Splicing Machinery Facilitates Post-Transcriptional Regulation by FBFs and Other RNA-Binding Proteins in Caenorhabditis elegans Germline

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ABSTRACT Genetic interaction screens are an important approach for understanding complex regulatory networks governing development. We used a genetic interaction screen to identify cofactors of FBF-1 and FBF-2, RNA-binding proteins that regulate germline stem cell proliferation in Caenorhabditis elegans. We found that components of splicing machinery contribute to FBF activity as splicing factor knockdowns enhance sterility of fbf-1 and fbf-2 single mutants. This sterility phenocopied multiple aspects of loss of fbf function, suggesting that splicing factors contribute to stem cell maintenance. However, previous reports indicate that splicing factors instead promote the opposite cell fate, namely, differentiation. We explain this discrepancy by proposing that splicing factors facilitate overall RNA regulation in the germline. Indeed, we find that loss of splicing factors produces synthetic phenotypes with a mutation in another RNA regulator, FOG-1, but not with a mutation in a gene unrelated to posttranscriptional regulation (dhc-1). We conclude that inefficient pre-mRNA splicing may interfere with multiple posttranscriptional regulatory events, which has to be considered when interpreting results of genetic interaction screens.

KEYWORDS germine splicing factor RNA-binding protein stem cells

Whole-genome synthetic interaction screens are used widely to identify functional partners of genes of interest. Large-scale analyses performed in Caenorhabditis elegans suggest that the majority of genes fail to produce a phenotype when singly depleted (Kamath et al. 2003), partially because of genetic redundancy. Synthetic phenotypes produced by simultaneous depletion of two genes and not observed in either single mutant often are interpreted as an indication of functional connections between genes. Synthetic interaction screens are a valuable tool to probe the complex regulatory networks. Here, we use synthetic interaction screen to identify factors contributing to regulation of the network that maintains the balance between stem cell proliferation and differentiation in the germline.

Caenorhabditis elegans germ cells undergo a stereotypical developmental program that ends in the production of mature gametes prepared for fertilization (Pazdernik and Schedl 2013). The germline functions as an assembly line, where stem cell proliferation and self-renewal occurs at the distal region in the stem cell niche supported by the activation of GLP-1/Notch signaling pathway (Kimble and Crittenden 2007). Meiotic differentiation is triggered as the germ cells are displaced from the niche (reviewed in Kershner et al. 2013). As germ cells move proximally, they transit through the stages of meiotic prophase and ultimately form fully differentiated gametes (sperm or oocytes). In a C. elegans hermaphrodite, germ cells of late larva develop along the male pathway and form sperm, and germ cells of the adult develop along the female pathway, forming oocytes. The balance between stem cell self-renewal and differentiation must be carefully maintained to support tissue development and maintenance. Regulation of stem cell proliferation and differentiation is characterized by multiple redundancies, feedback and feed-forward modules, and is also tightly integrated with regulation of germline sex determination.

In C. elegans germline, posttranscriptional mechanisms play a major role in the regulatory network determining the extent of germline proliferation (Hansen and Schedl 2013). For example, the PUF domain RNA-binding proteins FBF-1 and FBF-2 (collectively referred to as FBFs) maintain germline stem cell fate and prevent meiotic differentiation (Zhang et al. 1997; Crittenden et al. 2002; Lamont et al. 2004). FBFs repress differentiation-associated mRNAs, which include genes promoting differentiation/meiotic entry, genes supporting meiotic...
processes, and genes associated with spermatogenesis (Crittenden et al. 2002; Thompson et al. 2005; Merritt and Seydoux 2010). In addition to the FBFs, several splicing factors contribute to the regulation of the balance of proliferation and differentiation (Belfiore et al. 2004; Mantina et al. 2009; Kasturi et al. 2010; Kerins et al. 2010; Zanetti et al. 2011; Wang et al. 2012). The data to date suggest that an overall decrease in spicoseomal activity may induce overpolarization of germline, although the mechanism of splicing factor regulatory contribution remains unknown.

Germ cell differentiation into sperm or oocytes depends on the germline sex determination pathway. The developmental switch of C. elegans germline from spermatogenesis to oogenesis also is under posttranscriptional regulation that determines the number of sperm produced before the hermaphrodite switches to oogenesis (Francis et al. 1995; Crittenden et al. 2002; Zanetti and Puoti 2013). This decision depends on the relative abundance of proteins promoting male fate (such as FOG-1, FOG-3, and FEM-3) and the proteins promoting female fate (such as TRA-2 and TRA-3) (reviewed in Zanetti and Puoti 2013). In the L3/L4 larval stages, when C. elegans hermaphrodites produce sperm, proteins promoting male fate, including FOG-1, are expressed, whereas the female fate-associated tra-2 is translationally repressed. In the adult hermaphrodite, germ cells switch from spermatogenesis to oogenesis in response to the translation of the female fate mRNA tra-2 and translational repression of the male fate mRNA fem-3 (Ahringer and Kimble 1991). FOG-1 is one of the germline regulatory proteins necessary for sperm development and is an RNA-binding protein of the cytoplasmic polyadenylation element binding protein (CPEB) family (Jin et al. 2001b, Thompson et al. 2005). FOG-1 promotes proliferation and spermatogenesis during male as well as hermaphrodite larval development (Barton and Kimble 1990, Thompson et al. 2005). FOG-1 is one of the terminal regulators in the germline sex determination cascade, and loss-of-function mutations in fog-1 cause germline feminization, which is epistatic to a number of masculinizing mutations (reviewed in Zanetti and Puoti 2013).

Several factors coordinately regulate both the germline stem cell proliferation/differentiation switch and the spermatogenesis/oogenesis transition. For example, in addition to promoting stem cell renewal, the FBF proteins also repress protein production from fem-3 and fog-1 mRNAs (Zhang et al. 1997, Thompson et al. 2005). Indeed, fog-1 fbf-1 fbf-2 double mutant animals fail to make oocytes, which results in germline masculinization (Crittenden et al. 2002), fog-1 mRNA is a direct target of FBFs, its 3′-prime untranslated region (3′UTR) contains FBF binding sites that are necessary for silencing FOG-1 protein expression in the mitotic germ cells (Thompson et al. 2005). Similarly, loss-of-function mutations in a number of splicing factors cause masculinization of the germline, possibly through regulation of fem-3 translation (Graham and Kimble 1993; Puoti and Kimble 1999, 2000; Belfiore et al. 2004; Kawano et al. 2004; Konishi et al. 2008; Mantina et al. 2009; Kasturi et al. 2010; Kerins et al. 2010; Zanetti et al. 2011; Wang et al. 2012).

Splicing of pre-mRNA proceeds through the activity of the spicoseome, which is a large and dynamic protein–RNA complex that assembles on the mRNA in a characteristic step-wise fashion while progressing from recognition of 5′ and 3′ intron boundaries to eventual intron excision (Lee and Rio 2015). Efficient splicing is critical to generate a translatable open reading frame, and additionally plays a role in regulating multiple aspects of RNA metabolism including nuclear export, mRNA stability, localization, and translational activity (Nott et al. 2003; Hachet and Ephrussi 2004; Popp and Maquat 2014).

In this study, we set out to identify cofactors of FBF-2 by using genetic interaction screening. FBF-1 and FBF-2 are redundant, and although inactivation of a single gene does not produce a phenotype, simultaneous inactivation of both fbf-1 and fbf-2 leads to a loss of germline stem cells and sterility. Previously, we reported that FBF-1 and FBF-2 repress their target mRNAs using distinct mechanisms (Voronina et al. 2012), which now allows to identify genes required for FBF-2 function. Knockdown of such genes results in sterility only when fbf-1 function is compromised but not when fbf-2 function is compromised. In this study, we find that knockdown of splicing factors disrupted FBF function as well as compromised the function of at least one other RNA-binding protein. We conclude that in addition to their established role in mRNA biogenesis, the splicing factors act more broadly to maintain efficient translational control of germline mRNAs.

**MATERIALS AND METHODS**

**Nematode culture**

C. elegans strains (Table 1) were derived from Bristol N2 and cultured according to standard protocols (Brenner 1974) at 15°, 20°, or 24° as indicated.

**RNA interference (RNAi)**

RNAi was performed by feeding method, RNAi constructs were derived from Source BioScience RNAi library (Kamath and Ahringer 2003); all clones were verified by sequencing. Empty vector pL4440 was used as a negative control throughout the experiments. Three colonies of freshly transformed RNAi plasmids were combined for growth in LB/Cambenilin media for 4 hr and induced with 10 mM Isopropyl β-D-1-thiogalactopyranoside for 2 hr more at 37°. RNAi plates (NNGM plates containing 75 μg/mL carbenicillin and 0.4 mM Isopropyl β-D-1-thiogalactopyranoside) were seeded with the pelleted cells. RNAi treatments for genetic interactions with fbf-1, fbf-2, and fog-1 were performed by feeding the L1 hermaphrodites synchronized by bleaching with bacteria expressing double-stranded RNA for 70 hr at 24° (fbf-1, fbf-2) or for 144 hr at 15° (fog-1). RNAi on strains expressing green fluorescent protein (GFP)-tagged histone H2B was performed at 24°.

**Assessment of sterility, masculinization, and reporter deregulation**

Sterility of the treated worms was scored when no embryos were observed in the uterus at day 1 post L4. Masculinization of germlines was assessed after the treated worms were fixed, and chromatin was stained with 4′,6-diamidino-2-phenylindole (DAPI); germlines with sperm and no oocytes were scored as masculinized. Regulation of GFP::H2B::fog-1 3′UTR reporter was assessed by obtaining images of all germlines with identical exposure settings (2.8 sec). Epifluorescent images were acquired with an AxioCam MRm camera attached to a Zeiss Axioscop with a 63x Plan-Apochromat NA 1.4 objective using Zen Blue software (Zeiss). When expression of the fluorescent reporter was detected in the distal mitotic region, the germline was scored as “derepressing in stem cells.” To assess reporter overexpression, accumulation of nuclear GFP reporter was quantified in five transition zone nuclei per each germline and corrected to background using Zen Blue. Brightness values were normalized to the average intensity of the reporter in the rrf-1 background following control RNAi. Image processing was performed in Adobe Photoshop CS4.

**Embryonic lethality assessment**

RNAi treatments were performed at 15°. Wild-type (N2) or dhc-1 (or1950) animals at the fourth larval stage were placed on RNAi feeding plates and left overnight. The next day, the adult worms were transferred into a fresh RNAi plate and incubated for 5 hr before being
removed from the plate. After removal of the adult worms, plates were incubated for 48 hr at 15°C, and the number of unhatched eggs and larval worms on the plate was scored. Embryos were scored as dead or hatched after at least 2 d after being deposited on the plate.

**Data availability**

Strains are available upon request.

**RESULTS**

**Splicing factor RNAi results in enhanced synthetic sterility with mutants of either fbf-1 or fbf-2**

To identify possible FBF-2 cofactors and additional genes involved in regulation of the proliferation/differentiation transition in the germline, we performed an RNAi enhancer screen of 16 candidate genes predicted to contribute to FBF-2-mediated regulation (www.geneorienteer.org; Zhong and Sternberg 2006) as well as a subset of 34 genes predicted to function in RNA regulation or metabolism and highly expressed during oogenesis (Reinke et al. 2004). The oogenesis-enriched RNA regulators tested in this study are a part of an ongoing large-scale genetic interaction screen. We assayed for enhanced sterility in the fbf-1 mutant background compared with the control strain. Both strains carried a mutation in rrf-1 to preferentially direct RNAi to germline tissues (Sijen et al. 2001; Kumsta and Hansen 2012). Knockdown of three splicing factors, prp-17, lsm-4, and gut-2, resulted in enhanced sterility when depleted in rrf-1; fbf-1 mutant worms compared with the rrf-1 strain (Figure 1A and data not shown). All three splicing factors were present in the list of predicted FBF-2 cofactors. prp-17 and gut-2 also belong to the complete oogenesis-enriched RNA regulator gene set that was analyzed only partially in this study, but likely also had potential to recover splicing factors. The rest of the tested clones (47) failed to show enhanced sterility resulting either in completely fertile worms in both genetic backgrounds or in equal percentages of sterile worms across tested genetic backgrounds. These results suggest that multiple components of the spliceosome genetically interact with the fbf-1 mutant.

To test whether other components of the splicing machinery genetically interact with fbf-1, we used RNAi to deplete seven additional splicing factors distributed throughout the splicing reaction cycle. We chose the genes suggested in previous reports to function in splicing reaction and focused on those that have previously produced genetic interaction with glp-1, a regulator of germline proliferation (Mantina et al. 2009; Kerins et al. 2010). Knockdown of six of these genes resulted in enhanced synthetic sterility in the rrf-1; fbf-1 mutant (which reached statistical significance in four cases), whereas knockdown of the seventh (tec-4) induced 100% sterility even in the rrf-1 strain (Figure 1A).

Collectively, seven distinct components of the spliceosome significantly interact with fbf-1 and thus may contribute to FBF-2 function.

We next tested whether the synthetic sterility in the RNAi assays phenocopied that of fbf-1 fbf-2 double mutants, which fail to transition from spermatogenesis to oogenesis (Crittenden et al. 2002). We determined gamete chromatin morphology in the three treatments (mtr-4, F43G9.12, and prp-17(RNAi)) that produced high levels of enhanced sterility in the fbf-1 mutant background (Figure 1A). Similar to fbf-1 fbf-2 double mutants, the sterility of rrf-1; fbf-1 worms after splicing factor depletion was associated with an increased prevalence of masculinized germlines (Figure 2C; Table 2), in contrast to the fertile germlines containing both oocytes and sperm (Figure 2A). The other sterile phenotype was associated with degenerated endomitotic oocytes (Figure 2B) and was more prevalent in the rrf-1 background than in rrf-1; fbf-1 background. This phenotype is not relevant to sex determination or fbf function. These observations suggest that splicing factors may contribute to fbf-1 activity.

To test whether splicing factors were selective for fbf-2 or also contribute to fbf-1 function, we tested whether the splicing factor RNAi is synthetically sterile with the fbf-2 mutation. We found that knockdowns of two splicing factors, mtr-4 and prp-17, produced significant synthetic sterility with fbf-2 (Figure 1B). In contrast, knockdowns of five genes producing synthetic sterility with the fbf-1 mutation (rps-3, tef-1, gut-2, lsm-4, and lsm-7) failed to generate synthetic sterility with fbf-2, indicating either specific cooperation of these splicing factors with FBF-2 or a weaker overall FBF regulation in fbf-1 mutant leading to a greater sensitivity to synthetic interactions. The synthetic sterility in fbf-2 background was associated with an increased prevalence of masculinized germlines (Table 2). Together, these results suggest that the splicing machinery contributes to function of both FBF-1 and FBF-2, and depletion of splicing factors promotes sterility when either FBF-1 or FBF-2 are absent.

**Splicing factor RNAi affects FBF target regulation**

Next, we directly tested whether splicing factor RNAi affects FBF function by observing the effect of splicing factor depletion on an FBF target gene fog-1 (Thompson et al. 2005). Expression of a transgenic GFP::Histone H2B::fog-1 3’UTR reporter is silenced in the mitotic zones of wild-type, fbf-1, and fbf-2 worms, but it becomes derepressed in the mitotic zones of fbf-1 fbf-2 double-mutant germlines (Merritt et al. 2008). Upon splicing factor knockdown, 40–80% of sterile rrf-1; fbf-1 hermaphrodites derepressed fog-1 3’UTR reporter in the mitotic region (Figure 3, A and B). By contrast, control depletion of the splicing factors in the rrf-1 background did not result in significant reporter derepression in the mitotic region. These results indicate that depletion of splicing factors compromises FBF-2 activity in fbf-1 mutant background.

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**Table 1 Nematode strains used in the study**

| Genotype | Transgene Description | Strain | Reference |
|----------|-----------------------|--------|-----------|
| rrf-1(pk1417) I axls1772 [pcCM1.90] | pie-1 prom::GFP::h2b::3’UTR | UMT193 | This study |
| rrf-1(pk1417) I axls1772 [pcCM1.90] | pie-1 prom::GFP::h2b::3’UTR | UMT191 | This study |
| rrf-1(pk1417) I axls1772 [pcCM1.90] | pie-1 prom::GFP::h2b::3’UTR | UMT194 | This study |
| Mutant strains; no transgene | | | |
| dhtc-1(or195) I | | | |
| rnr-1(pk1417) I | | | |
| rnr-1(pk1417) I; fbf-1(ok91) II | | | |
| rnr-1(pk1417) I; fbf-2(q738) II | | | |
| fog-1(q523) rnr-1(pk1417) I | | | |
| | | EU828 | Hamill et al. 2002 |
| | | MAH23 | Kumsta and Hansen, 2012 |
| | | UMT186 | This study |
| | | UMT203 | This study |
| | | UMT220 | This study |
To determine whether the splicing factors affect FBF-1 activity, we repeated the same experiments in the fbf-2 mutant background (Figure 3, A and B). Although no treatments derepressed the transgenic reporter in the distal-most stem cell region, prp-17(RNAi) and mtr-4 (RNAi) resulted in a dramatic increase of fog-1 3’UTR reporter expression in the transition zone where the cells entered meiosis (Figure 3A). Transition zone nuclei expressing fog-1 3’UTR reporter in the rrf-1;fbf-2 background had on average 1.6 to 3.5 fold more GFP signal compared to the transition zone nuclei of the control germlines (Figure 3C; P < 0.01, Student’s t-test). Thus, knockdown of splicing factors may limit FBF-1 activity in the fbf-2 mutant background. These results are consistent with previous findings that splicing factors mog-1 and mog-6 repress expression of fem-3 3’UTR reporter in somatic cells (Gallegos et al. 1998).

**Splicing factor RNAi enhances feminization of fog-1(ts) mutant**

Our results indicate that loss of splicing factors enhances the single fbf mutant phenotype and that, like the fbf-2, splicing factors are required for stem cell maintenance. However, previous studies suggested that a decrease in splicing factor activity instead leads to the opposite phenotype: overproliferation and formation of synthetic germline tumors in combination with a weak gain of function allele of glp-1 (Mantina et al. 2009; Kerins et al. 2010; Wang et al. 2012). Because of these opposing combinatorial effects, we hypothesize that the role of splicing factors in germline stem cell proliferation and differentiation extends beyond generating specific splice isoforms of the stem cell maintenance regulators. We suggest the splicing act more broadly to maintain efficient translational control of germline mRNAs.

To test whether splicing factors are broadly required for RNA regulation, we took advantage of the fog-1(q253ts) mutant, which leads to failure of sperm production at the restrictive temperature of 25°C but permits spermatogenesis at 15°C (Barton and Kimble 1990; Jin et al. 2001a). The level of FOG-1 expression is tightly controlled and correlates with sperm number produced by the hermaphrodite (Barton and Kimble 1990; Lamont and Kimble 2007); therefore, any defect in FOG-1 function would be manifest in decreased or absent sperm production. If the normal function of splicing factors is to act with the fbf-2 to promote oogenesis, splicing factor knockdown would still cause masculinization in the fog-1(ts) background at the permissive temperature, where FOG-1(ts) is functional. Alternatively, if splicing factor knockdown disrupts RNA regulation in general rather than selectively affecting fbf function, it would produce synthetic feminization of the fog-1(ts) mutant at the permissive temperature.

Knockdown of splicing factors at permissive temperature failed to feminize rrf-1/fog-1(ts) strain. By contrast, RNAi of all tested splicing factors in rrf-1/fog-1 background produced some level of synthetic feminization; this feminization reached statistical significance in three cases (Figure 4E). Feminized phenotypes included arrested oocytes characteristic of fog-1 loss of function (sometimes disorganized) and ovulated unfertilized oocytes, indicating defects in spermatogenesis (Figure 4, B–D). In some cases, feminization was incomplete, and small amounts of sperm were produced before a switch to oogenesis detected by the presence of two to three embryos in the adult’s uterus followed by ovulated or arrested oocytes. None of these phenotypes was observed in fog-1(ts) worms exposed to control RNAi, in nonmasculinized rrf-1 mutant worms exposed to splicing factor RNAi, or in previous reports of splicing factor mutants. Because splicing factor knockdown may lead to either synthetic masculinization (fbf mutant background) or synthetic feminization (in fog-1(ts) background), the function of splicing factors in germline sex determination is not specific to the FBFs or oogenesis. Instead, we conclude that the functional splicing cascade facilitates RNA regulation carried out by multiple regulatory proteins in the germline.

**Splicing RNAi does not enhance embryonic lethality of dhc-1(or195ts)**

One potential consequence of splicing factor knockdown is general deterioration of all cellular functions; in that case, it would be expected to worsen the phenotype of any loss-of-function mutation, especially those that affect cell viability. To test whether a partial loss of function mutation would be nonselectively enhanced by depletion of splicing factors, we tested our panel of splicing factor RNAi in a strain carrying a temperature-sensitive S3200L mutation in the motor subunit of dynein, dhc-1(or195ts) (Hamill et al. 2002). This mutation causes embryonic lethality at 25°C because of failure of mitotic spindle alignment, chromosome congression defects, and mitotic spindle collapse within 1 min of temperature upshift; thus, the phenotype most likely does not involve changes in posttranslational regulation of gene expression (Schmidt et al. 2005). We expect that if splicing factor depletion causes nonspecific loss of viability and enhances reduction-of-function mutation phenotypes, the embryonic lethality of dhc-1(ts) would
be enhanced at the permissive temperature. Conversely, if splicing factor depletion primarily affects RNA regulation, the embryonic lethality of *dhc-1(ts)* would be equal either to the lethality of untreated *dhc-1(ts)* or to the lethality of splicing factor-depleted wild-type control.

RNAi knockdowns of *mtr-4*, *F43G9.12*, *bsm-4*, *bsm-7*, *gut-2*, and *tra-1* resulted in lethality similar to that observed in *dhc-1(ts)* treated with control RNAi. Knockdowns of *acac-1*, *ppr-17*, and *rps-3* showed pronounced embryonic lethality, albeit equal between N2 and *dhc-1(ts)* strains treated with splicing factor RNAi (Figure 5). *teg-4(RNAi)* caused small but statistically significant enhancement of embryonic lethality in the *dhc-1(ts)* mutant. Because the severity of the lethality caused by combined *teg-4(RNAi)* and *dhc-1(ts)* is close to the sum of the effects of the two perturbations individually, this effect appears additive rather than synthetic. We conclude that in the majority of cases splicing factor knockdowns do not exacerbate a developmental defect unrelated to RNA regulation.

**DISCUSSION**

Here, we demonstrate that reduction in the activity of the splicing pathway in *C. elegans* germline disrupts multiple processes that depend on posttranscriptional control of gene expression. This destabilization of RNA regulation is uncovered by genetic interaction assays that identify splicing factor knockdowns as genetic enhancers of partial loss-of-function mutations in RNA-binding proteins. We suggest that an important function of the splicing pathway is to facilitate RNA regulation in general, which includes regulation by PUF-family translational repressors FBFs. Regulation of germline stem cell balance between proliferation and differentiation as well as spermatogenesis to oogenesis transition is centered at the posttranscriptional level. Our hypothesis explains the observations that reduction of splicing factor function may exacerbate defects that lead to opposite phenotypic outcomes such as masculinization and feminization; or overproliferation and stem cell loss. In our study, the strains that are mutant for RNA-binding proteins don’t show sterility, sex determination, or reporter misexpression phenotypes unless splicing factors are knocked down. This suggests that the enhanced phenotypes resulting from a combination of RNA-binding protein mutation with splicing factor knockdown reflect a synthetic interaction rather than an additive effect.

Synthetic interactions observed in this and other studies likely do not result from missplicing of one specific transcript, because splicing factor knockdowns produce opposite synthetic phenotypes depending on the genetic background (tumor vs. loss of stem cells; masculinization vs. feminization). Indeed, so far, no specific missplicing events accounting for overproliferation or masculinization phenotypes of the majority of splicing factor mutants have been identified (Puoti and Kimble, 1999; Belfiore et al. 2004; Kasturi et al. 2010; Zanetti et al. 2011), although general defects in splicing have been suggested (Zanetti et al. 2011). Export of unspliced *tra-2* mRNA and aberrant cytoplasmic splicing resulting in accumulation of a dominant-negative protein is thought to cause masculinization after depletion of exon junction complex components *mag-1* and *Y14* (Shimori et al. 2013). However, cytoplasmic leakage of unspliced *tra-2* mRNA was not a consequence of a general splicing defect, and was not observed upon depletion of other splicing factors.

Despite the essential contribution of splicing to gene expression, splicing factor knockdowns change gene expression patterns in germline rather than cause tissue degeneration. This is likely due to a partial loss-of-function produced by splicing factor RNAi treatments.

**Translational repression**

The switch from spermatogenesis to oogenesis in the adult depends in part on translational repression of *fem-3* mRNA by FBF proteins (Zhang et al. 1997). Splicing factor genes *mag-1*, *mag-4*, and *mag-5* were isolated in the screen for mutations that disrupt the sperm to oocyte switch (Graham and Kimble 1993; Graham et al. 1993). A transgenic reporter expressed in the somatic tissues and regulated by *fem-3* 3’ UTR was used previously to assess the role of mogs in the translational control of *fem-3* (Gallegos et al. 1998). In wild-type animals, the reporter was expressed only weakly, but in the *mag* mutant background, significant derepression was observed in somatic tissues.

**Table 2** Germline masculinization in sterile worms after splicing factor knockdown

| RNAi                  | Strain     |
|-----------------------|------------|
|                       | *rrf-1*    | *rrf-1*, *fbf-1* | *rrf-1*, *fbf-2* |
| Control day 1         | 0          | 0                | 0                |
| day 3                 | 0          | 0                | 0                |
| mtr-4 day 1           | 43% (23)   | 97% (33)         | 80% (45)         |
| day 3                 | 37% (27)   | 89% (35)         | 48% (31)         |
| prp-17 day 1          | 62% (42)   | 100% (33)        | 98% (64)         |
| day 3                 | 47% (15)   | 87% (46)         | 93% (29)         |
| F43G9.12 day 1        | 4% (23)    | 41% (34)         | 52% (46)         |
| day 3                 | 0% (26)    | 56% (34)         | 25% (56)         |

Germline masculinization was scored after staining of dissected gonads of sterile worms with DAPI if formation of sperm but not oocytes was detected. The animals were fixed and stained on day 1 post-L4 stage (3d) and on day 3 post-L4 stage (5d). In several treatments, percent masculinized germlines decreased on day 3 post-L4, suggesting that some but not all observed masculinization on day 1 post-L4 was attributable to a delay in the switch to oogenesis. Control RNAi treatments did not have sterile worms. (n), number of germlines scored.
The conclusion that Mog genes contribute to fem-3 translational repression in the somatic tissues also was presumed true for the germline, although the mechanism of regulatory input by MOG proteins remained unclear (Gallegos et al. 1998).

We find that disruption of splicing factor genes by RNAi derepresses a germ-line-expressed fog-1 transgenic reporter, which is normally silenced by FBF activity in stem cells. We observed two types of derepression: expression of the reporter throughout distal mitotic region and up-regulation of the reporter expression in meiotic cells (typically along with reporter expression in some but not all mitotic cells). Up-regulation of the fog-1 reporter in meiotic cells is reminiscent of the regulation of another FBF target, FEM-3. Normally, FEM-3 is expressed in the primary spermatocytes, but several conditions disrupting fem-3 regulation by the FBFs lead to an expansion of FEM-3 expression to pachytene, but not to the stem cell region (Zanetti et al. 2012). We observed fog-1 reporter derepression in the backgrounds where one of two fbf genes was mutated, but rarely in the wild-type background worms subjected to splicing factor RNAi. We hypothesize that combined residual activity of FBF-1 and FBF-2 upon splicing factor depletion in the wild-type background is sufficient to maintain FBF-mediated target repression in germline stem cells. Why then did the previous study find somatic fem-3 reporter derepression in splicing factor mutants despite

Figure 3 Derepression of FBF target genes upon splicing factor RNA interference (RNAi) in sensitized backgrounds. (A) Distal gonads of the indicated genotypes expressing a GFP::Histone H2B fusion under the control of the fog-1 3' UTR after RNAi of the indicated splicing factor genes. Gonads are outlined; white brackets indicate the position of the transition zone as recognized by the “crescent-shaped” chromatin. All images were taken with a standard exposure. (B) The percentage of rrf-1 (light gray), rrf-1;fbf-1 (black), or rrf-1;fbf-2 (dark gray) gonads following indicated RNAi with GFP::H2B::fog-1 3’UTR expression extending to the distal end. N, number of germlines scored. (C) Background-corrected GFP intensity in transition zone nuclei (normalized to the average GFP intensity of control RNAi on rrf-1 strain) plotted for rrf-1 (light gray) and rrf-1;fbf-2 (dark gray) gonads after indicated RNAi treatments. Box plot whiskers indicate the minimum and maximum intensity values. N, number of germlines scored. Asterisks mark the treatments that caused significant increase in the reporter intensity of the double-mutant animals compared to the rrf-1 mutant (Student's t-test; P < 0.01). Note that the difference between reporter fluorescence after F43G9.12(RNAi) in rrf-1 and rrf-1;fbf-2 backgrounds is significant, although the absolute value of the increase is small (1.4-fold) and no germlines have fluorescence values twofold higher than the control. GFP, green fluorescent protein.
the presence of both FBF-1 and FBF-2 (Gallegos et al. 1998)? Both FBFs are predominantly expressed in the germline, and the baseline somatic activity of these proteins is much lower than the germline activity. This marginal activity of FBFs that represses fem-3 3’UTR reporter in somatic tissues is further reduced by mutation in splicing factors causing fem-3 reporter derepression. By contrast, in germline, the level of FBF protein and activity are greater, so that one of the genes has to be mutated for the splicing factor RNAi to have an effect. Combined, our and previous results suggest that deficient splicing activity leads to disruption of translational control by FBFs.

Splicing factors and sex determination
One of the synthetic phenotypes observed upon splicing factor RNAi in the fbf mutant background is masculinization of the germline. Germ-line masculinization was reported for single mutants of several splicing factors, including prp-17 (Kerins et al. 2010). In addition, we observed synthetic masculinization after mtr-4(RNAi) and F43G9.12(RNAi), that were not reported to produce masculinization when depleted singly (Kerins et al. 2010). If splicing machinery were specifically required to work with FBFs (directly or indirectly), splicing factor RNAi would result in masculinization independent of genetic background. Instead, we observed that splicing factor RNAi of fog-1(ts) animals at the permissive temperature was associated with weak but significant synthetic feminization of germline indicative of fog-1 loss of function. We hypothesize that the temperature-sensitive mutation in the RNA-binding domain of FOG-1 renders it sensitive to the ribonucleoprotein (RNP) assembly defects resulting from inefficient splicing activity. Previous studies of splicing factors in sex determination found that feminizing null mutations in fog-1, fog-3, and fem-3 are epistatic to masculinization of germline observed in splicing factor mutants (Graham and Kimble 1993; Kerins et al. 2010; Wang et al. 2012). Genetically, it suggests that splicing factors function upstream of the fog/fem genes. However, we find that knockdowns of splicing factors instead enhance weak fog-1 mutation, suggesting that in addition to regulating FOG-1 production, splicing machinery is important for FOG-1 function.

How do splicing factors contribute to gene regulation?
We propose that the splicing process contributes to efficient posttranslational control of mature spliced mRNA. Disruption of the splicing
cascade may lead to defects in the assembly of messenger RNPs, which then fail to undergo normal cytoplasmic regulation. Therefore, the effects of mild splicing disruption will be most pronounced in systems heavily reliant on the posttranscriptional control of gene expression, such as C. elegans germline, and readily manifest in the sensitized mutant backgrounds. Some splicing factors remain associated with the spliced transcript, such as the exon junction complex, or EJCs (Kataoka et al. 2000; Le Hir et al. 2000, reviewed in Le Hir and Séraphin 2008). Although the core of the EJC persists during RNP maturation, peripherally associated components change as the messenger RNP is exported from the nucleus and regulated in the cytoplasm. Splicing-dependent deposition of the EJC plays a profound role in mRNA metabolism, regulating nuclear export, nonsense-mediated decay, efficiency of translation, and RNA localization (Hachet and Ephrussi 2004; Ghosh et al. 2012, 2014; Popp and Maquat 2014). One possibility is that deposition of EJC or similar complexes is disrupted by the treatments reducing overall splicing efficiency.

**Splicing factor knockdown specifically enhances mutations affecting RNA regulation**

Our results suggest that down-regulation of splicing pathway enhances the phenotypes caused by defects in RNA regulation but not embryonic lethality resulting from disruption of cytoplasmic dynemin. Similarly, a whole-genome synthetic interaction screen for genes contributing to function of mel-28 failed to retrieve splicing factors as genetic interactors (Fernandez et al. 2014). MEL-28 is a conserved component of nuclear pores needed for reestablishment of nuclear envelope after cell division and is not expected to contribute to RNA regulation. In the same vein, mutation in splicing factor fog-3 does not enhance weak lin-12 mutations interfering with Notch signaling in the anchor cell/vulval precursor cell fate decision, despite showing genetic interactions with pathways regulating the balance between germ cell proliferation and differentiation (Mantina et al. 2009). By contrast, splicing factors were isolated as enhancing the phenotype of lin-35 Retinoblastoma homolog (Ceron et al. 2007), whose regulatory targets are under extensive posttranscriptional control (Grishok and Sharp 2005; Grishok et al. 2008). Additionally, splicing factors were isolated in synthetic screens for the enhancers of germline overproliferation phenotype in the sensitized backgrounds of weak glp-1(gf) (Mantina et al. 2009; Kerins et al. 2010; Wang et al. 2012). Together, these data suggest that the processes involving RNA regulation are likely to produce genetic interaction with splicing factors.

The broad contribution of splicing to posttranscriptional control needs to be taken into account when interpreting results of large-throughput genetic enhancer screens. We recommend to take genetic screen results identifying splicing factors as enhancers of a particular mutant phenotype as an indication that posttranscriptional gene regulation plays a major role in the process under investigation. However, in absence of other supporting evidence, genetic interaction most likely reflects a broad role for the splicing factors in maintaining efficient RNA regulation rather than specific contribution to the function of the gene mutated to sensitize a strain to genetic interaction.

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