures 2A and 2B; Table S3).

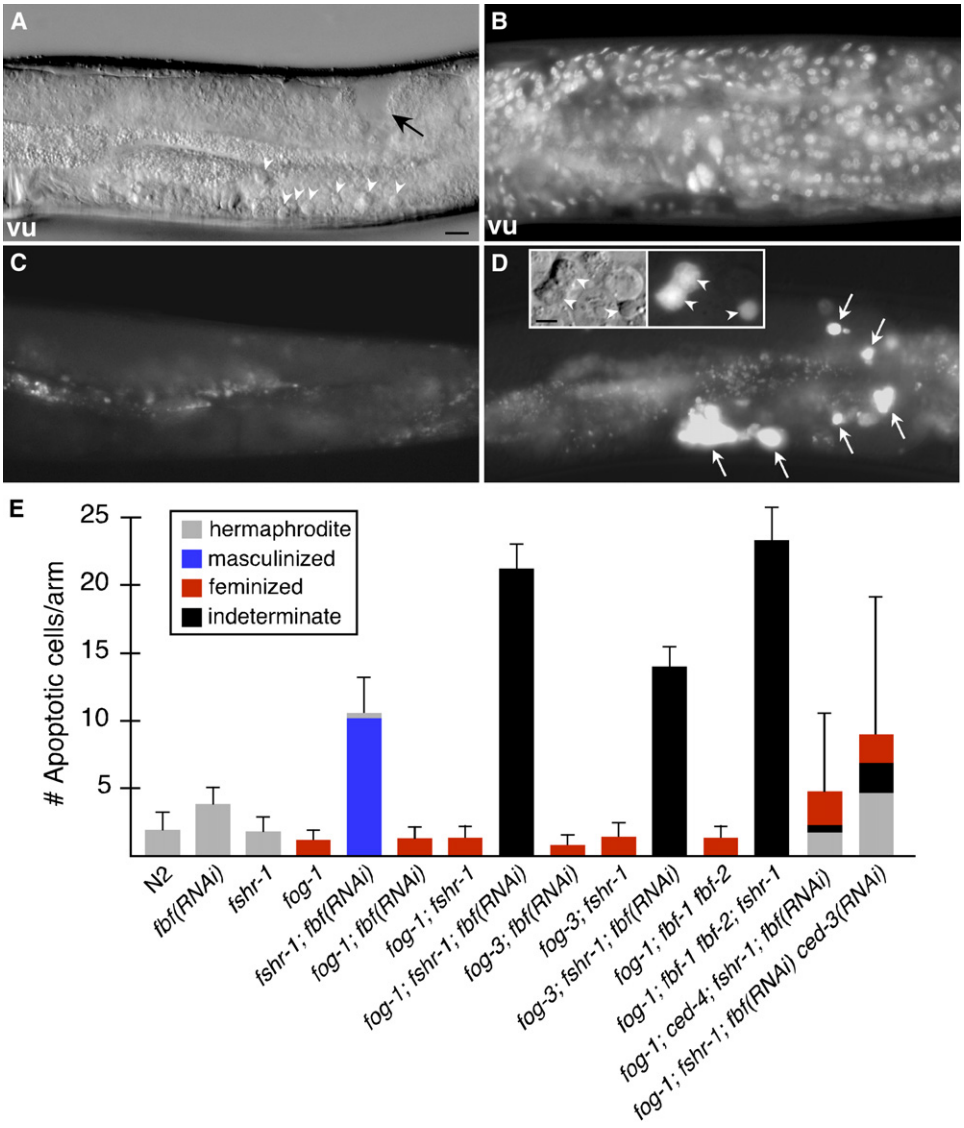


Figure 2. Synthetic Induction of Germline Apoptosis in Compound Mutants

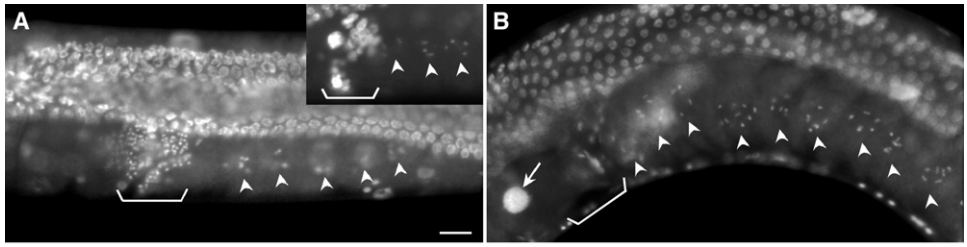
(A and B) DIC (A) and DAPI-stained (B) images of *fshr-1(0);fog-1(ts);fbf-1(RNAi)* adult hermaphrodites. The location of the vulva (vu) is indicated. In panel (A), white arrowheads indicate the position of germ cells with apoptotic-like morphologies; the black arrow indicates a region of germline degeneration.

(C and D) Staining with acridine orange marks apoptotic germline nuclei in *fog-1(q253ts);fbf(RNAi)* (C) and *fog-1(q253ts);fshr-1;fbf-1(RNAi)* (D) adults. Note the dramatic increase in the number of apoptotic nuclei in (D) versus (C). Panel (D) inset, comparative DIC (left) and acridine-orange staining (right) of germ cells undergoing apoptosis in a *fog-3;fshr-1(0);fbf(RNAi)* adult.

(E) Bar graph providing a quantitative comparison (based on acridine-orange staining) of germline apoptosis in relevant single- and compound-mutant backgrounds. A minimum number of 22 gonad arms was scored per strain. Bars are color coded based on germline sexual identities. Standard deviations for each strain were calculated with a t test. Results are shown for experiments with the *fshr-1(ok778)*, *fog-1(q253ts)*, *fog-3(q470)*, and *ced-4(n1162)* alleles. Similar results were also obtained with the null *fog-1(q241)* allele (also see Table S3). The scale bar in (A) represents 10 μm for (A)–(D); that in the (D) inset represents 5 μm.

The DIC and DAPI morphologies of germ cells in the compound mutants suggested that these germlines might be undergoing excessive apoptosis (Figures 2A and 2B). To test this possibility, we stained adult compound mutants and control animals with acridine orange, a vital dye that is highly specific to apoptotic cells [27–30]. Whereas most control strains tested (including the wild-type) exhibited relatively low levels of staining, compound mutants showed pronounced staining throughout the germline, indicating that these germlines are highly apoptotic (Figures 2C–2E). For example, whereas wild-type animals contained an average of 1.9 ± 1.3 (n = 31) positively staining cells per gonad arm, *fog-1(0);fbf-1(0) fbf-2(0);fshr-1(0)* compound mutants contained 22.9 ± 3.0 (n = 23). Intermediate levels of staining were also observed in *fshr-1(0);fbf(RNAi)* double mutants (Figure 2E; 10.5 ± 2.8; n = 30). The observed acridine-orange staining was also found to correlate strongly with apoptotic-germ-cell morphologies based on DIC analysis (Figure 2D, inset).

As a further test, we sought to reverse apoptosis in compound mutant germlines through direct inhibition



C

Genotype (XX)	RNAi injection	% Ind.	% ♀	% ♂	Total (n)
fog-1(0); fshr-1; fbf(RNAi)	No	100	0	0	154
	ced-3	43	19	38	142
	lin-15a	98	2	0	42
fog-1(0); fbf-1(0) fbf-2(0); fshr-1	No	92	0	8	48
	ced-3	40	4	56	50
fog-1(ts); fshr-1; fbf(RNAi)	No	98	2	0	146
	ced-3	24	22	54	83
	ced-4	29	21	50	70
fog-1(ts); ced-4; fshr-1	No	0	100	0	50
fog-1(ts); ced-4; fshr-1; fbf(RNAi)	No	8	56	36	72
fog-3; fshr-1; fbf(RNAi)	No	100	0	0	31
	ced-3	36	26	38	38

Figure 3. Compound Mutants Rescued from Germline Apoptosis Make Sperm

DAPI-stained images of (A) *fog-1(q253ts);ced-4(n1162);fshr-1; fbf(RNAi);* and (B) *fog-1(q253ts)* hermaphrodites. White brackets indicate the region of the spermatheca; white arrowheads indicate oocytes. Note the presence of sperm nuclei in the spermathecal region of (A) but not (B) (also see Figure 1G). The inset in (A) shows sperm that display abnormal nuclear morphologies in *fog-1(q253ts);fshr-1; fbf(RNAi); ced-3(RNAi)* (dsRNA-injected) animals. Panel (C) provides a quantitative summary of the observed phenotypes. XX refers to the hermaphrodite genotype. *fog-1(0)* designates the *fog-1(q241)* null allele. The female and hermaphrodite symbols indicate the sexual state of the germline based on DAPI staining. Similar results were also obtained through DIC analysis. Germlines designated as hermaphroditic contained multiple sperm-cell nuclei in addition to oocytes in the spermathecal region. "Ind." indicates animals with indeterminate or apoptotic germlines. dsRNA injection of *lin-15a* was used as a negative control for the *ced-3* and *ced-4* RNAi experiments. The scale bar in (A) represents 10 μm for (A) and (B).

of the cell-death pathway. Previous studies have shown *ced-3* and *ced-4* to be required for both somatic and germline cell death in *C. elegans* [31, 32]. To inhibit apoptosis in the compound mutants, we used either RNAi-injection methods or a *ced-4* loss-of-function mutation (*n1162*) to reduce *ced-3* and *ced-4* activities. We found that pretreatment with either *ced-3* or *ced-4* dsRNAs, or loss of *ced-4* by mutation, significantly reduced or abolished apoptosis in the majority of compound mutants and correspondingly reversed the germline-deterioration phenotype in these animals (Figures 2 and 3; data not shown). For example, *fog-1(ts);ced-4;fshr-1(0);fbf(RNAi)* animals contained an average of only 5.5 ± 7.4 (n = 22) positively staining cells per arm versus 21.2 ± 1.9 (n = 31) in *fog-1(ts);fshr-1(0);fbf(RNAi)* animals. Taken together, these results demonstrate that *fshr-1*, the *fbfs*, and *fog-1/3* integrate their functions to prevent aberrant apoptosis and to promote the survival of the germline.

Loss of Function in *fshr-1* and the *fbfs* can Bypass the Requirement for *fog-1/3* in the Expression of the Male-Germline Fate

The reversal of germline degeneration in the compound mutants through the inhibition of *ced-3* or *ced-4* provided us with the opportunity to determine the sexual fates of the compound-mutant germlines. Strikingly, the majority of apoptotically suppressed *fog-1(0);fshr-1(0);fbf(RNAi)* and *fog-3;fshr-1(0);fbf(RNAi)* germlines assayed contained substantial numbers of sperm (Figure 3). Moreover, 93% (n = 60) of *fog-1(0);fbf-1(0) fbf-2(0);fshr-1(0);ced-3(RNAi)* nondegenerate gonad arms were observed to contain sperm. In these animals, sperm were uniformly located within the region of the spermatheca and were discernable by DAPI staining and DIC (Figure 3 and data not shown). This finding strongly contrasts with results for *fog-1(0)* and *fog-3* single mutants, as well as all other binary combinatorial mutants that were tested (Figure 3 and Table S3). We note that despite the presence of sperm, animals that were suppressed for apoptosis via RNAi-injection methods were uniformly sterile and often contained sperm with abnormal nuclear morphologies (Figure 3A inset). In contrast, the sperm produced by *fog-1(ts);ced-4;fshr-1(0);fbf(RNAi)* animals was typically indistinguishable from that of the wild-type, and the large majority of these hermaphrodites were self-fertile (Figure 3 and data not shown).

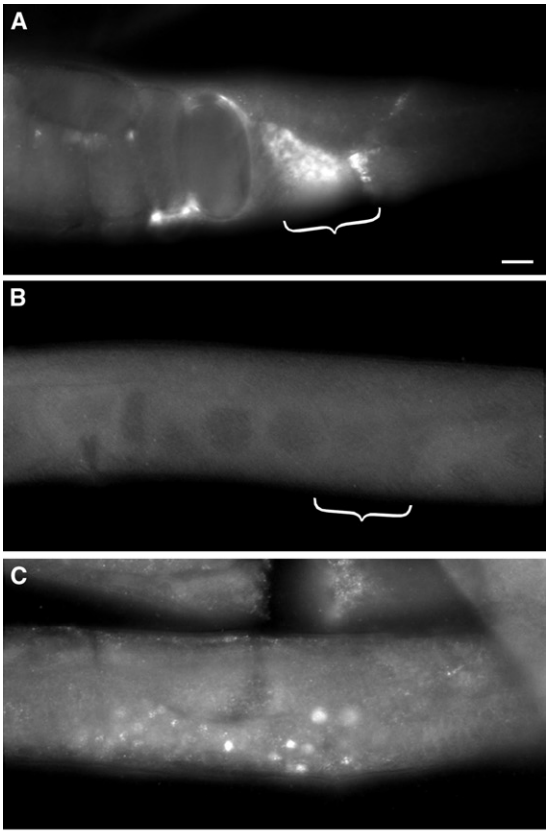
To assay expression of the male-germline fate in compound mutants further, we tested for the presence of Major Sperm Protein (MSP) by antibody staining of intact worms [33]. To rule out possible indirect effects

caused by inactivation of the cell-death pathway, we directly assayed MSP staining in compound mutants that had not been rescued from apoptosis. Notably, we observed clear MSP staining in about one-third to one-half of *fog-1(0);fshr-1(0);fbf(RNAi)*, *fog-1(0);fbf-1(0) fbf-2(0);fshr-1(0)*, and *fog-3;fshr-1;fbf(RNAi)* compound mutant germlines (Figure 4). MSP staining in compound mutants was typically discernible as dispersed bright puncta in the proximal region of the gonad arm and was generally associated with small round nuclei (Figure 4C and data not shown). In contrast, MSP staining was never detected in the feminized mutants assayed and was usually specific to the proximal gonad region containing sperm in the wild-type (Figures 4A and 4B).

The above findings strongly indicate that a substantial proportion of hermaphrodite compound mutants are capable of bypassing the normal requirement for *fog-1* or *fog-3* in the expression of the male-germline fate. In addition to playing a role in hermaphrodite germline sex determination, *fog-1* and *fog-3* have also been shown to be required for expression of the male-germline fate in males [22, 23]. We therefore sought to extend our findings by examining the germlines of *fog-1(0);fshr-1(0);fbf(RNAi)* male compound mutants. Although male compound mutants also displayed some germline abnormalities, these defects were markedly less pronounced than in hermaphrodites of the same genotype (consistent with the reported absence of cell death in wild-type male germlines [28]), and we could score sexual fates directly without having to suppress cell death. Strikingly, the large majority of male compound mutants contained sperm exclusively, and few or no males were strongly feminized (Figure 5). In contrast, 100% of control [*fog-1(0);fshr-1(0)* and *fog-1(0);fbf(RNAi)*] animals scored were strongly feminized in that these germlines contained multiple oocytes with few or no sperm. This result demonstrates that simultaneous loss of both *fshr-1* and the *fbfs* can circumvent the requirement for *fog-1* and *fog-3* in the expression of the male germ cell fate in males as well as hermaphrodites, indicating that *fshr-1* functions downstream of or in parallel to the known sex-determination pathway in *C. elegans*.

FSHR-1 Functions in the Soma to Control Germline Processes

To determine whether *fshr-1* acts in germline or somatic tissues to control germline sexual fates, we carried out a germline mosaic analysis on *fshr-1(0)* mutants containing the *fdEx31* (*fshr-1*+)-rescuing extrachromosomal array. This assay exploits the inherent mitotic instability of these arrays and facilitates the analysis of animals that specifically lack *fshr-1* function within the germline but retain *fshr-1* function in somatic tissues [34]. We first established a spontaneous germline loss frequency of 2.7% (n = 150) in the untreated *fshr-1;fdEx31* strain (germline mosaics were inferred based on the absence of GFP+ self-progeny). This number reflects the mitotic-loss frequency of the array during embryogenesis within the lineage that generates the germline precursor cell under nonselective conditions. We next determined the frequency of fertile germline mosaics in *fshr-1(0); fdEx31* animals exposed to *fbf(RNAi)*. Under these selective conditions, fertile animals that lack the rescuing array in the germline arose at a frequency of 2.1%



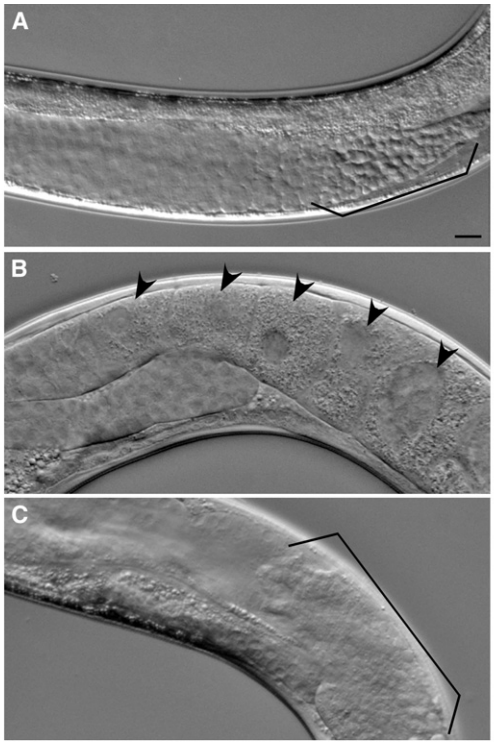
D

Genotype (XX)	*fbf(RNAi)*	% MSP staining	Total (n)
N2	No	100	76
fog-1(ts)	No	0	62
	Yes	0	66
fog-1(0)	No	0	44
	Yes	5	132
fshr-1	Yes	100	55
fog-1(ts); fshr-1	No	0	64
	Yes	28	111
fog-1(0); fshr-1	Yes	39	82
fog-1(0); fbf-1(0) fbf-2(0)	No	3	58
fog-1(0); fbf-1(0) fbf-2(0); fshr-1	No	47	68

Figure 4. Compound Mutants Synthesize Major Sperm Protein

Antibody staining of major sperm protein (MSP) in (A) wild-type, (B) *fog-1(q253ts);fbf(RNAi)*, and (C) *fog-1(q253ts);fshr-1;fbf(RNAi)* hermaphrodites. The white brackets (A and B) indicate the precise location of the spermathecae. Note the presence of the MSP antigen (including punctate staining) in panels (A) and (C). Panel (D) summarizes staining results for the relevant strains. XX refers to the hermaphrodite genotype. Null alleles for *fog-1(0)*, *fbf-1(0)*, and *fbf-2(0)* were *q241*, *ok91*, and *q704*, respectively. The scale bar in (A) represents 10 μm for (A)–(C).

(n = 677), close to the spontaneous germline loss rate in untreated animals. The efficacy of the *fbf(RNAi)* treatment in these experiments was confirmed by the high



D

Genotype (XO)	*fbf(RNAi)*	%♂	%♀	%⚥	Total (n)
fog-1(ts)	No	0	100	0	54
	Yes	0	100	0	58
fog-1(0)	Yes	0	100	0	42
fog-1(ts); fshr-1	No	0	100	0	68
	Yes	61	4	35	83
fog-1(0); fshr-1	No	0	100	0	60
	Yes	89	0	11	64

Figure 5. Compound-Mutant Males Make Sperm

DIC of (A) wild-type, (B) *fog-1(q241);fshr-1*, and (C) *fog-1(q241);fshr-1;fbf(RNAi)* males. Black brackets designate the regions containing sperm; black arrowheads indicate oocytes. Note the absence of oocytes and the presence of sperm (which appears slightly degenerative) in (C). Panel (D) quantifies the germline sexual identities of the genotypes assayed. XO refers to the male genotype. *fog-1(0)* designates the *fog-1(q241)* null allele. The male symbol indicates animals containing large amounts of sperm and no oocytes; the female symbol indicates animals with $\geq$5 oocytes with few or no sperm; the hermaphrodite symbol indicates animals containing 1 or 2 oocytes together with sperm. In addition to the scored animals, *fog-1(ts); fshr-1(0);fbf(RNAi)* and *fog-1(0);fshr-1(0);fbf(RNAi)* strains had 2% (n = 85) and 6% (n = 68) degenerative/indeterminate germlines, respectively. The scale bar in (A) represents 10 μm for (A)–(C).

percentage of sterility in *fshr-1(0);fbf(RNAi)* animals that failed to inherit the array (91%; n = 43) and by the highly penetrant masculinized-gonad phenotype exhibited by *fshr*$^{-}$ mutants (97%; n = 172). These data demonstrate that the large majority of *fshr-1(0);fbf(RNAi);fdEx31* germline-mosaic animals were efficiently rescued to fertility, despite the complete absence of wild-type *fshr-1* in the germline. In contrast, if *fshr-1* function were required in the germline, selective conditions should have produced a frequency of fertile germline mosaics of approximately 0.24% (0.09 × 2.7), 9-fold less than the observed frequency of 2.1%. These studies conclusively demonstrate that *fshr-1* is not required in the germline to control germline fates and that *fshr-1* must therefore control germline fates via the soma. We note that the slight reduction in frequency of viable germline mosaics observed under selective conditions is probably due to loss of the array in the somatic lineages that require *fshr-1* function as well as the expected induction masculinization by *fbf(RNAi)* in a low percentage of the array-positive (or wild-type) animals (Table 1 and data not shown).

Further evidence in support of a somatic role for *fshr-1* comes from the observation that repetitive high-copy extrachromosomal arrays containing sequences from the wild-type *fshr-1* locus consistently and robustly rescue the masculinization phenotype of *fshr-1(0);fbf(RNAi)* animals. For three independently derived arrays tested, masculinization was observed in 5%–25% of array-positive animals, versus 87%–98% of siblings that failed to inherit the array (Table S4). Because expression from repetitive arrays is often strongly silenced in the germline [35, 36], these results lend further support to our findings from the mosaic analysis showing that rescue by the extrachromosomal arrays does not require the expression of *fshr-1* in the germline.

To shed additional light on where FSHR-1 may function during germline sex determination, we engineered a construct expressing a full-length FSHR-1::GFP fusion protein under the control of the endogenous *fshr-1* regulatory sequences. High-copy extrachromosomal arrays expressing the fusion protein were found to rescue the defects of *fshr-1;fbf(RNAi)* animals strongly (data not shown), indicating that the fusion protein is functional and further supporting our previous findings regarding the ability of repetitive arrays to rescue the synthetic germline phenotype. We find that FSHR-1 is broadly expressed in a number of somatic tissues, including the pharynx, intestine, neurons, and the vulva, throughout development (Figures S4A–S4H). In addition, FSHR-1 was consistently expressed in the spermatheca, a somatic-gonad tissue that shares common lineal ancestors with several other somatic-gonad cell types, including the sheath cells (Figures S4I and S4J). These findings are further consistent with the model in which FSHR acts on the germline via the soma, possibly through a role in the somatic gonad.

In order to assay for expression of endogenous *fshr-1*, we carried out in situ hybridization. Similar to the findings described above for the GFP reporter, our results showed expression of *fshr-1* mRNA in a number of somatic tissues, with the strongest expression occurring in the intestine (data not shown). Furthermore, in contrast to several tested positive controls for germline expression, we were unable to detect any expression of *fshr-1* in the germline when we used probes to three different regions of the gene (data not shown). Although this assay could have failed to detect very low levels of *fshr-1* germline expression, these results, combined with the mosaic analysis demonstrating the absence of a functional requirement for *fshr-1* in the germline, suggest that *fshr-1* is neither required nor expressed in germ cells.

Table 2. Genetic Suppression of Mog by *gsa-1(gf)* and *acy-1(gf)*

Genotype	Percent Masculinized (n)
fbf(RNAi)	1 (205)
fshr-1(ok778);fbf(RNAi)	99 (301)
gsa-1(ce81);fbf(RNAi)	3 (117)
gsa-1(ce81);fshr-1(ok778);fbf(RNAi)	10 (150)
acy-1(md1756);fbf(RNAi)	2 (133)
acy-1(md1756);fshr-1(ok778);fbf(RNAi)[a]	90 (305)

Percentages were derived from the averages from two independent experiments. Untreated *gsa-1(ce81)* and *acy-1(md1756)* single mutants contained wild-type germlines (data not shown).
[a] Chi-squared test for statistical significance, $p < 0.01$.

FSHR-1 Probably Signals through a Canonical G Protein-Mediated Pathway

After ligand binding, the glycopeptide hormone receptors of mammals initiate signaling through heterotrimeric G proteins, primarily via $G\alpha_s$, to activate downstream targets that include adenyl cyclase, protein kinase A, and the cAMP-responsive transcription complex CREB-CBP [1, 37, 38]. To ascertain whether a similar pathway may function downstream of FSHR-1 in *C. elegans*, we tested for the ability of a gain-of-function (GOF) $G\alpha_s$ variant, *gsa-1(ce81)*, to suppress the Mog and Glp phenotype of *fshr-1;fbf(RNAi)* animals [39]. Strikingly, when present as a homozygous mutation, *gsa-1(ce81)* almost completely suppressed the germline-associated phenotypes of *fshr-1(0);fbf(RNAi)* animals, suggesting that GSA-1 may be the primary downstream target of FSHR-1, at least with respect to germline functions (Table 2). Consistent with this, we also observed significant, though weaker, suppression of *fshr-1(0);fbf(RNAi)* germline-associated defects when we used a GOF allele of the adenyl cyclase gene, *acy-1* (*md1756*; Table 2), indicating that stimulation of cAMP synthesis is also likely to be a conserved function of both FSHR-1 and the mammalian GPHRs [40]. In addition to reducing the penetrance of the Mog phenotype, both *gsa-1(ce81)* and *acy-1(md1756)* also led to a decrease in the severity of the observed Mog phenotypes (data not shown). We note that the enhanced suppressive ability of *gsa-1(ce81)* versus *acy-1(md1756)* may be due in part to the relative strength of these alleles because *gsa-1(ce81)* was previously shown to exert stronger suppression of *ric-8(md303)*-associated movement defects [39].

Discussion

Somatic Signaling in Germline Development

Our studies have revealed a role for FSHR-1, a structural ortholog of the human glycopeptide hormone receptors, in the control of germ-cell differentiation, proliferation, and survival in *C. elegans*. We also provide evidence that FSHR-1 functions within the soma to exert its effects on the germline. These findings are consistent with the well-established roles of mammalian FSHR and LHR, which act within the somatic support cells of the ovaries and testes to promote germ-cell development [5, 6]. We also show that the previously described genetic requirement for *fog-1* and *fog-3* in the execution of the male-germline fate can be bypassed in large part through the combinatorial removal of *fshr-1* and the *fbfs*. Given that these synthetic epistatic effects were observed for multiple alleles, including a presumed null allele of *fog-1* (*q240*), these results imply that *fshr-1* functions downstream of or in parallel to the known sex-determination pathway in *C. elegans.* These results also suggest that the *fbfs* may have targets outside of the known sex-determination pathway or that certain genes within the canonical sex-determination pathway may have functions that are independent of other downstream pathway components.

Our findings also build upon the growing body of knowledge regarding the role of the soma in controlling multiple aspects of germline development in *C. elegans*. Support for this model includes the well-characterized role of the gonadal distal-tip cell, which acts through a Delta–Notch signaling mechanism to maintain the distal-most germ cells in a nondifferentiated and proliferative state [41]. In addition to this, other cells within the somatic gonad influence the behavior of germ cells, although the mechanistic basis for these effects is currently unclear. For example, laser ablation of the precursors to the gonadal sheath and spermathecal (SS) cells during larval development leads to defects in meiotic progression and germline proliferation [42]. Furthermore, ablation of specific sheath cells later in development results in defective ovulation [42] and delays in the initial timing of meiosis [43]. This latter role for the sheath cells is further supported by the characterization of several genetic mutants, which display abnormal mitotic proliferation patterns as a result of impingement of sheath-cell functions [44, 45]. Of particular interest is the observation that partial loss of the SS lineage leads to a highly penetrant germline-feminization phenotype, indicating that the sheath or spermathecal cells can also influence germline sex [42]. Interestingly, a recent report indicates that the germline may also influence the development of the *C. elegans* somatic gonad [16], suggesting that signaling between the soma and germline may be reciprocal.

At present, relatively little is known with regard to the molecular nature of soma-germline controls in *C. elegans*. Our results indicate that signaling through the FSHR receptor and its downstream targets, including $G\alpha_s$ and adenyl cyclase, will be one component of this process. Consistent with our findings, signaling through $G\alpha_s$ has been recently shown to control meitotic maturation and microtubule reorganization in oocytes [46, 47]. Furthermore, similar to our findings on *fshr-1*, the focus for this activity is somatic and is likely to involve cells of the gonadal sheath. Thus, a common theme for soma-germline processes may be the involvement of signaling through pathways involving G proteins.

For future studies, it will be of significant interest to understand the basis for the observed genetic interaction between *fshr-1* and the *fbfs*. Although our data suggest that FSHR-1 and the FBFs act in parallel pathways, it is possible that FSHR-1 may regulate the FBFs more directly, possibly at the level of protein abundance or activity. It will also be of great interest to identify both the upstream and downstream effectors of the FSHR-1 pathway and to determine more precisely the tissue-specific requirements for FSHR-1 in germline processes. We note that the *C. elegans* genome does not

encode any obviously conserved glycopeptide hormone-receptor ligand subunits, although many of the known downstream targets of these receptors, including those involved in cAMP and inositol triphosphate signaling, are present. Interestingly, it is possible that FSHR-1 may not require ligand binding for activation because expression of FSHR-1 in mammalian cells leads to substantial induction in cAMP levels in the absence of ligand; these induction levels are similar to those produced by a constitutively activated form of the mammalian LH receptor [7]. In any case, our findings suggest a conserved role for glycopeptide-hormone receptors in germline development across widely separated species.

Novel Functions Revealed through Synthetic Genetic Interactions

The novel functions described here for FSHR-1 were uncovered through the nonbiased identification of synthetic genetic interactions. Loss of *fshr-1*, like many genes in *C. elegans*, does not produce an overt phenotype. In fact, inactivation of gene function by RNAi (or deletion mutations) suggests that the individual loss of approximately 70%–80% of genes in *C. elegans* will not result in readily discernable defects [17, 48]. A primary cause for this phenomenon is predicted to be genetic or functional redundancy, whereby specific proteins, pathways, or complexes are capable of compensating for the loss of unrelated gene products [49–51]. It is therefore likely that the functional characterization of many genes in *C. elegans* (and other systems) will require the identification of synthetic genetic interactions. Our findings further support the power of this approach and highlight its utility for identifying previously unknown regulators of important and well-studied developmental processes. Furthermore, we demonstrate that synthetic genetic interactions can be employed in epistasis analysis to provide unexpected results. We contend that synthetic genetic approaches will prove to be an essential means for dissecting the complexities of many biological and developmental processes.

Supplemental Data

Supplemental Data include Experimental Procedures, four figures, and four tables and are available online at http://www.current-biology.com/cgi/content/full/17/3/203/DC1/.

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