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Contributed Mini Review

A systematic mRNA control mechanism for germline stem cell homeostasis and cell fate specification

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Germline stem cells (GSCs) are the best understood adult stem cell types in the nematode *Caenorhabditis elegans*, and have provided an important model system for studying stem cells and their cell fate *in vivo*, in mammals. In this review, we propose a mechanism that controls GSCs and their cell fate through selective activation, repression and mobilization of the specific mRNAs. This mechanism is acutely controlled by known signal transduction pathways (e.g., Notch signaling and Ras-ERK MAPK signaling pathways) and P granule (analogous to mammalian germ granule)-associated mRNA regulators (FBF-1, FBF-2, GLD-1, GLD-2, GLD-3, RNP-8 and IFE-1). Importantly, all regulators are highly conserved in many multi-cellular animals. Therefore, GSCs from a simple animal may provide broad insight into vertebrate stem cells (e.g., hematopoietic stem cells) and their cell fate specification. [BMB Reports 2016; 49(2): 93-98]

C. elegans GERMLINE

Germline stem cells (GSCs) are characterized by their ability to both self-renew and generate gametes - sperm or eggs. In the adult gonads of many organisms, GSCs are maintained to replenish stocks of germ cells whose numbers are depleted by gamete production. GSCs are also responsible for transmitting genetic information across the generations. A systematic regulatory network, including extrinsic cues and intrinsic regulation, tightly regulates a balance between self-renewal and differentiation of GSCs (called "GSC homeostasis") (1, 2). Therefore, aberrant regulation of this network can result in either loss of a specific germ cell type (arrested gametogenesis resulting in sterility) or over-proliferation of undifferentiated germ cells, which are associated with germline tumors (3).

The nematode *C. elegans* is a very versatile reproductive model organism that has greatly contributed to the understanding of germline development (4). *C. elegans* exist as either hermaphrodites or males. Hermaphrodites produce a limited number of sperm in early larval stage (L3 to early L4) and switch to produce only oocytes in late larval stages (4). Thus, they are self-fertile (Fig. 1A). Infrequently occuring males produce sperm continuously without switching into oogenesis (Fig. 1B). Since hermaphrodites produce both sperm and oocytes from the same GSC, their germlines can be a good model system to study a mechanism for GSC homeostasis and cell fate specification. Moreover, the *C. elegans* germline is organized in a simple linear pattern that progresses from GSCs at the distal region to maturing gametes at the proximal region (Fig. 1A and 1B).

In the *C. elegans* gonad, a single mesenchymal somatic cell, called the distal tip cell (DTC), functions as a stem cell niche (also known as "microenvironment"), and drives the mitotic cell cycle in adjacent GSCs (4, 5) (Fig. 1C). Although specific individual GSC types have not been precisely defined in the *C. elegans*, genetic and cellular analyses suggest that GSCs are located in the distal mitotic region that directly contacts the DTC (4, 6) (Fig. 1C). As a GSC leaves the DTC niche, it enters the meiotic cell cycle and eventually differentiates into either sperm or oocytes (Fig. 1A and 1B). Notably, several RNA-binding proteins (e.g., PUF (Pumilio and FBF) (7)) control both GSC homeostasis and germ cell fate in the *C. elegans* germline. This observation suggests that regulatory mechanisms for the self-renewal/differentiation decision and sperm/oocyte decisions may be closely linked (8).

A comparative analysis of GSC regulatory mechanism in *Drosophila*, *C. elegans*, and mouse models has elucidated fundamental principles for self-renewal and differentiation (9). In these model systems, extrinsic factors (called niche signals) activate transcription for promoting the self-renewal of GSCs and preventing GSCs from differentiation. The extrinsic factors include Notch, Bone Morphogenic Protein (BMP) and Janus Kinase-Signal Transduction and Activator of Transcription (JAK/STAT) signaling pathways, more specific examples in-

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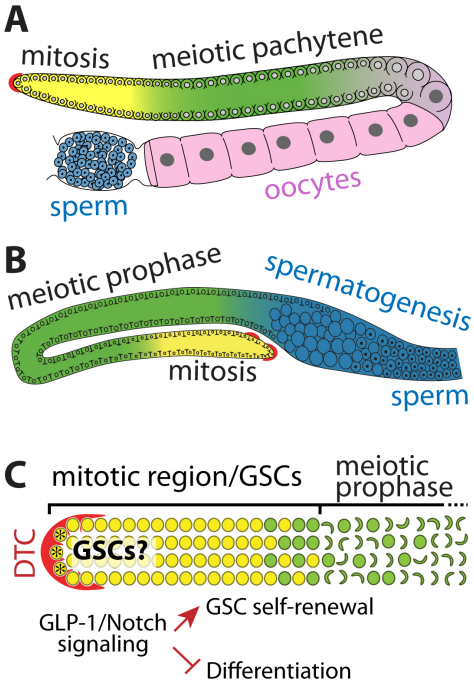

Fig. 1. *C. elegans* germline and GSC regulation. (A) Schematic of an adult *C. elegans* hermaphrodite gonad. Somatic DTC is located at the distal end. Cells at the distal end of the germline, including GSCs, divide mitotically (yellow). As cells move proximally, they enter meiosis (green) and differentiate into either sperm (blue) or oocytes (pink). (B) Schematic of an adult *C. elegans* male gonad. Two somatic DTCs reside at the distal ends of the adult male gonad. In the male germline, all GSCs differentiate into sperm (blue). (C) A simplified model for GSCs and their differentiation. GSC self-renew and differentiate into either sperm or egg. GLP-1/Notch signaling promotes GSC self-renewal and proliferation by inhibiting differentiation. *, inferred actual GSCs.

clude: Piwi/Yb in *Drosophila*, Notch signaling in *C. elegans* and Glial cell line-Derived Neutrophic Factor (GDNF) and possibly BMP signaling pathways in mouse testis (9).

In addition to these extrinsic factors several classes of intrinsic factors, including translational regulators (Pumilio, Nanos, Bam, SCF/c-kit, and Plzf), also control the self-renewal/differentiation of GSCs and cell lineage commitment (9). Although different combinations of extrinsic factors and intrinsic factors are need for GSC self-renewal and differentiation in different systems, a systematic mRNA control mechanism through an intimate interplay between extrinsic factors and intrinsic factors may be involved in controlling GSC homeostasis and cell fate specification.

In this review, we propose a mechanism to explain the control of GSC homeostasis and their cell fate specification through systematic and episodic mRNA selection. Most regulators identified in *C. elegans* are highly conserved in multi-cellular animals and have been implicated in stem cell control and cell fate specification. The streamlined *C. elegans* gonads may provide a useful platform to understand mechanisms underlying stem cell regulation and cell fate specification in higher model systems, including humans.

TRANSCRIPTIONAL ACTIVATION BY GLP-1/NOTCH SIGNALING IN GSCs

In *C. elegans*, the DTC functions as a GSC niche and promotes the mitotic cell cycle in germ cells through a signal transduction pathway initiated by GLP-1 (one of two *C. elegans* Notch receptors) (3-5) (Fig. 2A). The GLP-1/Notch signal maintains the germ cells in the undifferentiated state through the transcriptional activation of target genes (10-12) (Fig. 2B). The Notch signaling pathway and its core components in *C. elegans* are highly conserved: the Notch ligand "LAG-2 (a family of DSL ligands)" is expressed in DTCs and its receptor, "GLP-1", is expressed on the membranes of mitotically dividing germ cells (3, 13) (Fig. 2A).

In the absence of signaling or progression of meiotic cell cycle, the transcription factor, "LAG-1 (a family of CSL transcription effectors)", is associated with a repressor complex to inhibit the expression of GLP-1/Notch target genes. Upon signal activation, an ADAM-family metalloprotease and γ-secretase cleaves the GLP-1/Notch receptor, and its intracellular domain (NICD) translocates from membrane to the nucleus. In the nucleus, NICD interacts with LAG-1 and LAG-3 (a homolog of mastermind transcriptional co-activators) to activate the expression of Notch target genes (Fig. 2A). Therefore, identifying the direct GLP-1/Notch target genes driving GSC self-renewal is crucial for understanding the molecular mechanisms of normal stem cell regulation, as well as tumorigenesis mediated by aberrant Notch signaling.

Recently, bioinformatics has identified 163 putative GLP-1/Notch target genes, all harboring clusters of at least four LAG-1 binding sites (LBSs) (14). Among them, four genes were validated as *bona fide* GLP-1/Notch targets in the *C. elegans* germline. These include FBF-2 (a family of PUF RNA-binding proteins) (15), LIP-1 (a homolog of the dual-specificity phosphatase) (16), SYGL-1 (SYnthetic GLp-1) (14) and LST-1 (Lateral Signaling Target-1) (14) (Fig. 2B). These genes function redundantly to maintain GSCs in *C. elegans*, while *fbf-2*, *lip-1*, *sygl-1*, and *lst-1* single mutants appear normal (7, 14-17), the *lst-1 sygl-1* double mutant very nearly pheno-copies the *glp-1* loss-of-function mutant, which is unable to maintain GSCs (14). Notably, the *fbf-2*; *lip-1* double mutant displays a defect in germ cell fate specification, rather than GSC maintenance (Lee *et. al.*, unpublished results) (Fig. 2C). These observations suggest that GLP-1/Notch signaling and its targets may regulate both GSC maintenance and germ cell fate specification in the *C. elegans* germline.

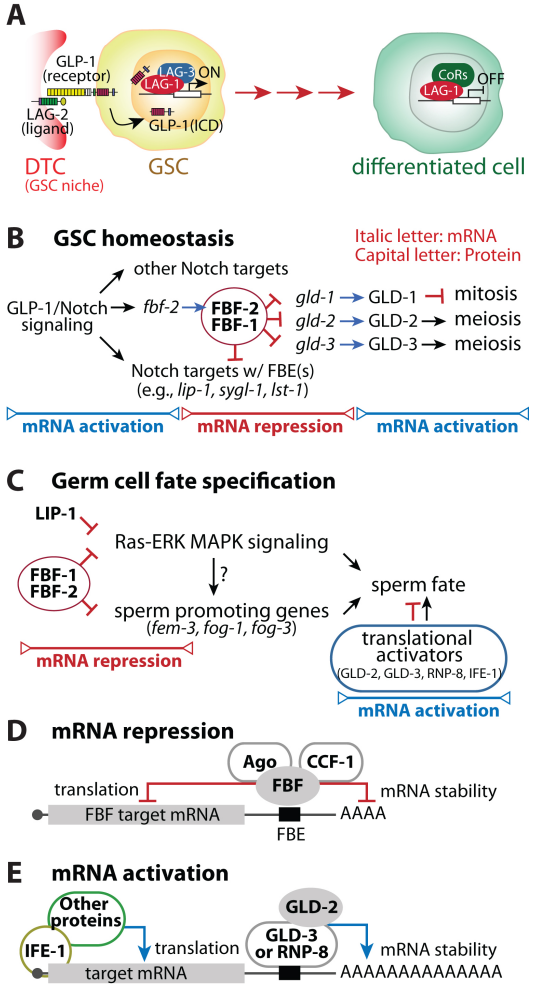

Fig. 2. A systematic RNA selection mechanism. (A) Transcriptional activation by GLP-1/Notch signaling. DTCs function a GSC niche. LAG-2, a Notch ligand, is expressed on the DTC membrane. Notch receptor, GLP-1, is expressed on the membrane of GSC and mitotically dividing germ cells. Upon Notch activation, the GLP-1 intracellular domain (ICD) is trans-located from membrane to nucleus and forms a ternary complex with transcription activators, "LAG-1 and LAG-3" to activate the expression of target genes. Once GSCs move away from a DTC, LAG-1 interacts with transcription corepressors (CoRs) to repress the expression of target genes. (B) GSC homeostasis. GLP-1/Notch signaling activates the expression of target genes, including FBF-2. FBF-2 acts as a regulatory hub for the proliferation and differentiation of GSCs. (C) Germ cell fate specification. FBF proteins inhibit the translation of the selected target mRNAs and translational activators (e.g., GLD-2, GLD-3, RNP-8, and IFE-1) promote the translation of the selected target mRNAs. (D) FBF proteins mediate mRNA repression. FBF proteins bind to the FBE(s) in target mRNAs and associate with CCF-1 or/and Ago to repress the stability or/and translation of target mRNAs. (E) GLD-2 and IFE-1-mediated mRNA activation. GLD-2 and its partners (GLD-3 or RNP-8) promote the stability of target mRNAs, as well as, IFE-1 and the translation initiation complex promote the translation of target mRNAs. Italic letters indicate mRNAs and capital letters indicate proteins.

FBF-2, A REGULATORY HUB FOR GSC HOMEOSTASIS

FBF-2 is expressed in the GSC region (15, 18). FBF-1 and FBF-2 (collectively known as FBF) are two, nearly identical PUF RNA-binding proteins that regulate the switch from mitosis to meiosis in the *C. elegans* germline (7, 15). Thus, in *fbf-1 fbf-2* double mutants, all GSCs leave the mitotic cell cycle, enter the meiotic cell cycle and undergo spermatogenesis (7). FBF proteins are translational repressors that specifically bind defined sequence element(s) in the 3'UTR (UnTranslated Region) of target mRNAs (4, 15, 16, 19, 20) (Fig. 2D). FBF proteins inhibit mRNA stability and its translation by recruiting CCF-1 (Pop2P deadenylase homolog) or/and Argonaute (Ago, microRNA-binding proteins) proteins (21, 22) (Fig. 2D).

A recent genome-wide study identified an FBF target list of 1,350 mRNAs, using immunoprecipitation of FBF with associated mRNAs followed by microarray analysis (19). Interestingly, several FBF targets are also targets of GLP-1/Notch activated transcription (14, 16). These two findings suggest that GLP-1/Notch target genes might be down-regulated post-transcriptionally by FBF proteins in the GSC region.

Why are mRNAs of GLP-1/Notch target genes also repressed by FBF-2? One possible explanation is that FBF-2 maintains a balance between proliferation and differentiation (called "GSC homeostasis") by moderately suppressing both cellular states. This regulation, called "dual negative regulation", maintains cellular homeostasis (2, 23). Therefore, single mutations do not affect either self-renewal or differentiation of GSCs. However, when multiple genes are affected, GSC fate is altered. In addition, FBF proteins repress the translation of mRNAs that normally promote differentiation of GSCs.

The repressed mRNAs include GLD-1 (a KH-motif containing RNA-binding protein) (7), GLD-2 (a cytoplasmic poly(A) polymerase) (24), and GLD-3 (a bicaudal homolog) (25) (Fig. 2B). The GLD proteins are critical for either promoting the differentiation of GSCs or inhibiting the proliferation of GSCs. Our model proposes that FBF proteins act a central regulatory hub for GSC homeostasis (Fig. 2B).

SELECTIVE mRNA REPRESSION/ACTIVATION MECHANISMS SPECIFY GERM CELL FATE

Once GSCs enter meiosis, dynamic changes in gene expression specify the germ cell fate (4). Normally, *C. elegans* hermaphrodites make sperm as larvae and oocytes as adults. This fate appears to be programmed in the early meiotic region (8). Here, sperm-promoting genes (e.g., *fem-3*, *fog-1*, and *fog-3*) are expressed in the fourth larval stage (L4), but are dramatically decreased when cell fate is switched to oogenesis in young adult stages (26-28). Notably, FBF proteins promote the sperm-to-oocyte switch by inhibiting the expression of sperm-promoting genes (19, 29) (Fig. 2C).

We recently reported that *C. elegans* Ras-ERK MAPK signaling promotes sperm fate specification (30) (Fig. 2C). One po-

tential target of Ras-ERK MAPK signaling is FOG-3 (a homolog of TOB/BTG anti-proliferative proteins) (26). In *C. elegans* germline, FOG-3 directs germ cells to adopt sperm fate at the expense of oogenesis (26, 31). We reported that unphosphorylated FOG-3 initiates the sperm fate program, but phosphorylated FOG-3 maintains continuous sperm production typical of males (26). Notably, FBF proteins inhibit the expression of both *mpk-1* (an ERK homolog) (20) and *fog-3* mRNAs (27, 32) (Fig. 2C). These findings suggest that FBF proteins selectively repress both sperm-promoting genes and the MPK-1/ERK MAPK signaling pathway to program sperm fate. We also found that FBF proteins promote spermatogenesis by inhibiting cell cycle regulators (Lee *et al.*, unpublished results). Notably, male germ cells appear to have faster cell cycle progression than female germ cells (33). This finding suggests an additional role for cell cycle regulators in germ cell fate specification.

In addition to selective mRNA repression, a few RNA regulators can alternately promote the translation of their target mRNAs. For example, *C. elegans* GLD-2 forms an active poly(A) polymerase (PAP) when it interacts with multiple RNA-binding proteins to promote the stability and translation of target mRNAs (34) (Fig. 2E). GLD-2 controls several developmental processes, including entry into the meiotic cell cycle, and progression through both spermatogenesis and oogenesis (35-38). Two RNA-binding partners (RNP-8 and GLD-3) associated with GLD-2 have been identified (36, 37) (Fig. 2E). RNP-8 has an RNA recognition motif (RRM) and binds purine-rich RNA sequences (37), whereas GLD-3 belongs to the Bicaudal-C family of RNA-binding proteins (25).

The GLD-2/GLD-3 PAP activates mRNA poly(A) elongation and translation of sperm fate-promoting genes (37) (Fig. 2C and 2E). In contrast, the GLD-2/RNP-8 complex activates mRNA translation of oogenic fate-promoting genes (37) (Fig. 2C and 2E). Mutants lacking GLD-2 are doubly defective in gametogenesis: aberrant spermatocytes occur proximally instead of mature sperm and no oocyte-like cells are observed (35).

In addition to the GLD-2-mediated polyadenylation role in gametogenesis, the translational recruitment of mRNAs mediated by *C. elegans* IFE-1 also regulate germ cell fate specification in *C. elegans* (39) (Fig. 2C and 2E). IFE-1 is a germline-specific isoform of translation factor eIF4E, one of five isoforms of the mRNA cap-binding protein in *C. elegans*, uniquely associates with the germ granule (called P granule in *C. elegans*). The germ granule shares components with the P bodies and stress granules in mammals (40). Interestingly, a mutant lacking IFE-1 shows a temperature sensitive arrest germ cells in secondary spermatocytes and a modest temperature-insensitive defect in oocyte development, resulting in sterility (39).

Several repressed germ cell determinant mRNAs, including *gld-1* and *glp-1*, are preferentially translated by IFE-1 in region-specific recruitment events both in early and late germ cell differentiation steps (39). These and other studies support a model in which translational repression and selective activation of mRNAs may coordinate germ cell fate in the premeiotic region of the *C. elegans* germline.

ABERRANT TRANSLATIONAL REGULATION AND ABNORMAL GERMLINE DEVELOPMENT

C. elegans GSCs are established in the early larval gonad and continuously maintain their population by controlling the balance between self-renewal and differentiation. Aberrant regulation of this balance is often associated with germline tumors and infertility (3). Therefore, studying the regulatory pathways controlling the balance between these two states is critical to understand how the aberrant regulation of GSCs causes such tumors. Although we have gained significant understanding of transcriptional regulation of GSCs and cell fate in vertebrates, little is known about how translational regulators control GSC fate.

In *C. elegans*, many translational regulators and RNA-binding proteins are identified genetically. For example, FBF (FBF-1 and FBF-2) and GLD (GLD-1, GLD-2, and GLD-3) proteins are critical for GSC self-renewal and differentiation (4). Mutants lacking FBF proteins do not maintain GSCs and all cells differentiate into sperm (7) (Fig. 2B and 2C). As such, FBF proteins are required for GSC maintenance and oogenic fate specification. Once GSCs enter the meiotic cell cycle, the GLD-1 protein represses mitosis by inhibiting GLP-1/Notch signaling. It also represses oogenic fate by inhibiting the translation of sperm-promoting gene mRNAs (e.g., *tra-2*) (41).

In parallel with the function of GLD-1, GLD-2 and GLD-3 together promote polyadenylation and translation of target mRNAs (37). One of GLD-2/GLD-3 targets is *gld-1* mRNA (42). All GLD proteins promote the meiotic entry of GSCs at the translational level. Therefore, mutations in *gld* genes promote germline tumors, with enhanced and uncontrolled germ cell proliferation (35). These translational regulators also control germ cell fate in the premeiotic germline.

One of key translational regulators is GLD-2. GLD-2 and its partners (GLD-3 and RNP-8) control the germ cell fate (sperm or oocyte) in a combinational faction: GLD-2/GLD-3 complex drives the sperm fate and GLD-2/RNP-8 complex drives the oocyte fate (37). Notably, GLD-3 and RNP-8 antagonize each other in the sperm/oocyte decision (37). Moreover, GLD-3 also binds FBF and inhibits its repression of target mRNAs (25).

How do GSC regulators govern germ cell fate? The answer is not yet clear, but we propose that these RNA-binding proteins, polyadenylation factors, and translation initiation factors regulate the translation of target mRNAs at different places and times in the germline. Moreover, regulatory mechanisms for GSC homeostasis and cell fate specification are closely linked (8). Furthermore, GSC regulators and their target mRNAs regulate each other. These dual reciprocal regulations appear to form a spatial boundary in germ cell fate decisions (mitosis/meiosis and sperm/oocyte). Disruption of this regulatory circuit leads to GSC loss, germline tumor, sperm/oocyte switching, or other abnormal germ cell fate, which in turn, result in infertility.

CONCLUSIONS

In this review, we describe a new mechanism for *C. elegans* GSC homeostasis and their cell fate specification through several mRNA selection processes. In the GSC region, GLP-1/Notch signaling activates the expression of target genes. FBF-2, one of *C. elegans* GLP-1/Notch targets, likely controls GSC homeostasis by inhibiting both the proliferation and differentiation of GSCs. Once GSCs enter pre-meiotic cell cycle, FBF-2 selectively represses its target mRNAs, associated with sperm fate specification. In addition, positive translational regulators selectively activate mRNAs, associated with oogenic fate specification. These multistep mRNA selections lead germ cells progressively to a designated cell fate, preventing abnormal development.

Interestingly, most regulators involved in this mechanism are localized to *C. elegans* P-granules (analogous to germ granule in mammals) (18, 43). *C. elegans* P-granules are highly enriched for RNA and RNA-binding proteins and are key centers for specialized translational control (43, 44). Importantly, these nematode RNA regulators are highly conserved in invertebrate and vertebrate organisms, including humans. For example, the function of the PUF RNA-binding proteins is conserved throughout many species in evolution (45). Mammalian PUF proteins (e.g., PUM1 and PUM2) bind to the Pumilio binding element (PBE) in the 3′UTR of the target mRNAs. Importantly, several PUF target mRNAs are themselves conserved among *C. elegans*, *Drosophila*, and humans (19). Mammalian PUM2 is also expressed in their embryonic stem cells (46), hematopoietic stem cells (47), and germ cells (46). It is suggested that Pum2 plays a vital "identity role" in all of these stem cells. We propose that the systematic activation/repression of discrete mRNA pools may be a conserved mechanism that broadly influences both stem cell homeostasis and cell fate specification in multicellular organisms other than *C. elegans*.

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