The Puf RNA-binding proteins FBF-1 and FBF-2 inhibit the expression of synaptonemal complex proteins in germline stem cells

Christopher Merritt and Geraldine Seydoux*

SUMMARY

FBF-1 and FBF-2 (collectively FBF) are two nearly identical Puf-domain RNA-binding proteins that regulate the switch from mitosis to meiosis in the C. elegans germline. In germline stem cells, FBF prevents premature meiotic entry by inhibiting the expression of meiotic regulators, such as the RNA-binding protein GLD-1. Here, we demonstrate that FBF also directly inhibits the expression of structural components of meiotic chromosomes. HIM-3, HTP-1, HTP-2, SYP-2 and SYP-3 are components of the synaptonemal complex (SC) that forms between homologous chromosomes during meiotic prophase. In wild-type germlines, the five SC proteins are expressed shortly before meiotic entry. This pattern depends on FBF binding sites in the 3' UTRs of the SC mRNAs. In the absence of FBF or the FBF binding sites, SC proteins are expressed precociously in germline stem cells and their precursors. SC proteins aggregate and SC formation fails at meiotic entry. Precocious SC protein expression is observed even when meiotic entry is delayed in fbf mutants by reducing GLD-1. We propose that parallel regulation by FBF ensures that in wild-type gonads, meiotic entry is coordinated with just-in-time synthesis of synaptonemal proteins.

KEY WORDS: Germ cells, Meiosis, Post-transcriptional regulation, C. elegans

INTRODUCTION

A crucial step in the development of the germline is the transition from an undifferentiated germ cell that divides by mitosis to a differentiating germ cell that is ready to begin meiosis. In animals, this transition often takes place via an intermediate cell type that still divides by mitosis but has begun to express factors required for meiosis. For example, in C. elegans a group of ~220 mitotic germ cells are maintained throughout life at the distal end of each gonad (‘mitotic zone’, Fig. 1A) (Byrd and Kimble, 2009; Hubbard, 2007). The progeny of these cells differentiate in a distal-to-proximal gradient along the length of the gonad. The mitotic zone contains two cell types (Fig. 1A): distal-most cells, including the germline stem cells, which remain undifferentiated throughout the life of the animal; and proximal cells, which begin to express meiotic genes and are likely to include transit-amplifying cells and cells in meiotic S phase (Cinquin et al., 2010; Hubbard, 2007). Upon exit from the mitotic zone, cells initiate the chromosome dynamics required for meiotic pairing and synopsis (‘transition zone’, Fig. 1A). In preparation for this transition, proximal cells in the mitotic zone activate the expression of both regulators of meiotic entry (e.g. the RNA-binding protein GLD-1) and chromosomal proteins required for synopsis (e.g. HIM-3) (Hansen et al., 2004). The mechanisms that coordinate meiotic entry with the synthesis of meiotic chromosomal proteins are not known and are the focus of this study.

Meiotic entry is regulated by a complex network of RNA-binding proteins (Byrd and Kimble, 2009). Central to the network are FBF-1 and FBF-2, two highly similar Puf-domain RNA-binding proteins known collectively as FBF (Crittenden et al., 2002). FBF prevents premature meiotic entry in the mitotic zone at least in part by inhibiting the expression of GLD-1 (Crittenden et al., 2002). FBF and GLD-1 are expressed in roughly reciprocal patterns, with high FBF/low GLD-1 distally and low FBF/high GLD-1 proximally (Crittenden et al., 2002; Lamont et al., 2004) (Fig. 1A). FBF inhibits GLD-1 expression in distal cells via the gld-1 3' UTR, which contains two predicted FBF-1 binding sites (Crittenden et al., 2002). A reporter containing the gld-1 3' UTR reproduces the GLD-1 protein expression pattern (Merritt et al., 2008). Mutations that eliminate FBF-1 binding in vitro (Crittenden et al., 2002) cause the gld-1 reporter to be expressed at an evenly high level throughout the mitotic zone (Merritt et al., 2008). Reducing the dose of GLD-1 by half is sufficient to maintain a mitotic zone in fbf mutants, consistent with GLD-1 promoting premature meiotic entry in the absence of FBF (Crittenden et al., 2002).

What regulates the expression of meiotic chromosomal proteins is not known. In a survey of gene expression in the germline (Merritt et al., 2008), we found that the him-3 3' UTR blocks expression in distal cells in a pattern similar to that observed with the gld-1 3' UTR (Fig. 1D). HIM-3 is a component of the synaptonemal complex that forms between homologous chromosomes upon entry into meiosis (Zetka et al., 1999). In this study, we demonstrate that HIM-3 and four other synaptonemal proteins are regulated by FBF. Our findings suggest that parallel regulation by FBF coordinates meiotic entry with the timely production of meiotic chromosomal proteins.

MATERIALS AND METHODS

Nematode strains

C. elegans strains (Table 1) were maintained using standard procedures (Brenner, 1974).

Transgene construction and transformation

Transgenes were constructed using the Multisite Gateway cloning system (Invitrogen) as described (Merritt et al., 2008). See Table 1 and Table S1 in the supplementary material for lists of plasmids and oligos used in this...
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### Table 1. Continued

#### 3' UTR fusions

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#### Heat-shock fusions

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*3' UTR fusions with mutations in predicted FBF-1 binding site elements are named M1 and/or M2 (where M1 is the 5'–most element).
study. 3’ UTR reporters contain the pie-1 promoter (5’ entry, pCG142), GFP::histone H2B (middle entry, pCM1.35) and gene-specific 3’ UTRs (Table 1). Heat-shock fusions contain the hsp16-41 heat shock promoter (5’ entry, pCM1.55), GFP (middle entry, pCM1.53) and gene-specific ORF:3’ UTRs (3’ entry, Table 1). ORF fusions contain gene-specific promoters (5’ entry, Table 1), GFP (middle entry, pCM1.53) and gene-specific ORF:3’ UTRs (3’ entry, Table 1). QuikChange site-directed mutagenesis (Stratagene) or PCR fusion was used to create FBE mutations in 3’ entry clones (for oligos used, see Table S1 in the supplementary material). FBE sites were mutated from UGURHHAU to acacRHHHAU as described (Merritt et al., 2008). All transgenes contain an unc-119 rescue fragment and were transformed into unc-119(e3) worms by microparticle bombardment as described (Merritt et al., 2008; Pruits et al., 2001).

Motif search
Motifs overrepresented in the him-3, htp-1, htp-2, sup-2 and sup-3 3’ UTRs of C. elegans, C. briggsae, C. remanei and C. bremieri were identified using Multiple Em for Motif Elicitation (MEME, http://meme.sdsc.edu) (Bailey and Elkan, 1994). Search settings were: search of given strand only; motif width, 6-50; number of motifs per sequence, any number; maximum number of motifs to find, five. The search yielded one highly significant and highly represented motif (Fig. 2A; E-value=9.5×10−23, sites=26) and four less significant and rarer motifs (E-value=2.8×10−4, sites=4; E-value=15, sites=7; E-value=74, sites=3; E-value=130, sites=4).

Staining of dissected gonads
Immunostaining was performed as described (Couteau et al., 2004). Synchronized htf-1/2/miRNA and htf-1/2 L1 larvae were grown until the L4 stage, dissected and stained on the same slides. Primary antibodies used were: rabbit anti-HIM-3 at 1:200 (from Monique Zetka et al., 1999) and rabbit anti-HTP-1/2 at 1:200 (from Abby Dernburg et al., 2008). Cy3-goat anti-rabbit (Jackson ImmunoResearch) was used as secondary antibody. FITC-conjugated anti-phospho-histone H3 (Ser10), clone 3H10 (Upstate Cell Signaling Solutions), was used at 1:50 to mark mitotic germ cells.

RNA immunoprecipitation (IP) and immunoblots
Worm lysate preparation and IPs were performed as described (Cheeseman et al., 2004), with the following modifications. Beads were washed with IP buffer (50 mM Hepes, 1 mM EGTA, 150 mM KCl, 10% glycerol, 0.05% NP40, pH 7.4) containing protease inhibitors and 0.5% SDS-PAGE loading buffer. A detailed IP protocol is available upon request.

Primary antibodies for western blots were Living Colors anti-GFP A. v. monoclonal antibody (JL-8) (Clontech) at 1:1000, and anti-α-tubulin monoclonal antibody produced in mouse (clone DM1A) (Sigma) used at 1:1000. Secondary antibodies used for western blots were HRP-goat anti-mouse IgG1a and IgG2a (Jackson ImmunoResearch), both used at 1:5000.

Quantitative RT-PCR (qPCR)
Bound RNA from IPs was extracted in TRIZOL, eluted in water, treated with Turbo DNase (Ambion), and converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed using SYBR Green and an iCycler thermocycler (Bio-Rad) with the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). PCR primer pairs were designed with at least one primer spanning an exon junction and PCR primer pair efficiencies were determined for use in the final quantification of fold change. PCR primers were also tested to assure that no primer dimers were present in the qPCR reaction. Each qPCR reaction contained: 8.7 μl water, 10 μl 2× iQ SYBR Green Supermix (Bio-Rad), 0.4 μl sense primer at 20 μM, 0.4 μl antisense primer at 20 μM, and 0.5 μl of cDNA (this allowed for at least 80 qPCR reactions per IP). Cycling protocol was: 95°C for 5 minutes (denaturation); 95°C for 15 seconds, 60°C for 1 minute (46 times, amplification and quantification); 95°C for 1 minute, 55°C for 1 minute; 55-95°C for 15 seconds (81 times, melting curve); 4°C hold (cooling). For all qPCR reactions, melting curves of final PCR products were determined to ensure a single product and no primer dimers. All qPCR reactions were performed in triplicate. Ct values were determined with iQ5 Optical System Software (Bio-Rad) and fold change was determined using Pfaffl’s method (Pfaffl, 2001): Etarget=ACt target (anti-GFP IP-IgG IP)/Eactin (anti-GFP IP-IgG IP). Error bars represent standard error.

RNAi
RNAi was performed by feeding as described (Merritt et al., 2008), using the empty feeding L4440 (a gift from Andy Fire, available at www.addgene.org) as a negative control. htf-1/2, mex-3, him-3 and sup-2 RNAi feeding constructs were obtained from the Ahringer RNAi Feeding Library (Kamath et al., 2003). Worms were fed RNAi bacteria on NGM plates containing 1 mM IPTG and 100 μg/ml ampicillin. For RNAi of adults with 3’ UTR reporters (Fig. S3A and see Fig. S2 and Table S2 in the supplementary material), L4 larvae were fed for either 12 hours (htf-1/2) or 18 hours (L4440 and mex-3). Hermaphrodites treated for only 12 hours with htf-1/2 RNAi retain a mitotic zone (Merritt et al., 2008). For htf-1/2 and gld-1 RNAi of adults with GFP::ORF fusions, L1 larvae were fed for 60 hours or longer (to adult stage) (Fig 6B and see Fig. 5AB in the supplementary material). For htf-1/2 RNAi feeding of L2 and L4 stage animals (Fig. 3B, Fig. 5AB, Fig. 6A and see Fig. S3 in the supplementary material), L1 larvae were fed for 24 hours (to L2 stage) or for 40 hours (L4 stage).

Microscopy
For whole adult gonads (see Fig. S1 in the supplementary material), images were acquired at 400× with a CoolSNAP HQ digital camera (Photometrics) attached to a Zeiss AX10 microscope. Images were acquired and normalized linearly with IPLab software (Scanalytics). All other images were acquired at 1000× (except 1000× for Fig. 6A) with a Cascade QuantEM:512 SC camera attached to a CSUX-A1 spinning-disc confocal system (Yokogawa Electric) mounted to a Zeiss imager Z1. An L23 405-50 mW, 491-50 mW, 561-50 mW LaserStack laser system (Intelligent Imaging Innovations) was used for illumination. Images were acquired and normalized linearly with Slidebook (Intelligent Imaging Innovations). Images were taken in a single focal plane for GFP::histone H2B strains and somatic heat-shock GFP::ORF strains (Fig. 1, Fig. 3, Fig. 5C and see Figs S1-S3 in the supplementary material). Images were taken in a single focal plane (Fig. 6B) or as collapsed z-stacks for germline GFP::ORF strains and antibody staining (Fig. 5AB, Fig. 6A and see Fig. S4B in the supplementary material). Normalization for all images was performed with the intensity of the non-worm background area set to black and the brightest germline GFP signal set to white. Normalized images were imported and saved as .tiff files in Photoshop CS2 (Adobe).

RESULTS

3’ UTRs from htp-1, htp-2, sup-2 and sup-3 are sufficient to downregulate gene expression in germline progenitors
To determine whether meiotic proteins depend on 3’ UTR sequences for regulation in the mitotic zone, we screened the 3’ UTRs from 15 C. elegans genes that encode proteins implicated in early meiotic chromosome dynamics (Fig. 1B). Each 3’ UTR was cloned downstream of a GFP::histone H2B germline reporter and transformed into worms by microparticle bombardment (see Materials and methods) (Fig. 1C). Four 3’ UTRs (htp-1, htp-2, sup-2, sup-3) strongly blocked reporter expression in distal-most cells (Fig. 1B). As with the him-3 3’ UTR, expression was first detected in the proximal half of the mitotic zone and reached peak levels in the transition zone (meiotic entry) (Fig. 1D). All other 3’ UTRs allowed some reporter expression throughout the mitotic zone, including the distal-most cells (Fig. 1B). Consistent with these findings, HIM-3, HTP-1/2, SUP-2 and SUP-3 proteins have been reported to be absent from distal-most cells, whereas HTP-3, RAD-51, ZIM-1, ZIM-2, ZIM-3, REC-8 and COH-1 have been detected
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throughout the mitotic zone (Colaiacovo et al., 2003; Goodyer et al., 2008; Pasierbek et al., 2001; Phillips and Dernburg, 2006). The only exceptions were RAD-50 and MSH-5 (no antibody staining reported) and SYP-1 and ZHP-3, SYP-1 and ZHP-3 are absent from distal-most cells (Bhalla et al., 2008; MacQueen et al., 2002), but showed an even level of expression throughout the mitotic zone in our reporter assay. The syp-1 3’ UTR reporter was expressed at a moderate level in the mitotic zone and was upregulated in mid-pachytene (see Fig. S1 in the supplementary material), raising the possibility that this 3’ UTR is at least partially inhibited in distal germ cells (see Discussion).

We conclude that although 3’ UTR regulation may not be sufficient to account for the distribution of all meiotic proteins, a subset of meiotic 3’ UTRs is sufficient to suppress expression in the mitotic zone. Interestingly, all five proteins in this subset are components of the synaptonemal complex (SC), the zipper-like structure that links homologous chromosomes. HIM-3, HTP-1 and HTP-2 are HORMA-domain proteins that localize to the lateral elements of the SC, whereas SYP-2 and SYP-3 are coiled-coil-domain proteins that localize to the central region of the SC (reviewed by Mlynarczyk-Evans and Villeneuve, 2010). We refer to him-3, htp-1, htp-2, syp-2 and syp-3 collectively as SC genes.

FBF activity and FBF binding sites are required to silence SC 3’ UTR reporters in the mitotic zone

To determine whether the SC genes are co-regulated, we searched for a sequence shared among the 3’ UTRs of the SC genes and their homologs in three other nematode species (C. briggsae, C. remanei and C. brenneri). Using MEME (see Materials and methods), we identified the motif UCnUGUnnAU (Fig. 2A). This motif (Fig. 2B, blue boxes) is present in one or more copies in all the C. elegans SC 3’ UTRs with the exception of syp-2, which has two versions of the motif missing the first U (CCUUGUUUAU, ACAUGUAUCAU) (conserved bases underlined; data not shown). UCnUGUnnAU matches the FBF-response element (FBE) in the fem-3 3’ UTR, a known FBF target (Crittenden et al., 2002; Zhang et al., 1997). UGUUnnAU is the minimal sequence required for FBF binding in vitro (Bernstein et al., 2005), and the preferred in vitro-defined FBF-1 binding site is UGURHHAU (R is A or G, and H is A, C or U) (Fig. 2B, red boxes) (Bernstein et al., 2005; Lee et al., 2006).

To test whether the SC 3’ UTRs are regulated by FBF, we first examined all reporters in adult hermaphrodites treated for 12 hours with fbf-1/2 RNAi. In all cases, the 3’ UTR reporters were derepressed in the mitotic zone (Fig. 3A; see Fig. S2 and Table S2 in the supplementary material). To control for specificity, we also examined two other 3’ UTRs that block expression in the same region of the distal gonad (rme-2) or in a broader region that includes part of the pachytene zone (puf-5) (Merritt et al., 2008). Neither 3’ UTR was derepressed following treatment with fbf-1/2 RNAi (Fig. 3A; see Fig. S2 and Table S2 in the supplementary material). rme-2 is a known target of MEX-3, another translational repressor expressed in the distal region (Ciosk et al., 2004). In mex-3(RNAi), the rme-2 3’ UTR was derepressed in the distal region but none of the other reporters was affected (see Fig. S2 and Table S2 in the supplementary material).

We conclude that the SC 3’ UTRs depend on FBF for repression, but that not all 3’ UTRs inhibited in the mitotic zone depend on FBF for regulation.

To test whether the predicted FBF-1 sites are functional, we first mutated those matching the consensus UGURHHAU (Fig. 2B, red boxes) to acaRHHAU, which does not bind FBF-1 in vitro (Bernstein et al., 2005; Lee et al., 2006). Such mutations were sufficient to derepress the him-3, htp-1, htp-2 and syp-2 3’ UTR reporters throughout the mitotic zone (Fig. 3A). To derepress the syp-3 3’ UTR reporter, it was necessary to also mutate a nearby motif (UCAUGUCGAUAU) that does not match the in vitro-defined
FBF-1 consensus at positions 4 and 5, but which matches the MEME motif (Fig. 2B, blue box). The puf-5 3′ UTR contains two predicted FBF-1 binding sites (data not shown), but mutations in these sites did not affect expression of the puf-5 reporter, consistent with the observation that this reporter is not affected in fbf-1/2(RNAi) (Fig. 3A). We conclude that the SC 3′/H11032 UTRs depend on predicted FBF-1 binding sites (or related sites) for repression, but that not all predicted FBF-1 sites are essential for regulation.

**FBF activity and FBF binding sites are required to silence SC 3′ UTR reporters in germline progenitors**

Our results so far show that FBF is required to inhibit SC reporter expression in the distal mitotic zone of adult hermaphrodites, which include the germline stem cells. To determine whether FBF also functions in the progenitors of the germline stem cells, we examined the SC 3′ UTR reporters in L2 larvae. At this stage, all germ cells are proliferating and none have initiated meiosis. Wild-type SC 3′/H11032 UTR constructs were not expressed at this stage (Fig. 3B). By contrast, the SC 3′ UTR constructs with mutations in the predicted FBF sites were expressed in all L2-stage germ cells (Fig. 3B). Expression was also seen in all cells when wild-type SC 3′ UTR constructs were examined in hermaphrodites that were double mutant for fbf-1 and fbf-2 [fbf-1(ok91);fbf-2(q704)] or hermaphrodites treated with fbf-1/2 RNAi from the L1 stage (Fig. 3B). We conclude that FBF is required both in adult germline stem cells and in their larval progenitors to block the expression of SC 3′ UTR reporters.

**FBF-1 and FBF-2 function redundantly to repress the him-3 3′ UTR**

FBF-1 and FBF-2 function redundantly, but also have unique functions (Crittenden et al., 2002; Lamont et al., 2004). To determine whether FBF-1 and FBF-2 function redundantly to regulate the SC genes, we compared expression of the him-3 3′ UTR reporter in mutants lacking FBF-1 [fbf-1(ok91)], FBF-2 [fbf-2(q738)], or both [fbf-1(ok91);fbf-2(q704)]. We examined early L4 larvae because at this stage all three genotypes have a normal mitotic zone. We observed weak derepression of the him-3 reporter in the mitotic zone of fbf-1(ok91) larvae, no derepression in fbf-
2(q738) larvae, and complete derepression in the fbf-1(ok91);fbf-2(q704) double mutant (Fig. 3C). We conclude that FBF-1 and FBF-2 function redundantly to inhibit him-3.

**gld-1** and **fog-1**, two well-characterized FBF targets (Crittenden et al., 2002; Thompson et al., 2005), behaved similarly to him-3, except that the gld-1 3’ UTR reporter was expressed only very weakly in fbf-1;fbf-2 larvae (Fig. 3C). This result is consistent with the fact that GLD-1 is expressed at low levels in sperm-producing germlines (Jones et al., 1996) and with the analyses of Suh et al. (Suh et al., 2009), who proposed that FBF is required both to repress and activate gld-1 (Suh et al., 2009). FBF-dependent activation, however, is unlikely to be a characteristic of all FBF targets, as the him-3, fog-1, syp-2 and syp-3 reporters were all strongly expressed in fbf-1(ok91);fbf-2(q704) hermaphrodites (Fig. 3C and see Fig. S3 in the supplementary material).

**FBF-1 and FBF-2 interact with SC mRNAs in vivo**

The finding that FBF and its predicted binding sites are essential to repress the SC 3’ UTRs suggests that FBF-1 and FBF-2 inhibit the SC mRNAs via direct binding. To test this prediction further, we examined whether FBF-1 and FBF-2 associate with the SC mRNAs in vivo. We immunoprecipitated FBF-1 and FBF-2 as GFP fusions from worm extracts (Fig. 4A). In parallel, we also immunoprecipitated GFP::tubulin as a negative control (Fig. 4A). RNA abundance in the immunoprecipitates was determined by quantitative real-time PCR (qRT-PCR), and expressed as a ratio over RNA immunoprecipitated by a control antibody (IgG). Results were normalized to the extent of actin mRNA enrichment in each immunoprecipitation. We found that the five SC mRNAs bound preferentially to GFP::FBF-1 and GFP::FBF-2 as compared with GFP::tubulin (Fig. 4B). Binding efficiency varied widely, with htp-1 mRNA binding most efficiently (34.6- and 28.9-fold enrichments) and syp-2 mRNA binding least efficiently (4.4- and 3.1-fold enrichments). syp-1 also bound robustly to GFP::FBF-1 (18.8- and 7.4-fold enrichments). The syp-1 3’ UTR reporter was expressed at low levels throughout the mitotic zone (Fig. 1 and see Fig. S1 in the supplementary material), raising the possibility that this mRNA might also be under FBF regulation. All mRNAs that showed strong expression throughout the mitotic zone in the 3’ UTR reporter assay exhibited weak enrichment (3.3- and 2.9-fold enrichments for zim-2) or no significant enrichment (less than 2.1-fold). Interestingly, these mRNAs include four that contain predicted FBF-1 binding sites in their 3’ UTRs (Fig. 4B). These results confirm that SC mRNAs are direct FBF targets, and suggest that not all predicted FBF-1 binding sites are recognized by FBF in vivo.

**In the absence of FBF, SC proteins are expressed precociously in germline stem cells and their progenitors**

To examine the distribution of the SC proteins in fbf-1;fbf-2 mutants, we used antibodies against HIM-3 and HTP-1/2 (Martinez-Perez et al., 2008; Zetka et al., 1999), and GFP fusions.
with HIM-3, HTP-1, HTP-2, SYP-2 and SYP-3 (antibodies against SYP-2 and SYP-3 were not specific enough in our hands for this analysis). In wild-type gonads, all proteins were absent from the distal half of the mitotic zone, showed increased expression through the proximal half of the mitotic zone, and were most highly expressed in the transition and pachytene regions (Fig. 5A).

In the proximal half of the mitotic zone, the SC proteins were mostly nuclear and overlapped with chromatin (most evident for GFP::SYP-2 and GFP::SYP-3), but had not yet coalesced into the bright nuclear threads that are characteristic of the fully formed SCs of pachytene nuclei. We also observed GFP::SC fusions in bright nuclear puncta (Fig. 5A, arrows). Similar SYP-3 aggregates have been reported in wild type, in fbv-1;fbf-2 gonads (Smolikov et al., 2007). By the mid-L4 stage, all pachytene nuclei had an abnormal morphology in fbv-1;fbf-2 mutants (Smolikov et al., 2007). By the mid-L4 stage, all pachytene nuclei had an abnormal morphology in fbv-1;fbf-2 mutants (Smolikov et al., 2007). By the mid-L4 stage, all pachytene nuclei had an abnormal morphology in fbv-1;fbf-2 mutants.

In fbv-1;fbf-2 mutant or RNAi-treated gonads, we detected the distribution of GFP::HIM-3 in all germ nuclei, however, were progressively more abnormal, with stunted synaptonemal threads and large aggregates of SC proteins (Fig. 6A, arrows). Similar SYP-3 aggregates have been reported in wild type, in fbv-1;fbf-2 mutants grown at 20°C (Crittenden et al., 2002). We and others (T. Schedl, personal communication) have observed that gld-1 mutants that have all entered meiosis and progressed through spermatogenesis to form mature sperm (Crittenden et al., 2002).

Lowering the dose of factors that promote meiosis, such as gld-1, can suppress this phenotype: fbv-1;fbf-2;gld-1 worms maintain a mitotic zone into adulthood (Crittenden et al., 2002). We and others (T. Schedl, personal communication) have observed that fbv-1;fbf-2 mutants grown at 25°C also maintain a mitotic zone into adulthood (see Fig. S4 in the supplementary material). To determine whether these mitotic zones misexpress SC proteins, we examined the distribution of GFP::HIM-3 in fbv-1;fbf-2 mutants grown at 25°C and in fbv-1;fbf-2 mutants grown at 20°C but treated with partial (incomplete) gld-1 RNAi (Fig. 6B). We also examined GFP::SYP-3 in fbv-1/2(RNAi) hermaphrodites grown at 25°C (see Fig. S4 in the supplementary material). In all cases, mitotic zones were present in the adult stage and were positive for GFP::SC expression (Fig. 6B and see Fig. S4 in the supplementary material). We conclude that misregulation of SC proteins occurs in fbv-1;fbf-2 mutants regardless of whether mitotic germ cells are fated to enter meiosis precociously.

We also tested whether reducing HIM-3 or HTP-1/2 levels could suppress the loss of a mitotic zone in fbv-1;fbf-2 mutants. We found that, unlike fbv-1;fbf-2;gld-1(RNAi) hermaphrodites, fbv-1;fbf-2;him-
Ectopic SC expression is observed as early as the L2 stage and ectopically in all mitotic germ cells of
\textit{fbf-1/2} (RNAi). We conclude that premature meiotic entry in \textit{fbf-1/2} mutants does not depend on HIM-3 or HTP-1/2 expression or the ability to form an SC. Consistent with these results, loss of SC proteins in wild-type gonads blocks SC formation but does not affect the timing of meiotic entry (Colaiacovo et al., 2003; Couteau and Zetka, 2005; Smolikov et al., 2007; Zetka et al., 1999).

We conclude that meiotic entry and SC protein expression are not necessarily linked, and that FBF is required for their coordination.

**DISCUSSION**

In this study, we identify five new mRNAs regulated by FBF. The mRNAs encode components of the SC, the expression of which is inhibited by FBF in mitotic germ cells. Our results indicate that regulation by FBF ensures that SC protein synthesis is coordinated with meiotic entry.

**Expression of SC mRNAs is silenced post-transcriptionally by FBF in germline stem cells and their progenitors**

Four lines of evidence indicate that the SC mRNAs (him-3, htp-1, htp-2, syp-2 and syp-3) are direct FBF targets. First, the SC 3' UTRs confer the same pattern of regulation in the mitotic zone as the \textit{gld-1} 3' UTR: no or very low levels in distal-most cells, increasing levels in proximal cells, and high levels at the transition zone and into pachytene. Second, the SC 3' UTRs contain one or more predicted FBF-1 binding sites, and these sites are required for repression in the mitotic zone. Third, SC mRNAs are in a complex with FBF-1 and FBF-2 in vivo. Finally, SC 3' UTR reporters and SC proteins are expressed ectopically in all mitotic germ cells of \textit{fbf-1/2} mutants.

Ectopic SC expression is observed as early as the L2 stage and can be induced in adult animals by a brief exposure to \textit{fbf-1/2 RNAi}, suggesting that FBF is required continuously to block SC expression in germline stem cells and their larval progenitors. Consistent with these observations, we previously showed that the \textit{him-3} promoter is active in all germ cells from the L2 stage onward (Merritt et al., 2008).

Our mutational analysis defined four sites in which mutations unambiguously caused derepression in vivo (one site each in \textit{him-3}, \textit{htp-2}, \textit{syp-2} and \textit{syp-3}). By comparing these sites to the mutationally defined FBE in \textit{fem-3} (Ahringer and Kimble, 1991) and to the in vitro-defined preferred FBF-1 binding site [UGURHHAU (Bernstein et al., 2005)], we derived a 'repressive FBE' consensus (CNUUGVNUHAU; Fig. 2C). The repressive FBE differs from the in vitro-defined FBF-1 binding site consensus in two ways: (1) relaxation of the middle base consensus from RHH to VNH to accommodate the \textit{gld-1} UGU cytosine; (2) the addition of a 5' cytosine at the –2 position. Mutations that disrupt this cytosine in the endogenous \textit{fem-3} 3' UTR (Ahringer and Kimble, 1991; Zhang et al., 1997) or in a \textit{fem-3} 3' UTR reporter (C.M., unpublished) disrupt \textit{fem-3} regulation. Structural studies of yeast Puf3 have shown that this FBF homolog recognizes an additional 5' cytosine in the same position relative to the core UGUNNNAAU motif (Zhu et al., 2009). Consistent with a similar recognition for FBF-1, FBF-1 binding in vitro is sensitive to mutations upstream of the core element, including at the –2 position (Bernstein et al., 2005). We note that, with one exception (\textit{syp-2}), the mutationally defined repressive FBEs also have a uracil immediately preceding the 5' cytosine (Fig. 2C). Mutation in this base disrupts \textit{fem-3} regulation (Ahringer and Kimble, 1991) (C.M., unpublished), suggesting that a 5' uracil also contributes to recognition by FBF.

Since it is based on only five mutationally defined sites, the repressive FBE consensus is unlikely to describe all sites that are functional in vivo, but it is a useful reference for prioritizing searches.
for additional new sites. A recent genome-wide microarray analysis of mRNAs immunoprecipitated by GFP::FBF-1 identified 1350 candidate FBF targets (Kershner and Kimble, 2010). This list includes the five SC mRNAs described here, and also syp-1, rec-8, zim-2, rad-51 and msh-5, which did not behave like the SCs in the 3′ UTR reporter assay (see Table S3 in the supplementary material). With the exception of msh-5, all have at least one predicted FBF-1 binding site, and syp-1 and zim-2 have one site matching a repressive FBE. Predicted FBF-1 binding sites and repressive FBEs are overrepresented in the 3′ UTRs of meiotic genes (see Table S3 in the supplementary material), raising the possibility that most meiotic genes are in fact FBF targets. So why were only five recovered in our survey? Our 3′ UTR reporter assay might not be sensitive enough to detect the full range of FBF regulation, and some FBF targets might not be regulated in the pattern typified by gld-1 and him-3. The latter might be the case for syp-1, which was only partially repressed in the mitotic zone (see Fig. S1 in the supplementary material), yet was enriched in the FBF-1 immunoprecipitates as robustly as him-3 (Fig. 4). We also found some mRNAs that contained repressive FBEs but were not enriched in FBF-1 immunoprecipitates and showed no evidence of regulation in 3′ UTR reporters (htp-3, zim-1 and zhp-3; see Table S3 in the supplementary material). Clearly, further studies are needed to define the full sequence requirements necessary for regulation by FBF.

**Premature expression of SC proteins leads to aggregation and defective SC formation**

Why inhibit the expression of SC proteins in immature germ cells? fbf-1;fbf-2 mutants exhibit a defect in SC formation. Consistent with defective synapsis, fbf-1;fbf-2 oocytes contain more than the expected six bivalents, and eggs fertilized by fbf-1;fbf-2(RNAi) sperm are not viable (Luitjens et al., 2000; Thompson et al., 2005). We suggest that defective synapsis is caused by premature expression of the SC proteins. In premeiotic germ cells (and when expressed ectopically in embryonic cells), SC proteins form aggregates. Aggregation might be a conserved property of synaptonemal proteins, as the yeast and mammalian functional homologs of SYP-2 and SYP-3 have also been reported to aggregate (Ollinger et al., 2005; Sym and Roeder, 1995; Yuan et al., 1996). SC aggregates could reduce the pool of SC proteins available to form SCs and cause synapsis and meiotic chromosomal segregation to fail. We suggest that parallel regulation by FBF of the SC proteins and of meiotic entry regulators (such as GLD-1) reduces non-productive SC aggregation by linking SC synthesis to the production of synapsis-competent chromosomes. We note, however, that not all SC proteins appear to be regulated by FBF. For example, HTP-3 and REC-8 have been detected on chromosomes throughout the mitotic zone (Goodyer et al., 2008; Pasierbek et al., 2001) and do not show obvious regulation by FBF in our 3′ UTR reporter analysis (this study). Also, because FBF is likely to regulate hundreds of mRNAs (Kershner and Kimble, 2010), we cannot exclude the possibility that failed synapsis in fbf-1;fbf-2 mutants is caused by the misregulation of other proteins besides those analyzed here.

In the absence of FBF, we detected SC protein expression as early as the L2 stage. Premature SC protein expression does not appear to interfere with the proliferation of larval germ cells, as fbf-1;fbf-2 L4 larvae have normal germ cell numbers (Crittenden et al., 2002). At 25°C, fbf-1;fbf-2 mutants even maintained mitotic cells into adulthood. fbf-1;fbf-2 mitotic zones, however, were smaller than in wild type and eventually degenerated in older adults (see Fig. S4 in the supplementary material). These observations suggest that accumulation of meiotic proteins in proliferating germ cells eventually erodes cell renewal and cell viability. One possibility is that true germline stem cells are never formed in fbf-1;fbf-2 mutants. Instead, descendents of the primordial germ cells differentiate directly at the L2 stage into a premeiotic transitional fate that is characteristic of cells normally found in the second half of the mitotic zone. These ‘transit-amplifying’ cells retain proliferative potential and the ability to respond to niche signals that balance mitosis and meiosis, but have lost the ability to self-renew for extended periods of time and at all temperatures.

**Fig. 6. Defective synaptonemal complexes in C. elegans fbf-1;fbf-2 mutants.**

(A) Fluorescence photomicrographs of pachytene-stage germ cells in early and middle L4 gonads stained with anti-HTP-1/2 or expressing GFP::SYP-3, comparing hermaphrodites heterozygous (fbf-1/2/min1) or homozygous (fbf-1/2) for mutations in fbf-1 and fbf-2 or wild-type hermaphrodites treated with control feeding vector (L4440) or fbf-1/2 RNAi. Arrows point to representative large foci not seen in controls. (B) Distal gonads expressing GFP::HIM-3 in adult hermaphrodites of the indicated genotypes. All germ cells undergo spermatogenesis in fbf-1;fbf-2 homozygotes grown at 20°C (GFP::HIM-3 is downregulated during spermatogenesis so no fluorescence is visible); gld-1 RNAi or growth at 25°C restores a mitotic zone in fbf-1;fbf-2 adults, but this zone still misexpresses GFP::HIM-3.
Coordinate regulation of meiosis genes by post-transcriptional mechanisms: parallels between C. elegans and mouse

The regulation of meiotic gene expression is best understood in S. cerevisiae, in which a transcriptional cascade controls the temporally staggered expression of six classes of meiosis genes (Chu et al., 1998; Kassir et al., 2003; Primig et al., 2000). At the top of the cascade is the master regulator Ime1. Ime1 is a transcription factor that directly activates the transcription of early meiosis genes, including genes required for premeiotic DNA replication and genes required for homolog pairing, such as the yeast homolog of HIM-3/HTP-1/HTP-2 (Kassir et al., 2003). Thus, in yeast, synaptonemal complex protein expression is also coordinated with meiotic entry, but regulation occurs at the level of transcription. By contrast, in mice, post-transcriptional mechanisms have been implicated in the regulation of early meiotic genes. The RNA-binding proteins DAZL and CPEB have been implicated in the regulation of early meiosis gene transcription. By contrast, in mice, post-transcriptional mechanisms have been implicated in the regulation of early meiotic genes. The RNA-binding proteins DAZL and CPEB (cytoplasmic polyadenylation element binding protein) are both meiotic genes. CPEB repressors. Consistent with this possibility, the mammalian FBF complexes are dispensable for loading strand-exchange proteins but critical for proper completion of recombination. Dev Cell 5, 475-484.


chromosome axis composition that is linked to two-step loss of sister chromatid cohesion. Genes Dev. 22, 2886-2901.


