

Discovery of two GLP-1/Notch target genes that account for the role of GLP-1/Notch signaling in stem cell maintenance

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A stem cell's immediate microenvironment creates an essential "niche" to maintain stem cell self-renewal. Many niches and their intercellular signaling pathways are known, but for the most part, the key downstream targets of niche signaling remain elusive. Here, we report the discovery of two GLP-1/Notch target genes, *lst-1* (lateral signaling target) and *sygl-1* (synthetic Glp), that function redundantly to maintain germ-line stem cells (GSCs) in the nematode *Caenorhabditis elegans*. Whereas *lst-1* and *sygl-1* single mutants appear normal, *lst-1 sygl-1* double mutants are phenotypically indistinguishable from *glp-1/Notch* mutants. Multiple lines of evidence demonstrate that GLP-1/Notch signaling activates *lst-1* and *sygl-1* expression in GSCs within the niche. Therefore, these two genes fully account for the role of GLP-1/Notch signaling in GSC maintenance. Importantly, *lst-1* and *sygl-1* are not required for GLP-1/Notch signaling per se. We conclude that *lst-1* and *sygl-1* forge a critical link between Notch signaling and GSC maintenance.

Stem cell self-renewal requires signaling from a specialized microenvironment, or stem cell niche (1, 2). Well-defined stem cell niches include the distal tip cell (DTC) for *Caenorhabditis elegans* germ-line stem cells (GSCs) (3), the cap and hub cells for *Drosophila* GSCs (e.g., refs. 4 and 5) and Paneth cells for murine intestinal stem cells (6). These niches use a variety of intercellular signaling pathways (e.g., Notch, BMP, JAK/Stat, Wnt), all of which have been implicated broadly in stem cell regulation throughout the animal kingdom (1). The identification of direct molecular links between niche signaling and downstream targets driving stem cell self-renewal is crucial for understanding niche function. However, few such links have been established.

Here, we focus on how Notch signaling controls stem cell self-renewal. Notch signaling typically occurs between adjacent cells, one signaling and the other receiving, and activates target genes using a transcription factor complex that includes the DNA binding protein CSL [human CBF1/RBPJK, *Drosophila* Su(H), and *C. elegans* LAG-1] (7). Although Notch signaling regulates stem cells in vertebrates, including those in the mammalian muscle, brain, and intestine (reviewed in ref. 8), its use in the nematode *C. elegans* to maintain GSCs provides the best defined and most tractable paradigm (Fig. 1A). In this case, a single mesenchymal cell, the DTC, forms the niche for GSCs. The DTC uses GLP-1/Notch signaling to maintain GSCs, and when GSCs leave their DTC niche they are triggered to enter the meiotic cell cycle and begin differentiation (3, 9). Regardless of sex or developmental stage, laser ablation of the DTC or genetic ablation of GLP-1/Notch signaling causes all GSCs to cease self-renewal and differentiate, the so-called Glp (germ-line proliferation defective) phenotype (3, 9, 10). No other signaling pathway has the same profound effect on *C. elegans* GSC maintenance. Therefore, *C. elegans* GLP-1/Notch provides an unequalled entrée into understanding Notch regulation of stem cells.

The key direct targets of GLP-1/Notch signaling that promote GSC self-renewal remain an open question. A partial answer is that GLP-1/Notch signaling activates *fbf-2* transcription (11). FBF-2, a PUF family mRNA binding protein, is a broad-spectrum inhibitor of differentiation and a regulator of GSC self-

renewal together with its nearly identical paralog FBF-1 (see ref. 12 for review). FBF-1 and FBF-2 function redundantly to maintain GSCs in late larvae and adults (13). GLP-1/Notch signaling also activates *lip-1*, which encodes a dual specificity phosphatase and ERK/MAPK inhibitor (14). LIP-1 promotes robust germ-line proliferation but has not been implicated in GSC self-renewal per se (15). Therefore, two GLP-1/Notch target genes are known, but neither accounts for GLP-1/Notch regulation of GSC self-renewal.

Additional GLP-1/Notch targets must exist to drive GSC self-renewal, but such genes have been elusive. Thirty years of genetics have not found them, either by screening for mutants with a Glp phenotype or by isolating suppressors and enhancers of genes in the GSC control pathway. Here, we report that *lst-1* and *sygl-1* function redundantly as pivotal GSC regulators with a double-mutant GSC phenotype indistinguishable from that of *glp-1/Notch* mutants. We also provide evidence that both genes are GLP-1/Notch targets but not components of GLP-1/Notch signaling per se. The *lst-1* and *sygl-1* genes establish a previously unidentified and important link between niche signaling and stem cell maintenance.

Results

GLP-1/Notch Targets Essential for Larval GSC Self-Renewal Not Yet Known. Wild-type adults possess a total of ~2,000 germ cells, with ~1,000 in each of two gonadal arms (Fig. 2A and Fig. S1A and E) (e.g., ref. 16), whereas *glp-1* null mutants make a total of only ~four to eight germ cells that all differentiate precociously (Fig. S1B and E) (9). Previous studies identified *fbf-2* and *lip-1* as germ-line targets of GLP-1/Notch signaling, but neither on its own was essential for GSC self-renewal (see Introduction). One

Significance

Stem cells generate tissues, and they can be hijacked in cancer. A fundamental mechanism of stem cell regulation is signaling from their immediate microenvironment, or niche. Although many niches and signaling pathways have been identified, much less is known about how stem cells respond. Here, we report the discovery of two key niche signaling effector genes. These genes are activated in stem cells by Notch signaling, a conserved and clinically significant pathway, and together are essential for the stem cell state. Surprisingly, neither was previously implicated in stem cell regulation and their sequences yield few clues to their functions. We suggest that these newly discovered genes may be pioneers for a new class of stem cell regulators.

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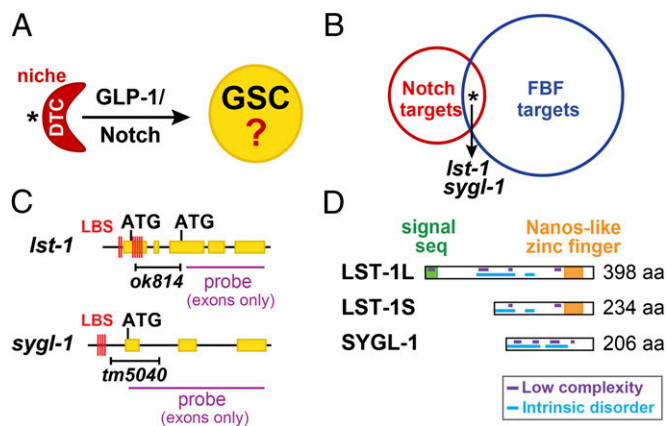


Fig. 1. Identification of *lst-1* and *sygl-1* as candidate GSC regulators. (A) DTC (red) uses GLP-1/Notch signaling to maintain GSCs within the niche. Asterisk marks distal end. (B) Fifteen genes (asterisk) are shared between lists of putative Notch and FBF targets. Double RNAi of two common genes, *lst-1* and *sygl-1*, caused a Glp phenotype. Also see Tables S1 and S2. (C) *lst-1* and *sygl-1* genes. Yellow, exons; red lines, LAG-1 binding sites (LBS); black bar, deletions; purple line, probe for in situ hybridizations (exons only). (D) LST-1 and SYGL-1 proteins and their predicted motifs.

possibility might have been that *fbf-2* and *lip-1* function redundantly to maintain GSCs. To test this idea, we examined *fbf-2; lip-1* double mutants, but they were self-fertile with essentially normal germ lines (Fig. S1E). Another possibility might have been that *lip-1* functions redundantly with the two nearly identical *fbf-1* and *fbf-2* genes, but *fbf-1 fbf-2; lip-1* triple mutant germ lines were similar to *fbf-1 fbf-2* double-mutant germ lines (Fig. S1C–E). Therefore, we reasoned that one or more additional GLP-1/Notch target genes must await discovery.

Identification of *lst-1* and *sygl-1* as Functionally Redundant Stem Cell Regulators. Our strategy to identify the key GLP-1/Notch targets essential for GSC maintenance was as follows. We hypothesized that (i) such genes are targets of both GLP-1/Notch signaling and the FBF RNA-binding protein, (ii) they are expressed in GSCs, and (iii) their functions in GSC self-renewal are masked by redundancy or pleiotropy. The idea that such genes might be targets of FBF regulation was entirely speculative and based simply on *fbf-2* and *lip-1* being FBF targets (11, 15); the idea that they might function redundantly or have pleiotropic effects was based on the failure to find them previously despite many genetic screens.

We compared lists of putative Notch and FBF-1 targets. The Notch target list of 163 genes was derived bioinformatically with all harboring clusters of at least four LAG-1 binding sites (LBSs) (17); the FBF-1 target list of 1,350 mRNAs was obtained experimentally using immunoprecipitation of FBF-1 with associated mRNAs followed by microarray analysis (18). Fifteen genes were common to both lists (Fig. 1B and Table S1). We depleted each of the 15 using RNAi, but none caused a Glp phenotype (Table S2). Among those 15 genes, T27F6.4 was expressed within the GSC niche, according to a database of mRNA in situ hybridizations to ~10,000 *C. elegans* genes (Nematode Expression Pattern DataBase; <http://nematode.lab.nig.ac.jp>); the other 14 were either not in the database or did not stain detectably in the niche. With the idea that T27F6.4 might function redundantly with one of the other 14 genes, we performed double RNAi against T27F6.4 and other genes in the common pool. The double RNAi knockdown of T27F6.4 plus *lst-1* caused a Glp phenotype but others did not (Tables S2 and S3); the double RNAi of T27F6.4 plus *lst-1* gave similar results in either a wild-type strain or the *rf-1* mutant, which is sensitive to RNAi in only

some tissues, including the germ line (19) (Table S3). We therefore named T27F6.4 *sygl-1* (synthetic Glp).

Molecularly, the *lst-1* gene is predicted to produce two mRNAs and polypeptides (Fig. 1C and D, Upper), whereas *sygl-1* is predicted to produce a single mRNA and polypeptide (Fig. 1C and D, Lower). BLAST analysis of *lst-1* and *sygl-1* sequences revealed homologs in closely related nematodes, including *Caenorhabditis briggsae* and *Caenorhabditis remanei*, but not in more distantly related organisms. We queried LST-1 and SYGL-1 amino acid sequences using a variety of algorithms. A structure prediction program [Phyre2 (20)] predicted a single Nanos-like zinc finger in LST-1 (Fig. 1D), but no motifs or folded domains in SYGL-1; a signal sequence prediction program [SignalP (21)] predicted a signal sequence at the N terminus of LST-1L (Fig. 1D), but not in LST-1S or SYGL-1; algorithms designed to identify low complexity [SEG (22)] or intrinsically disordered sequences [MFDp2 (23)] predict that both proteins harbor multiple low complexity sequences and are largely disordered

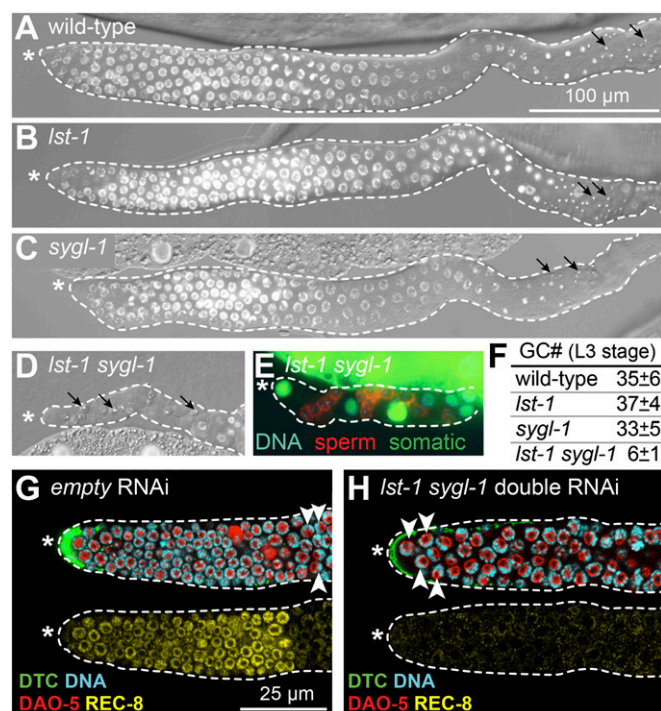


Fig. 2. *lst-1* and *sygl-1* function redundantly to promote GSC self-renewal in larvae and adults. (A–D) DIC and DAPI-stained images of gonads dissected from L4 hermaphrodites. Asterisk marks distal end; dotted line outlines germ line plus somatic gonadal cells; arrows mark mature sperm. (A–C) Wild type (100% non-Glp; $n > 100$), *lst-1(ok814)* (100% non-Glp; $n = 146$), and *sygl-1(tm5040)* (100% non-Glp; $n = 159$) all produce normal germ lines. (D) *lst-1 sygl-1* double mutants produce Glp germ lines with only a few differentiated sperm (100% Glp; $n = 76$). (E) Gonad from *lst-1 sygl-1* L4 with a somatic GFP marker (green), a sperm marker (red) and DNA staining (blue). All nonsperm cells expressed somatic GFP. Each gonadal arm contained 14 ± 3 sperm ($n = 9$) on average (from three to four premeiotic germ cells). (F) Total premeiotic germ cells (GC#) in entire L3 gonad, scored with PGL-1 germ cell marker. For wild-type and each single mutant, $n = 5$; for *lst-1 sygl-1* double mutant, $n = 9$. (G–H) Representative confocal images of wild-type late L4 larval germ lines treated with RNAi for 48 h; DTC expresses GFP (green). Same conventions as in A–D; mitotic zone scored by presence of mitotic marker REC-8 (yellow) and absence of crescent-shaped DAPI staining typical of early meiotic prophase nuclei (white arrowheads). Anti-DAO-5 (red, nuclear marker) counter stain facilitates scoring of DAPI crescents. (G) Germ line treated with empty RNAi vector possesses a mitotic zone. (H) Germ line treated with *lst-1 sygl-1* double RNAi lacks a mitotic zone and hence lacks GSCs.

(Fig. 1D). We conclude that *lst-1* and *sygl-1* encode novel proteins with a central role in stem cell regulation.

***lst-1* and *sygl-1* Function Redundantly to Promote GSC Self-Renewal.** To investigate more rigorously the effects of *lst-1* and *sygl-1* on GSC self-renewal, we obtained two deletion mutants: *lst-1(ok814)* and *sygl-1(tm5040)* (Fig. 1C and Fig. S2). The *lst-1* deletion removes most exons and introns unique to the longer *lst-1* isoform; the *sygl-1* deletion removes the first exon, including the ATG translational start codon. These two mutants are likely loss-of-function alleles, because they reproducibly mimic the strongest defect observed with RNAi (see below).

Both *lst-1* and *sygl-1* single deletion mutants possessed germ lines comparable in size and organization to wild type (Fig. 2A–C and F), and they were both self-fertile as hermaphrodites and cross-fertile as males. By contrast, all *lst-1 sygl-1* double deletion mutants displayed a dramatic Glp defect, regardless of sex (Fig. 2D–F and Fig. S3A–J). When *lst-1* or *sygl-1* single mutants were targeted with RNAi against the other [e.g., *lst-1(ok814) sygl-1(RNAi)*], the resultant germ lines were also Glp (Table S3). We conclude that *lst-1* and *sygl-1* function redundantly to promote GSC self-renewal in larvae of both sexes.

We observed no obvious somatic defect in either single mutant or the double mutant. For example, all were viable with normal body shape. Nonetheless, we note that *lst-1* plays roles in neurons and the developing vulva, indicating that at least *lst-1* likely functions in tissues other than the germ line (17, 24).

***lst-1 sygl-1* Glp Defect Comparable to *glp-1* Glp Defect.** Mutants lacking the GLP-1/Notch receptor make only four to eight germ cells, which differentiate precociously to produce 16–32 sperm (each germ cell makes 4 sperm) (9). To ask whether *lst-1 sygl-1* Glp sterility mimicked that of a *glp-1* mutant, we used a germline marker (PGL-1) to count germ cell number (Fig. 2F and Fig. S3K–N) and also asked whether germ cells differentiated precociously during development. Newly hatched *lst-1 sygl-1* L1 larvae contained two germ cells, as did wild-type controls; late *lst-1 sygl-1* L1s had made a total of five germ cells on average ($n = 10$; range, 4–7), and that number was essentially unchanged in early L3s, which contained only six germ cells on average ($n = 9$; range, 4–8). By contrast, wild-type early L3 larvae contained ~35 germ cells on average, as did *lst-1* and *sygl-1* single mutants (Fig. 2F). Moreover, *lst-1 sygl-1* germ cells differentiated as sperm during the third larval stage (L3), which is one stage earlier than normal and therefore precocious (Fig. S4). Importantly, sperm number in double mutants was consistent with the number of PGL-1–positive undifferentiated germ cells, and no evidence for cell death was seen. We conclude that the *lst-1 sygl-1* GSC defect is indistinguishable from that of a *glp-1* null mutant (9).

GLP-1/Notch signaling is also required to maintain GSCs throughout larval development and in adults (9). To ask whether *lst-1* and *sygl-1* were similarly required in later development, we treated wild-type L4 larvae with RNAi and then scored their germ lines as adults (2 d later) (Fig. S5A). Features diagnostic of GSCs were scored as follows: (i), presence of a mitotic zone (25); (ii), presence of mitotic marker REC-8 (26) in distal germ cells (Fig. 2G and H); and (iii), absence of meiotic marker HIM-3 (27) in the same germ cells (Fig. S5C and D). When treated with either control RNAi (empty vector), *lst-1* RNAi, or *sygl-1* RNAi (each mixed 1:1 with empty vector), all adult distal germ lines maintained a mitotic zone (Fig. 2G and Fig. S5B and C). However, when treated with *lst-1 sygl-1* double RNAi, adult germ lines lost their mitotic zone and instead contained only differentiating germ cells in the meiotic cell cycle (Fig. 2H and Fig. S5B). In addition, these *lst-1 sygl-1* double RNAi adult germ lines lost REC-8 staining and gained HIM-3 staining to the distal end (Fig. 2H and Fig. S5D). We conclude that *lst-1* and *sygl-1* function in adults to promote GSC self-renewal. Therefore, like GLP-1/Notch

signaling, *lst-1* and *sygl-1* are essential for GSC self-renewal and function in larvae, adults, and both sexes.

***lst-1 sygl-1* Is Epistatic to *glp-1(gf)* but Not *gld-2 gld-1* Germ-Line Tumors.** If *lst-1* and *sygl-1* are critical GLP-1/Notch targets for GSC self-renewal, they should act downstream of GLP-1/Notch signaling and upstream of GLD regulators promoting meiotic differentiation (Fig. S6A). We first explored these predictions genetically, asking whether the *lst-1 sygl-1* Glp phenotype was epistatic to the germ-line tumorous phenotype of two key mutants. First was the constitutively active gain-of-function allele *glp-1(oz112gf)*, which encodes an unregulated GLP-1/Notch receptor and drives germ-line tumors independently of signaling ligand (28). All *glp-1(gf)* mutants made germ-line tumors when treated with control RNAi (Fig. S6B and F), but >90% of *glp-1(gf); lst-1(RNAi) sygl-1(RNAi)* animals produced tiny germ lines with only sperm (Fig. S6C and F). Because *lst-1* and *sygl-1* are required for *glp-1(gf)* to drive tumor formation, they likely do not act upstream, but instead act either downstream or in parallel to GLP-1/Notch signaling. Second was the *gld-2 gld-1* double null mutant, which makes germ-line tumors because of a failure to enter the meiotic cell cycle and differentiate (29). Like the *gld-2 gld-1* double mutant, all *gld-2 gld-1 lst-1 sygl-1* quadruple mutants formed germ cell tumors (Fig. S6D–F). Therefore, *lst-1* and *sygl-1* likely work upstream of *gld-1* and *gld-2*. These epistasis results are consistent with the idea that *lst-1* and *sygl-1* are critical GLP-1/Notch targets for GSC self-renewal.

GLP-1/Notch Signaling Activates *lst-1* and *sygl-1* Expression Within the Niche. To test the idea that *lst-1* and *sygl-1* function downstream of GLP-1/Notch signaling, we asked whether GLP-1/Notch signaling activates their expression. When assayed by mRNA in situ hybridization, both *lst-1* and *sygl-1* mRNAs localized to the distal-most germ line, within the GSC niche (Fig. 3A and B, arrows); both genes are also expressed more proximally in developing oocytes (Fig. 3A and B), which became useful as a control in later experiments. To ask whether their niche expression relies on GLP-1/Notch signaling, we took advantage of the *gld-2 gld-1* double mutant, which generates a tumorous germ line independently of GLP-1/Notch signaling (29) and also independently of *lst-1* and *sygl-1* (Fig. S6D–F). When GLP-1/Notch signaling was normal and active, *lst-1* and *sygl-1* mRNAs were both expressed in germ cells within the niche of *gld-2 gld-1* double mutants, but when GLP-1/Notch signaling was eliminated, both mRNAs became undetectable (Fig. 3C and D). Therefore, *lst-1* and *sygl-1* expression in the GSC niche depends on GLP-1/Notch signaling.

LAG-1 Binding Sequences in *sygl-1* Promoter Required for Expression Within Niche. GLP-1/Notch signaling activates transcription via the LAG-1/CSL DNA-binding protein and its binding to the LAG-1 binding sequence (LBS) (30). The *lst-1* LBSs were previously shown to mediate Notch activation in the soma (17), and, given the reliance of *lst-1* expression on GLP-1/Notch signaling in the distal germ line (Fig. 3C), it seemed likely that the *lst-1* LBSs would also be critical for Notch activation in the distal germ line. We therefore turned to *sygl-1* and asked whether its LBSs were critical for expression within the niche. For this experiment, we generated a *Mos1*-mediated single copy insertion of a Histone 2B::GFP reporter transgene driven by the *sygl-1* promoter, which harbors a cluster of four LBSs (Figs. 1C and 3E) (*Materials and Methods*). The wild-type *sygl-1* promoter drove expression of Histone 2B::GFP in the distal-most germ cells (Fig. 3E), but that expression was eliminated when all four LBSs were mutated from the consensus RTGGGAA to RACGGAA, a sequence that LAG-1 cannot bind in vitro (30) (Fig. 3E). The lack of distal expression was not attributable to gene silencing, as the LBS mutant reporter supported proximal expression (Fig. S7).

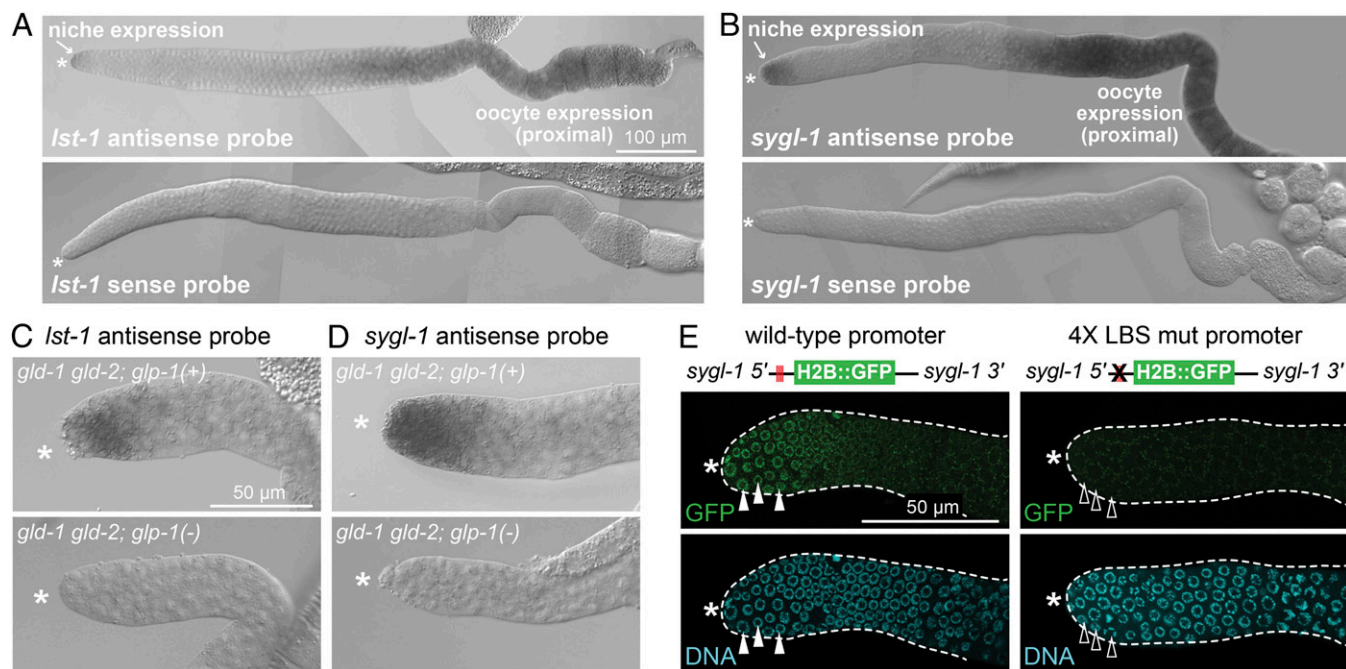


Fig. 3. *lst-1* and *sygl-1* are targets of GLP-1/Notch activation. (A and B) *lst-1* and *sygl-1* mRNA expression in wild-type young adult gonads. (C and D) Expression of *lst-1* and *sygl-1* in GSCs within the niche requires GLP-1/Notch. Shown are L4 gonads. GLP-1(+): *lst-1*, 97% positive ($n = 37$); *sygl-1*, 97% positive ($n = 36$). GLP-1(-): *lst-1*, 0% positive ($n = 28$); *sygl-1*, 0% positive ($n = 33$). (E) Wild-type *sygl-1* promoter drives reporter expression in germ cells within the niche (100% GFP-positive; $n = 45$), whereas 4 \times LBS mut *sygl-1* promoter does not (0% distal positive; $n = 46$). Filled white triangles mark nuclei positive for H2B::GFP; empty white triangles mark nuclei negative for H2B::GFP. Because reporters were occasionally silenced, only germ lines expressing GFP proximally were scored (SI Materials and Methods). (A–E) Asterisk marks distal end.

Therefore, the *sygl-1* LBSs are responsible for expression distally in the niche but not proximally in oocytes.

lst-1 and *sygl-1* Are Not Required for GLP-1/Notch Signaling Per Se.

One potential caveat to the model that *lst-1* and *sygl-1* function downstream of GLP-1/Notch to regulate GSCs might have been that they encode essential components of Notch signaling rather than encoding key downstream stem cell regulators. To explore this concern, we asked whether GLP-1/Notch signaling remains functional in the *lst-1 sygl-1* double mutant.

Our assay took advantage of the *gld-2 gld-1 lst-1 sygl-1* germline tumor (Fig. S6E), which carries an ideal reporter of GLP-1/Notch signaling: the *sygl-1(tm5040)* deletion mutant retains its LBS cluster and generates a truncated *sygl-1(tm5040)* mRNA that lacks its translational start codon and lacks wild-type SYGL-1 activity (Figs. 1C and 4A and Fig. S2B). The *gld-2 gld-1 lst-1 sygl-1* quadruple mutant expressed this GLP-1/Notch reporter in germ cells within the niche as expected (Fig. 4B). More importantly, that reporter expression required GLP-1/Notch signaling: when signaling was eliminated, reporter expression was no longer detected in the niche (Fig. 4C). Therefore, GLP-1/Notch signaling is active and functional in animals harboring both *lst-1* and *sygl-1* deletions. We conclude that *lst-1* and *sygl-1* deletions do not affect GLP-1/Notch signaling per se even though their double-mutant phenotype mimics a complete loss GLP-1/Notch signaling. Therefore, these two pivotal stem cell regulators are targets of GLP-1/Notch signaling and function downstream of GLP-1/Notch signaling to promote GSC self-renewal (Fig. 4D).

Discussion

A central question in stem cell biology is how niche signaling maintains stem cells. Here, we identify two genes, *lst-1* and *sygl-1*, which are activated by GLP-1/Notch signaling and function redundantly to maintain the stem cell state. Remarkably, these two genes fully account for the stem cell function of GLP-1/Notch

niche signaling. Both *lst-1* and *sygl-1* encode novel proteins and therefore may represent a new class of stem cell regulators. Here, we place this discovery in context and discuss its implications.

The DTC Niche and GLP-1/Notch Signaling Maintain GSCs by Activation of Two Redundant Genes, *lst-1* and *sygl-1*. The DTC niche was discovered decades ago and its use of GLP-1/Notch signaling to maintain GSCs was found soon thereafter (see Introduction). A

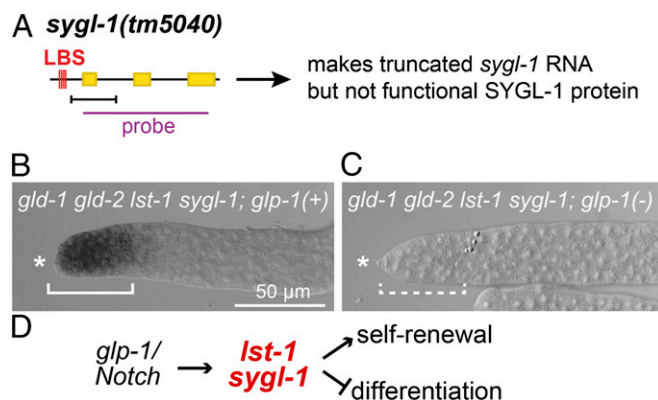


Fig. 4. *lst-1* and *sygl-1* do not affect GLP-1/Notch signaling. (A) GLP-1/Notch reporter assay exploits *sygl-1(tm5040)* deletion mutant (see Results). Conventions same as Fig. 1C. (B and C) L4 hermaphrodite gonads were probed for GLP-1/Notch reporter by in situ hybridization. Shown are representative images of distal gonads. Both mutants have tumorous germ lines that appear the same. Asterisk marks distal end. (B) The *gld-2 gld-1 lst-1 sygl-1* quadruple mutant stains positively for the GLP-1/Notch reporter (solid bracket) (91% positive; $n = 35$). (C) The *gld-2 gld-1 lst-1 sygl-1; glp-1* quintuple mutant does not stain positively for the GLP-1/Notch reporter (dashed bracket) (0% positive; $n = 27$). (D) Model for *lst-1* and *sygl-1* function in the GSC self-renewal pathway.

longstanding question has been how the DTC niche and GLP-1/Notch signaling promote GSC self-renewal. A partial answer is *fbf-2* activation (11), but FBF only maintains GSCs in adults, whereas the DTC niche and GLP-1/Notch signaling maintain them in both larvae and adults (9, 13). Therefore, a larval GSC-promoting activity was predicted to exist—an activity that would complement the previously found adult GSC-promoting activity of FBF. Our search for this additional GSC-promoting activity was guided by several predictions.

Two straightforward predictions were that this gene would be a direct target of GLP-1/Notch signaling and that its RNA would be expressed in the distal-most GSCs within their niche. Another prediction, which was based on the striking failure to find this gene despite extensive mutant screens, was that it likely functioned redundantly with at least one other gene. A final and more risky prediction was that it might be an FBF target, a highly speculative prediction that turned out to be crucial. A comparison of candidate Notch and FBF targets identified 15 in common, one of which was expressed in germ cells within the niche according to an extensive *in situ* hybridization database; RNAi directed against that 1 gene plus the 14 others led to identification of *lst-1* and *sygl-1*, two genes required for GSC self-renewal in larvae. Follow-up experiments demonstrated that a double mutant harboring deletions of both *lst-1* and *sygl-1* abolished GSC self-renewal in a manner indistinguishable from a *glp-1* null mutant. However, removal of either gene alone, using either RNAi or a deletion mutant, did not affect GSCs, confirming the prediction of redundancy for genes driving the larval GSC-promoting activity.

Surprisingly, *lst-1* and *sygl-1* are not larval-specific but instead are essential for the GSC state in both larvae and adults. Thus, in addition to the dramatic GSC loss found upon deletion of the two genes, adult GSCs fail to self-renew when treated later in development with RNAi against both *lst-1* and *sygl-1*. The *lst-1* gene was previously identified as a target of Notch signaling in the soma (17). Here, we show that *lst-1* and *sygl-1* are also regulated by GLP-1/Notch signaling in GSCs within the niche and that *lst-1* and *sygl-1* function downstream of GLP-1/Notch signaling rather than as an integral component of signaling *per se*. Together these findings demonstrate that the niche and GLP-1/Notch signaling maintain GSCs by activation of *lst-1* and *sygl-1* and that these two genes are essential effectors of niche signaling for GSC self-renewal. We do not yet know whether overexpression of *lst-1* or *sygl-1* is sufficient for GSC self-renewal, because transgenes driving them in an unregulated fashion could not be generated. Nonetheless, their dramatic loss-of-function phenotype demonstrates unambiguously that *lst-1* and *sygl-1* are critical downstream targets of niche signaling that are essential for all GSC self-renewal.

How Might LST-1 and SYGL-1 Promote GSC Maintenance? A major question emerging from this work is how LST-1 and SYGL-1 proteins promote GSC self-renewal. Few clues come from their amino acid sequences. LST-1 harbors a single C-terminal Nanos-like zinc finger, suggesting RNA binding activity. However, LST-1 is not a canonical Nanos protein, which possesses two zinc fingers in tandem (31); moreover, zinc fingers can perform many molecular functions in addition to RNA binding (32). The LST-1L protein also harbors a predicted signal sequence, implying a possible membrane association or secretion of this isoform. The only other features of note are low complexity sequences (LCSs) in both LST-1 and SYGL-1. In other proteins, LCSs can promote assembly of RNA-protein granules (33), consistent with the possibility of a role in RNA regulation. However, ideas for LST-1 and SYGL-1 molecular activities remain highly speculative at the current time.

The *lst-1* and *sygl-1* amino acid sequences are not conserved outside of nematodes. As such, these two key stem cell regulators may have evolved recently and represent a new mechanism for

stem cell regulation, or they may represent a broadly conserved mechanism of stem cell regulation that relies on proteins whose amino acid sequences are not recognizable across evolution. Instructive cases of proteins not recognizable by amino acid sequence similarity have been found in other pathways. For example, *C. elegans* SYS-1 encodes a β -catenin whose amino acid sequence bears little resemblance to canonical homologs; its discovery therefore relied on genetic phenotype and X-ray crystal structure rather than amino acid similarity (34). Here, discovery of LST-1 and SYGL-1 required a well-defined niche/stem cell system in a genetically tractable organism, sophisticated knowledge of Notch and FBF targets and the motivation to probe for redundant functions. To our knowledge, similar searches have not been applied broadly. Although not yet definitive, we favor the idea that LST-1 and SYGL-1 are pioneers for a previously unidentified class of stem cell regulators.

Notch Target Genes and Stem Cell Self-Renewal. In addition to *C. elegans* GSCs, other types of stem cells also depend on Notch signaling for self-renewal (see Introduction) (reviewed in ref. 8). Critical downstream targets have also been identified, the most well-studied being genes encoding the Hes/Hey family of bHLH transcription factors. For example, mouse embryos with a conditional brain-specific CSL knockout lose virtually all neural stem cells (NSCs) (35) and *Hes1*; *Hes3*; *Hes5* triple-mutant embryos similarly lose NSCs in many parts of the developing brain (36), consistent with the idea that these transcription factors are key targets of niche signaling. However, a brain-specific CSL knockout has a broader effect on NSCs than found in the *Hes1*; *Hes3*; *Hes5* triple mutant (35, 37), and a muscle-specific CSL knockout similarly has a more dramatic defect than a mutant lacking key *Hes* genes (38, 39). Therefore, removal of known downstream Notch targets in brain and muscle stem cells does not fully recapitulate the effect of removing the DNA-binding protein responsible for most Notch signaling. This discrepancy may be explained by additional targets or by a lack of congruence between Notch signaling and CSL activity (reviewed in ref. 40). Regardless, our work reports two GLP-1/Notch targets that do in fact fully recapitulate the effect of this canonical signaling pathway on stem cell maintenance. Indeed, *lst-1* and *sygl-1* provide a previously unidentified example of Notch target genes that fully explains the effects of Notch signaling on stem cells of a particular tissue. A fascinating possibility is that similarly pivotal targets of niche signaling exist in other stem cell systems.

Materials and Methods

Nematode Culture and RNAi. Strains were maintained at 20 °C following standard protocols (41), except for H2B::GFP reporter transgenic lines, which were maintained at 25 °C. Wild type was the N2 Bristol strain. See *SI Materials and Methods* for a full list of strains and alleles used in this study. RNAi feeding experiments were carried out following established protocols (42). For multiple gene knockdowns, HT115 bacteria containing *lst-1*, *sygl-1*, and empty (pL4440) RNAi vectors were grown separately in overnight cultures and then seeded to RNAi plates in equal volumes.

Immunocytochemistry and mRNA *In Situ* Hybridization. Antibody staining of dissected gonads was carried out as described (15), and staining of whole animals was carried out as described (43). See *SI Materials and Methods* for protocols. mRNA *in situ* hybridization was performed on dissected gonads from either adults grown to 24 h post L4 stage or L4 larvae, using digoxigenin-labeled DNA probes (44). See *SI Materials and Methods* for protocols.

Transgenic *C. elegans*. Transgenes were inserted into the genome using the *Mos1*-mediated single copy insertion method (45) (*SI Materials and Methods*). The presence of H2B::GFP was scored in unfixed dissected gonads in PBS plus 0.1% Tween-20, 0.25 mM Levamisole, and Hoechst 33342 (Invitrogen) diluted 1:10,000 and visualized using the Zeiss Axiomager microscope.

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