The molecular basis of LST-1 self-renewal activity and its control of stem cell pool size

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ABSTRACT

PUF RNA-binding proteins have diverse roles in animal development, with a broadly conserved role in stem cells. Two paradigmatic PUF proteins, FBF-1 and FBF-2, promote both self-renewal and differentiation in the C. elegans germline. The LST-1 protein is a pivotal regulator of self-renewal and is oncogenic when mis-expressed. Here, we demonstrate that LST-1 self-renewal activity resides within a predicted disordered region that harbors two KXXL motifs. We find that the KXXL motifs mediate the binding of LST-1 to FBF, and that point mutations of these motifs abrogate LST-1 self-renewal activity. The LST-1-FBF partnership is therefore crucial to stem cell maintenance and is a key element in the FBF regulatory network. A distinct region within LST-1 determines its spatial expression and size of the GSC pool. Most importantly, the molecular understanding of how an IDR-rich protein works in an essential partnership with a conserved stem cell regulator and RNA-binding protein suggests broad new avenues for combinatorial control.

KEY WORDS: Intrinsically disordered region, PUF RNA-binding protein, PUF partnership, Stem cell self-renewal, Zinc finger, Stem cell pool

INTRODUCTION

A central paradigm in stem cell biology is that niche signaling regulates key target genes to promote self-renewal. Examples of niches and niche signaling pathways abound (e.g. Lander et al., 2012), but direct targets of niche signaling – the key genes activated in stem cells to drive self-renewal – have been elusive, with a handful of exceptions. One such is the Myc gene, a target of Notch, BMP and Wnt signaling in mammalian stem cells (Moore and Lemischka, 2006). Another is the lst-1 (lateral signaling target 1) gene, a target of GLP-1/Notch signaling in nematode germline stem cells (GSCs) (Fig. 1A) (Kershner et al., 2014; Lee et al., 2016). The LST-1 protein stands out as being pivotal for self-renewal and acts redundantly with another target of niche signaling, SYGL-1 (Kershner et al., 2014). LST-1 protein is normally restricted to the GSC pool region but drives a germline tumor when ubiquitously expressed (Shin et al., 2017). Although the biological significance of LST-1 is unambiguous, the challenge now is to understand, in molecular terms, how it executes its key role in stem cell self-renewal and how it is regulated.

In addition to LST-1, PUF RNA-binding proteins are central to self-renewal of germline stem cells (Crittenden et al., 2002; Forbes and Lehmann, 1998; Lin and Spradling, 1997; Wickens et al., 2002). This role is best understood in Caenorhabditis elegans, where two PUF proteins, FBF-1 and FBF-2 (collectively FBF), control self-renewal and differentiation by regulating hundreds of target RNAs (Kershner et al., 2013; Porter et al., 2019; Prasad et al., 2016). LST-1 was recently proposed to work as an FBF partner to repress differentiation-promoting RNAs and thus to promote self-renewal (Fig. 1B) (Shin et al., 2017). This idea was based on several lines of evidence. LST-1 protein harbors a predicted Nanos-like zinc finger (Kershner et al., 2014) and is cytoplasmic and granular (Shin et al., 2017), features that are consistent with a role in RNA regulation; LST-1 interacts in yeast with both FBF-1 and FBF-2 (Shin et al., 2017); LST-1 cannot form tumors in the absence of FBF, revealing dependence on FBF for its self-renewal activity (Shin et al., 2017); and LST-1 contributes to repression of gld-1, an established FBF target mRNA (Brenner and Schedl, 2016; Shin et al., 2017). Although this model is attractive, it has not yet been tested in vivo in nematodes, nor is it known whether LST-1 must bind FBF directly to exert its self-renewal activity.

The restriction of LST-1 expression to the GSC pool region suggested that its expression might help determine the size of the GSC pool (Fig. 1C) (Shin et al., 2017). Consistent with that idea, ubiquitous expression of full-length LST-1 throughout the germline drove formation of a tumor. One established lst-1 regulator is Notch signaling, which activates lst-1 transcription within the niche (Kershner et al., 2014; Lee et al., 2016). However, prior to this work, little was known about how LST-1 protein is spatially restricted or whether that restriction was biologically significant.

The LST-1 amino acid sequence provides few clues to the molecular basis of its self-renewal activity or its regulation (Kershner et al., 2014). Here, we identify one region sufficient for stem cell self-renewal and another region required for spatial regulation. Within the self-renewal region, we find two short sequence motifs that mediate direct binding to FBF and that are essential for LST-1 self-renewal activity. The regulatory region includes a Nanos-like zinc finger and loss of this zinc finger expands LST-1 distribution and generates a larger than normal GSC pool. Thus, LST-1 works within the stem cell regulatory network as a key FBF partner, and its spatial regulation helps determine the size of the GSC pool.

RESULTS

LST-1L isoform is critical for self-renewal

The lst-1 locus encodes two transcripts, which generate longer LST-1L and shorter LST-1S proteins (Fig. 1D) (Kershner et al., 2014).
The LST-1L and LST-1S amino acid sequences overlap extensively and harbor multiple predicted intrinsically disordered regions (IDRs; regions with a high proportion of polar and charged amino acids and a low proportion of nonpolar amino acids (Dyson, 2016)) plus a CCHC Nanos-like zinc finger (Fig. 1D, Fig. S1A) (Kershner et al., 2014). To differentiate between the roles of LST-1L and LST-1S, we compared self-renewal activities of wild-type LST-1, \textit{lst-1}(wt) (Fig. 1E), with a mutant LST-1, \textit{lst-1}(frameshift) that harbors a single base pair deletion in the \textit{lst-1L} start codon, and only makes LST-1S (Fig. 1F). For protein visualization, both \textit{lst-1}(wt) and \textit{lst-1}(fs) carried a V5 epitope tag at the shared C terminus (Fig. 1E,F), which we indicate henceforth in superscript.

We assayed self-renewal activities in \textit{lst-1}(wt)V5 and \textit{lst-1}(fs)V5 animals in the absence of SYGL-1, where GSC maintenance relies on LST-1 alone (Kershner et al., 2014). Virtually all \textit{lst-1}(wt)V5 animals make a healthy fertile germline in the absence of SYGL-1, while strong loss-of-function \textit{lst-1} mutants are all sterile with no GSCs without SYGL-1 (Fig. S1B), as shown previously (Kershner et al., 2014; Shin et al., 2017). \textit{lst-1}(fs)V5 were also all sterile with no GSCs in the absence of SYGL-1 (Fig. 1E,F, Fig. S1B). Western blot demonstrated that LST-1L was nearly eliminated in \textit{lst-1}(fs)V5, while LST-1S remained (Fig. 1I, Fig. S1C).

Immunostaining showed that LST-1 V5 proteins were similarly restricted in \textit{lst-1}(wt)V5 and \textit{lst-1}(fs)V5 germlines (Fig. S1D-G). We conclude that LST-1L is necessary for GSC self-renewal activity, and that LST-1S not sufficient despite expression in GSCs.

Consistent with its role in GSC self-renewal, we expected LST-1L to be expressed in GSCs. To visualize this isoform specifically,
we introduced a FLAG epitope tag at the unique LST-1L N-terminus to create lst-1(L)FLAG. FLAG was used because attempts to insert an N-terminal V5 failed (Fig. 1G). As a control, we made C-terminal lst-1(L/S)FLAG to visualize LST-1L and LST-1S collectively (Fig. 1H). Both N-terminal and C-terminal LST-1FLAG variants maintained GSCs in the absence of SYGL-1 and therefore were functional (Fig. 1G,H, Fig. S1B). Upon immunostaining, both LST-1(L)FLAG and LST-1(L/S)FLAG were expressed in the GSC region with similar subcellular localization (Fig. 1J,K). We conclude that the LST-1L isoform is present in GSCs and that it harbors self-renewal activity.

**N-terminal LST-1 fragment is sufficient for stem cell maintenance**

To delineate the region within LST-1 required for self-renewal activity, we generated a series of lst-1 variant alleles (Fig. 2). Each was introduced into the lst-1(wt) locus (Fig. 2A). Because all lst-1 variants from this point carry V5, we henceforth omit the V5 superscript in allele designations. As a control, we created an lst-1(α) protein null mutant by deleting the entire open reading frame at the endogenous locus (Fig. 2B). As expected (Kershner et al., 2014; Shin et al., 2017), all lst-1(α) homozygotes maintained GSCs in the presence of SYGL-1 due to redundancy, but none maintained GSCs in the absence of SYGL-1 (Fig. 2B, Fig. S2A).

To test lst-1 variants for function in vivo, each was assayed for GSC maintenance with and without SYGL-1. Two variants harboring N-terminal regions, lst-1(1-210) and lst-1(1-152), maintained GSCs in the absence of SYGL-1 (Fig. 2C,D, Fig. S2A). Complementary variants harboring C-terminal regions, lst-1(211-328) and lst-1(153-328), did not maintain GSCs (Fig. 2E, F, Fig. S2A) (see Fig. 4 and Fig. S5 for confirmation of germline expression). Analogous transgenic experiments performed prior to the CRISPR/Cas9 revolution produced similar results (Fig. S2B-E). Although both sets of truncation experiments showed that the zinc finger was dispensable for self-renewal, we explored this domain specifically by mutating two cysteine residues required for its architecture (Hashimoto et al., 2010; Weidmann et al., 2016). The lst-1(C260S C263S) missense mutant, dubbed lst-1(ZnF), maintained GSCs in the absence of SYGL-1, confirming that the zinc finger is not crucial for GSC maintenance (Fig. 2G). We conclude that LST-1 self-renewal activity resides in the N-terminal half of the protein.

Interestingly, although both lst-1(1-210) and lst-1(1-152) maintain GSCs, these two variants differ with respect to the hermaphrodite sperm/oocyte switch: in the absence of SYGL-1, all lst-1(1-210) animals made the switch and were self-fertile, whereas no lst-1(1-152) animals made the switch and had a sterile Mog (Masculinization of Germline) phenotype (Fig. S2A). A role for LST-1 in the sperm/oocyte decision was known: indeed, even with SYGL-1 present, a few lst-1(α) homozygotes (≤5%) failed to make the switch and had a Mog phenotype (Fig. S2A), as previously shown for two other lst-1 strong loss-of-function alleles (Kershner et al., 2014; Shin et al., 2017). A low penetrance Mog defect can be associated with an increased brood size (Lamont et al., 2004), but this was not the case for lst-1 strong loss-of-function alleles (Shin et al., 2017). This LST-1-mediated effect on germline sex determination likely reflects the common molecular basis for regulation of germline self-renewal and germline sex determination as both rely on FBF RNA regulation (Crittenden et al., 2002; Zhang et al., 1997). Yet for the purposes of this study, we focus on LST-1 regulation of GSC maintenance via its N-terminal half.

**Two FBF-binding motifs are essential for LST-1 self-renewal activity**

LST-1 has been proposed to function with FBF for self-renewal (Fig. 1B, see Introduction). We therefore investigated the N-terminal half of LST-1 for FBF interaction. Two other FBF partners, CPB-1 and GLD-3, possess a consensus KTXL motif that is crucial for FBF binding, where X is any amino acid (Fig. 3A) (Menichelli et al., 2013; Wu et al., 2013). We scanned LST-1(1-152) for a KTXL

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**Fig. 2. Identification of LST-1 region required for GSC maintenance.** (A-G) Left, alleles. Only lst-1L is depicted for simplicity. All variants were created at the endogenous locus in lst-1 wild-type)V5; an otherwise wild-type allele carrying 3× V5 at its C terminus (Fig. 1E). Internal deletion boundaries (brackets). Middle, protein products. For amino acid changes in each variant, see Fig. S1A. Right, GSC maintenance was scored positive (+) if the vast majority (>90%) produced many progeny and negative (−) if all lacked GSCs. For result details, see Fig. S2A. Alleles: lst-1(wild-type)V5 is q1004; lst-1(α) is q869 and removes entire open reading frame plus 139 bp upstream of start codon and 228 bp downstream of stop codon; lst-1(1-210)V5 is q1115; lst-1(1-152)V5 is q1060; lst-1(211-328)V5 is q869; lst-1(153-328)V5 is q1119; and lst-1(ZnF)V5 is q1032 and harbors missense mutations in two amino acids that are crucial for zinc finger architecture (black asterisks) (Hashimoto et al., 2010; Weidmann et al., 2016).
Fig. 3. FBF-binding regions in LST-1 and their role in stem cell regulation. (A) KTXL motif identified in vitro for two FBF partner proteins: CPB-1 and GLD-3 (Menichelli et al., 2013; Wu et al., 2013). PDB ID for FBF is 3K5Q (Wang et al., 2009). (B) Two predicted FBF interaction motifs, A and B (light blue), in the LST-1L-specific sequence. Top: nucleotides encoding the A motif span an intron. Bottom: positions of A and B in LST-1L (see also Fig. S1A). (C) Yeast interaction between LST-1 and FBF-2. Red letters indicate missense mutations. Binding: strong (+++), weak (+) or none (−) (see Fig. S3 for data). (D) GSC maintenance scored in LST-1 A and B motif variant nematodes. For result details, see Fig. S4B. (E-G) Representative z-projected confocal images of extruded gonads immunostained using an anti-SP56 sperm antibody (red) (Ward et al., 1986) and DAPI (cyan). (E) lst-1(AB)V5 in a strain harboring wild-type SYGL-1. The germline and progenitor zone (white arrowhead, boundary) are both of normal size. (F) lst-1(AB)V5 in a strain lacking SYGL-1. The germline is tiny and has only a few sperm (red). Other DAPI-stained nuclei belong to the somatic gonad. (G) lst-1(ø) sygl-1(ø) mutants have tiny germlines and only a few sperm. (H) Quantitation and comparison of the number of germ cells (# GC) per animal. Total number of GCs in lst-1(AB)V5 sygl-1(ø) is indistinguishable from lst-1(ø) sygl-1(ø). (I,J) Representative single confocal z-slice from the middle plane of the distal region of an extruded gonad, stained with anti-V5 antibodies to detect LST-1 (magenta) and DAPI (cyan). Inset shows perinuclear staining (scale bar: 2 µm). (I) LST-1(wt)V5 protein is restricted to distal germline, with distribution similar to wild type (compare with I). (J) LST-1(AB)V5 protein is restricted to distal germline, as previously reported (Shin et al., 2017). (K) Quantitation of LST-1(wt)V5 and LST-1(AB)V5 protein as a function of distance from the distal end (by Fiji/ImageJ; see Materials and methods for details). Lines represent mean value of three independent replicates, each with at least seven gonads; shading shows s.e.m. Sample sizes: LST-1(wt)V5, 25 germlines; LST-1(AB)V5, 36 germlines. After background from wild-type N2 control was subtracted for each replicate, LST-1(wt)V5 was set to 1.0 at its peak and LST-1(AB)V5 was normalized to this value. The x-axis shows distance from distal end in μm at the bottom and germ cell diameters (gcd) at the top. (D-K) Alleles: lst-1(wt)V5 is q1004; lst-1(A)V5 is q1124; lst-1(B)V5 is q1086; lst-1(AB)V5 is q1125; lst-1(ø) is q869; and sygl-1(ø) is q828.
FBF-binding motif but found none. Instead, we found two similar sequences: KQLL (amino acids 32-35) and KLHL (amino acids 80-83), which we dub A and B potential motifs, respectively (Fig. 3B, Fig. S1A). Intriguingly, A and B reside in the region unique to LST-1L.

We first tested the A and B motifs for FBF binding in yeast (Fig. S3A). Because full-length, wild-type LST-1 bound to FBF-1 and FBF-2 similarly in a yeast two-hybrid assay (Shin et al., 2017), we focused on FBF-2 here. We first found that full-length LST-1(wt) and LST-1(1-152) interacted similarly with FBF-2 in yeast (Fig. S3B) and therefore used LST-1(1-152) for subsequent assays. To test the A and B sites, we made site-specific mutants, changing their first and fourth amino acids to alanine (Fig. 3C, Fig. S3B). Mutation of site A had no appreciable effect on the yeast interaction, mutation of site B reduced the interaction, and mutation of A and B abolished the interaction (Fig. 3C, Fig. S3B) both in yeast growth assays (Fig. S3C) and β-gal assays (Fig. S3D). We conclude that the LST-1 self-renewal fragment has two FBF interaction motifs, and that, at least in yeast, the B motif is quantitatively more important than the A motif.

To probe the significance of LST-1 A and B sites for self-renewal activity in nematodes, we engineered point mutations at the lst-1 endogenous locus, both individually and together (Fig. 3D). The nematode B motif mutation was identical to that made in yeast (K80A L83A), but the nucleotide sequence encoding the A motif straddles an intron (Fig. 3B, top), making simultaneous mutation of both K32 and L35 challenging. Because the leucine at the fourth position stood out as crucial for CPB-1 and GLD-3 interactions with FBF (Menichelli et al., 2013; Wu et al., 2013), we chose to disrupt L35 alone to test the importance of the A motif in nematodes.
We introduced mutations into \textit{lst-1(wt)}\textsuperscript{Z3} (Fig. 3D) and made three alleles, \textit{lst-1(A)}[L35A], \textit{lst-1(B)}[K80A L83A] and \textit{lst-1(AB)}[L35A K80A L83A]. All were fertile and healthy when SYGL-1 was present [Fig. 3D,E,H for \textit{lst-1(AB)}; Fig. S4B]. When SYGL-1 was removed, \textit{lst-1(A)} and \textit{lst-1(B)} mutants maintained GSCs and were fertile, but \textit{lst-1(AB)} did not maintain GSCs and was sterile (Fig. 3D, Fig. S4B). Indeed, the \textit{lst-1(AB)} \textit{sygl-1(a)} germline was indistinguishable from that of \textit{lst-1(a)} \textit{sygl-1(a)} (Fig. 3F-H); it was tiny and differentiated to sperm (Fig. 3D,F,H, Fig. S4B).

Importantly, \textit{lst-1(AB)} protein was present with an abundance and distribution similar to \textit{lst-1(wt)} (Fig. 3I-K). We conclude that \textit{LST-1} depends on its two KXXL FBF-binding motifs for self-renewal activity.

**The C-terminal region controls spatial restriction**

We next examined the \textit{lst-1} variants for more subtle effects on GSC maintenance. Specifically, we assayed size of the progenitor zone (PZ) in all variants (Fig. 4A), because PZ size is a rough measure of the switch from stem cell state to differentiation (Crittenden et al., 2006). All PZ measurements were carried out in the presence of wild-type SYGL-1 to ensure healthy germline size and organization. Most variants had a PZ size similar to wild type, but \textit{lst-1(1-210)} and \textit{lst-1(ZnF)} PZs were larger than normal (Fig. 4B).

We reasoned that longer PZs in \textit{lst-1(1-210)} and \textit{lst-1(ZnF)} might reflect aberrant \textit{LST-1} expression. To test this idea, we immunostained for \textit{LST-1}, again with wild-type SYGL-1 present [Fig. 3D,E,H for \textit{lst-1(AB)}; Fig. 3F-H].

LST-1(wt) was present with an abundance and controls its extent, likely through an effect on protein turnover (see Discussion).

The increased abundance of \textit{LST-1(1-210)} and \textit{LST-1(ZnF)} proteins could be due to increased \textit{lst-1} mRNA stability, to increased translation or to changes in \textit{LST-1} protein turnover. To quantify protein abundance as a function of position within the distal gonad, we generated \textit{lst-1(AB)} \textit{sygl-1(ø)} mutants. Extruded gonads were stained for \textit{LST-1} and \textit{SYGL-1} in situ hybridization (smFISH) with probes designed against sequences that were identical in the three variants tested, but absent in the control (Fig. 5A). The \textit{lst-1} RNAs in \textit{lst-1(wt)}, \textit{lst-1(1-210)} and \textit{lst-1(ZnF)} were all restricted to the distal-most four or five rows of germ cells and absent in the \textit{lst-1(a)} control (Fig. 5B-E). Therefore, expansion of \textit{LST-1} protein was not due to expansion of \textit{lst-1} RNA. Curiously, quantitation revealed that \textit{lst-1(1-210)} and \textit{lst-1(ZnF)} RNAs were modestly more abundant (∼30% higher) than \textit{lst-1(wt)} RNA (Fig. 5F,G), suggesting that the \textit{LST-1} zinc finger has an autoregulatory effect on \textit{lst-1} mRNA abundance. Together, these analyses indicate that the \textit{LST-1} C-terminal region mediates two distinct regulatory activities: the zinc finger downregulates \textit{lst-1} RNA abundance and a broader region, yet to be defined but including the zinc finger, downregulates \textit{LST-1} protein abundance and controls its extent, likely through an effect on protein turnover (see Discussion).
Spatial extent of LST-1 determines GSC pool size

Finally, we asked whether the increased PZ size in *lst-1(1-210)* and *lst-1(ZnF)* mutants reflects a shift in the regulatory network from self-renewal to differentiation. To this end, we conducted emb-30 assays (Fig. 6A) (Cinquin et al., 2010). Briefly, this assay blocks the cell cycle and stops migration through the progenitor zone so that germ cells reveal their naïve or differentiated state in situ. This is the only functional assay available for GSC pool size, and provides a rough size estimate. We focused on *lst-1(wt)* and *lst-1(1-210)* for this experiment, and tested both CRISPR-induced *lst-1* mutants (Fig. 2A,C) as well as single-copy transgenic variants inserted at a MosSCI site (Fig. 2B,C). While the *lst-1* fragments assayed were identical, two differences existed between these experiments, for historical reasons. First, the endogenous alleles were assayed in a *sygl-1(a)* background so that GSCs were dependent on *lst-1* alone, while transgenes were assayed in an *lst-1(a) sygl-1(+) background so that all LST-1 protein came from the transgenic allele. Second, the endogenous alleles were tagged with V5 while transgenes were tagged with HA. Remarkably, these two experiments gave virtually the same result: *lst-1(wt)* possessed an average of ∼40 germ cells in its GSC pool, whereas *lst-1(1-210)* had an average of ∼55-75 (Fig. 6B). Thus, *lst-1(1-210)* increases GSC pool size, suggesting that *lst-1* extent modulates size of the GSC pool.

To further interrogate the potency of the *lst-1(1-210)* protein for GSC maintenance, we assayed its effect when ubiquitously expressed. This assay was essentially the same as that carried out earlier with full-length LST-1(wt) protein, which makes a massive germine tumor when placed under control of a ubiquitous germine promoter, *mex-3* and the *tbb-2 3’ UTR* (Shin et al., 2017). To ask whether *lst-1(1-210)* is similarly oncogenic, we made an analogous transgene, placing LST-1(1-210) under the same regulatory elements (Fig. 6C). The strain was created and maintained with *lst-1(RNAi)* to prevent expression of the potentially oncogenic LST-1. Upon removal from *lst-1(RNAi)*, ubiquitous LST-1(1-210) drove formation of germine tumors, as evidenced by M-phase nuclear morphology and PH3-marked cells (Fig. 6D). We conclude that ubiquitous LST-1(1-210) mimics LST-1(wt) in its oncogenicity. This result dovetails with the more
DISCUSSION
C. elegans LST-1 provides a key link between niche signaling and an RNA regulatory network driving stem cell self-renewal (Kershner et al., 2014; Shin et al., 2017). Here, we report the molecular basis of the LST-1–FBF partnership, the significance of the partnership for self-renewal activity and the significance of LST-1 spatial restriction to GSC pool size.

Dual FBF-binding motifs may provide plasticity to the RNA regulatory network
The LST-1 self-renewal region harbors two short KXXL motifs that mediate FBF binding (Fig. 7A). Each motif has biological activity in nematodes: LST-1 self-renewal activity remains intact when either is mutated, but when both are mutated, self-renewal activity is lost. The discovery of two motifs was unexpected, because other FBF partners GLD-3 and CPB-1 possess only a single KXXL motif (Campbell et al., 2012; Menichelli et al., 2013; Wu et al., 2013). Yet the two LST-1 motifs are conserved throughout the Caenorhabditis (Fig. S4A), suggesting biological significance.

The existence of two KXXL motifs may afford plasticity to the LST-1–FBF complex and its role in the FBF regulatory network. We do not yet know where within FBF these motifs bind, but clues exist. The motifs in GLD-3 and CPB-1 interact in vitro at the loop between PUF repeats 7 and 8, dubbed the R7/8 loop (Menichelli et al., 2013; Wu et al., 2013). By analogy, the dual LST-1 motifs may each be able to bind the same loop. Indeed, a small purified fragment harboring one LST-1 motif, called in this work the B site, binds to the R7/8 loop in the crystal structure of an FBF-2/LST-1B-site/RNA complex (Qiu et al., 2019). However, the LST-1 A and B motifs are unlikely to bind the same site simultaneously, raising the possibility that these dual motifs provide other opportunities. For example, some LST-1–FBF complexes may rely on the A site binding to the R7/8 loop (Fig. 7B), while others rely on B site binding to the same loop (Fig. 7C). This scenario introduces the possibility of considerable plasticity in the regulation and molecular configuration of the two complexes. Alternatively, the dual motifs may bind at distinct sites in FBF (Fig. 7D). More radically, they may link two FBF proteins together, with LST-1 binding at each of their R7/8 loops. These various scenarios have important implications for configuration, stability and regulation of this critical LST-1–FBF partnership and thus for FBF combinatorial control of RNAs and stem cell self-renewal.
FBF-binding elements regulate poly(A) tail length (Ahringer and Kimble, 1991). Second, FBF, like other PUF proteins, interacts in vitro with the CCR4-Not deadenylase complex (Suh et al., 2009), suggesting that FBF represses RNAs, at least in part, via deadenylation. Third, LST-1 promotes destabilization of an FBF target mRNA in vivo (Shin et al., 2017), suggesting that it works with FBF to promote deadenylation. Indeed, yeast PuF3 (yPuF3), a PUF RNA-binding protein from S. cerevisiae, possesses an N-terminal tail composed largely of IDRs and critical for interactions with the CCR4-Not complex. Remarkably, the longer the yPuF3 tail, and hence the more IDRs, the greater the deadenylase activity in vitro (Webster et al., 2019) (Fig. 7F). We suggest that trans-acting LST-1 protein may work similarly to stabilize the interaction with an effector protein or complex (Fig. 7G). The CCR4-Not complex is a strong candidate because of its in vitro interaction with FBF (Suh et al., 2009). Other possibilities exist and they are not mutually exclusive. For example, LST-1 may prevent recruitment of a positive-acting regulatory factor, such as the GLD-2/GLD-3 poly(A) polymerase (Wang et al., 2002). Regardless, the discovery of an IDR-rich fragment that works in trans with an RNA-binding protein suggests new avenues for combinatorial control.

LST-1 downregulation and the molecular switch from stem cell state to differentiation

Normally, lst-1 mRNA and LST-1 protein are restricted to a germline region corresponding roughly to the GSC pool, but ubiquitous LST-1 expression drives formation of a germline tumor (Kershner et al., 2014; Shin et al., 2017; this work). Therefore, spatial distribution of LST-1 must be highly regulated. Here, we report one key aspect of that spatial regulation. Two LST-1 variants, LST-1(1-210) and LST-1(ZnF), both lacking a functional zinc finger, are more abundant and expand more proximally than normal. Moreover, both variants increase the size of the progenitor zone (Fig. 4B) and LST-1(1-210) increases the size of the GSC pool (Fig. 6B,D). Our interpretation is that downregulation of LST-1 protein is essential for proper cell fate determination and the switch between self-renewal and differentiation (Fig. 6E).

The primary mechanism of LST-1 downregulation is likely regulated protein instability. RNAs encoding LST-1(1-210) and LST-1(ZnF) were restricted spatially as in wild type, but the proteins were dramatically expanded (Fig. 5G). Germ cells move proximally at a rate of about 0.5 to 1 cell per hour in the progenitor zone (Rusu and Cohen-Fix, 2017), which provides a useful space-time axis. Wild-type lst-1 RNA and LST-1 protein disappear at about the same position along this axis (Shin et al., 2017; this work), suggesting a tight and precise regulation with RNA and protein turned over about the same time. By contrast, LST-1(1-210) and LST-1(ZnF) proteins persist for 10-20 cells rows further proximally than their RNA, and thus protein turnover is delayed up to 20 h. This change in protein turnover was particularly noticeable with LST-1(1-210). Therefore, loss of the zinc finger is likely to affect LST-1 stability, but loss of the C-terminal third is more dramatic. Earlier studies found that decreased proteasome activity leads to increased germline proliferation (Gupta et al., 2015; Macdonald et al., 2008; Mohammad et al., 2018). However, identification of the crucial E3 ligase or ligases for LST-1 protein turnover remains a challenge for the future.

Regulation of protein stability as a determining factor in the fate switch from stem cell to differentiation is likely a broadly used mechanism (Werner et al., 2017). Although few cases are thoroughly understood, examples exist in flies and human cells in addition to nematodes. In flies, cyclin A protein is downregulated by the Bam-dependent deubiquitinase complex to promote differentiation (Ji et al., 2017); in human embryonic stem cells, Nanog is downregulated by ERK MAP kinase to promote differentiation (Kim et al., 2014). As more examples are uncovered, the regulation of protein stability may emerge as a ubiquitous mechanism for triggering fate switches.

LST-1 and its role in FBF combinatorial control of stem cell regulation

We have found that LST-1 partnership with the FBF RNA-binding protein is pivotal to GSC self-renewal. This remarkable protein therefore provides an important new window into FBF combinatorial control of stem cell regulation. Indeed, two FBF partners, LST-1 and SYGL-1, drive GSC self-renewal (Shin et al., 2017; this work). Each partner is sufficient, and at least one must be present to maintain stem cells (Kershner et al., 2014). Intriguingly, both full-length SYGL-1 protein and the LST-1(1-210) self-renewal fragment consist largely of IDRs and are of comparable size. The SYGL-1 protein possesses one KXXL motif, although its significance has not yet been tested. Nonetheless, we suggest that LST-1 and SYGL-1 are both trans-acting FBF partners that bring IDRs to their respective complexes. Analogous short, trans-acting RNA regulators may be more common than appreciated.

The existence of two IDR-rich FBF partners might simply reflect redundancy but also might have a more interesting role and expand the FBF repertoire for combinatorial control. Their functional redundancy is well established (Kershner et al., 2014), but in favor of individual roles, the LST-1 and SYGL-1 amino acid sequences bear no similarity to each other, and some genetic interactions differ between the two (Brenner and Schedl, 2016; Shin et al., 2017). Moreover, LST-1 localizes to perinuclear granules while SYGL-1 localizes to smaller cytoplasmic puncta, and spatial regulation of LST-1 is tighter than SYGL-1 (Shin et al., 2017). We therefore suggest that additional layers of regulation remain to be discovered. Accordingly, we note that stem cell maintenance must proceed under widely divergent physiological and environmental circumstances. Study of the factors that orchestrate responses to those circumstances, likely including LST-1 and SYGL-1, provide a tantalizing entrée to the complexities of regulation in metazoans.

MATERIALS AND METHODS

Nematode strains and maintenance
C. elegans were maintained at 20°C on Nematode Growth Medium (NGM) plates spotted with E. coli OP50, following established protocols (Brenner, 1974), except that strains containing emb-30(m377ts) were maintained at 15°C and the strain containing the qSi291 transgene was maintained on lst-1(RNAi) plates (see germline tumor assays section). Wild-type was N2 Bristol strain. See Table S1 for list of strains used in this study. We also used the balanced LGI; LGIII hT2[qls48] (Siegfried and Kimble, 2002).

CRISPR/Cas9 genome editing to generate lst-1 alleles
See Table S2 for list of CRISPR-induced alleles, and Tables S4 and S5 for additional details about their generation. We used two CRISPR/Cas9 editing methods to create alleles at the endogenous lst-1 locus. Three alleles, lst-1(q869), lst-1(q867), and lst-1(q869) and lst-1(q296), were generated using a DNA-based CRISPR/Cas9 approach with a co-conversion strategy (Arribere et al., 2014; Dickinson et al., 2013). Briefly, the following components were microinjected into wild-type germlines: an lst-1 sgRNA plasmid (25 ng/µl), a repair oligo designed to incorporate the desired lst-1 mutations (500 nM) and a plasmid encoding Cas9 (pPD162, 50 ng/µl) (Dickinson et al., 2013) along with a dpy-10 sgRNA (pJAS8, 10 ng/µl) and repair oligo targeting the dpy-10 locus (AF-ZF-827, 500 nM) (Arribere et al., 2014). Progeny of injected hermaphrodites were visually screened for co-injection marker editing and subsequently screened by PCR and Sanger sequencing for editing at the lst-1 locus.
Other alleles, lst-1(q895), lst-1(q1032), lst-1(q1044), lst-1(q1060), lst-1(q1086), lst-1(q1115), lst-1(q1119), lst-1(q124), lst-1(q125) and lst-1(q1198), were generated using RNA-protein complex CRISPR/Cas9 editing with a co-conversion strategy (Arribere et al., 2014; Paix et al., 2015). The following were microinjected into wild-type N2 (for q895), JK6154 (for q1125), JK5596 (for q1198) or JK5929 [ls1-1(q1004)], which we call lst-1[w3] for simplicity [all other alleles]: lst-1 crRNAs (10 µM), dpy-10 or unc-58 co-CRISPR crRNAs (4 µM) and tracrRNA (13.6 µM) (all Alt-R from Integrated DNA Technologies); repair oligos encoding the desired lst-1 mutation (4 µM) and targeting the respective co-CRISPR locus (1.34 µM); and recombinant Cas9 protein (24.5 µM). Progeny of injected hermaphrodites were first visually screened for co-injection marker editing (Alt-R from Integrated DNA Technologies); repair plasmids containing the gene of interest flanked by sequence targeting the tti5605 insertion site were cloned using the Gibson assembly method (Gibson et al., 2009). The repair plasmids were microinjected at 50 ng/µl together with Mos1 transposase and co-injection marker plasmids into JK9450. At least three successful insertions were isolated and analyzed in our experiments, and we report one representative line in this work. During strain generation and maintenance, lst-1 [Si290] [F<sub>max</sub>:'-lst-1(1-210)-:GSGSG linker::3xFLAG::tbb-2 3' UTR, unc-119[+]+] and related strains were grown on lst-1[RNAi] feeding bacteria to prevent germline tumorigenesis (see RNA interference).

RNA interference
RNA interference (RNAi) was performed by feeding as described previously (Timmons and Fire, 1998). We used sygl-1 or lst-1 clones from the Ahringer RNAi library (Fraser et al., 2000) and L4440 plasmid lacking a gene of interest flanked by sequence targeting the tti5605 insertion site were cloned using the Gibson assembly method (Gibson et al., 2009). The repair plasmids were microinjected at 50 ng/µl together with Mos1 transposase and co-injection marker plasmids into JK9450. At least three successful insertions were isolated and analyzed in our experiments, and we report one representative line in this work. During strain generation and maintenance, lst-1 [Si290] [F<sub>max</sub>:'-lst-1(1-210)-:GSGSG linker::3xFLAG::tbb-2 3' UTR, unc-119[+]+] and related strains were grown on lst-1[RNAi] feeding bacteria to prevent germline tumorigenesis (see RNA interference).

DNA staining
To visualize nuclear morphology, we stained extruded gonads with DAPI (4',6-diamidino-2-phenylindole) as described previously (Crittenden et al., 2017), with minor modifications. Briefly, we dissected animals in PBSst [PBS+0.1% (v/v) Tween-20] with 0.25 mM levamisole to extrude gonads, then fixed at room temperature for at least 15 min in ~2% (v/v) paraformaldehyde diluted in PBSst. Samples were incubated overnight in ~2% (v/v) paraformaldehyde diluted in PBSst. Samples were incubated overnight in ~20°C methanol, washed with PBSst, then incubated with 0.5 ng/µl DAPI in PBSst to stain DNA. We mounted in either Vectashield (Vector Laboratories) or ProLong Gold (Thermo Fisher Scientific).

GSC maintenance and masculinization assays
For Figs 1E,F, 2, 3D, Figs S1B, S2A, S2E, S4B, mid-L4 hermaphrodites were placed on NGM plates at 20°C. After 3-4 days, their F1 progeny were assayed for embryo production, which requires a functional germline. All fertile animals made many embryos and young larvae and were scored positive for GSC maintenance. Sterile animals were analyzed further with DAPI staining and compound microscopy. Two types of steriles were found: Mog (for Masculinization of Germline) and Glp (Germline proliferation defective). Mog germelines had a roughly normal size, harbored mitotically dividing GSCs in the distal germline, but made only sperm (no oocytes); Mogs were scored positive for GSC maintenance. Glp steriles had a very small germline made of only a few sperm. In Glp animals, we counted sperm number after DAPI staining and divided by four to estimate germ cell number. We removed sygl-1 in some cases by feeding RNAi and in others by crossing into a sygl-1 loss-of-function or null mutant. For RNAi, strains were plated onto sygl-1[RNAi] plates as mid-L4 hermaphrodites at 20°C and their F1 progeny were scored for GSC maintenance as described. In the case of Glp germelines, we quantitated the number of germ cells by DAPI staining, counting the number of mature sperm, and dividing by four (since one germ cell differentiates into four sperm).

Progenitor zone size
Progenitor zone (PZ) size was assessed in nematodes staged to 24 h past mid-L4 at 20°C. Extruded gonads were DAPI stained and imaged with a confocal microscope (see Microscopy). We examined nuclear morphology to determine PZ size, according to convention (Crittenden et al., 2006; Seidel and Kimble, 2015). Briefly, when germ cells exit the PZ and begin meiotic prophase, their nuclear morphology takes on a distinctive crescent shape (see Fig. 4A). We selected a central focal plane in the distal gonad and then counted the number of cells along each edge of the tissue until we reached the distal-most cell with crescent morphology. We counted manually using the FIJI/ImageJ multi-point tool, calling each DAPI-stained nucleus a unique cell row. We averaged the two values from the two edges of the gonad together to determine PZ size.

Immunostaining
We performed immunostaining of extruded gonads as described previously (Crittenden et al., 2017) with minor modifications. All strains (except the strain containing the qSi297 tumor transgene, see Germline Tumor Assays section) were grown at 20°C and staged to 24 h past mid-L4 stage, then dissected in PBSst with 0.25 mM levamisole to extrude gonads. Tissue was fixed in 2.5% (w/v) paraformaldehyde diluted in PBSst for 10 min, then permeabilized with PBSst+0.2% (v/v) Triton-X for 10-15 min. Samples were blocked for at least 1 h and not more than 4 h in 0.5% (w/v) bovine serum albumin diluted in PBSst, except o-FLAG which was blocked in 30% (v/v) goat serum diluted in PBSst. Next, samples were incubated overnight at 4°C with primary antibodies diluted in blocking solution as follows: mouse anti-FLAG (M2, 1:1000, F1804-1MG, Millipore Sigma), rabbit anti-GLD-1 (1:100, a gift from E. Goodwin, University of Wisconsin, Madison, USA); mouse anti-phospho-histone H3 (Ser10) (6G3, 1:200, 9706L, Cell Signaling Technology), mouse anti-V5 (SV5-Pk1, 1:1000, MCA1360, Bio-Rad) and mouse anti-SP56 (1:200, a gift from Susan Strome, University of California, Santa Cruz, CA, USA). Secondary antibodies were diluted in blocking solution and incubated with samples for at least 1 h and not more than 4 h as follows: Alexa 488 donkey anti-mouse (1:1000, A21202, Thermo Fisher Scientific), Alexa 647 goat anti-rabbit (1:1000, A21245, Thermo Fisher Scientific). To visualize DNA, DAPI was included at a final concentration of 0.3-1 ng/µl during a final PBSst wash performed after secondary antibody incubation. Samples were mounted in ProLong Gold (Thermo Fisher Scientific) and allowed to cure overnight before imaging. All steps were performed at room temperature unless otherwise indicated.

smFISH
Single molecule fluorescence in situ hybridization (smFISH) (Raj et al., 2008; Voronina et al., 2012) was performed as described previously (Lee et al., 2016). Custom Stellaris FISH probes were designed using the Stellaris Probe Designer Tool (Biosearch Technologies). The lst-1 probe set contains 40 probes targeting the 5'UTR of lst-1L, the coding sequence for amino acids 1-210 and the 3'UTR. Probes were labeled with CAL Fluor Red 610 and used at a final concentration of 0.25 µM. Probe sequences are provided in Table S6.

emb-30 assay
The assay was performed as previously described (Cinquin et al., 2010; Shin et al., 2017) with minor modifications. Briefly, strains were maintained in a programmable incubator at 15°C until 36 h beyond L4, then transitioned to 25°C for an additional 12 h. Gonads were extruded, fixed and stained using anti-PH3 and -GLD-1 antibodies, and DAPI (see Immunostaining). We imagined gonads by confocal microscopy (see Microscopy). To analyze the images, we used the DAPI channel to determine the ‘M-phase boundary’.
between presence and absence of arrested M-phase cells. In cases where a single M-phase cell was found more than three cell rows proximal to all other M-phase cells, that cell was disregarded for determining the boundary. We counted all cells distal to the M-phase boundary, including arrested M-phase cells and cells likely still arrested but not with a typical M-phase morphology, using the multipoint tool in Fiji/ImageJ (Scheidentrüppel et al., 2012). Germelines with excessively fragmented distal nuclei were excluded from the counts as cell numbers could not be determined (20-60% per experiment).

**Germine tumors assay**

To induce ubiquitous expression of LST-1(1-210) using the qS29I tumor transgene, L4 P0 animals were transferred from *lst-1* RNAi bacteria to OP50-seeded NGM plates. Experiments were performed at 15°C to maximize tumor penetrance (Shin et al., 2017). After removal from RNAi, subsequent generations were assayed under a dissection microscope and showed increasing tumor penetrance (n>100 for all): in F1, we observed no animals with germine tumors; in F2, ~60% had tumors; in F3, ~90% had tumors. For Fig. 6D, we dissected and stained F3 generation L4 staged animals.

**Microscopy**

All gonad images were taken using a laser scanning Leica TCS SP8 confocal microscope fitted with Photomultiplier (PMT) and Hybrid (HyD) detectors, and run with LAS software version 3.3.1 or X. A 63×/1.40 CS2 HC Plan Apochromat oil immersion objective was used. All images were taken using the standard scanner with 400-700 Hz scanning speed and 100-300% zoom. To prepare figures, Adobe Photoshop was used to equivalently and linearly adjust contrast among samples to be compared.

**Fluorescence quantitation**

Immunostaining quantitation in Figs 3K and 4H was performed using Fiji/ImageJ (Schindelin et al., 2012) with images taken under identical conditions across all samples. In Fig. 3K, we performed three independent experiments consisting of at least seven gonads per genotype for a total of at least 21 gonads per genotype. In Fig. 4H, we performed at least three independent experiments consisting of at least five gonads per genotype, with a total of at least 23 gonads per genotype. To collect intensity data from our images, we adapted a workflow from the literature (Brenner and Schedl, 2016). First, the sum of all z-slices for each gonad was projected onto a single plane. A freehand line, 50 pixels wide and at least 80 µm long that bisected the gonad, was drawn manually starting from the distal tip of the tissue. Next, the pixel intensity data for the V5 channel along the line was obtained using the Plot Profile feature. We averaged the raw pixel intensity at every x value to generate an average protein expression plot for each genotype in a given experiment. Next, to adjust for non-specific background staining, we subtracted the average intensity of the respective negative control from the average expression curves at each x value. We then normalized each average expression curve using the maximum and minimum values of the respective average wild-type [5*st-l (wt+)] plot. Finally, to generate the plots shown, the adjusted (background subtracted and normalized) protein expression plots for each genotype were averaged among at least three experiments. Standard error at each x value was calculated among the three independent replicates for each genotype. The number of germ cell diameters (gcd) along the x-axis were calculated using a conversion factor of 4.4 gcd/µm (Lee et al., 2016).

smFISH quantitation in Fig. 5F was performed similarly to the immunostaining quantitation described above, with minor modifications. After z-projection, an average gonad-specific background level was also collected and subtracted from the raw values. This was carried out by using the rectangle tool to create a 2 µm square box that was manually placed on the image where no transcripts could be seen by eye. This was repeated for three separate locations along the gonad: one distally within 50 µm of the distal tip, one centrally between 50-100 µm from the distal tip and one proximally between 100-150 µm from the distal tip. For each location, the measure feature was used to collect the average pixel intensity within the 2×2 µm box. The values obtained for each location were then averaged together to yield the final background value for the individual gonad. This gonad-specific background value was subtracted from the raw values of the respective gonad and we proceeded with quantitation as described above (i.e. plot profile, averaging, background subtraction and normalization). We performed three independent experiments consisting of at least nine gonads per genotype for a total of 30 gonads per genotype analyzed. To compare between datasets that were collected using different zoom factors, we condensed each average RNA expression plot by calculating a rolling average of either four or five x- and y-values. After adjustment, the respective x-values across all data sets were essentially equal and differed by no more than 0.02 µm. For smFISH, the genotype of the negative control was *lst-1* (q869), which harbors a deletion in the *lst-1* locus spanning from 139 bp upstream of the start codon to 228 bp downstream of the stop codon. Of note, five of the 40 smFISH probes used were predicted to anneal in the *lst-1* (neo) negative control.

**Yeasted two-hybrid**

Modified yeast two-hybrid assays were performed as described previously (Bartel and Fields, 1997). Briefly, LST-1 variants were amplified from cDNA and cloned into the Gal4 activation domain plasmid pACT2 using the Gibson assembly method (Gibson, 2009). We also used plasmid pJK2017, which is FBF-2 cDNA (amino acids 121-632) fused to the LexA-binding domain in the pBTM116 backbone (Shin et al., 2017). Activation and binding domain plasmid pairs were co-transformed into L40-ura3 strain [MATa, ura-3, 52, leu2-3,112, his3Δ200, trp1Δ1, ade2, LYS2::(LexA-op)–His3, ura3::(LexA-op)–LacZ] using the LiOAc method (Gietz and Schiestl, 2007). His3 reporter activity was assayed on synthetic defined medium –Leu–Trp–His plates supplemented with varying concentrations of 3-amino-1,2,4-triazole (3-AT) (Millipore Sigma) and compared with –Leu–Trp plates as controls. We measured LacZ reporter activity using the Beta-Glo Assay System following the commercially available protocols and the yeast literature (Promega) (Hook et al., 2005) and luminescence was quantitated using a Biotek Synergy H4 Hybrid plate reader with Gen5 software. A complete list of plasmids used in yeast two-hybrid assays is available in Table S5.

**Western blots**

For Fig. 1I, samples were prepared by boiling ~50 untagged adult worms in sample buffer [60 mM Tris (pH 6.8), 25% glycerol, 2% SDS, 0.1% bromophenol blue with 700 mM β-mercaptoethanol]. For Fig. 3E, we grew yeast transformants in –Leu–Trp liquid media and prepared samples by boiling yeast in sample buffer. Subsequent analysis was conducted on a 12% SDS-PAGE gel and blots were probed with either mouse anti-V5 (SV5-Pk1, 1:1000, MCA1360, Bio-Rad), mouse anti-HA (HA.11, 1:1000, MMS-101P, Covance) or mouse anti-actin (C4, 1:40,000, MAB1501, Millipore Sigma) followed by donkey anti-mouse horseradish peroxidase (1:10,000, 715-035-150, Jackson ImmunoResearch). Immunoblots were developed using SuperSignal West Pico Femto Sensitivity substrate (Thermo Fisher Scientific) and developed using a Konica Minolta SX-101A medical film processor. For final figure preparations, contrast of the blot was linearly adjusted in Adobe Photoshop. For Fig. 1I, Fiji/ImageJ was used for quantitation.

**Statistics**

Where appropriate, statistical analyses are described in figure legends. Homogeneity of variance was established using Levine’s test. Where appropriate, statistical analyses are described in figure legends. Where appropriate, statistical analyses are described in figure legends. Where appropriate, statistical analyses are described in figure legends.
Competing interests
The authors declare no competing or financial interests.

Author contributions


in Caenorhabditis spermatogenic or oogenic germlines. *G3* (Bethesda) 9, 153-165. doi:10.1534/g3.118.200300


